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# Canine osteosarcoma as a model in comparative oncology: Advances and perspective

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# Canine osteosarcoma as a model in comparative oncology: Advances and perspective

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# Editorial: Canine osteosarcoma as a model in comparative oncology: Advances and perspective

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**comparative oncology, dog, model, osteosarcoma, spontaneous tumors**

Editorial on the Research Topic
Canine osteosarcoma as a model in comparative oncology: Advances and perspective

Canine osteosarcoma (OSA) is an aggressive malignancy, sharing biological and clinical similarities with the human counterpart. The prognosis of patients with high-grade OSA still remains relatively poor in both species, with survival rates not having significantly improved during the recent decades. Thus, novel biomarkers of disease progression and response to treatment, as well as molecular targets for development of novel therapeutics, are urgently needed to improve the outcome of both human and canine OSA. Given the similarities between human and canine OSA, and the higher incidence rates of OSA in dogs, the canine population is a valid natural model of human disease. Therefore, the identification of specific altered pathways in canine OSA could facilitate the establishment of improved treatment strategies and provide the basis for the development of a personalized approach to OSA therapy in comparative oncology.

In this respect, the present Research Topic features original studies and reviews relevant to our theme of "*Canine osteosarcoma as a model in comparative oncology: Advances and perspective*" by bringing together scientific contributions from multiple experts in this field of study.

By an integrated analysis of whole-exome and RNA sequencing, the original research of Gola et al. provided the molecular characterization of a large number of canine OSA cell lines, allowing future investigations on their functional implications and drug response, and representing excellent translational models. In fact, cell lines constitute one of the most suitable and reproducible pre-clinical models and therefore, the knowledge of their molecular network is essential to explore oncogenic mechanisms and drug response (1). In particular, mutations in eight genes, previously described as human OSA drivers and including TP53, PTCH1, MED12, and PI3KCA, were detected in the investigated cell lines (Gola et al.).

MicroRNAs (miRNAs) are small non-coding RNAs involved in the regulation of gene expression, and a growing body of literature exists exploring the significance of their expression changes in OSA (2–10). miRNAs are also attractive molecules for biomarker/target discovery efforts (11–13). In this respect, Dailey et al. successfully identified miRNA expression changes associated with patient outcome in both canine OSA tumors and patient serum samples. Focusing on tumor-derived miRNAs associated with poor outcome, pathway and miRNA target prediction analyses were used to integrate miRNA and gene expression data to identify potential aberrant pathways contributing to OSA progression. These integrated analyses suggested that the interaction between OSA cells and the primary tumor microenvironment may contribute to the metastatic phenotype of aggressive tumors.

The importance of glucose transporter member 1 (GLUT-1, also known as SLC2A1), matrix metallopeptidase 3 (MMP3) and nuclear factor erythroid 2–related factor 2 (NFE2L2/NRF2) is also well-established in human OSA (14–17). For this purpose, Rutland et al. investigated the immunohistochemical expression of these cancer promoting proteins, that have been shown to be upregulated at the gene level in canine OSA compared to normal bone tissue (18). The study of Rutland et al. confirmed the expression of GLUT1, MMP3 and NRF2 in canine OSA, suggesting them as good potential candidates for prognostication and therapeutic targets, and encouraging clinical trials using drugs targeting these proteins.

Studies have also demonstrated the roles of parathyroid hormone-related protein (PTHrP) and its receptor (PTHR1) in the development, progression and metastasis of several tumors, including OSA. In this respect, the review of Al-Khan et al. highlighted the latest findings about functions of PTHrP and PTHR1 in normal and neoplastic tissues by focusing on their roles in OSA progression and discussing the possible related pathways in humans and canines.

Vasculogenic mimicry (VM) is a unique property of malignant cancer cells to create their own fluid-conducting microvascular channels without the involvement of endothelial cells, and has emerged as a potential target for anti-tumor therapy (19, 20). For this reason, the review of Massimini et al. illustrated the main findings concerning VM process in human OSA, as well as the related current knowledge in canine pathology and oncology, in order to provide a basis for future investigations on VM in canine tumors.

As well, in order to accelerate the understanding of the molecular basis of OSA, potentially facilitating a more rapid identification of novel therapeutic targets relevant to both people and dogs, the review of Simpson et al. focused on the shared molecular mechanisms between human and canine OSA, also presenting key differences revealed in comparative studies.

Evidence also suggests that OSA is an immunogenic tumor, and development of immunotherapies for the treatment of pulmonary micrometastases might improve long-term outcomes. The core hypothesis of adoptive natural killer (NK) cell therapy is the existence of a natural defect in innate immunity that can be restored by adoptive transfer of NK cells in cancer patients (21). In this respect, the perspective article of Kisseberth and Lee described the rationale for adoptive NK cell immunotherapy, NK cell biology, TGFβ and the immunosuppressive microenvironment in canine OSA, also illustrating the manufacturing of *ex vivo* expanded canine NK cells and providing perspectives on the present and future clinical applications of adoptive NK cell immunotherapy in spontaneous OSA and other tumors in dogs. The review of Razmara et al. also focused on the recent literature characterizing NK and T cell infiltration in OSA tumors and their prognostic significance in humans and dogs.

Finally, in the study of Flesner et al., a multimodal pain assessment methodology was used to evaluate pain relief after therapeutic intervention in dogs with primary bone cancer, suggesting that an improved assessment of pain severity and relief in dogs with cancer may allow a better evaluation of the efficacy of therapy. A direct benefit for people with cancer-induced bone pain was also highlighted, by potentially decreasing the amount of subtherapeutic novel drugs entering human clinical trials.

In conclusion, the studies collected in this Research Topic further support spontaneous OSA in dogs as a valuable model system to inform the development of new prognostic and therapeutic tools for both human and canine OSA. We hope that the contributing articles will inspire and encourage future studies on OSA pathogenesis, disease progression and therapeutic management in comparative oncology.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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# Roles of Parathyroid Hormone-Related Protein (PTHrP) and Its Receptor (PTHR1) in Normal and Tumor Tissues: Focus on Their Roles in Osteosarcoma

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Osteosarcoma (OS) is the most common primary bone tumor and originates from bone forming mesenchymal cells and primarily affects children and adolescents. The 5-year survival rate for OS is 60 to 65%, with little improvement in prognosis during the last four decades. Studies have demonstrated the evolving roles of parathyroid hormone-related protein (PTHrP) and its receptor (PTHR1) in bone formation, bone remodeling, regulation of calcium transport from blood to milk, regulation of maternal calcium transport to the fetus and reabsorption of calcium in kidneys. These two molecules also play critical roles in the development, progression and metastasis of several tumors such as breast cancer, lung carcinoma, chondrosarcoma, squamous cell carcinoma, melanoma and OS. The protein expression of both PTHrP and PTHR1 have been demonstrated in OS, and their functions and proposed signaling pathways have been investigated yet their roles in OS have not been fully elucidated. This review aims to discuss the latest research with PTHrP and PTHR1 in OS tumorigenesis and possible mechanistic pathways.
This review is dedicated to Professor Michael Day who died in May 2020 and was a very generous collaborator.

**Keywords: canine, osteosarcoma, parathyroid hormone, parathyroid hormone related protein, prognostic factor**

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## INTRODUCTION

Osteosarcoma (OS) or osteogenic sarcoma is defined as the malignancy that originates from bone-forming mesenchymal cells (1–5). This tumor is also known as the "growing bone tumor" (6). OS is the primary malignant tumor of the skeleton in which tumor cells directly form immature bone or osteoid (7). OS is the most prevalent type of primary bone cancer in both humans and dogs (8–11). OS occurs more frequently in children, adolescents, taller humans, and large breeds of dogs (9, 12). In both species, OS mostly affects the ends of long bones near the metaphyseal regions (9, 13). The femur, tibia and humerus are the locations that are most often affected by OS in humans (14).

OS is not a modern disease. A recent study revealed that dinosaurs also were affected by OS (15). Ekhtiari et al. confirmed this grossly, radiographically, and histologically in a fibula from a *Centrosaurus* in Canada. The dinosaur dates from around 77 to 75.5 million years ago (15).

Previously, paleontologists found periosteal OS using micro-computerized tomography (CT) in the hindleg of a fossilized turtle (16). This was the oldest OS to be found in an amniote indicating that OS was present in this fossil that has been dated to 240 million years old.

There has been little improvement in the treatment of OS and its prognosis in the last 40 years, especially for those patients with metastatic OS (17–21). The reason behind this could be the unavailability of novel biomarkers. Perhaps if there were confirmed prognostic tumor markers, this might assist in categorizing patients for risk-based treatment. Furthermore, the complexity of OS is such that no two tumors look alike (22).

The current treatment strategy for human OS involves neoadjuvant chemotherapy followed by surgical removal of the tumor and adjuvant chemotherapy (23). Standard chemotherapy uses a combination of doxorubicin and cisplatin with a high dose of methotrexate in the neoadjuvant and adjuvant regimens (24). This treatment procedure can improve the five-year survival rate by 60-65% (23, 25). However, early surgical removal of the tumor is the most successful treatment method (26, 27).

Canine and human OS share several key features such as presence of micrometastatic disease at diagnosis, p53 mutations, abnormal expression of several proteins (e.g., activator of transcription 3, tensin homolog, Met, phosphatase, signal transducer and ezrin), affected site and development of chemotherapy-resistance (28). Furthermore, OS in dogs and humans share similar DNA copy number aberrations and show overlapping transcriptional profiles, suggesting that these two diseases are similar at the molecular level. In addition, the metastatic rate of OS without chemotherapy is 90% for dogs and 85–90% for humans and occur mostly in lung, bone and soft tissues, in both species (28).

The high metastasis rate of OS results from the primary bone tumor spread via hematogenous path to other secondary locations (28). The most common cause of death in OS patients is the development of pulmonary metastasis (28). Metastasis occurs most frequently in lungs but rarely occurs in the surrounding pleura. There is one case report where this happened and the authors suggested it was due to the direct contact of pleura with the lungs (29).

Even though <15% of OS metastases in canine and human patients are detected at diagnosis radiologically, 85 to 90% of patients develop gross metastases regardless of effective management of the primary bone tumor (28). This shows that microscopic metastases arise in the early stages of the disease (30). The overall 5-year survival rate for OS in humans is around 60 to 70% in patients with no metastases and 10 to 30% in patients with metastases at diagnosis (24, 31–33). On the other hand, long-term survival rates for OS in dogs is only 10 to 15% (34), supporting the idea that the canine OS may be more aggressive compared to human OS (28).

One study found that overexpression of membrane-cytoskeleton linker ezrin is involved with early development of OS metastases in dogs (35). In line with canine OS data, it has been found that increased expression of ezrin is significantly associated with poor prognosis in OS cases in children (28). Using canine OS cell lines, Hong et al. found that there is an association between PKC and ezrin-radixin-moesin (36). They showed that PKC inhibitor stops ezrin phosphorylation and tumor cell migration (36). Jaroensong et al. reported that overexpression of p-ezrin-radixin-moesin occurred early in the development of pulmonary micrometastases of OS using orthotopic xenograft mouse model of canine OS (37). This expression decreased at later stages suggesting that ezrin is involved in roles related to the survival of cancer cells after their arrival at secondary metastatic sites (37).

Development of metastatic OS is the major cause of death in dogs and humans. So, the identification of new and significant treatments are crucial for the prevention of tumor metastasis which would lead to the reduction of the number of deaths in both dogs and humans (28).

The only basic prognostic indicators of human OS are the patient's response to chemotherapy, the presence of metastases and satisfactory surgical margins (38). Other prognostic indicators such as histological subtype, age, high concentration of serum lactate dehydrogenase or alkaline phosphatase (ALP), tumor size and site are still contentious (38). Recently, it has been shown that the expression of parathyroid hormone receptor 1 (PTHR1) is a prognostic indicator in canine OS (39). Although several studies have been carried out to elucidate the molecular pathogenesis and related signaling pathways of OS using human tissue, murine, canine models and cell lines, the disease remains an unsolved puzzle.

Parathyroid hormone-related protein (PTHrP) was first discovered as a causative factor of humoral hypercalcemia of malignancy syndrome (40, 41). This syndrome occurs because of increased secretion of PTHrP from tumor cells resulting in elevated levels of calcium in serum and increasing cyclic adenosine 3′,5′-monophosphate (cAMP) excretion in urine (42, 43). In humans, PTHrP is synthesized as a protein with either 139, 141, or 173 amino acids due to differences in mRNA splicing (44). PTHrP shares homology of its N-terminal amino acid sequence (1–34) with parathyroid hormone (PTH) (41). This allows both hormones to act through a common receptor (PTH/PTHrP receptor or PTHR1) (45).

PTHR1 is a seven-transmembrane class B G-protein-coupled receptor (GPCR) (46). Examples of receptors included in this family are the receptors for secretin, glucagon, pituitary adenylate cyclase-activating peptide, growth hormone-releasing hormone, vasoactive intestinal peptide, corticotrophin-releasing factor, glucagon-like peptide, calcitonin, and gastric inhibitory peptide (47). Structurally, PTHR1 contains N-terminal extracellular domain (ECD) of ∼100–160 amino acid residues, a transmembrane domain (TMD) containing the seven membrane-spanning α-helices and a C-terminal tail (48).

PTHR1 is activated by the binding of the N-terminal (1–34) amino acids of PTH or PTHrP (47). The $NH_2$-terminal part of PTH/PTHrP binds to the extracellular connecting loops and the TMD α-helices of PTHR1 (49, 50). This interaction induces conformational changes in PTHR1, which initiates intracellular signaling (51, 52). However, the COOH-terminal part of PTH/PTHrP binds to the N-terminal ECD of PTHR1 (53, 54).

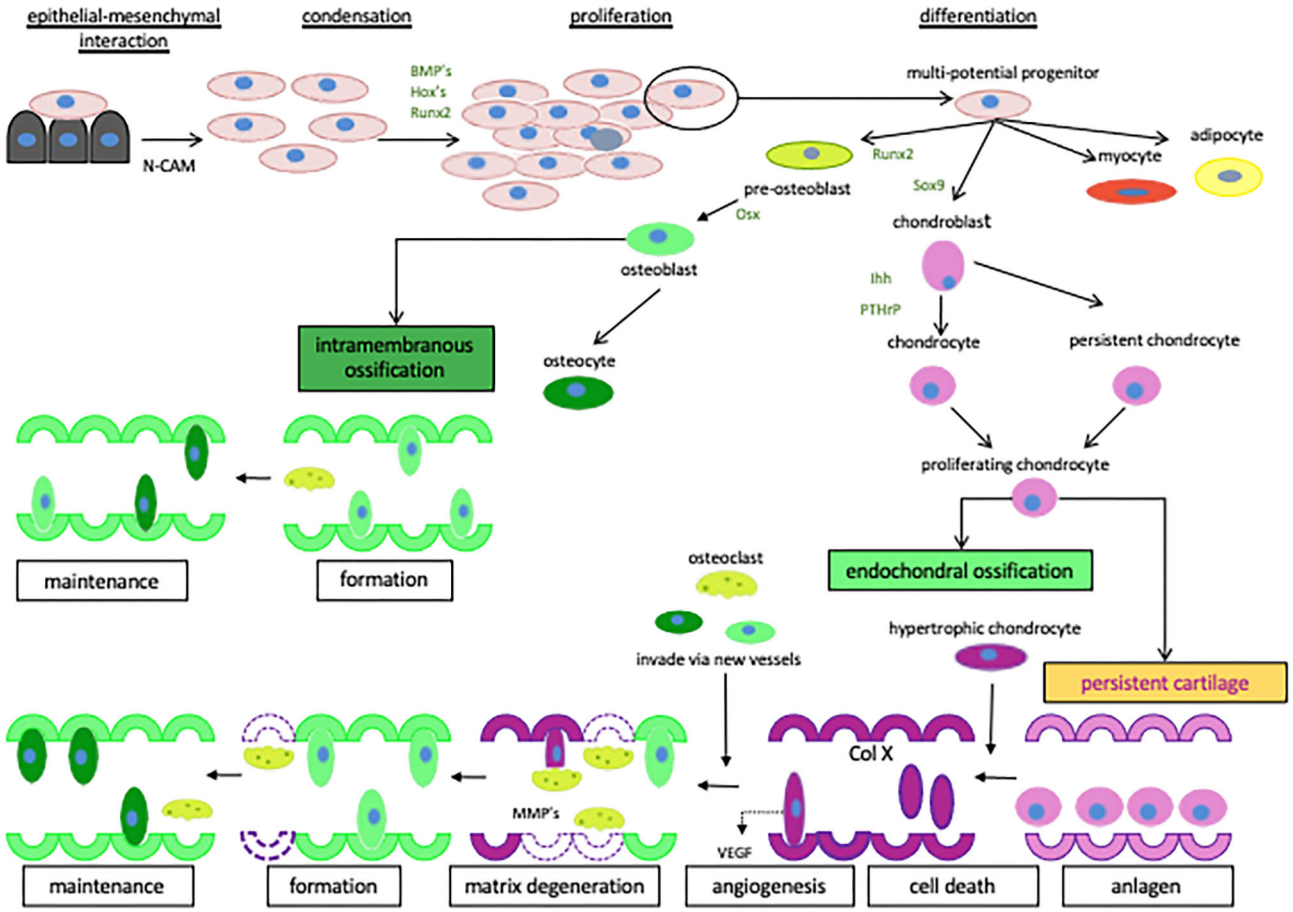


**FIGURE 1 |** The regulation of cartilage and bone formation. Diagrammatic representation of the network of signaling factors involved in cartilage and bone formation. Starting with the creation of mesenchymal condensations and their subsequent transition to differentiated cartilage and bone. The cells are represented as osteoblasts, pre-osteoblasts, chondroblasts, chondrocytes, osteoclasts, hypertrophic chondrocytes, adipocytes and myocytes. Reproduced with permission from reference (59).

Activation of PTHR1 initiates events of intracellular processes by signaling through the stimulatory G-protein α-subunit (Gsα) (55). Subsequently, the synthesis of cAMP is stimulated and PKA is triggered (56). However, PTHR1 can be activated by another signaling pathway through the Gq class of G-protein α-subunits (Gqα) (57). This activation results in triggering phospholipase C (57) which in turn activates PKC and raises inositol triphosphate and intracellular calcium in tissues (56, 58).

Numerous studies have established the roles of PTHrP and PTHR1 in bone formation, remodeling (**Figures 1**, **2**) and regulation of calcium transport (60–64). In addition, these molecules play a role in the progression and metastasis of many human tumor types such as lung and breast cancers (65, 66). The aim of this review is to highlight the latest findings about functions of PTHrP and PTHR1 in normal and neoplastic tissues by focusing on their roles in the progression of OS and discuss the possible related pathways.

## ROLES OF PTHrP IN NORMAL AND TUMOR TISSUES

PTHrP acts as an autocrine or paracrine factor and has a role in a number of significant physiological processes in bone, such as the regulation of chondrocyte and osteoblast differentiation and the proliferation (**Figure 1**) in the growth plates of developing long bones (60, 61). In bone tissue, PTHrP maintains the columnar organization of the chondrocytes and slows down their differentiation (61). Garcia-Martin et al. (67) suggested that PTHrP promotes proliferation of osteoblasts and matrix mineralization via three partially redundant mechanisms. These mechanisms are an intracrine nuclear localization signal-dependent mechanism, an autocrine/paracrine signal-peptide/PTHR1-dependent mechanism, and mixed mechanism (67). Thus, secretion of PTHrP and subsequent activation of PTHR1 would induce proliferation and mineralization of osteoblastic cells (67) (**Figure 1**).

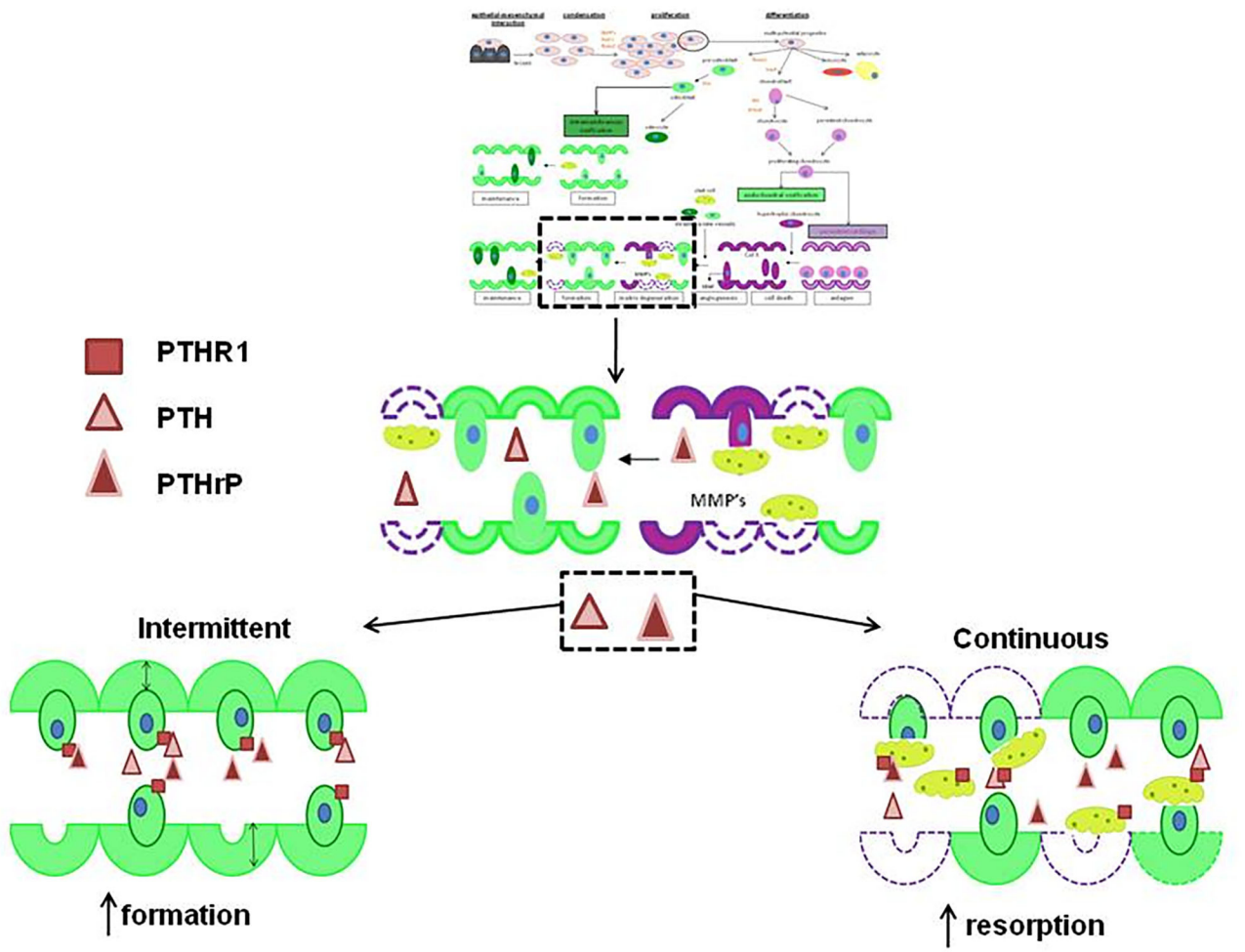


**FIGURE 2 |** The actions of PTH and PTHrP on new bone formation. If PTHrP and PTH are given intermittently to patients, they increase the formation of new bone but if either are given continuously, they increase resorption by stimulating osteoclasts to remodel bone. Both act via PTHR1. Reproduced with permission from reference (59).

In addition, PTHrP is involved in significant processes in other tissues including breast (62, 68) and placenta (63, 64). In the breast, PTHrP is abundant in milk, produced via the lactating breast and has an important role in branching morphogenesis of the mammary glands (62, 68). The concentration of PTHrP in plasma is increased during lactation resulting in the regulation of calcium transport from blood into the milk (62) and stimulation of calcium mobilization from bone (68). In the placenta, PTHrP has a role in regulating the direct transport of maternal calcium to the fetus across the placental membrane (63, 64).

Over and above its normal roles, increasing evidence has indicated that PTHrP plays critical roles in tumorigenesis (69–72). It has been found that PTHrP has a role in the activation of protein kinase A (PKA) and C (PKC) pathways (73), regulation of primary tumor growth and in metastasis (72). Luparello et al. (69) found that PTHrP stimulates cell invasion using the 8701-BC human primary breast ductal infiltrating carcinoma cell line. Further data obtained from immortalized human mammary epithelial cell lines (S1T3, S2T2, and NS2T2A1) indicated that PTHrP stimulates proliferation of tumor cells (70). In addition, it has been found that knockdown of PTHrP reduced tumor growth, induced apoptosis of osteoblasts and stimulated the formation of autophagosomes using human MDA-MB-231 breast cancer cell line (74). The authors suggested that blocking of PTHrP in the tumor cells might be a possible targeted therapy for breast cancers, particularly those with skeletal metastases (74). Similarly, Li et al. showed that PTHrP promotes breast tumor initiation, progression and metastasis in mice and it could be a novel therapy target (75). Together, these studies revealed that PTHrP plays a critical role in the initiation of breast cancer (74, 75).

**TABLE 1 |** Roles of PTHrP in progression of OS.

| Role of PTHrP in OS | Type of tissue | Species | References |
|---|---|---|---|
| Increased expression of *PTHrP* is associated with reduced tumor growth and cell proliferation | Cell line | Rat | (95) |
| Increased expression of *PTHrP* is correlated with decreased cell proliferation and tumor growth | Cell line | Mouse | (96) |
| Overexpression of *PTHrP* caused tumor chemoresistance | Cell line | Human | (97) |
| Overexpression of *PTHrP* stimulates migration of tumor cells | Cell line | Human | (98) |
| Inhibition of *PTHrP* reduced cell growth and invasion | Cell line | Mouse | (99) |
| Knockdown of *PTHrP* increased apoptosis and growth inhibition | Tissue | Mouse | (99) |
| Presence of PTHrP protein in tumors was not a prognostic marker | Tissue | Dog | (39) |

A retrospective study found that increased circulating PTHrP levels might be prognostic with shorter survival time and bone metastases in patients with lung carcinoma (71). Recently, Hastings et al. (65) also examined whether N-terminus or C-terminus of PTHrP correlated with different lung carcinoma type and prognosis. They established that C-terminus of PTHrP may reduce the effect of N-terminus PTHrP on tumor growth and progression (65). Iguchi et al. (76) established the role of PTHrP in bone metastasis in mice models using human lung squamous cell carcinoma-derived cells. Breast and lung cancers usually cause osteolytic metastases in bone (77). This osteolytic process depends on osteoclast-mediated bone resorption via up-regulated osteoclastogenesis (77). Osteoclast differentiation factors, which play a significant role in this process are receptor activator of nuclear factor-jB (RANK), its ligand (RANKL) and the decoy receptor, osteoprotegerin (OPG) (77). In humans, positive PTHrP staining was seen in 60% of primary breast tumors (78) and 92% of bone metastases (79). Recently Kim et al. (66) showed that activation of the calcium-sensing receptor (CaSR), a GPCR, up-regulated the production of PTHrP in breast cancer *in vitro*. As a result, this enhanced proliferation of breast cancer cells and reduced apoptosis (66). It was observed that reducing the expression of CaSR *in vivo* and *in vitro* inhibited the production of PTHrP and reduced the growth of the breast cancer (66).

In addition to breast and lung cancers, PTHrP has been found to stimulate tumor cell survival and proliferation in other cancers including chondrosarcoma (80), anaplastic thyroid cancer (81), medulloblastoma (82), adrenocortical cancer (83), oral squamous cancer (84), colon cancer (85), prostate cancer (86) and renal cancer (87). It has also been found that PTHrP is an essential growth factor for human clear cell renal carcinoma (CCRC) and acts as a novel target for the von Hippel-Lindau tumor suppressor protein *in vitro* (88). Talon et al. (87) demonstrated that apoptosis could be induced in the human CCRC cell line via the induction of PTHrP-neutralizing antibodies followed by the inhibition of PTHR1. Furthermore, Danilin et al. (89) showed that the mRNA-binding protein HuR is involved in increased expression of *PTHrP* and in mRNA stabilization in CCRC. A number of case studies reported a strong expression of PTHrP in pancreatic adenocarcinoma (90), intrahepatic cholangiocarcinoma (91), pancreatic neuroendocrine cancer and that PTHrP levels were elevated in the patient serum (92).

In addition to its role in tumorigenesis, Kir et al. (93) showed that PTHrP is involved in cancer cachexia, a wasting disorder of adipose and skeletal muscle tissues that leads to intensive weight loss resulting in reduced survival time in patients with cancer. PTHrP drives the expression of genes that are involved in thermogenesis in adipose tissue (93). It was demonstrated that the genes responsible for fat and muscle tissue loss were neutralized by anti-PTHrP antiserum (93). In summary, PTHrP is appearing to be a crucial factor in the pathogenesis of a large range of epithelial and non-epithelial tumors.

## ROLES OF PTHrP IN OS

The first attempt to understand the role of PTH in OS was by Martin et al. (94) by inducing OS in rats using radioactive phosphorous isotopes. Several later studies found that PTHrP also plays a role in pathogenesis of OS (**Table 1**, **Figure 3**) (96, 101–104). Suda et al. (102) demonstrated the expression of PTHrP mRNA in all investigated rat UMR 106-01 and UMR 106-06 OS cell lines. Ho et al. (99) revealed that PTHrP is also expressed by murine OS cells. Recently, PTHrP was detected in all primary canine OS tissues ($n = 50$) using immunohistochemistry staining (39). The findings showed that 50% of these canine OS tissues had weak staining intensity and 50% strong staining intensity. The study also found that there was not significant correlation between the staining intensity and the prognosis of OS in dogs (39).

In fact, the immunohistochemical (IHC) staining of PTHrP demonstrated the presence of the protein in the OS at the time of staining, but it does not tell us how much PTHrP is produced and secreted over the time (39). This association between the presence of PTHrP protein and prognosis has not yet been investigated in humans

In contrast, PTHrP mRNA was not detected in aggressive human OS xenografts (105). It has also been found that increased expression of the *PTHrP* gene is associated with reduced tumor growth and cell proliferation (**Table 1**) using a murine OS cell line (96) and a rat OS cell line (106). Previous findings discussed above showed that over-expression of *PTHrP* could be correlated with a better prognosis for OS (**Figure 4**).

However, Gagiannis et al. (97) noted that PTHrP caused tumor cells of SaOS2 human OS cell line to be chemoresistant (**Table 1**). This was observed after inhibiting major apoptosis

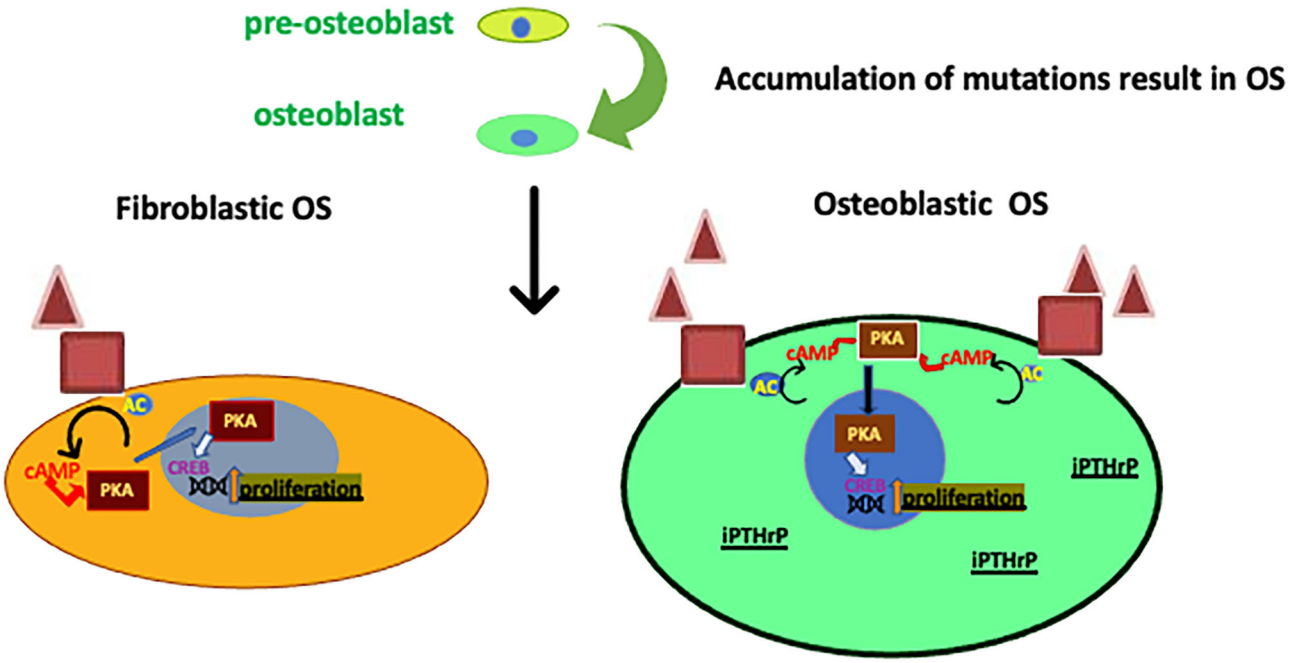


**FIGURE 3 |** Roles of PTHrP on two of the OS subtypes. Subtypes of OS arise from pre-osteoblasts that accrue mutations (possibly in Rb or p53). PTHrP, PTHR1 and CREB activity are increased in osteoblastic OS influencing proliferation (99, 100) when compared to fibroblastic OS. Also, the intracrine PTHrP (iPTHrP) may contribute to this process.

signaling pathways via blocking the death receptor and mitochondria-mediated apoptosis signaling (97). It has also been found that PTHrP stimulates migration of SaOS-2 and MG-63 human OS cell lines (98). These two studies suggest that overexpression of PTHrP could be correlated with a poorer prognosis of OS (97, 98). These conflicting data may be related to the use of different portion of PTHrP sequences in these different studies (67, 107). If PTHrP influences chemoresistance then it would be a good therapeutic target. Blocking this action could improve patient survival with current treatments.

Ho et al. (99) found that the three major OS subtypes (osteoblastic, chondroblastic and fibroblastic OS) produce PTHrP, which act through the PTHR1 to activate adenylyl cyclase, PKA, and the transcription factor cAMP responsive element binding protein 1 (CREB1) (**Figure 3**) (99). The osteoblastic subtype had an increased level of PTHR1 compared with the fibroblastic subtype but the PTHrP levels were no different (99). The knockdown of PTHrP in OS reduced cell growth and invasion *in vitro* and increased apoptosis and growth inhibition *in vivo*, while the knockdown of CREB1 had much greater growth inhibition and apoptosis (99). Moreover, Walia et al. (108), found that PTHrP is a key factor for initiation of OS in p53-deficient osteoblasts. The production of cAMP is stimulated by PTHrP (108). This stimulation is followed by PTHR1 activation, then, phosphorylation and transcription of CREB1 is activated in p53-deficient OS (**Figure 3**) (108). It was suggested that PTHrP-cAMP-CREB1-axis is essential for the initiation and progression of OS in p53-deficient osteoblasts (108). These findings are significant because P53 deficiency is a common event in OS and understanding of this pathway could lead to a better elucidation of this disease (108).

All of the above data showed that PTHrP is crucial for tumorigenesis of OS and increased expression could be linked with poor prognosis in mice (**Table 1**). However, further *in vivo* studies are necessary to clarify the exact roles of PTHrP in the progression of OS, possibly to be undertaken in dogs.

## ROLES OF HUMAN PARATHYROID HORMONE IN OS

The active portion of human parathyroid hormone is a 34-amino acid peptide (109). Studies demonstrated that PTH (1–34) and the native 84-amino acid hormone have identical spectrum of biological responses in bone (110, 111). It has been shown that single-daily subcutaneous administration of PTH (1–34) accelerates the production of new bone matrix on the endocortical, trabecular and periosteal surfaces via the stimulated osteoblasts (**Figure 2**) (110). This leads to significant elevation of bone mineral density, bone mass and strength of the bones (112, 113). Because of this, PTH (1–34) or teriparatide has been used in the management of adult patients with osteoporosis to increase bone mass and prevent bone fracture (114–117).

The Food and Drug Administration (FDA) approved teriparatide Eli Lilly & Co. (Indianapolis, IN, USA) as a treatment for osteoporosis under the name "Forteo" in November 2002 (118). The approval of this drug came after preclinical and clinical trials produced some conflicting results. Data from preclinical

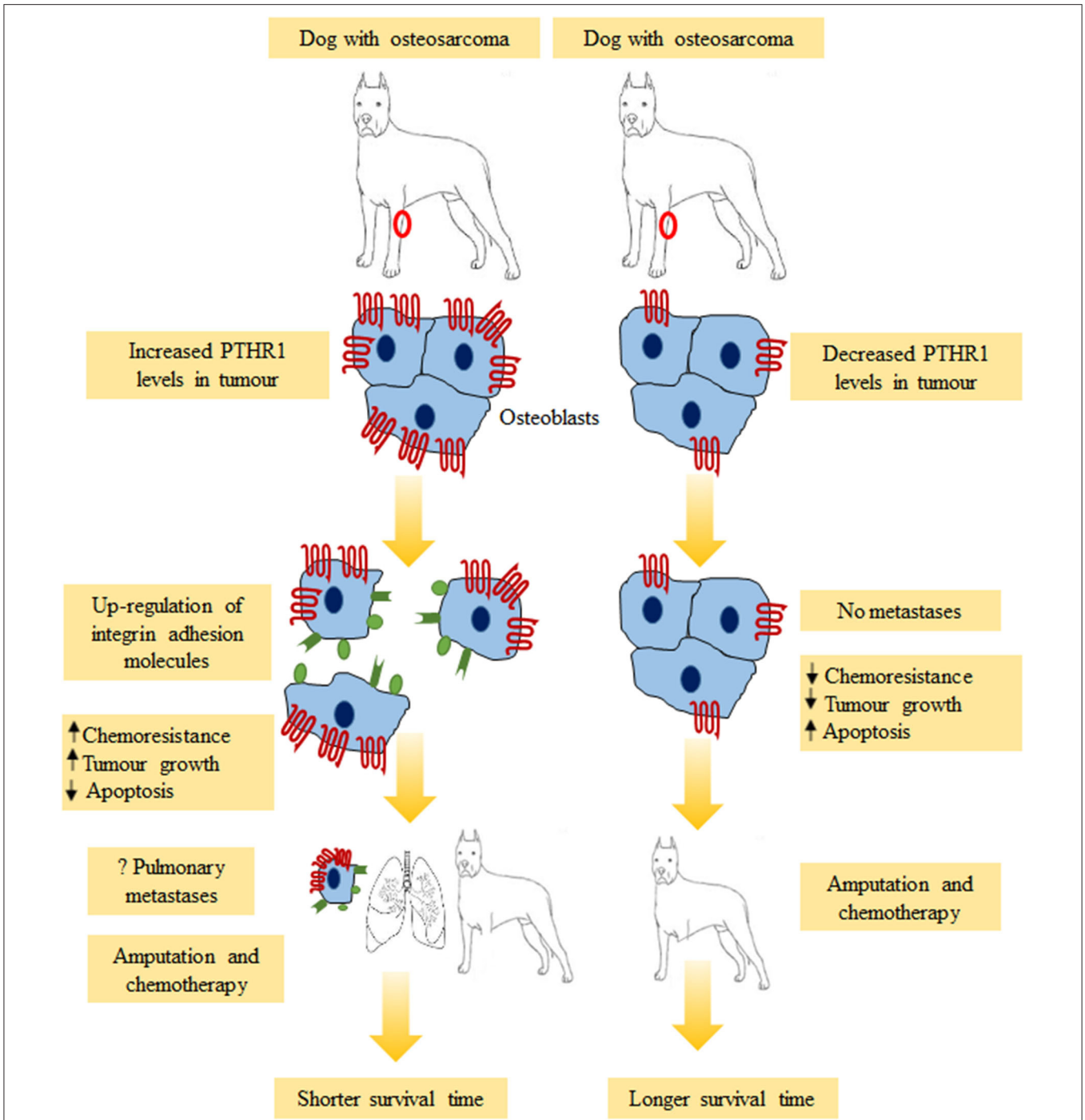


**FIGURE 4 |** Possible outcomes for dogs with osteosarcoma. Dogs with strong PTHR1 immunostaining tumors had shorter overall survival times compared to those with weak immunostaining. Overabundance of PTHR1 could activate neoplastic osteoblasts to detach via up-regulation of integrin adhesion molecules (αvβ3, β1, α2β1, α5β1, α6β1), resulting in pulmonary metastases. Other possible mechanisms which could explain the effects of PTHR1 expression including increased chemoresistance, increased tumor growth and decreased apoptosis. This might result in shorter survival time.

trials revealed that a high number of rodents developed OS after the treatment with very large doses of teriparatide for most of their lifespan. For this reason, the FDA was required to balance the possible side effects with the vital benefits of this distinctive product (118). In addition, teriparatide is not used to treat patients affected by primary malignant and metastatic bone tumors (119), Paget's disease (120) or who have had radiotherapy (121). All these conditions may increase

the probability of OS development in patient treated with teriparatide (122).

Watanabe et al. showed that the induction of OS in rats treated with teriparatide depends on the duration and dose of treatment (123). In 2004, Vahle et al. described a safe regime of teriparatide for rats (124), starting with 5 μg/kg at 6 months of age and continued for either six or 20 months (up to 70% of life span) resulted in significant increase in bone mass with no development of neoplasms (124).

In humans, two cases of OS after treatment with teriparatide have been reported in the USA (122). Nevertheless, in the first case, the connection between teriparatide and the OS was not clear (121). In the second case, the patient was treated with radiotherapy before treated with teriparatide; thus, it is uncertain whether the teriparatide treatment or radiotherapy was associated with development of OS (119). Recently, another patient developed OS after administration of teriparatide (122). This patient had no history of Paget's disease and had never received any radiotherapy. According to Ogawa et al. (122), this case was the first case with definite correlation between teriparatide and acceleration of growth of a pre-existing malignant tumor in humans.

Hyaluronan (HA) is a glycosaminoglycan component of the extracellular matrix. It is involved is regulation of cancer cell function (125, 126). It has been found that PTH increases the production of HA in osteoblast-like OS cell line (UMR 106-01 BSP) (127). Furthermore, as a response to PTH, endosteal and periosteal osteoblastic cells exhibited metabolic variances in their HA synthesis (128). It is suggested that PTH (1–34) has a role in an administration mode-dependent manner, on HA metabolism that is vital for migration of OS cell (98). This role is correlated with OS cell differentiation and behavior (98). Treatment of aggressive and poorly differentiated MG-63 cells with intermittent PTH (1–34) was found to increase expression of their HA-synthase-2, which lead to enhanced high-molecular size HA deposition in the pericellular matrix and increased migration of these cells. Continuous treatment of well-differentiated Saos2 cell with PTH (1–34) also increased the production of HA and modestly stimulated their migration (98). Another study showed that the anabolic effect of PTH (1–34) on bone metabolism was associated with changes in fibroblast growth factor-2 (FGF-2) expression (129). These FGF variations could modify the nuclear accumulation and subsequent action of runt-related transcription factor 2 (Runx-2) and CREB transcription factors which are important in the regulation of osteoblast differentiation and growth (129).

Although the mechanism responsible for the rodent bone neoplasms is still a puzzle, it was suggested that the incidence of bone tumors is increased as a result of the prolonged treatment period in these rats in conjunction with an extreme response of the skeleton to the elevated bone formation effect of daily administration of teriparatide (110). Moreover, as mentioned previously, PTHR1 is activated by the binding of the N-terminal (1–34) amino acids of PTH or PTHrP (47). The abundant production of PTHrP which can bind to PTHR1 and promote the formation of cAMP could result in induction of OS as it will be discussed in the section "Roles of PTHR1 in OS" (130). Hypothetically, treatment with teriparatide and blocking of PTHR1 at the same time could reduce the possibility of OS induction. More studies are warranted to clarify the correlation between PTH, PTHR1, and OS.

## ROLES OF PTHR1 IN NORMAL AND TUMOR TISSUES

PTHR1 is found mainly in bones and kidneys (131), and is involved in mineral ion homeostasis, bone turnover and skeletal development (132). In bone, PTHR1 regulates function, differentiation and proliferation of chondrocytes and osteoblasts (**Figure 1**) (133–135). It also controls calcium release from the matrix (136, 137).

In the kidney, PTHR1 has a role in the reabsorption of calcium in the distal convoluted tubule (46, 138) and in the maintenance of blood phosphate levels via inhibiting phosphate reabsorption in the distal and proximal tubules (139, 140). It also increases the activity of 1α-hydroxylase, resulting in increased calcium absorption from the intestine through increasing levels of 1,25-dihydroxycholecalciferol (46, 138).

Expression of PTHR1 protein has been detected in human primary tumors, including melanoma (100%), prostate adenocarcinoma (100%), colorectal carcinoma (100%), OS (50%), renal cell carcinoma (23%), and breast carcinoma (17%) (141). Studies showed that expression of PTHR1 was also detected in several human breast cancer cell lines (70, 142). Previously, Linforth et al. (143) found that expression of PTHR1 is correlated with poor prognosis in patients with primary breast cancer whilst Hoey et al. (144) reported that PTHR1 was highly expressed in human breast cancer bone metastases samples compared to primary breast cancer. The overexpression of PTHR1 in MCF-7 cells stimulated tumor cell proliferation through autocrine signals, which are mediated by cAMP and extracellular signal-regulated kinase (ERK) pathways (144).

In addition to PTHR1, recent studies have shown that overexpression of other GPCRs were associated with poor prognosis in pancreatic, breast and prostate cancers (145–147). Li et al. (146) found that increased expression of purinergic receptor *P2Y2*, a class A GPCR, correlated with a poor prognosis in prostate cancer. Moreover, protease-activated receptor 1 (*PAR1*), a second-class A GPCR, was reported to be highly expressed in aggressive breast tumors (146). Wang et al. (147) found that overexpression of GPR87, another class A GPCR, was linked with reduced survival for patients with pancreatic cancer. Furthermore, *GPR87* was reported to promote aggressiveness in primary cell lines derived from the above patients' tumors (147). These data might support the carcinogenicity of PTHR1 and other GPCRs.

PTHR1 was not well-studied in cancers other than breast and OS. The next section highlights the critical roles of PTHR1 in OS.

## ROLES OF PTHR1 IN OS

Numerous studies using human cell lines (105), murine (99), human (141) and canine (39) tissues have reported

**TABLE 2 |** Roles of PTHR1 in progression of osteosarcoma.

| Role of PTHR1 in OS | Type of tissue | Species | References |
|---|---|---|---|
| Overexpression of *PTHR1* is linked with increased invasion and proliferation | Cell line | Human | (105) |
| Knockdown of *PTHR1* stimulated tumor differentiation and decreased invasion and growth | Tissue | Mouse | (99) |
| Blocking of PTHR1 reduced metastatic cell invasion, proliferation, migration and adhesion | Cell line | Human | (148) |
| Patients with strongly staining for PTHR1 OS tumors had reduced survival times compare to those with weak immunostaining intensity OS tumors | Tissue | Dog | (39) |
| Decreased mRNA expression of *PTHR1* inhibited proliferation, migration and invasion | Cell line | Human | (149) |

the association between overexpression of PTHR1 and OS progression (**Table 2**). Mutsaers et al. (100) detected PTHR1 in primary and metastatic OS of osteoblastic and fibroblastic subtypes *in vivo* from two different types of transgenic mice. It has been suggested that increased expression of PTHR1 in OS could stimulate progression by formation of a more aggressive subtype (105).

*PTHR1* mRNA is highly expressed in metastatic human OS compared with primary tumors (105). Overexpression of *PTHR1* was linked with increased invasion and proliferation in 143B, U2OS, SaOD-2 and HOS cell lines (105). In addition, Ho et al. (99) reported that knockdown of *PTHR1* in murine OS cells stimulated tumor differentiation and decreased invasion and growth. It has been found that reduced expression of PTHR1 *in vivo* enhanced mineralization and differentiation in OS (99).

Recently, immunostaining for PTHR1 was detected in all canine OS tissues (n = 50) (39). The findings showed that dogs with PTHR1 strongly staining OS tumors had significant shorter survival time compared to those with weakly staining tumors (39). According to this study, dogs with appendicular OS showing PTHR1 strong immunostaining lived for 212 days compared to those with weak immunostaining who lived for more than double the time (459 days). The conclusion was that expression of PTHR1 could be a significant prognostic indicator in canine OS (39). As was mentioned previously, the relationship between the expression of PTHR1 and survival time of OS patient has not yet studied in humans. However, recent experiments by the group at Liaoning Cancer Hospital showed that treatment of human Saos-2 and U2OS cell lines with mangiferin, a xanthonoid, decreased mRNA expression of *PTHR1 in vitro* (149). This study suggested that the inhibition of proliferation, migration and invasion of OS cells that resulted from this treatment are correlated with inhibition of PTHR1 (149). Moreover, a recent evidence revealed that blocking of PTHR1 in human Saos-2 and U2OS cell lines by using of Quercetin, a flavonoid found in vegetables, fruits, and grains, reduced metastatic cell invasion, proliferation, migration, and adhesion (148). These findings suggest that PTHR1 could be a novel and promising therapeutic target for OS.

The pathway of PTHR1 in tumorigenesis of OS was suggested by Walkley et al. (130). Under normal conditions, PTHrP binds and activates PTHR1which is located on the surface of osteoblasts. Activation of PTHR1 leads to the synthesis of cAMP from ATP via adenylyl cyclase. Consequently, cAMP induces the detachment of cAMP-dependent PKA from its α regulatory subunit of PKA type 1 (PRKAR1A) (130). Activated PKA translocates into the nucleus to phosphorylate and activates CREB. As a result, target genes downstream of *PTHR1* signaling are activated (130). In OS, several abnormalities in the PTHrP-PTHR1-PKA pathway increased the activity of PKA pathway. This includes an elevated number of PTHR1 on the cell surface and increased expression of the *Prkaca* gene that encodes the catalytic component of PKA (130). Other abnormalities are increased production of PTHrP, which can bind to PTHR1 and promote the formation of cAMP and mutations in *PRKAR1A* gene, which result in an increase in the PKA activity (130).

A recent study carried by Li et al. (150) proposed that the effects of PTHR1 could be mediated by angiogenesis, inflammation and the Wnt pathway through altering the expression of the crucial enriched genes (*Dkk1*, *Lef1*, *Agt-CCR3*, and *Agt-CCL9*) using mouse OS cells.

Previous studies have reported that integrin adhesion molecules are involved in the migration of OS cells (151–153). Up-regulation of integrins including α5β1, α2β1, α6β1 (151), β1 (152) and αvβ3 (153) was associated with aggressive metastastic OS. PTHR1 could have a role in down-regulation or up-regulation of cell-cell or cell-extracellular matrix adhesion molecules. Integrins might be upregulated by PTHR1 in aggressive OS (**Figure 4**). To validate the current hypothesis and to further understand OS, future studies should investigate the correlation between PTHR1 and integrins in OS.

The results from all these studies taken together, show that detection of PTHR1 in OS could predict prognosis and therefore may be a potential therapeutic target.

The obvious question that may arise from this review is, why increased immunostaining of PTHR1 is correlated with reduced survival time, although dogs studied by Al-Khan et al. (39). had no clear evidence of metastasis at presentation in the smaller group ($n = 20$ dogs). This suggests that increased amounts of PTHR1 may activate tumor cells later to detach and metastasize to the lung, which leads to a reduced survival time (see **Figure 4**). The increase in PTHR1 in OS could be correlated with increasing the capability of tumor cells to metastasize and this was supported by a recent study (99). Knockdown of *PTHR1* in OS reduced invasion of tumor cells *in vitro* (99). In addition, Yang et al. (105) revealed that overexpression of *PTHR1* increased invasion and showed that metastatic OS had increased expression of *PTHR1* mRNA compared to the primary tumor.

## CYTOPLASMIC AND NUCLEAR LOCALIZATION OF PTHrP AND PTHR1 IN OS

It has been found that full length PTHrP has a nuclear localization signal (NLS) that allows transport into the nucleus after binding to the transport regulatory protein, importin β in the cytoplasm (154). PTHR1 binds to both importin α1 and importin β (155). PTHR1 overexpression has been found in the nucleus during early interphase stage (G0/G1, S, and G2 phases) of the cell cycle in the following cell lines; SaOS-2 human OS, MC3T3-E1 mouse non-transformed osteoblasts and ROS 17/2.8 rat OS (155). At G0/G1, S, and G2 phases, DNA is more open to transcriptional activity compared to the later phases where DNA is compact, transcriptional activities are reduced and the immunofluorescent staining of PTHR1 was weaker (155).

The localization of PTHrP was observed in the cytoplasm of canine primary OS cells in 66% cases and in the nucleus plus the cytoplasm in 34% cases ($n = 50$ dogs) (39). Similarly, PTHrP was detected in the cytoplasm and nucleus using murine OS tissue (99) and human metastatic bone lesions in patients with prostate carcinoma (156). In contrast, PTHR1 was localized to the cytoplasmic plus nucleus of canine OS cells in 100% cases. Another study detected PTHR1 in the cytoplasm of murine OS cell (99), while it was detected also in the nucleus and cytoplasm of normal rat liver cells (157). The study of Al-Khan found that there was no significant correlation between the localization of PTHrP and PTHR1 and prognosis of OS in dogs (39). According to this study, the increased nuclear localization of PTHR1 in OS cells could be linked to the high rate of mitosis. Moreover, most of these cells are at stage G0 and G1.

On the other hand, it has been found that nuclear localization of PTHrP is correlated with inhibition of apoptosis using nine human and rat prostate cancer cell lines [PC-3, PC-3 MB, LNCaP, DU-145, AT-2.1, MLL, AT-3.1, MAT-Lu (ML), and GP9F3] (156). It is suggested that PTHrP has a vital role in the promotion of prostate tumor growth and/or progression (123). Another study revealed that nuclear localization of PTHrP promotes survival of chondrocytes under conditions that stimulate cell death using COS-7 cell line (158).

The only study that investigated the immuno-localization of PTHR1 in human OS cells did not mention the pattern of the immunostaining and they used only four cases of OS (141). The study of Al-Khan et al. (39) is the only immunohistochemical study that investigated the localization of PTHR1 in canine OS. More studies are warranted to confirm the present findings.

## CONCLUSION

In conclusion, this review has shown that canine OS is a good model for the human disease and highlighted the roles of PTHrP and PTHR1 in normal tissue and in OS. Both PTHrP and PTHR1 are crucial factors for induction of OS. Increased expression of these two proteins in OS is correlated with a poor prognosis. PTHrP and PTHR1 play critical roles in pulmonary metastasis, chemoresistance, tumor growth and decreased apoptosis in OS patients. Although the function of these two proteins in bone, breast, placenta, and kidney has been described, their evolving roles in the pathogenesis of OS requires further investigation. This review supported the proposition that PTHR1 could be a novel and significant prognostic indicator in OS and both PTHrP and PTHR1 could be targets for novel therapeutics for OS. Also, future studies on the correlation between increased expression of PTHR1 and integrins may improve our understanding of OS progression via the discovery of novel signaling pathways that could be manipulated to improve patient outcomes.

## AUTHOR CONTRIBUTIONS

AA-K reviewed the literature and wrote the manuscript. JD, SR, and NA wrote and edited the manuscript. All authors read and approved the final version of the manuscript.

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# MicroRNA Expression Changes and Integrated Pathways Associated With Poor Outcome in Canine Osteosarcoma

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MicroRNAs (miRNA) are small non-coding RNA molecules involved in post-transcriptional gene regulation. Deregulation of miRNA expression occurs in cancer, and miRNA expression profiles have been associated with diagnosis and prognosis in many cancers. Osteosarcoma (OS), an aggressive primary tumor of bone, affects ∼10,000 dogs each year. Though survival has improved with the addition of chemotherapy, up to 80% of canine patients will succumb to metastatic disease. Reliable prognostic markers are lacking for this disease. miRNAs are attractive targets of biomarker discovery efforts due to their increased stability in easily obtained body fluids as well as within fixed tissue. Previous studies in our laboratory demonstrated that dysregulation of genes in aggressive canine OS tumors that participate in miRNA regulatory networks is reportedly disrupted in OS or other cancers. We utilized RT-qPCR in a 384-well-plate system to measure the relative expression of 190 miRNAs in 14 canine tumors from two cohorts of dogs with good or poor outcome (disease-free interval >300 or <100 days, respectively). Differential expression analysis in this subset guided the selection of candidate miRNAs in tumors and serum samples from larger groups of dogs. We ultimately identified a tumor-based three-miR Cox proportional hazards regression model and a serum-based two-miR model, each being able to distinguish patients with good and poor prognosis via Kaplan–Meier analysis with log rank test. Additionally, we integrated miRNA and gene expression data to identify potentially important miRNA–mRNA interactions that are disrupted in canine OS. Integrated analyses of miRNAs in the three-miR predictive model and disrupted genes from previous expression studies suggest the contribution of the primary tumor microenvironment to the metastatic phenotype of aggressive tumors.

**Keywords: osteosarcoma, bone cancer, prognosis, miRNA, microRNA, predictive signature, canine (dog)**

## INTRODUCTION

Despite increased survival in osteosarcoma (OS) patients resulting from the addition of chemotherapy to standard treatment protocols, only about one-fourth of canine OS patients will survive longer than a year (1). New treatment strategies are needed to manage this disease and will likely include integration of targeted therapies with standard chemotherapeutics in an individualized medicine setting. To facilitate this effort, biomarkers of disease progression and response to treatment are needed to optimize the stratification of patients into groups most likely to benefit from various treatments and identify targets for development of novel therapeutics.

Previous gene expression studies in our laboratory identified the activation of the Notch signaling pathway in OS but suggested that Notch-independent changes in HES1expression resulted in low HES1 expression in the most aggressive tumors. We also identified upregulation of insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), an oncofetal protein and known target of the let-7 tumor suppressor family of miRNAs that has been implicated in various cancers (2–4). We hypothesized that the disconnect between the HES1 and Notch pathway activation, as well as the escape of IGF2BP1 from inhibitory mechanisms present in normal adult cells, likely involved the disruption of post-transcriptional regulation by miRNAs.

miRNAs are small non-coding RNAs involved in the regulation of gene expression, providing fine-tuning of multiple cellular processes involved in the development and maintenance of homeostasis. In general, miRNAs suppress the expression of their target genes, and it is estimated that half of mammalian genes are subject to miRNA regulation via 3′ UTR binding sites (5, 6). Since a 2002 report from the Croce laboratory, the involvement of miRNA dysregulation in cancer has been well-established (7). Molecular genomic techniques such as cDNA microarrays and next-generation sequencing have been adapted to facilitate miRNA expression biomarker and novel target discovery efforts (8, 9).

A growing body of literature exploring the significance of miRNA expression changes in OS exists. Several comprehensive reviews have been written to summarize the involvement of miRNAs in OS (10–18). Major findings in OS miRNA studies include suggested or experimentally demonstrated oncogenic- or metastasis-promoting roles for miR-17-92 cluster (19–21), miR-181 family (22–24), miR-27a (23), and miR-21 (25, 26) as well as tumor-suppressive roles for miR-15/16 family members (23) and miR-34 (20, 27, 28). The roles of other miRNAs are less clear, such as the miR-29 family with reports of both elevated and decreased expression in osteosarcoma cell lines and tumors compared to "normal" controls for each sample type (20, 21, 23, 24). Significant bodies of work have explored the association of miRNAs in OS with prognosis. Loss of miRNAs located in the 14q32 locus has been associated with poor patient outcome in both human and canine OS, with the findings in human OS confirmed by multiple groups (29–31). In addition to confirming the oncogene- and tumor-suppressive roles of mir-27a and mir-16, respectively, both *in vitro* and *in vivo*, Jones et al. (23) identified tumor-based signatures associated with "osteosarcomagenesis," metastasis, and response to chemotherapy. Several reports have included functional experiments confirming interactions between miRNAs of interest and genes previously identified as dysregulated in OS, such as loss of 14q32 miRNAs and miR-135 with upregulation of c-MYC, miR-34 with RUNX2, and miR-20a of the miR-17-92 cluster and Fas (19, 28, 31, 32).

miRNAs are attractive molecules for biomarker discovery efforts due primarily to increased stability in biologic fluids and in formalin-fixed tissues compared to other RNA molecules (33–35). These features exemplify the clinical utility of miRNA, particularly in healthcare settings where stringent sample collection and storage requirements necessary for the analysis of mRNA are not always possible. A handful of studies focusing on human OS have identified associations between miRNA expression and outcome, including studies utilizing paraffin-embedded, formalin-fixed tissues, and blood fluids (23, 29, 36). Consequently, we explored the hypothesis that cancer-associated miRNAs would be measurable in tumor and serum and associated with outcome.

Our first objective was to identify candidate biomarker miRNAs differentially expressed in tumors from different outcome groups and in all tumors relative to normal bone. Candidate miRNAs were measured in a larger group of tumors and similarly sized set of serum samples to determine associations between miRNA expression changes and patient outcome. Finally, pathway and miRNA target prediction analyses were used to integrate miRNA and gene expression data to identify potential miRNA–gene regulatory networks important for OS progression.

## MATERIALS AND METHODS

### Patient and Tissue/Fluid Selection

Tumors in disease-free interval (DFI) cohorts from dogs with DFI >300 or <100 days treated with limb amputation followed by doxorubicin or platinum-based chemotherapy were collected as previously described (2). Normal bone was obtained from dogs with osteosarcoma from limbs post-amputation and harvested so that "normal" bone included in the study was distant from the tumor site and separated from the tumor by a joint (e.g., a femoral tumor would have matched a distal tibia bone collected). A 1–2-cm section of normal bone was collected for each sample; marrow and medullary fat were removed at collection. **Supplementary Table 1** shows patient data for these groups of tumors. Thirty-three additional tumors were selected from the Colorado State University Flint Animal Cancer Center's tissue archive with post-treatment data to document disease progression and matched serum or plasma samples available for miRNA extraction and expression analysis (**Supplementary Table 2**, COS33). Dogs from both cohorts were confirmed to be free of metastatic lung disease at diagnosis and surgery. Following RNA extraction and quality checks of the serum or plasma samples from the second cohort, 24 of these patients were included in circulating miRNA expression analysis.

## Total RNA Isolation, Quantification, and Quality Assessment (Tissues)

RNA was extracted from frozen samples using a freeze fracture device, followed by homogenization and separation of RNA from DNA and protein fractions using TRIzol® Reagent (Life Technologies, Grand Island, NY). The freeze fracture device and the samples were placed in liquid nitrogen to chill for 15–20 min. Approximately 1 $cm^3$ of tumor tissue and up to 4 $cm^3$ of normal metaphyseal bone were used for RNA extraction. Pulverized tissue was transferred into 2 ml/$cm^3$ of tissue of TRIzol in 15-ml conical tubes. The tissue/TRIzol mixture was then homogenized at medium to high speed for 1 min. Homogenized samples were gently shaken, centrifuged for 1 min at 2,000 RPM, and then incubated for 5 min at room temperature. The supernatant was collected into two 1.5-ml tubes and carried forward using the TRIzol reagent manufacturer's protocol for RNA extraction.

After resuspension of the extracted RNA pellet in nuclease-free water, the mirVana™ miRNA extraction kit (Life Technologies, Grand Island, NY) was used for additional RNA purification. RNA was eluted in 50 μl nuclease-free water and treated in 20-μl batches with DNAse (2 μl 10× DNAse buffer and 2 μl DNAse-I; DNA-free™ kit, Life Technologies) to eliminate genomic DNA contamination. RNA concentration and purity were determined using the NanoDrop 1000 spectrophotometer (NanoDrop Products, Thermo Scientific, Wilmington, DE). The quality of isolated total RNA was determined by RNA integrity number using a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA) with a RNA 6000 Nano chip. Only samples with RNA integrity number >6 were used. All samples were stored at −80°C.

## Total RNA Isolation (Serum)

Archived serum samples stored at −80°C were thawed at room temperature, transferred to RNAse/DNAse Free 2-ml microcentrifuge tubes, and centrifuged for 5 min at 4°C and 16,000 × g. Exactly 200 μl of the supernatant was moved to a fresh 2-ml tube for extraction of RNA using the miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA) following the manufacturer's directions. Synthetic ce-miR-39 mimic ($1.6 \times 10^8$ copies) was spiked in to each sample prior to addition of chloroform. Strict preset volumes of reagents and sample RNA were used following the manufacturer's recommendations.

## Real-Time Reverse Transcriptase Quantitative PCR

cDNA synthesis of small non-coding RNAs was performed using the miScript Reverse Transcription kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, reverse transcription (RT) was performed in 20-μl reactions containing 1 μg total RNA in nuclease-free water, 5× miScript RT Buffer (Mg, dNTPs, and oligo-dTprimers), and 1 μl miScript Reverse Transcriptase Mix [poly(A)polymerase and reverse transcriptase]. Generated cDNAs were stored at −20°C until analysis. Quantitative PCR measurements were performed in 384-well PCR plates in a 6-μl reaction containing 2× Quantitect SYBR Green master mix (Qiagen, Valencia, CA), 10 μM miRNA-specific forward primer (MWG Biotech), 10× Universal Reverse Primer (Qiagen, Valencia, CA), 2 ng equivalent cDNA, and nuclease-free water. miRNA-specific primers were designed based on sequences of mature miRNA from MirBase (**Supplementary Table 8**). Samples were run in duplicate with non-template and reverse transcriptase-free (no RT) controls.

Modifications to this protocol for measurement of serum miRNA were as follows: cDNA synthesis was carried out in 10-μl reactions containing 2 μl 5× HiSpec Buffer, 1 μl Nucleics mix, 1 μl nuclease-free water, and 5 μl total serum RNA. The serum cDNA was diluted 1:10 in nuclease-free water, and a consistent volume (0.15 μl), rather than a consistent concentration, was included in each 6-μl RT-qPCR reaction.

## Data Analysis

For analysis of RT-qPCR data from tumor samples, both GeNorm (37) and NormFinder (38) were used to identify the best candidate reference miRNAs from 10 options, and data was normalized to the geometric mean of miR-30a, miR-27b, and miR-185. The $2^{-\Delta\Delta Ct}$ method was used for differential expression analysis in the initial set of 14 tumors. Statistical analysis of survival data was performed using normalized and transformed expression data from 19 miRNAs in the test set of 33 tumors and 13 miRNAs in 31 serum samples using a combination of Prism and the coxph and survfit functions from the survival package in R.

## Statistical Analysis

Associations between miRNA expression levels and DFI were evaluated using Cox proportional hazards linear regression. Multivariable Cox regression was then performed on a subset of candidate miRNAs ($p < 0.25$ from univariate analysis), utilizing both forward and backward stepwise models based on the Akaike information criterion (AIC). A risk score was calculated for each sample based on the best multivariate model, and Kaplan–Meier method was used to determine median DFI for low- and high-risk groups based on the median risk score. Comparison between groups was made with the log rank analysis, and a $p$-value of $<0.05$ was considered significant.

This analysis pipeline was modified slightly for serum samples. Raw Ct values were first adjusted based on the expression of synthetic cel-miR-39 (33, 39). Then, two miRNAs, miR-16 and miR-21, were selected as reference miRNAs and normalized using a variation of mean centering, termed concordance correlation restricted, as described in Wylie et al. (40). This method was found to be well-suited for biofluid samples.

# RESULTS

## Differentially Expressed miRNAs in Tumors From Dogs With Poor Response Compared to Those With Good Response

Expression of 188 miRNAs was measured in 14 tumors—seven tumors from dogs with DFI >300 days (good responders) and seven tumors from dogs with DFI <100 days (poor responders)—using RT-qPCR. Four miRNAs were differentially expressed in tumors from poor responders relative to those from

**TABLE 1 |** Results of univariate Cox proportional hazard regression analysis for expression of miRNAs in canine osteosarcoma tumors ($n = 33$; disease-free interval range, 20–937 days).

| miRNA name | *p*-value | HR | 95% CI |
|---|---|---|---|
| *mir.223.3p* | *0.001* | *2.25* | *1.38–3.68* |
| *mir.181b.5p* | *0.028* | *0.65* | *0.44–0.96* |
| *mir.130a.3p* | *0.107* | *0.70* | *0.46–1.08* |
| *mir.199a.5p* | *0.158* | *0.75* | *0.50–1.12* |
| *let.7b.5p* | *0.171* | *0.60* | *0.29–1.25* |
| *mir.451a* | *0.194* | *1.24* | *0.90–1.71* |
| *mir.7.5p* | *0.236* | *1.22* | *0.88–1.69* |
| mir.26a.5p | 0.315 | 0.80 | 0.52–1.24 |
| mir.30c.5p | 0.369 | 0.85 | 0.61–1.20 |
| mir.142.3p | 0.423 | 1.19 | 0.78–1.83 |
| mir.206 | 0.583 | 0.91 | 0.65–1.27 |
| mir.18a.5p | 0.617 | 1.10 | 0.76–1.58 |
| mir.16.5p | 0.648 | 0.93 | 0.67–1.29 |
| mir.196b.5p | 0.668 | 0.92 | 0.63–1.35 |
| mir.9.5p | 0.742 | 0.94 | 0.65–1.36 |
| mir.135a.5p | 0.788 | 0.96 | 0.69–1.32 |
| mir.128.3p | 0.796 | 0.96 | 0.70–1.32 |
| mir.210.3p | 0.964 | 1.01 | 0.70–1.46 |
| mir.17.5p | 0.981 | 0.10 | 0.73–1.35 |

*The italicized rows ($p < 0.25$) were selected for multivariate analysis.*

good responders using a cutoff of $p < 0.05$ for significance. Nineteen miRNAs were selected based on $p < 0.1$, fold change >2.0, or biological interest based on human OS studies for additional expression analyses in a larger set of tumors (**Supplementary Table 3**, bold).

Cox proportional hazard univariable regression analysis of expression of 19 miRNAs in 33 tumors from patients with DFI ranging from 20 to 937 days identified miRNAs associated with patient outcome (**Table 1**). The goal of multivariate Cox proportional hazard analysis in this study was to identify the best combination of candidate miRNAs whose expression explained a significant proportion of the variability of patient outcome in this group of tumors and which would be likely to predict outcome in an independent set of canine tumors. Thus, expression values for seven miRNAs with $p < 0.25$ based on the univariate analysis were included in both forward and backward step-wise multivariate Cox proportional hazard regression analysis. A three-miRNA model was selected as the best model based on AIC, a measurement of model selection that takes into account the goodness-of-fit of the model with penalties for increased complexity (**Table 2**).

## Three-miRNA Signature for Patient Outcome (DFI)

The Cox proportional hazard multivariate model with three miRNAs—miR-223-3p, miR-130a-3p, and let-7b-5p—was used to calculate the risk scores for each sample. The median risk score was used as a cutoff to discriminate samples considered high or low risk. Kaplan–Meier survival analysis with the log rank test using the three-miRNA model-based risk score distinguished patients with high risk and low risk with respective median DFIs of 123.5 and 392 days (**Figure 1A**, $p = 0.0002$, hazard ratio 3.2, 95% confidence interval 2.5–12.9). Relative expression of each miRNA in the signature (**Figure 1B**) indicated that Let-7b was not significantly elevated in the low-risk group, while miR-103a was significantly elevated in the low-risk group ($p = 0.008$), and miR-223 was significantly reduced in the low risk group ($p = 0.003$). Additionally, if samples were separated into cohorts of good and poor responders based on mean DFI, the three-miRNA model signature had an accuracy, based on area under the curve of 0.86 (**Figure 1C**).

**TABLE 2 |** Three-miRNA model with lowest Akaike information criterion *via* both forward and backward step-wise Cox proportional hazard regression ($R^2 = 0.413$, concordance = 0.73).

| miRNA name | *p*-value | HR | 95% CI |
|---|---|---|---|
| mir.223.3p | 0.0003 | 2.676 | 1.57–4.57 |
| mir.130a.3p | 0.0229 | 0.5718 | 0.35–0.93 |
| let.7b.5p | 0.1451 | 0.6034 | 0.31–1.19 |

## Pathway Analysis of Dysregulated miRNAs and Genes Suggests Roles for Tumor Microenvironment and IGF2BP1 Regulatory Network in Aggressive OS

We used the mirPath tool from the Diana Tools website (41, 42) with species set to human to identify the top pathways enriched for genes that are targets of the three miRNAs in our Cox proportional hazards model. The top 20 significant pathways using the microT-CDS database and the genes union function include: FoxO signaling, ECM–receptor interaction, signaling pathways regulating pluripotency of stem cells, TGF-beta signaling, cytokine–cytokine receptor interaction, and p53 signaling (**Table 3**). The let-7 family, being among the earliest miRNAs discovered and more widely studied, shows 682 targets in this tool, while miR-223 and miR-130a list only 367 and 552 genes, respectively. Since loss of let-7b-5p and mir-130a-3p was associated with a shorter DFI, we explored the pathways that they regulate separately from those pathways regulated by mir-223-3p which was elevated in the tumors with higher risk of metastasis. Mir-223-3p is specifically involved in transcriptional misregulation in cancer and cytokine–cytokine receptor interaction. Taken together, this suggests a role for these miRNAs in the regulation of the extracellular environment, immune system, and developmental pathways.

We next used multiMiR, a miRNA–target interaction R package and database out of the Theodorescu lab (43), to identify either experimentally validated or predicted miRNA–mRNA interactions based on data from this study and previous studies in our laboratory (**Supplementary Table 6**). MultiMiR predicted potential interactions between miR-223, over-expressed in tumors from dogs with shorter DFI, and both dystonin (DST) and catenin (cadherin-associated protein), Alpha

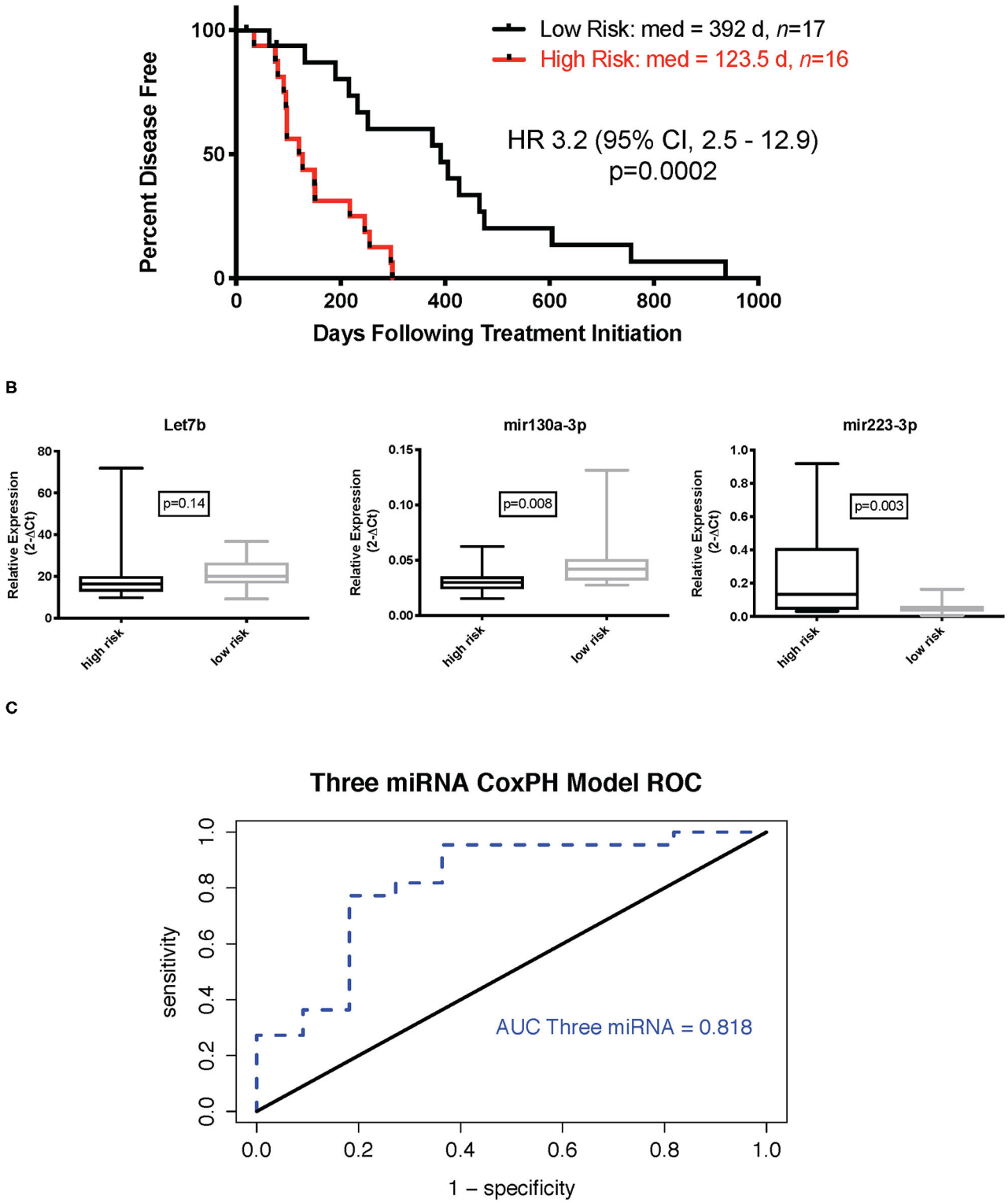


**FIGURE 1 |** Three-miRNA tumor-based predictive model. **(A)** Kaplan–Meier survival curve with log rank test (cutoff is median risk score: 0.8897). **(B)** Relative expression ($2^{-\Delta Ct}$) of individual miRNAs in low- and high-risk groups (Mann–Whitney test). **(C)** Receiver operator characteristic curve for three-miRNA Cox proportional hazard-based risk score dividing outcome groups based on mean disease-free interval.

**TABLE 3 |** Top pathways ($p < 0.05$) enriched for genes targeted by let-7b-5p, miR-223-3p, and/or miR-130a-3p.

| KEGG pathway | *p*-value | #genes | #miRNAs |
|---|---|---|---|
| Prion diseases | 4.76 × 10E-19 | 1 | 1 |
| Mucin type O-glycan biosynthesis | 3.83 × 10E-16 | 9 | 3 |
| FoxO signaling pathway | 9.91 × 10E-05 | 27 | 3 |
| Extracellular matrix–receptor interaction | 9.91 × 10E-05 | 12 | 3 |
| Signaling pathways regulating pluripotency of stem cells | 1.02 × 10E-04 | 29 | 3 |
| TGF-beta signaling pathway | 9.52 × 10E-04 | 19 | 3 |
| Cytokine–cytokine receptor interaction | 3.86 × 10E-03 | 30 | 3 |
| Amoebiasis | 0.011 | 16 | 3 |
| p53 signaling pathway | 0.046 | 13 | 3 |
| Transcriptional misregulation in cancer | 0.048 | 28 | 3 |

*#genes, number of genes targeted by analyzed miRNAs in the pathway.*
*#miRNAs, number of analyzed miRNAs that have targets in the pathway.*

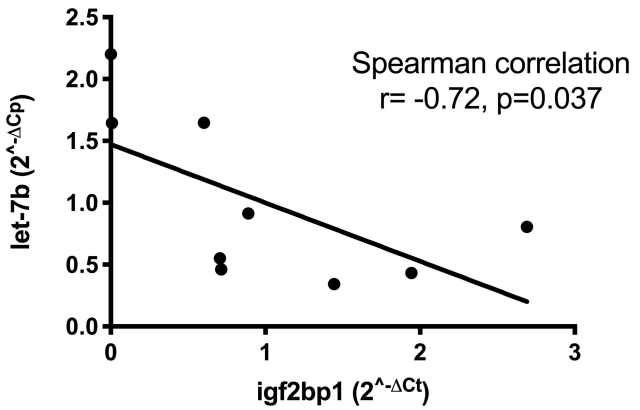


**FIGURE 2 |** Correlation between low let-7b expression and high expression of IGF2BP1 in eight osteosarcoma tumors as determined by RT-qPCR.

2 (CTNNA2). Both are adhesion proteins interacting with the cytoskeleton, potentially implicating disruption of the tumor microenvironment in the aggressiveness of OS. Interactions between let-7b and six other under-expressed miRNAs and IGF2BP1 confirm that miRNA expression changes likely play a role in the high expression of this gene in tumors from dogs with poor outcome. Relative expression of let-7b and IGF2BP1 in OS tumors with a short disease-free interval via RT-qPCR confirms a statistically significant correlation, suggesting that this interaction occurs in canine OS and may contribute to outcome (**Figure 2**).

## Differentially Expressed miRNAs in OS Tumors Relative to Normal Bone Support Dysregulation of the Notch Pathway in OS

Expression of 188 miRNAs was also measured via RT-qPCR in seven normal bone samples. As has been our experience with gene expression, more differentially expressed miRNAs were identified with higher statistical significance and larger fold changes. Forty differentially expressed miRNAs were identified using cutoffs of $p < 0.05$ for significance and fold change >2; 21 miRNAs had a lower expression in tumors than normal bone, while 19 miRNAs were over-expressed in tumors (**Supplementary Tables 4**, **5**).

Based on our previous work associating the Notch signaling pathway with OS and again using multiMiR, we sought validated interactions between 21 downregulated miRNAs and 30 upregulated Notch/HES1-associated genes as well as between 19 upregulated miRNAs and 14 downregulated Notch/HES1-associated genes. The pool of Notch/HES1-associated genes was a subset of the genes previously published (44). MultiMiR identified experimental, protein-based evidence for interactions between 21 of 41 miRNAs and 17 of 44 genes or roughly half of the miRNAs and genes entered into the analysis (**Figure 3**). This data supports the hypothesis that dysregulation of the Notch signaling pathway contributes to the pathogenesis of OS and likely involves disruption of miRNA regulation of Notch pathway-associated genes.

## Serum miRNA Changes Associated With OS Patient Outcome

Expression of 13 miRNAs in 31 serum samples from patients with DFI ranging from 20 to 772 days was analyzed using a similar Cox proportional hazard regression pipeline described for tumor miRNA expression data. The 13 miRNAs evaluated comprised a combination of 10 miRNAs selected from our analysis of tumor-derived miRNA expression and three miRNAs commonly highly expressed in human serum samples. Forward and backward stepwise Cox multivariable proportional hazard regression analysis identified a two-miRNA model (miR-23a-3p and miR-30c-5p) with the best fit based on AIC (**Table 4**, **Figures 4A–C**). The risk score based on this model separated the samples into groups, with mean DFI of 272 days for the low-risk group and 123.5 days for the high-risk group ($p = 0.004$, hazard ratio 2.6, 95% confidence interval 1.6–8.5).

## Tumor-Based miRNA Signature Compared to Clinical Predictors

One measure of the value of a new prognostic biomarker is its usefulness compared to other predictive markers including clinical parameters (45). For OS, the most consistent clinical indicators of outcome are proximal humerus location, weight, and serum ALP (1, 46–48). We had access to an expanded set of curated, quality-checked clinical data for a subset of our tumors ($n = 24$) that were included in a large retrospective study by Selmic et al. (46). Multivariate Cox proportional hazard regression of the three miRNA expression-based risk score and other clinical parameters ($p < 0.25$ on univariate analysis) showed that, when adjusting for these indicators, the miRNA expression based risk score remains a significant predictor of outcome (**Table 5**). This suggests that incorporation of miRNA expression signatures would improve the estimation of prognosis for canine patients.

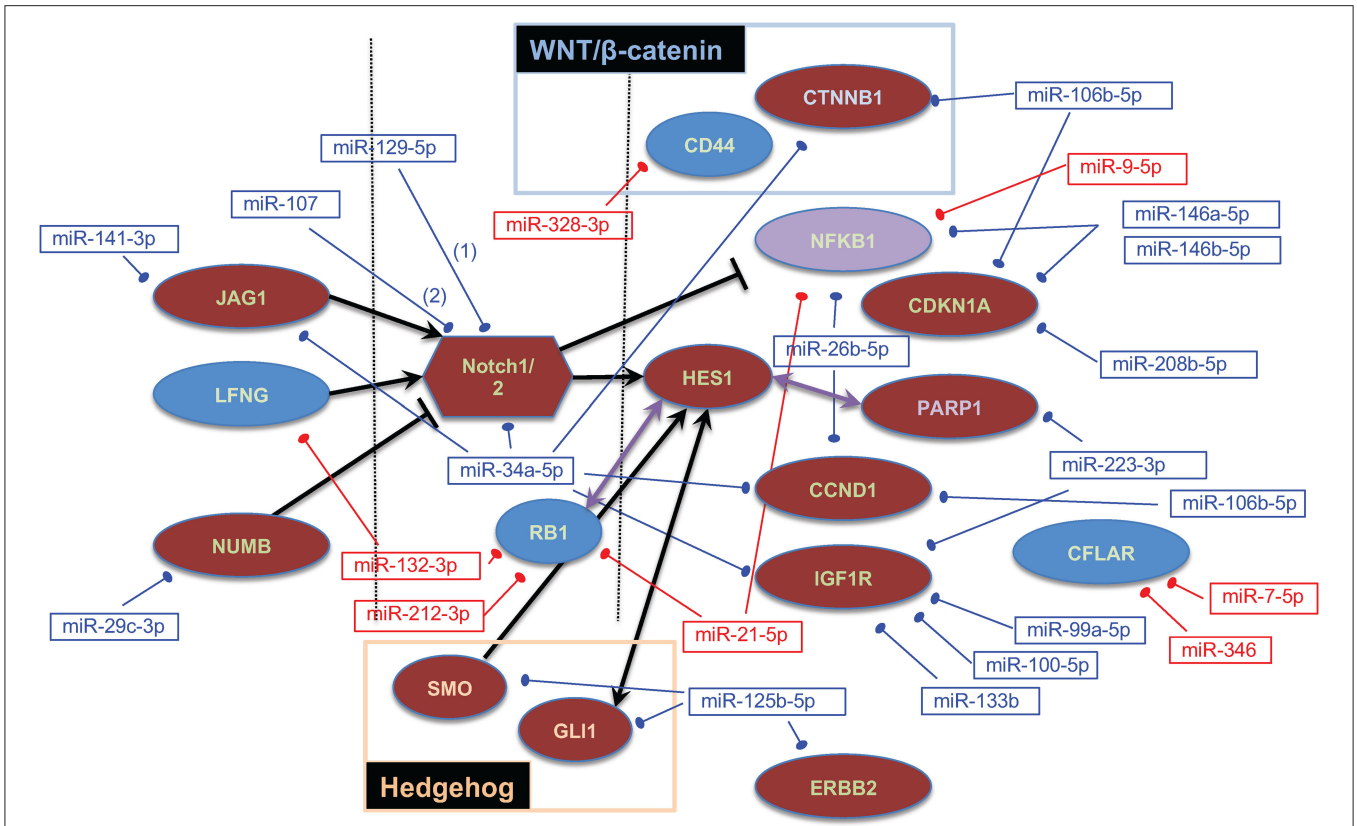


**FIGURE 3 |** Notch/HES1-associated miRNA–mRNA interactions. Dysregulated genes are shown as ovals or polygons, dysregulated miRNAs are shown in text boxes. In both cases, red indicates expression that is higher in tumors than in normal bone, blue indicates expression that is lower in tumors, and purple indicates that one probe in the Affymetrix array showed NFKB1 as upregulated and another as downregulated. Genes on the left are ligands or inhibitors of Notch; genes on the right are downstream targets of the Notch signaling pathway and/or specifically interact with HES1.

**TABLE 4 |** Two miRNA models after step-wise Cox proportional hazard regression ($R^2 = 0.278$, concordance = 0.69).

| miRNA name | *p*-value | HR | 95% CI |
|---|---|---|---|
| mir.23a.3p | 0.0209 | 0.5652 | 0.35–0.92 |
| mir.30c.5p | 0.0099 | 0.5487 | 0.35–0.87 |

## DISCUSSION

Aberrant miRNA expression patterns have been associated with patient outcome for a variety of human tumors. Combined with their stability in fixed tissues and less invasively obtained body fluids, miRNAs make attractive candidates for biomarker discovery efforts. In this study, we identified miRNA expression signatures from both canine OS tumor and patient serum samples that associated significantly with outcome following surgical amputation of the affected limb and standard-of-care chemotherapy. Pathway and miRNA–gene interaction analyses focused on tumor-derived miRNAs associated with poor outcome, suggesting that the interaction between OS cells and the primary tumor microenvironment may be a major determinant in the ultimate metastatic capabilities of OS tumor cells. Additional miRNA–gene interaction analyses combining expression changes identified in this study with gene expression changes from earlier studies suggest that miRNA dysregulation contributes to both (1) disruption of the Notch pathway in OS compared to normal bone and (2) deregulation of the growth-promoting oncofetal protein IGF2BP1 in the most aggressive OS tumors. Finally, we demonstrated that the tumor-based three-miRNA signature remains an independent predictor of outcome when we control for possible effects of other clinical parameters such as tumor location, patient weight, and age at diagnosis.

Although previous studies have established grading systems for canine OS (49, 50), a limitation of the current study is the lack of grading for the tumors in this data set. Meta-analysis and direct comparisons have shown limited utility for these grading systems in prognosis with simplified high- or low-grade models suggested (48, 51). Variability within tumors as well as the complexity of the criteria in the proposed grading schemes may contribute to high subjectivity. Furthermore, more than 80% of tumors will fall into high-grade histologic categories, within which variable patient outcomes may be achieved. For OS, the most consistent clinical indicators of outcome are proximal humerus location, weight, and serum ALP (1, 46–48). Among these clinical parameters (scoring $p < 0.25$ on univariate analysis), only location of the

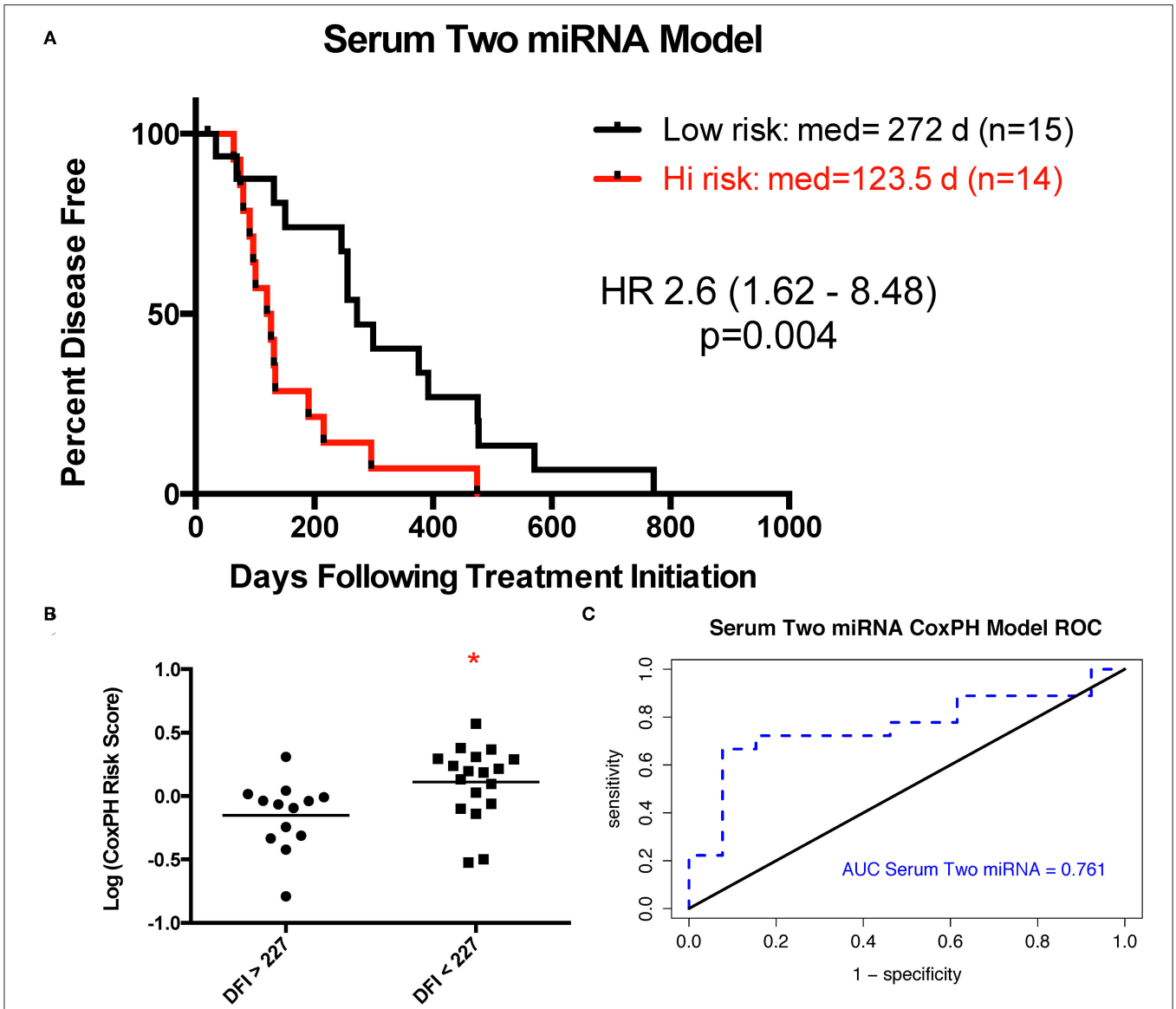


**FIGURE 4 |** Two-miRNA serum-based predictive model. **(A)** Kaplan–Meier survival curve with log rank test (cutoff is median risk score: 1.0372). **(B)** Scatter plot of risk scores in two outcome groups based on mean disease-free interval (DFI) for all 33 samples (*$p = 0.014$, Mann–Whitney test). **(C)** Receiver operator characteristic curve for serum two-miRNA Cox proportional hazard-based risk score dividing outcome groups based on mean DFI.

tumor in the proximal humerus and the three-miRNA risk score were significant predictors of disease outcome. This suggests that incorporation of miRNA expression signatures would improve the estimation of prognosis for canine patients.

Our first goal of this study was to identify miRNAs associated with progression of OS despite standard-of-care treatment including surgical amputation and doxorubicin and/or platinum-based chemotherapy. We identified a three-miRNA expression signature that separated patients into two distinct outcome groups. Within this signature, elevated expression of miR-223 and decreased expression of let-7b and miR-130a were associated with increased risk and ultimately shorter median DFI. Of these three, miR-223 was the most significantly associated with DFI based on *p*-value in both the univariate and multivariate regression analyses. Interestingly, the expression of miR-223 is nearly 20 times lower in OS tumors compared to normal bone (**Supplementary Table 5**), which is consistent with two reports in human OS (21, 23). miRNA expression analyses performed in canine cancer cell lines conducted in our laboratory showed that miR-223 expression is similarly uniformly low across canine osteosarcoma cell lines. We performed pathway and miRNA–gene regulatory analyses to identify pathways potentially affected by expression changes in miR-223. These analyses suggest that the significant increase in miR-223 expression in canine OS tumors may be either originating from or influenced by interactions with the tumor microenvironment.

**TABLE 5 |** Results of univariate/multivariate analysis of factors associated with clinical outcome, including a three-miRNA expression-based risk score (tumor-derived miRNA expression).

| | | Med DFI (days) | HR | *P* | 95% CI |
|---|---|---|---|---|---|
| **Univariate analysis** | | | | | |
| Three-miRNA risk score | Low | 392 | 0.18 | 0.00061 | 0.070–0.484 |
| | High | 123.5 | | | |
| Weight | | | 1.05 | 0.046 | 1.001–1.103 |
| Age at diagnosis | | | 0.785 | 0.10 | 0.587–1.051 |
| Proximal humerus | Yes | | 3.055 | 0.057 | 0.969–9.628 |
| | No | | | | |
| **Multivariate analysis** | | | | | |
| Three-miRNA risk score | | | 0.185 | 0.0067 | 0.055–0.626 |
| Proximal humerus | | | 5.63 | 0.016 | 1.38–23.06 |

Pathways enriched for both miR-223 and miR-130a included hematopoietic cell development and osteoclast differentiation. Several lines of evidence support a role for miR-223 as an important regulator of the immune response inhibiting the differentiation of classically activated (M1) macrophages and promoting anti-inflammatory and pro-tumor (M2) polarization (52–54). Notch signaling, a pathway we have found to be significantly dysregulated in aggressive canine OS, is also important for pro-inflammatory M1 polarization (55). Normal differentiation and function of osteoclasts, which are derived from bone marrow monocyte precursors, are also reliant on the expression of miR-223 (56). Given that miR-223 is highly expressed by both M2 macrophages and osteoclasts, it is possible that the increased expression of miR-223 in tumors from poor responders is originating from or induced by an interaction with the increased numbers of these cells in the tumor microenvironment. For example, Yang et al. (57) demonstrated that M2-polarized macrophages can shuttle miR-223 via exosomal transport to breast cancer cells, increasing their invasive ability. In addition, miR-223 may suppress the maturation and immunogenicity of dendritic cells to promote a tolerogenic environment (58, 59).

The role of both osteoclasts and macrophages in OS remains controversial due to a variety of factors, including the potentially different behaviors of these cells depending on the level of differentiation, polarization, and response to external stimuli (60–63). Despite this uncertainty, macrophage-activating agents (promoting pro-inflammatory M1 polarization), such as muramyl tripeptide phosphatidylethanolamine, have consistently shown promise for treatment of OS (64, 65).

In further support of the influence of the tumor microenvironment on miRNA expression changes in OS, miRNA–gene interaction analysis identified potential interactions between miR-223 and adhesion proteins DST and CTNNA2. Both are involved in actin cytoskeletal remodeling, a pathway commonly associated with metastasis (66) and identified as enriched for dysregulated genes in our previous gene expression studies (2). Changes in actin cytoskeletal remodeling are commonly triggered by cell–cell interactions, including those that may occur between tumor cells and supporting stromal cells. The decreased expression of CTNNA2 in tumors from our poor responders supports a pro-metastatic role for miR-223 as CTNNA2 acts as a tumor suppressor in both endometrial and laryngeal carcinomas (67, 68). Additional evidence for an association between miR-223 and metastasis or chemotherapy resistance has been demonstrated in recurrent ovarian tumors, renal cell metastases, and gastric cancer (69–71).

In contrast, recent reports support a potential tumor suppressor role for miR-223 *in vitro* (72–74). Low miR-223 expression combined with elevated expression of its target gene, epithelial cell transforming sequence 2, in OS tissues is associated with poor outcome (72, 73). It is worth noting that only one of these studies utilized patient tissues, and this included a mix of pediatric and adult tumors (age range 8–66 years) (73). Canine osteosarcoma most closely resembles the pediatric disease, while human adult OS is frequently associated with Paget's disease and may thus involve different underlying molecular mechanisms of progression (1, 75).

Another goal of this study was to integrate miRNA and gene expression data to identify key aberrant pathways contributing to pathogenesis and progression of OS. miRNA–gene expression analysis revealed seven miRNAs with low expression in aggressive tumors predicted or known to target IGF2BP1, an oncogene of interest to our lab. IGF2BP1 has a 3′ UTR that is thousands of kilobases long and with multiple well-conserved binding sites for various miRNAs. This extended 3′ UTR contains multiple polyadenylation sites, with alternate use of polyadenylation sites to produce a shortened 3′ UTR serving as a mechanism by which the gene may avoid miRNA regulation, including at least four sites for the miRNA let-7 (3). We found a statistically significant correlation between low expression of let-7b and increased expression of IGF2BP1 via RT-qPCR in eight OS tumors (**Figure 2**). Identification of potential miRNA regulators of this protein will facilitate additional functional studies. In addition to IGF2BP1, let-7b targets a variety of oncogenes and has been proposed to act as a tumor suppressor in human osteosarcoma by targeting insulin-like growth factor-1 receptor (76). Despite challenges with effective delivery to target tissues, restoration of tumor-suppressor miRNAs remains a rapidly growing area of research. Studies such as ours may identify new therapeutic miRNAs.

We did not identify any potential interactions between our most dysregulated miRNAs and HES1 nor was Notch signaling identified in our pathway analyses involving miRNAs aberrantly expressed between our DFI cohort tumors. This is consistent with our findings and those of Poos et al. (26) that Notch activation likely contributes to the proliferative response but does not appear to drive metastasis. To further explore the role of miRNAs in Notch activation in OS, we utilized miRNA expression changes identified by comparing tumors to normal bone. We found experimental evidence for interactions between nearly half of the dysregulated miRNAs and one or more Notch/HES1 associated. A handful of these pathways are targetable *via* small molecule inhibitors including Notch, Hedgehog, HER2/ERRB and PARP. Several of these have been or are under investigation for potential use in the treatment of OS (77, 78). Expression studies like ours

might identify biomarkers to help stratify patients for optimal therapeutic benefit or monitor therapeutic response.

The last aim of this study was to identify expression changes of presumed tumor-associated miRNAs in the serum associated with patient outcome. Reliable, repeatable RT-qPCR results for measurement of serum are challenging due to typically low miRNA yield, which inhibits efforts to identify and control for poor-quality samples. This has contributed to inconsistencies between circulating biomarker studies and remains a considerable roadblock to the clinical utility and reliability of such screens (79).

Despite these challenges, we set out to identify a data analysis pipeline utilizing the same relatively affordable SYBR green RT-qPCR platform to measure the relative expression of serum miRNAs. We were able to ultimately identify a two-miRNA signature which successfully stratified patients into distinct outcome groups. The most significantly altered miRNA in this signature was miR-30c, which shows a progressively decreased expression from normal bone to tumors and from tumors from dogs with good outcome to tumors from dogs with poor outcome (**Supplementary Tables 3–5**). While promising, we acknowledge that, for all of our miRNA signatures, predictive capability in an independent tumor set remains to be established.

## CONCLUSIONS

In conclusion, we successfully identified miRNA expression changes associated with patient outcome in both OS tumor and patient serum samples. miRNA–gene interactions of the disrupted miRNAs in tumors with genes identified as aberrantly expressed by previous studies (2) can be used to identify targetable pathways disrupted in OS. These studies support the value of miRNA expression studies in biomarker/target discovery efforts for OS.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/ (GSE24251).

## ETHICS STATEMENT

The animal study was reviewed and approved by Colorado State University Animal Care and Use Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

DDa isolated RNA, conducted RT-qPCR and data analysis, prepared the figures, and wrote the manuscript. AH provided assistance in the statistical analysis of data. GB directed the RT-qPCR analysis of miRNAs. DDu directed the study design and sample acquisition and helped with data analysis and writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.637622/full#supplementary-material

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# Genomic and Transcriptomic Characterization of Canine Osteosarcoma Cell Lines: A Valuable Resource in Translational Medicine

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Osteosarcoma (OSA) represents the most common primary bone tumor in dogs and is characterized by a highly aggressive behavior. Cell lines represent one of the most suitable and reproducible pre-clinical models, and therefore the knowledge of their molecular landscape is mandatory to investigate oncogenic mechanisms and drug response. The present study aims at determining variants, putative driver genes, and gene expression aberrations by integrating whole-exome and RNA sequencing. For this purpose, eight canine OSA cell lines and one matched pair of primary tumor and normal tissue were analyzed. Overall, cell lines revealed a mean tumor mutational burden of 9.6 mutations/Mb (range 3.9–16.8). Several known oncogenes and tumor suppressor genes, such as *ALK*, *MYC*, and *MET*, were prioritized as having a likely role in canine OSA. Mutations in eight genes, previously described as human OSA drivers and including *TP53*, *PTCH1*, *MED12*, and *PI3KCA*, were retrieved in our cell lines. When variants were cross-referenced with human OSA driver mutations, the E273K mutation of *TP53* was identified in the Wall cell line and tumor sample. The transcriptome profiling detected two possible p53 inactivation mechanisms in the Wall cell line on the one hand, and in D17 and D22 on the other. Moreover, *MET* overexpression, potentially leading to MAPK/ERK pathway activation, was observed in D17 and D22 cell lines. In conclusion, our data provide the molecular characterization of a large number of canine OSA cell lines, allowing future investigations on potential therapeutic targets and associated biomarkers. Notably, the Wall cell line represents a valuable model to empower prospective *in vitro* studies both in human and in dogs, since the *TP53* driver mutation was maintained during cell line establishment and was widely reported as a mutation hotspot in several human cancers.

**Keywords: dog, osteosarcoma, cell line, next generation sequencing, whole-exome sequencing, RNA sequencing**

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## INTRODUCTION

Canine osteosarcoma (cOSA) represents the most common primary malignant bone tumor in dogs (1, 2) and is characterized by a natural history of disease and molecular abnormalities similar to human osteosarcoma (hOSA) (3, 4). cOSA is locally aggressive and highly metastatic (5), and despite significant improvements of surgical and chemotherapeutic treatments, most dogs perish

within a year from the diagnosis (6), indicating a need for identification of specific tumor targets to develop novel treatment strategies. Recently, two whole-exome sequencing (WES) studies revealed that several pathways and driver genes, such as *TP53*, *RB1*, *DLG2*, *PTEN*, *MYC*, and *MET*, were equally mutated in both cOSA and its human counterpart (7, 8). Moreover, such genes have been previously identified as key players in cOSA pathogenesis (4, 9) and potential therapeutic targets (10–12). Two further studies characterized the genomic profile of several canine cancer cell lines, including cOSA cell lines, and investigated their relevance in comparative oncology (13, 14). Notably, driver mutations in MAPK/ERK and PI3K/AKT signaling pathways were identified in cOSA cell lines, and an anti-proliferative target inhibition using trametinib showed encouraging results, while alterations of the *TP53* pathway were detected in non-sensitive cell lines (13).

These data highlight the importance of canine cancer cell lines as effective and reproducible pre-clinical models to provide crucial insights on pathogenetic mechanisms and drug response (14). Even though canine cancer cells have been used in oncologic research over decades, their mutational profiles were never investigated thoroughly (15–17). Consequently, a deep mutational analysis of such *in vitro* models will allow the identification of new targets and offer valuable tools in translational medicine (18, 19), considering that integration of genomic data with drug screening is fundamental for the development and pre-clinical evaluation of novel treatments that would equally benefit canine and human patients.

The purpose of the current study was to describe the mutational landscape and determine variants and putative driver genes as well as gene expression aberrations by an integrated analysis of whole-exome and RNA sequencing of eight cOSA cell lines and one matched pair of primary OSA and normal tissue.

# MATERIALS AND METHODS

## Sample Collection and Cell Culture

Eight primary canine OSA cell lines and one matched pair of FFPE primary OSA and normal tissue were analyzed.

Penny, Wall, Desmond, Sky, Lord, and Pedro cell lines were previously established and validated by Maniscalco et al., while D17 (ATCC® CCL-183™) and D22 (ATCC® CRL-6250™) were obtained from American Type Culture Collection.

These were cultured in Dulbecco's modified Eagle's medium (DMEM; D17 and D22) and Iscove's standard medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% $CO_2$. The FFPE samples were obtained from the same OSA from which the Wall cell line was established.

## DNA and RNA Isolation From Cell Lines and FFPE Tissues

Genomic DNA (gDNA) was isolated and purified from cell lines and the FFPE samples (**Supplementary Table 1**) using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and GeneRead DNA FFPE kit (Qiagen, Hilden, Germany), respectively. gDNA concentration was determined using the Qubit 2.0 Fluorometer (Thermo Fischer, Foster City, CA, USA). Total RNA was extracted from six cell lines (Penny, Wall, Desmond, Sky, D17, and D22; **Supplementary Table 1**) using QIAzol Lysis reagent (Qiagen, Hilden, Germany) and purified. The total RNA concentration was determined using the NanoDrop ND-1000 UV-Vis spectrophotometer, and its integrity was measured by the Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). RNA samples with an RNA integrity number (RIN) ≥8 were considered for the RNA-seq library preparation.

The isolated DNA and RNA were stored at −20 and −80°C, respectively, until further use.

## WES and RNA-Seq Library Preparation and Sequencing

High-quality whole-genome libraries from 10 samples (eight cells lines and two FFPE samples) were prepared using the KAPA HyperPlus Library Preparation Kit (Roche Sequencing and Life Science, Wilmington, MA). Exome capture was executed using Roche's SeqCap EZ Share Prime Developer Kit (Roche Sequencing and Life Science, Wilmington, MA) for non-human genomes following the SeqCap EZ HyperCap Workflow User's Guide. Probes for the exome capture were designed on the target enrichment design of 150 megabases developed by Broeckx et al. (20). The developer's reagent (06684335001) was used in place of COT-1, and index-specific hybridization enhancing oligos were also used. The final concentration and size distribution were tested with the Bioanalyzer 2100 workstation (Agilent Technologies, Santa Clara, CA, USA). The libraries (fragments ranging from 300 to 400 bp) were then sequenced on an Illumina NovaSeq 6000 platform in a paired-end (150 PE) mode. The chosen target sequencing coverage was 200×. Non-normalized libraries for RNA sequencing experiments were prepared using NEBNext® Ultra™ II Directional RNA Library Prep (New England Biolabs) with Sample Purification Beads and NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs).

A single-end sequencing (75 SE) was carried out on a NextSeq 500 platform (Illumina Inc., San Diego, CA, USA).

## WES Data Analysis

Quality control prior to alignment was performed on output from Illumina software and was processed by FastQC v.0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/download.html) software. Trimmomatic was used to select high-quality reads and remove adapter sequences.

Filtered reads were mapped to the canine reference genome (CanFam3.1; Broad Institute, release 99) using BWA software (21). To verify coverage in the exonic regions, the resulting BAM files were manually inspected using Integrative Genomic Viewer (IGV) (22). Pre-processing for variant calling was performed following the Genome Analysis Toolkit (GATK) v.4.1 Best Practices (https://gatk.broadinstitute.org/hc/en-us/articles/360035894731-Somatic-short-variant-discovery-SNVs-Indels). Briefly, single-nucleotide variants (SNVs) and small insertion and deletions (indels) were identified with the GATK Mutect2

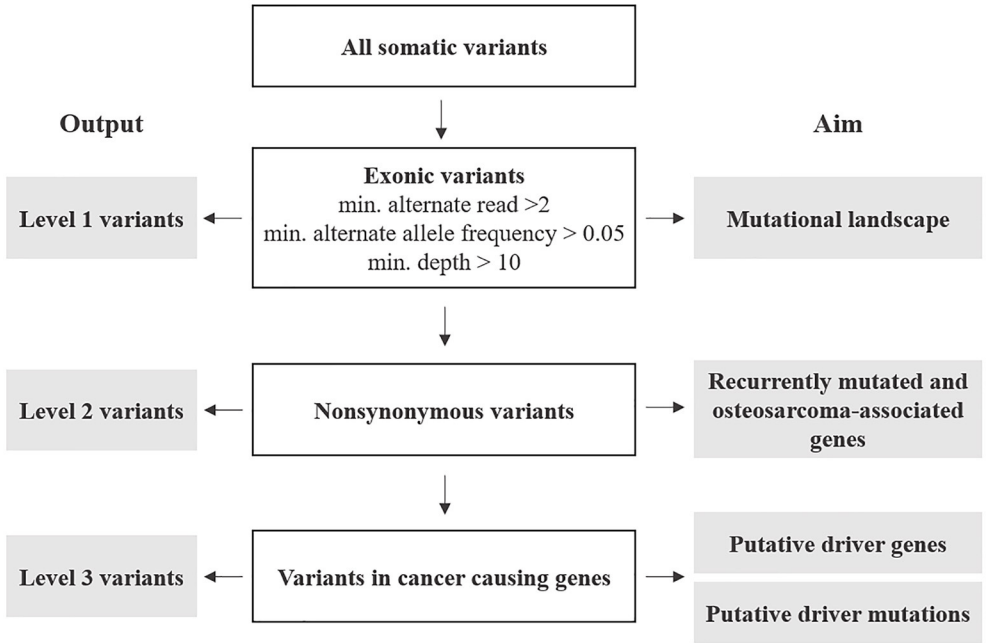


**FIGURE 1 |** Post-processing of somatic variants: outline of selection criteria, categorization, and analyses.

tool (23) and filtered for standard parameters of a min-alternate-count of 2, a min-alternate-frequency of 0.05, and a read depth > 10. To reduce germline artifacts, a panel of Normals (PON) was built using the GATK CreateSomaticPanelOfNormals tool by downloading public available WES data from 18 non-tumor-bearing and unrelated dogs (normal stroma and blood samples) from the NCBI SRA database (**Supplementary Data 1**) (13, 24). An additional filter was added to exclude known single-nucleotide polymorphisms as annotated in the dbSNP 146 using the Dog Genome SNP Database (http://dogsd.big.ac.cn/) (25). Distribution and functional consequences of variants were assessed using ANNOVAR. Additionally, missense mutations were categorized according to their pathogenicity using FidoSNP (26).

The detailed WES workflow applied to both canine OSA cell lines and the FPPE samples is summarized in **Supplementary Figure 1**.

## RNA-Seq Data Analysis

All RNA-seq analyses were performed using conventional RNA-seq analysis tools (27). Detailed information is provided in **Supplementary Figure 2**. Briefly, post-alignment quality parameters of RNA-seq (insert length, gene-mapping bias, RNA junctions) were evaluated using RSeQC (28) in standard mode. Next, the counts of aligned reads per gene were obtained using htseq-count from the HTSeq (29) software package in single-stranded mode. Only reads that were uniquely aligned were retained. Finally, count filtering and normalization were performed using EdgeR R package (30).

## Recurrent Variants and Putative Driver Mutations Identification

Annotated variants were subjected to three filtering levels with increasing stringency and designated as follows (**Figure 1** and **Supplementary Data 2**).

Level 1: variants included the totality of exonic-only SNV and indels retrieved from the variant call described above. These were further filtered for number of reads (min. 2), alternate allele frequency (min. 0.05), and each variant's depth of coverage (min. 10). The resulting variants were analyzed to describe the mutational profile of cOSA cell lines.

Level 2: these were non-synonymous exonic variants selected from Level 1 to identify recurrently mutated genes having a likely role in cOSA pathogenesis. Furthermore, variants encoding for genes commonly mutated in human and canine OSA were also prioritized (**Supplementary Data 3**) (7). Level 3: these were selected from Level 2 protein-coding variants of genes listed in COSMIC Cancer Gene Census, (version 92, https://cancer.sanger.ac.uk/census) (31). 5′ UTR and splice site variants COSMIC-listed genes were also included in the analysis due to their potential impact on protein expression and function. These variants were also manually cross-checked against known oncogenic variants in hOSA available on the IntOgen platform (https://www.intogen.org/search?cancer=OS) (32) to identify putative driver mutations.

## Validation of TP53 Mutation

E273K mutation of *TP53* identified in the Wall cell line and FFPE tumor sample was validated by Sanger dideoxy sequencing on Wall samples gDNA and Penny cell line (negative control).

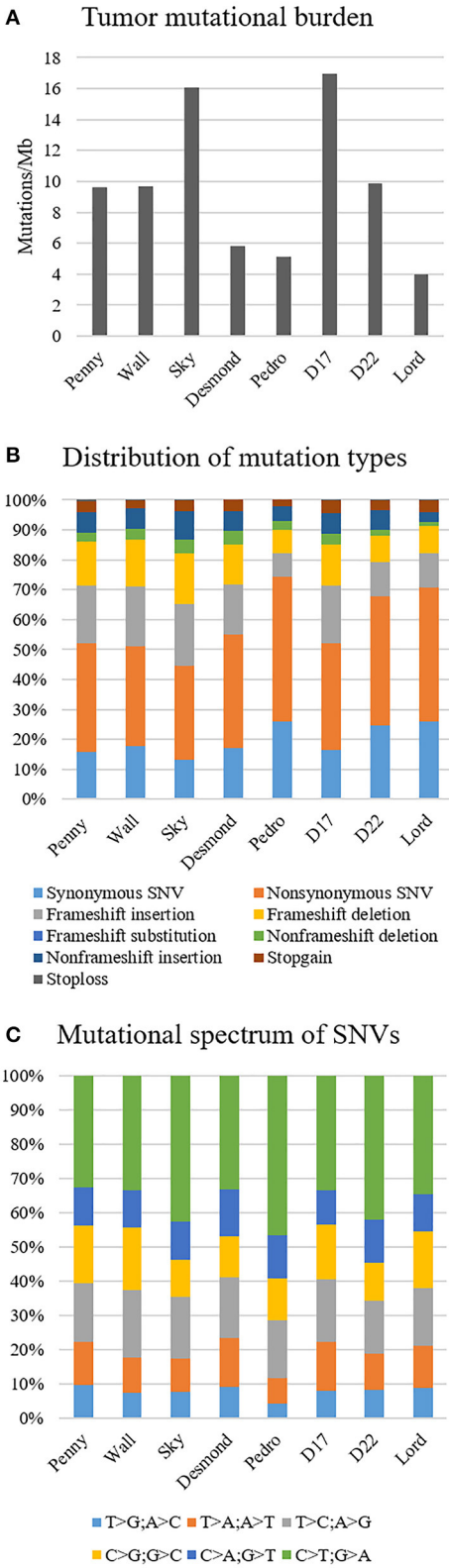


**FIGURE 2 |** Mutational landscape of canine osteosarcoma cell lines, Level 1 variants. **(A)** Tumor mutational burden. **(B)** Distribution of mutation types. **(C)** Mutational spectrum of single-nucleotide variants.

Briefly, two primers (sense 3′-ATGAGGGTGGCTAGGAGTCA-5′) and (antisense 5′-CAGTGCTGGGAAAGAGAGGA-3′) spanning the mutated region were designed by PRIMER3 Express software, and PCR on gDNA was performed using HotStar Taq (Qiagen) at 58°C (annealing temperature) for 35 cycles. After evaluation of agarose gel, amplification products were purified by QIAquick PCR Purification Kit (Qiagen).

# RESULTS

## The Mutational Profile of Primary Canine Osteosarcoma Cell Lines

DNA extracted from eight canine OSA cell lines underwent WES, and an average of 158 million reads per sample (range 143–164) was obtained.

The mean depth of reads mapping to the canine reference genome (CanFam3.1) was 187.7 (range 128–219), with over 99% of the targeted exome uniquely aligned. The optimal coverage was achieved for six out of eight cell lines. For each cell line, all reads passed the quality control (Phred quality score) $\geq$ 30. The FFPE samples (tumor and matched normal) achieved a mean depth of 54.71.

The median tumor mutational burden of all Level 1 somatic variants was 9.6 mutations/Mb (range 3.9–16.9); in the Wall FFPE sample, the mutational burden reached 17.7 mutations/Mb (**Figure 2A**). On average, 19.6% (range 13.3–26%) and 17.1% of Level 1 variants of canine OSA cell line and Wall FFPE sample, respectively, were annotated as synonymous and were consequently excluded from downstream analyses.

In all our cell lines, missense mutations were the most frequently represented somatic coding mutation type, accounting for an average of 38.7% across all exonic variants. Frameshift insertion and deletions were 15.8 and 12.5% of the variants, respectively (**Figure 2B**). In Wall FFPE sample non-synonymous variants, frameshift and non-frameshift deletions were the most represented mutation types (36.3, 20.5, and 10.3%, respectively).

The most common base change identified in all samples was C > T transition (**Figure 2C**). The analysis of the WES data using Mutect2 revealed a total of 11,554 exonic variants (Level 1); 7,981 of these were identified as non-synonymous (Level 2) and encoded for 4,045 genes (**Figure 3**). The number of genes in each sample ranged from 318 (Lord) to 1,345 (D17) and reached the maximum of 1,533 genes in the Wall FFPE sample (**Figure 4**).

Using the FidoSNP pathogenicity prediction tool, 50.5% (1,929/3,819 SNVs) of all missense point mutations were categorized as deleterious.

## Canine Osteosarcoma Cell Lines Show Mutations in Several Known Oncogenes and Tumor Suppressor Genes

As mentioned above, 4,045 genes with 7,981 protein-modifying variants were identified (**Supplementary Data 2**). Overall, 483 genes were recurrently mutated in at least three cell lines. Taking into account the recurrent variants across all cell lines, a total of 234 variants were retrieved in three or more samples, and 51.4% of all SNVs (54/105) were categorized as

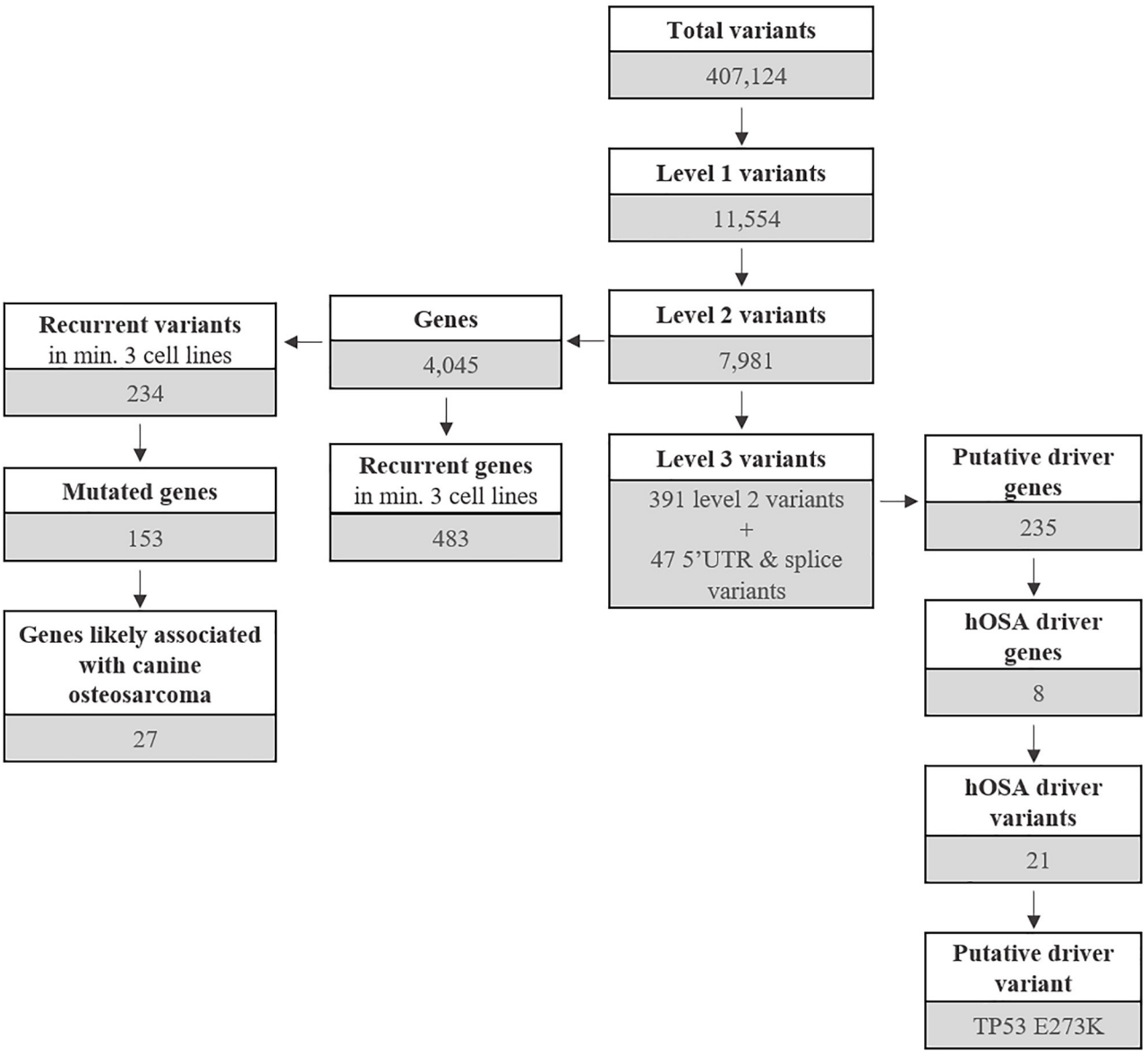


**FIGURE 3 |** Outline of mutated genes and variants distributed across Level 1, 2, and 3 variants.

pathogenic. When recurrent variants were collapsed to genes, 153 recurrently mutated genes were identified (**Figure 3**). In addition, genes were filtered using the list of osteosarcoma-associated genes retrieved from literature. Finally, a total of 27 genes likely implicated in OSA pathogenesis were identified across all our cell lines. Among them, *PDGFRB*, *PTCH1*, and *WRN* were retrieved in at least three cell lines, whereas oncogenes and tumor suppressor genes, such as *TP53*, *ALK*, *MYC*, and *MET*, were retrieved in only one cell line each (**Figure 5**).

The number of genes ranged from two (Desmond and Lord) to 10 genes (Sky) (**Supplementary Figure 3**). Overall, these cancer genes were encoded by 51 variants, and 53.3% of all SNVs (16/30) were categorized as pathogenic.

Comparing these 27 genes to the top 20 most frequently mutated genes in human cancers (Cancer Genome Atlas; https://portal.gdc.cancer.gov/), four overlapping genes were identified, namely, *PIK3CA*, *KRAS*, *APC*, and *NF1*, which ranked 2nd, 6th, 10th, and 13th, respectively. At last, four genes were also identified in the Wall FFPE sample but did not overlap those of the corresponding cell line (**Supplementary Table 2**).

## Canine Osteosarcoma Cell Lines Share Several Driver Genes With Human Osteosarcoma

COSMIC Cancer Gene Census was used to identify candidate driver variants (Level 3) in known cancer-causing genes in humans.

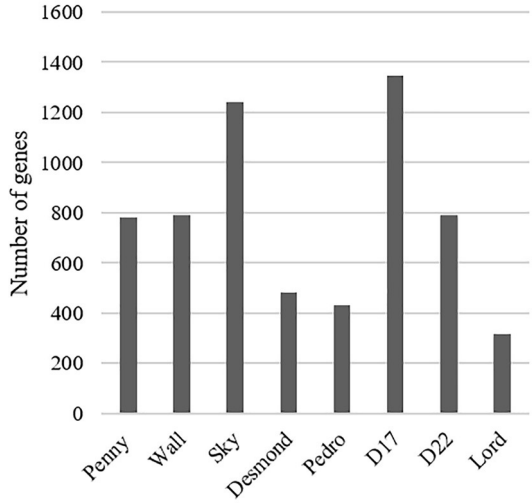


**FIGURE 4 |** Distribution of the mutated genes across the cell lines.

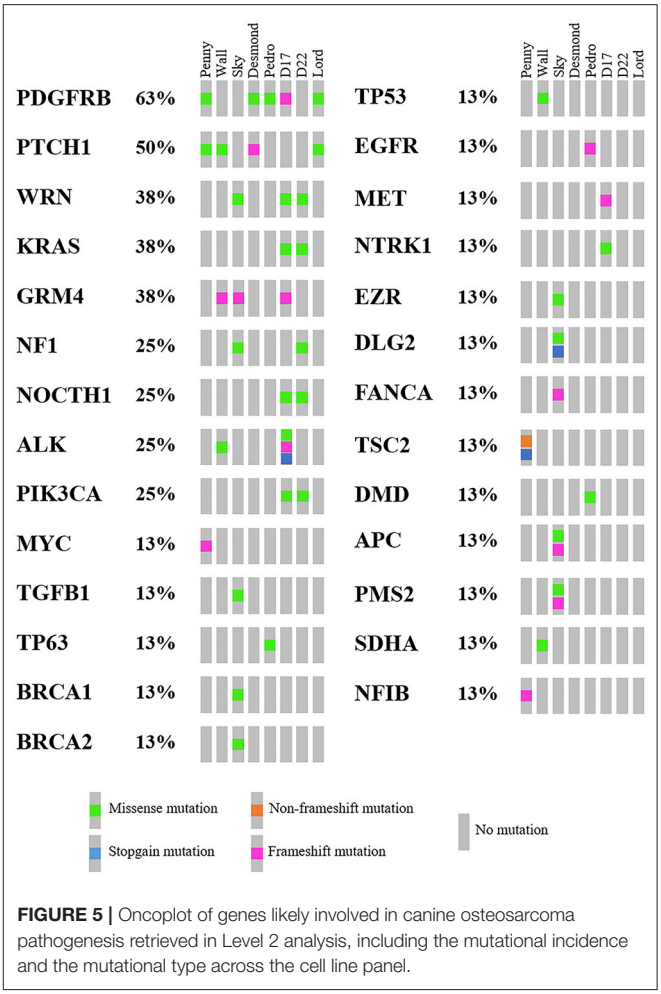


**FIGURE 5 |** Oncoplot of genes likely involved in canine osteosarcoma pathogenesis retrieved in Level 2 analysis, including the mutational incidence and the mutational type across the cell line panel.

Level 3 included 438 variants coding for 235 genes, regardless of their incidence across the cell line panel (**Figure 3** and **Supplementary Data 2**). A total of 19 genes were uniquely encoded by 5′ UTR or splice variants.

Overall, 190 genes were indicated as having a relevant and documented activity that promotes oncogenic transformation. In particular, 28 were designated as fusion genes, 74 as tumor suppressors, and 63 as oncogenes, and 25 functioned as both. The distribution of putative driver mutations across all cell lines is depicted in **Figure 6**.

About 88% of the SNVs encoding for these genes were categorized as pathogenic.

When compared to the top 20 cancer driver genes involved in human OSA (COSMIC Cancer Browser), eight genes, encoded by 21 variants, were retrieved in Level 3 genes (**Table 1**). Among these, well-known oncogenes and tumor suppressor genes, such as *TP53*, *PTCH1*, *MED12*, and *PI3KCA*, were identified.

## Canine Osteosarcoma TP53 Putative Driver Mutation Matches a known Human-Equivalent Mutation Hotspot

All putative driver variants were cross-referenced with human OSA driver mutations available on IntOgen. Only the Wall cell line and FFPE tumor sample harbored a putative driver mutation, namely, the $TP53^{E273K}$ mutation (c.818C>T) corresponding to the human $TP53^{E285K}$ mutation hotspot.

This putative driver mutation was further validated in homozygosis in the Wall cell line and tumor sample by Sanger sequencing (**Figure 7**).

## The Oncogenic Potential of TP53 and MET Gene Expression Aberrations

RNA sequencing generated over 10 million reads per sample. Quality control and trimming procedures retained the vast majority of the sequences, and unique alignment to the canine reference genome was successful for 86% of the cleaned reads (**Supplementary Table 3**).

The normalized gene expression of the osteosarcoma-associated genes described above was then profiled within the same gene across all samples.

In particular, *TP53* gene expression was increased in the Wall cell line, which harbored a hotspot mutation, as well as in D17 and D22, which retained a wild-type gene status. Interestingly, D17 and D22 showed a 9-fold and 5.5-fold increase of *MDM2* and *MDM4* transcript levels, respectively, compared to the other cell lines. Conversely, the *MDM2* transcript level was decreased by 2 to 20 times in the Wall cell line compared to the rest of the cell lines.

Transcriptional upregulation of *MET* by 19-fold was observed in the D17 cell line, which harbored a frameshift insertion on this gene, and to the same extent in D22 which retained a wild-type gene status. Notably, genes involved in the downstream

MAPK/ERK pathway, such as *MAPK1*, *MEK*, and *MYC*, showed increased transcript levels, although no mutations were detected in WES analysis.

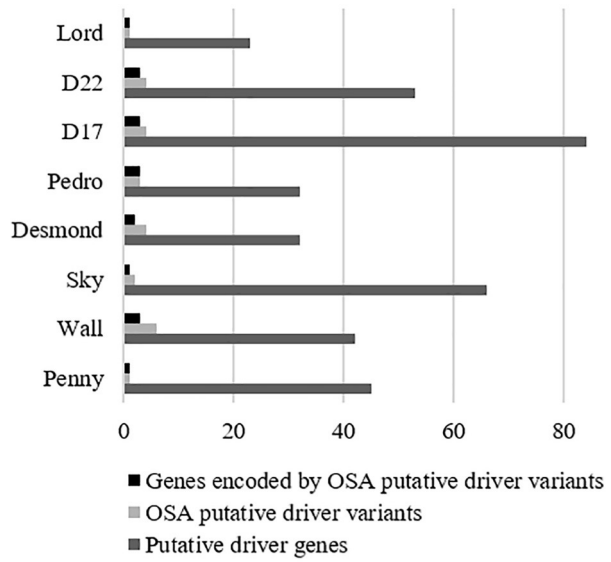


**FIGURE 6 |** Distribution of putative driver variants and encoded genes across canine osteosarcoma cell lines.

Conversely, downstream signaling components of mutated cancer-associated genes, such *as PTCH1*, *MED12*, *PDGFRB*, and *PIK3CA*, did not show any transcript level change.

## DISCUSSION

Cancer cell lines are considered valuable models in basic cancer research, drug discovery, and translational medicine (14, 33, 34).

The recent characterization of a large panel of human cancer cell lines with omics technologies has empowered data-driven precision medicine (34, 35). Despite the substantial number of studies, an analogous dataset modeling canine cancer cell lines is currently unavailable (14).

In the present study, we integrated data from whole-exome and RNA sequencing of eight cOSA cell lines to obtain genomic and molecular data recapitulating *in vivo* cOSA pathogenesis and identifying suitable targets for drug discovery. To date, this dataset represents one of the largest explored for a single tumor in dogs.

Specifically, two commercial and six primary cOSA cell lines established and validated in our laboratory were analyzed. The assorted cell lines were previously used in several studies to investigate cOSA pathogenetic mechanisms and response to therapies (11, 12, 14, 36–38). So far, only the D17 cell line has been characterized at the genomic level by Das et al. in 2019 (13).

About exome sequencing, variants were analyzed using three levels of increased stringency: first, describing the mutational

**TABLE 1 |** List of putative driver genes and variants across all the cell lines.

| Gene | Mutation | Cell lines | | | | | | | FFPE | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Penny | Wall | Sky | Desmond | Pedro | D17 | D22 | Lord | Wall |
| PTCH1 | c.17A>G | - | - | - | - | - | - | - | X | - |
| | c.3850C>T | X | - | - | - | - | - | - | - | - |
| | c.4014insT | - | X | - | - | - | - | - | - | X |
| | c.4023delA | - | X | - | - | - | - | - | - | X |
| | c.4200_4201insAGTCCCCG | - | X | - | X | - | - | - | - | X |
| | c.4203_4210del | - | X | - | X | - | - | - | - | X |
| LRP1B | c.12056A>T | - | - | - | X | - | - | - | - | - |
| | c.3112A>C | - | - | - | - | - | - | X | - | - |
| | c.3105_3106insATTGGGCCTGTGATGGTGA | - | - | - | - | - | - | X | - | - |
| ARID1A | c.6276A>T | - | X | - | - | - | - | - | - | X |
| | c.4863_4862insCCCCCCA | - | - | X | - | - | - | - | - | - |
| | c.4858_4852del | - | - | X | - | - | - | - | - | - |
| | c.1877G>A | - | - | - | - | X | - | - | - | - |
| NFATC2 | c.510G>A | - | - | - | - | X | - | - | - | - |
| TET2 | c.1349G>C | - | - | - | - | - | X | - | - | - |
| | c.2817_2818insCTGTGACTTCCTCCCTGGTCAGACA | - | - | - | - | - | X | - | - | - |
| | c.2894_2897del | - | - | - | - | X | - | - | - | - |
| PIK3CA | c.2217G>T | - | - | - | - | - | X | X | - | - |
| TP53 | c.818C>T | - | X | - | - | - | - | - | - | X |
| MED12 | c.2089_2090insATGGACTGCCCTTCCCCTCAC | - | - | - | X | - | - | - | - | - |
| | c.2581G>A | - | - | - | - | - | X | X | - | - |

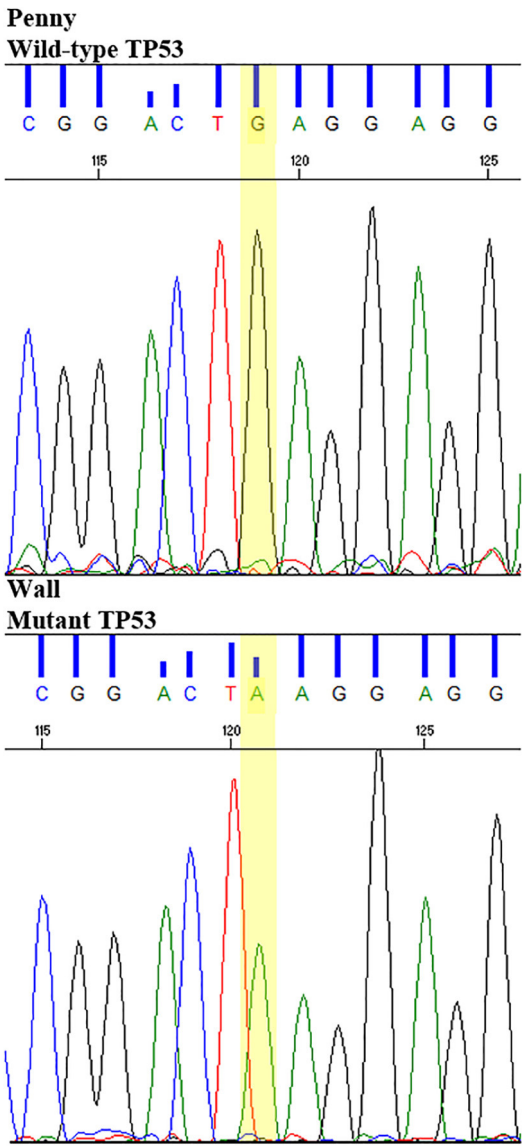


**FIGURE 7 |** Sanger sequencing of TP53$^{E273K}$ mutation in the Wall cell line and primary FFPE tumor, compared to wild-type TP53 Penny cell line and the reference canine TP53 sequence.

profile of each cell line; second, identifying recurrently mutated genes; and third, unraveling putative driver genes having a likely role in cOSA pathogenesis.

Overall, the mutational burden in our cOSA cell lines ranged between 3.9 and 16.9 mutations/Mb but was lower than the one described by Das et al. (13). However, the diversity of the cell lines and the differences in library preparation kits, exome capture designs, and downstream stringency filters may have caused this discrepancy.

Consistent with previous reports in cOSA cell lines and hOSA, the mutational type distribution showed a prevalence of missense mutations, and C>T transitions dominated this mutational spectrum (13, 39).

Across genes identified in Level 2 analysis, the two tyrosine kinase receptors *PDGFRB* and *MET* were retrieved. Both genes are known to play an important role in the development and progression of many canine cancers and were thoroughly investigated in cOSA as well (4, 11, 36, 40).

In five cell lines, *PDGFRB* harbored both frameshift and missense mutations, but only Desmond showed an increased gene transcript level. Nevertheless, no overexpression of *PDGFRB* downstream signaling molecules was detected, suggesting that these mutations did not affect gene transcription in Desmond. Gardner et al. reported previously that *PDGFRB* loci are affected by copy number gains rather than point mutations; however, no correlation with gene expression was found (7). The *MET* oncogene was highly expressed in D17 and D22 cell lines but resulted to be mutated only in the D17 cell line. However, the frameshift insertion mutation was unlikely associated with overexpression, since several stop codons were retrieved in the transcript analysis. Nevertheless, *MET* is regulated by several mechanisms, including amplifications and epigenetic aberrations (41). In D17 and D22 cell lines, overexpression of *MET* downstream genes, including *MAPK1* and *MEK*, was observed, suggesting a possible activation of the MAPK/ERK pathway (36, 42, 43).

The *MYC* gene was mutated in the Penny cell line only. However, increased transcript levels were identified in D17 and D22 cell lines and likely related to the aforementioned *MET* signaling. In hOSA cell lines, *MYC* overexpression promotes cell invasion *via* MAPK/ERK signaling and is correlated with a poor prognosis *in vivo* (44, 45). Such data are currently unavailable in cOSA, but aberrant activations of *MYC* and MAPK pathway genes have been reported (7, 13).

Mutations of *TP53* in Wall and *WRN* in the Sky, D17, and D22 cell lines were also retrieved. In particular, the putative driver *TP53*$^{E273K}$ mutation was identified in the Wall cell line and tumor sample and further validated by Sanger sequencing. As most of the *TP53* mutations, *TP53*$^{E273K}$ occurred in the mutational hotspot corresponding to the DNA-binding domain (46) and matched the human pathogenic hotspot mutation E285K (7). Both canine *TP53*$^{E273K}$ and its human equivalent were previously reported in cOSA and hOSA (7). According to the IARC TP53 database (47), this mutant allele is listed among the top 15 most common mutations in human cancers predicted to disrupt protein structure and function (48). In our experiment, the presence of this mutation in both Wall tumor tissue and the derived cell line demonstrates a genetic fidelity with the primary tumor that remained stable during cell line establishment (34, 49).

Looking at gene expression, the mutant *TP53* transcript levels in Wall were twice as high as in Penny, Sky, and Desmond cell lines. This is in accordance with the literature where *TP53* missense mutations are reported to moderately affect the transcription but produce a full-length protein with a scarce ability to bind specific DNA sequence motifs and activate downstream target genes (48, 50). Concurrently, *MDM2*, a well-known *TP53* transcriptional target, showed a lower expression in Wall compared with the other wild-type *TP53* cell lines. This data suggests that *TP53* mutation in the Wall cell line might cause a

loss of function rather than an altered mRNA expression (51). Overall, this indicates that $TP53^{E273K}$ is a likely pathogenic driver mutation providing a spontaneously inactivated *TP53 in vitro* model for specific biological and reactivation assays (52).

Remarkably, D17 and D22 cell lines showed an increase of the *TP53* transcript compared to Wall, while retaining a wild-type gene status. In association, *MDM2* and *MDM4* transcript levels were increased, suggesting that *TP53* function might be impaired by their oncogenic and deregulated inhibiting activity in these cell lines (53). In accordance with our findings, *TP53* overexpression in D17 was also detected in a recent report by Modesto et al. (38).

Besides *TP53*, putative driver genes such as *PTCH1*, *MED12*, and *PIK3CA* were identified in Level 3 analysis. *PTCH1* was mutated in four out of eight cell lines. Physiologically, Hh ligand binding to the Ptch1 receptor relieves its inhibitory effect on the canonical Hedgehog pathway, whose activation plays a role in both hOSA and cOSA (10, 54). Despite a low *PTCH1* transcript level in these cell lines, no constitutive expression of Hedgehog pathway target genes was detected.

Similarly, *MED12* and *PIK3CA*, which are known to contribute to hOSA initiation and progression *via* the Wnt and PI3K/Akt pathways (55, 56), did not show gene expression aberrations in the mutated cell lines, suggesting a biological irrelevant role in our cell lines.

Contrary to previous reports in dogs, no somatic mutations neither gene expression aberrations affecting *CDKN2A* and *SETD2* were identified (7, 8). Regarding *CDKN2A*, it is generally affected by germline mutations and copy number variations, which were not investigated here (7, 8, 57), whereas *SETD2* mutations were only recently identified in hOSA and cOSA, and their biological role remains to be elucidated (7, 8, 58).

In conclusion, these data provide valuable insights into the molecular mechanisms of a large number of cOSA cell lines, allowing future investigations of their functional implications and drug response. Since similarities were identified with hOSA, these cell lines may also represent excellent translational models. In future, the addition of new primary cOSA cell lines and the integration of new sequencing approaches, such as methylation analysis and single-cell RNA-seq, are needed to provide an accurate characterization of these models and explore the underlying oncogenic mechanisms.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: https://www.ncbi.nlm.nih.gov/, PRJNA701141.

## AUTHOR CONTRIBUTIONS

RD and LA contributed conception and design of the study. PB and EM enrolled the canine osteosarcoma cases used for cell line establishment. CG, PM, SI, and RD cultured the cell lines and extracted DNA and RNA from canine osteosarcoma cell lines and FFPE samples. AR performed the NGS sequencing. DG performed the computational analyses. CG performed data post-processing. CG and SI performed data visualization. CG and DG wrote the manuscript. RD edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.666838/full#supplementary-material

**Supplementary Figure 1 |** Workflow for whole-exome sequencing short variant discovery, sequential use of tools in evaluation of canine osteosarcoma cell lines and FFPE DNA samples.

**Supplementary Figure 2 |** Workflow for RNA sequencing analysis, sequential use of tools in evaluation of canine osteosarcoma cell lines.

**Supplementary Figure 3 | (A)** Distribution of recurrently mutated genes and variants across all the canine osteosarcoma cell lines. **(B)** Distribution of osteosarcoma-associated genes and corresponding variants across all the canine osteosarcoma cell lines.

**Supplementary Table 1 |** NGS sequencing technologies applied in the analysis of eight canine osteosarcoma cell lines and one matched FFPE tumor sample.

**Supplementary Table 2 |** Variants retrieved in genes likely involved in canine osteosarcoma pathogenesis ($n = 27$; Level 2 analysis) in Wall cell line and matching FFPE tumor sample.

**Supplementary Table 3 |** RNA sequencing quality control, trimming and alignment data.

**Supplementary Data 1 |** Panel of Normals: public available WES data from 18 non-tumor bearing and unrelated dogs (normal stroma and blood samples) from NCBI SRA database.

**Supplementary Data 2 |** Whole-exome sequencing results of 8 canine osteosarcoma cell lines and one matched FFPE tumor sample: Level 1, Level 2, and Level 3 variants.

**Supplementary Data 3 |** List of known osteosarcoma-associated gene in dogs and humans.

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# Adoptive Natural Killer Cell Immunotherapy for Canine Osteosarcoma

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Osteosarcoma is the most common primary bone tumor in both humans and dogs. It is a highly metastatic cancer and therapy has not improved significantly since the inclusion of adjuvant chemotherapy into disease treatment strategies. Osteosarcoma is an immunogenic tumor, and thus development of immunotherapies for its treatment, especially treatment of microscopic pulmonary metastases might improve outcomes. NK cells are lymphocytes of the innate immune system and can recognize a variety of stressed cells, including cancer cells, in the absence of major histocompatibility complex (MHC)-restricted receptor ligand interactions. NK cells have a role in controlling tumor progression and metastasis and are important mediators of different therapeutic interventions. The core hypothesis of adoptive natural killer (NK) cell therapy is there exists a natural defect in innate immunity (a combination of cancer-induced reduction in NK cell numbers and immunosuppressive mechanisms resulting in suppressed function) that can be restored by adoptive transfer of NK cells. Here, we review the rationale for adoptive NK cell immunotherapy, NK cell biology, TGFβ and the immunosuppressive microenvironment in osteosarcoma, manufacturing of *ex vivo* expanded NK cells for the dog and provide perspective on the present and future clinical applications of adoptive NK cell immunotherapy in spontaneous osteosarcoma and other cancers in the dog.

**Keywords: canine, immunotherapy, osteosarcoma, NK cell, TGFβ**

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## INTRODUCTION

Osteosarcoma is the most common primary bone tumor in both humans and dogs, with the disease incidence being as much as 30–50 times higher in the latter (1). The similarities of the disease in humans and dogs are well-described and include commonalities in underlying molecular biology, including gene expression and genetic mutations, histopathology, clinical presentation, disease progression, and response to therapy (2). Spontaneous osteosarcoma in the dog has been used extensively as a preclinical large animal model to evaluate new therapies for osteosarcoma in humans, including limb-sparing procedures (3–5), chemotherapy delivery (6, 7), targeted therapeutics (8), and immunotherapeutics, including therapeutic vaccines and others (9–11). New immunotherapeutic approaches to cancer treatment have emerged and are now making a significant clinical impact for large numbers of cancer patients (12, 13). The development of new immunotherapeutics can be greatly facilitated by the use of well-characterized and validated animal models and spontaneously occurring osteosarcoma in pet dogs is exceptionally well-suited

for this purpose. In addition to the similarities described above, other features of the disease in dogs are particularly relevant and important for evaluating immunotherapeutic interventions. Notably, cancers in dogs occur in a relatively outbred population that generally shares similar environmental exposures with humans and the spontaneously occurring tumors are heterogeneous, existing in a complex microenvironment and in a host with an intact immune system; all critical features that are poorly addressed in most rodent models (14).

## RATIONALE FOR ADOPTIVE NK CELL IMMUNOTHERAPY

The core hypothesis of adoptive natural killer (NK) cell immunotherapy is that there exists a natural defect in innate immunity (a combination of cancer-induced reduction in NK cell numbers and immunosuppressive mechanisms resulting in suppressed function) that can be restored by adoptive transfer of NK cells (15). The immunosuppressive tumor microenvironment suppresses NK cell function (16), and although many drugs and radiation can sensitize tumors for recognition by NK cells, chemotherapy, anesthesia, and radiation therapies can also adversely affect NK cell numbers and function (17–21). While much effort has gone into T-cell based approaches for immunotherapy, including chimeric antigen receptor (CAR) T-cells and immune checkpoint inhibition, these approaches can have significant problems that may impede their application such as graft-vs. host disease (GVHD), cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), or severe on-target off-tissue toxicities (22, 23). Adoptive NK cell therapy is not associated with GVHD (24), thus making it potentially safer than T-cell based therapies and because allogeneic transfer is tolerated, NK cell products can be manufactured and stored for later use in patients as needed, rather than manufacturing "on-demand" for patient-specific use.

## NK CELL BIOLOGY

NK cells are lymphocytes of the innate immune system. NK cells can recognize a variety of stressed cells in the absence of major histocompatibility complex (MHC)-restricted receptor ligand interactions. NK cells are non-T, non-B lymphocytes, and are known for their cytotoxicity and cytokine effector functions. Importantly, they can kill target cells without prior antigen sensitization. Also, NK cells can cross-talk with dendritic cells in different ways, thus participating in the shaping of the subsequent immune response. NK cells have a role in controlling tumor progression and metastasis and are important mediators of different therapeutic interventions, including cytokines, antibodies, immunomodulatory drugs, and stem cell transplantation.

### NK Cell Receptors and Function

The number of NK cells as a percentage of peripheral lymphocytes varies widely in humans (1–32.6%, median 7.6%) and in dogs (2.5–15%) (25–29). In humans, NK cells are identified by the lack of CD3 and the presence of CD56 and/or CD16, and make up 85% of the large granular lymphocyte (LGL) population (30). The phenotypic characteristics of NK cells in the dog are not as clearly defined; however, distinct phenotypic NK cell subsets have been described (31). NK cells express many different cell surface receptors that can be grouped as activating, inhibitory, adhesion, cytokine, or chemotactic receptors. Although many of the cell surface molecules involved in the regulation of NK cell function are found in both humans and mice, only a small subset has been validated in the dog. Canine NK cells do express at the mRNA level several genes classically associated with NK cells, such as NKp30, NKp44, NKp46, NKG2D, CD16, DNAM-1, perforin, and granzyme B (25).

The regulation of NK cell function relies on a complex interplay of activating and inhibitory signals. Unlike T-cells, whose activation is highly restricted to an antigenic peptide presented in the groove of MHC proteins, NK cell activation is not antigen specific. NK cell activation and tolerance are accomplished through a large variety of activating receptors for recognition of danger, balanced with an equally large number of inhibitory receptors that identify self. The balance between these signals determines whether NK cells will activate their effector function (e.g., FasL/TRAIL-mediated killing, perforin/granzyme release, or cytokine production). In humans, there are several families of activating receptors, including CD126 (FcRγIIIa), natural cytotoxicity receptors (NCRs), NK Group 2 (NKG2) lectin-like receptors, DNAM-1, and 2B4; however, most of these have not been well-characterized in the dog (32). Activating receptors generally recognize proteins that are upregulated by cell stress or are of non-self-origin, whereas inhibitory receptors primarily bind MHC for self-recognition (33). Inhibitory receptors provide control for NK cell activity against healthy tissue. The primary inhibitory receptors in human NK cells are killer-cell immunoglobulin-like receptors (KIRs) and NKG2A, both of which bind to HLA class I molecules, preventing NK cell-mediated lysis of cells with normal HLA expression (33). MHC class-I deficient targets have heightened sensitivity to NK cell killing. This biology is reflected and summarized by the "missing self" hypothesis, which states that the presence of MHC class I, ubiquitously expressed by healthy cells, provides NK cells with a "self" signal that is recognized by NK cell inhibitory receptors and thus prevents NK cell self-reactivity (34).

### Canine NK Cells

While human NK cells are distinguished by the absence of surface expression of CD3 and the presence of variable levels of expression of CD56 and CD16, depending on differentiation state (35), the phenotypic characterization of canine NK cells is still evolving. Morphologically, canine NK cells are medium- to large-sized lymphocytes containing electron-dense intracytoplasmic granules that contain granzyme B and perforin and lack expression of CD4 and CD20, T-cell and B-cell markers, respectively (36). However, CD8 may be expressed by a subset of these cells (37, 38). Canine NK cell populations have also been defined based on density of

CD5 surface marker expression, with $CD5^{dim}$ representing a NK cell population (28), especially in the setting of IL-2 stimulation. Further, under *ex vivo* expansion conditions with cytokine stimulation, the majority of cytotoxic large granular lymphocytes expressed a $CD5^{dim}CD3^{+}CD8^{+}TCR\alpha\beta^{-}TCR\gamma\delta^{-}CD4^{-}CD21^{-}CD11c^{+/-}CD11d^{+/-}CD44^{+}$ phenotype that highly upregulated NKp46, and expressed traditional T-cell lineage markers, but lacked T-cell receptors (39). NCR1/NKp46, a NK-specific activating molecule, is considered a "pan-species" NK cell marker (40). One study concluded that canine NK cells are comprised of both $CD3^{-}GranzymeB^{+}NCR1^{+}$ and $CD3^{-}GranzymeB^{+}NCR1^{-}$ populations cells, with the presence of NCR1/NKp46 positive cells representing an activated state (27, 31). Similarly, a canine-specific antibody to NKp46 identifies $CD3^{-}NKp46^{+}$ and $CD3^{-}NKp46^{-}$ NK subsets that vary in cytotoxicity, with $CD3^{-}NKp46^{-}$ population being less cytotoxic, but could be induced to express NKp46 (25). Another putative marker for canine NK cells is the C-type lectin-like CD94 (KLRD-1). Experiments with a canine-specific anti-CD94 identifies a candidate NK cell population representing ∼7.7% of PBMCs and subsets within the $CD5^{dim}$ population (26). It should be noted that the KIR family of surface receptors described above for humans has not been identified in dogs. One gene of a similar paralogous family found in mice, the Ly49 family, has been identified in the canine genome (39, 41).

## MECHANISMS OF NK CELL KILLING

NK cells exert direct and indirect antitumor activity and kill target tumor cells via release of granules containing perforin and granzyme, secretion of cytokines such as IFNγ and other effector molecules, ligation and activation of death receptors, and antibody-dependent cellular toxicity (ADCC) mediated through CD16 when combined with anti-tumor antibodies. Further, the release of pro-inflammatory cytokines enhances the recruitment and maturation of adaptive immune responses (42, 43). The mechanism by which NK cells induce apoptosis in osteosarcoma cells may depend on both the activation state of the NK cells and the death receptor and apoptotic pathways present and functional in the target cell (33). For example, *in vitro*, direct NK cell lysis of osteosarcoma cells is mediated via direct release of granzyme B (44); however, granule-independent mechanisms may be more relevant *in vivo*, as losing Fas and TRAIL may be simpler mechanisms of escape than redundant downstream death pathways (45). Degranulation of NK cells is mediated by the balance of activating and inhibitory receptors, which in turn is influenced by the expression of ligands on the tumor cell. This suggests that NK cells isolated, expanded, and activated using different techniques may differ as to which activating receptors are highly expressed and important for recognizing a particular tumor (33). For example, in one study IL-15 stimulated NK cells targeted osteosarcoma predominantly through DNAM1, whereas in another study IL-2 stimulated NK cells targeted osteosarcoma predominantly through NKG2D (44, 46).

## OSTEOSARCOMA, THE IMMUNOSUPPRESSIVE MICROENVIRONMENT, AND TGFβ

Tumors, especially solid tumors, have evolved mechanisms to actively suppress the immune system. These include induction of inhibitory receptors on NK and T-cells, recruitment of Tregs, myeloid derived suppressor cells and tumor associated macrophage, and production of immunosuppressive cytokines and other factors, including TGFβ (47). Overexpression of TGFβ is a hallmark of many cancers, including osteosarcoma. It inhibits NK cell activity through several mechanisms-suppressing NKG2D and CD16 expression, decreasing perforin, and inhibiting cytokine release (48–51). TGFβ is highly expressed in cancer cell lines and notably, is more highly expressed in osteosarcoma than most other solid tumor cell lines, suggesting that TGFβ is an important contributor to the immunosuppressive tumor microenvironment for osteosarcoma in particular (52). TGFβ signaling is a crucial factor in cross-talk between osteosarcoma cells and stroma cells. Secretion of TGFβ by tumor cells or stroma cells can act in a paracrine manner to regulate the tumor microenvironment, promoting angiogenesis, bone remodeling and cell migration, and by inhibiting immunosurveillance. TGFβ secretion and TGFβ receptor expression has been demonstrated in canine osteosarcoma cells (53). Our group has developed a NK cell expansion technique that confers relative TGFβ-resistance to NK cells in an attempt to improve their function in the hostile immunosuppressive tumor microenvironment (54). TGFβ resistance, or imprinting, is achieved by chronic exposure of NK cells to IL-2 and TGFβ during the expansion process. TGFβ-imprinted NK cells secrete more IFNγ and TNFα than non-imprinted NK cells in the absence, or presence, of TGFβ. Furthermore, TGFβ-imprinted NK cells have increased cellular toxicity compared to non-imprinted cells and are more resistant to TGFβ-mediated decreases in cellular cytotoxicity (54). *Ex vivo* expanded canine NK cells cultured under similar conditions are likewise conferred relative TGFβ-resistance (Lee, unpublished).

## MANUFACTURING OF *EX VIVO*-EXPANDED CANINE NK CELLS FOR ADOPTIVE IMMUNOTHERAPY

NK cells for clinical use can be obtained through apheresis with T-cell depletion, or by *ex vivo* expansion. In humans, NK cells have been successfully expanded from peripheral blood, cord blood, and pluripotent or embryonic stem cells (55). Various methods for expanding purified NK cell populations have been developed in people, using exposure to different cytokines and co-culture with feeder cell lines (15, 55). Several of these methods have been extrapolated to and modified for canine studies (25, 56). In general, superior expansion is achieved when recombinant canine cytokines are used vs. recombinant human cytokines. In humans, the incorporation of IL-21 cytokine exposure by co-culture with the K562 feeder

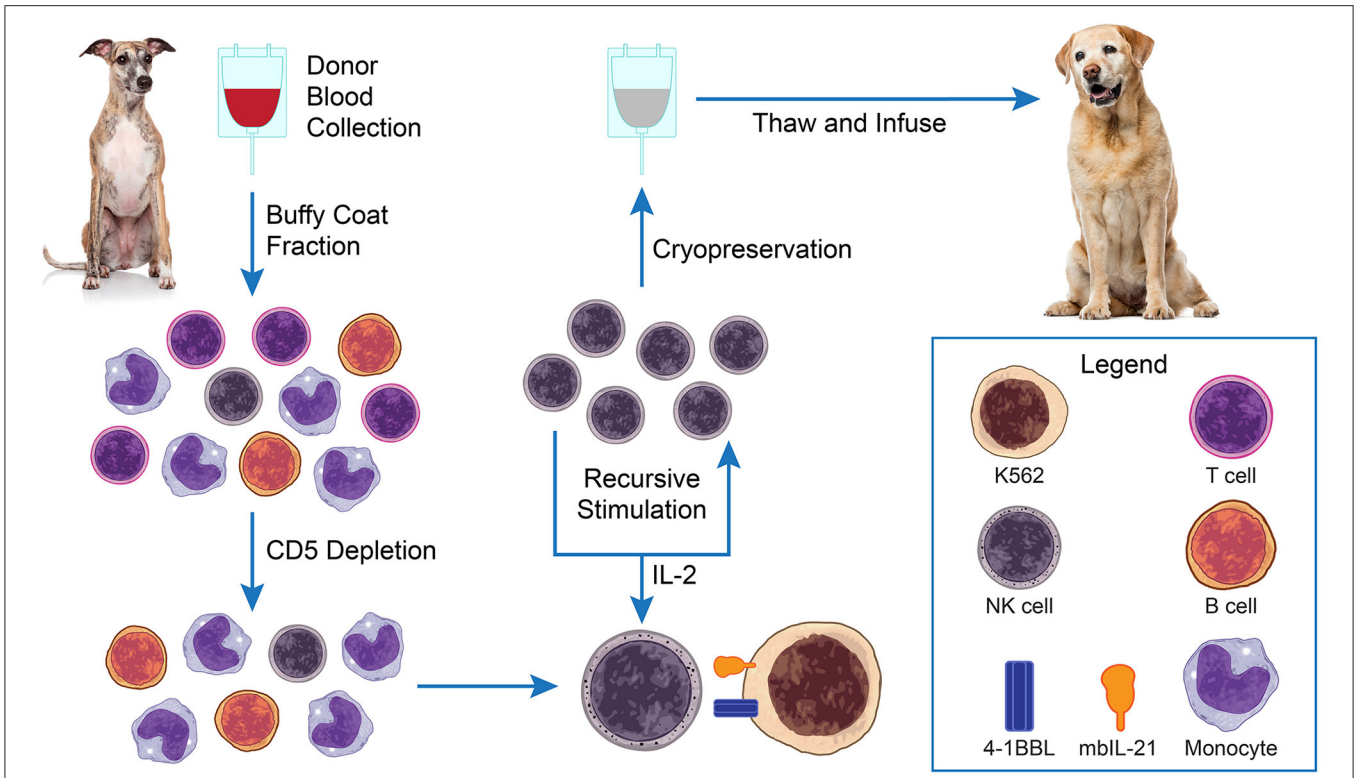


**FIGURE 1 |** Adoptive natural killer (NK) cell therapy. PBMCs are isolated from blood buffy coats of healthy blood donor dogs by Ficoll separation. The separated cells undergo CD5 cell depletion and are then co-cultured and recursively stimulated in the presence of IL-2 (±TGFβ) 3× over a 3-week period with irradiated K562 feeder cells expressing membrane bound IL-21. Expanded NK cells can be used immediately, or cryopreserved for later use.

cell line, significantly enhanced NK cell expansion in IL-15/IL-2 expanded NK cells (55).

These techniques have been modified by our group to manufacture *ex vivo* expanded canine NK cells with similarly robust results (25). Clinical grade expanded NK cells are produced using good manufacturing practices (GMP) principles including closed-system processes and standardized release testing and certification criteria. For our studies, the primary donor NK cells used for expansion are obtained from the buffy coats of routine whole blood donations from healthy volunteer canine blood bank donors at our veterinary medical center. Peripheral blood mononuclear cells (PBMCs) are isolated by Ficoll separation from the buffy coats. The separated cells undergo CD5 cell depletion and are then co-cultured and recursively stimulated in the presence of IL-2 three times over a 3 week period with irradiated human K562 feeder cells expressing the co-stimulatory ligand 4-1BBL and membrane bound IL-21 (**Figure 1**). The final *ex vivo* expanded NK cell product release criteria include: ≥70% viability, CD3+ cells <5%, NKp46+ cells as reported, endotoxin <5 EU/kg, and mycoplasma negative. Expanded NK cells can be used immediately, or cryopreserved for later use.

The influence and importance of the donor on the final NK cell product is largely unexplored, although there does appear to be individual donor variability in the robustness of expansion and *in vitro* cytotoxicity of the final expanded NK cell product. The influence of donor breed is also unknown. A preliminary survey of NK cell numbers (NKp46+, CD3–) and expression of DNAM-1 and TIM-3 receptors in the four most common donor breeds (greyhound, pit bull, golden retriever, and Labrador retriever) in our blood bank showed minor breed differences with no one breed being a clearly superior donor source (Peck, unpublished). Ultimately, for our initial clinical trials, we chose to use greyhound donors exclusively, as they are the most common breed in our blood donor population and by doing so, any unknown breed associated variability in the final product could be excluded.

## CLINICAL APPLICATION OF ADOPTIVE NK CELL IMMUNOTHERAPY IN CANINE OSTEOSARCOMA

As described above, spontaneous osteosarcoma in pet dogs provides an ideal large animal translational model for studying new immuno-oncology approaches for treating this cancer, including adoptive NK cell therapy. The more recent development of canine-specific antibodies for identifying canine NK cells and subsets, adaptation and development of *ex vivo* NK cell expansion techniques, and overall gradually increasing availability of canine-specific reagents and analysis techniques, now makes clinical trials of adoptive NK cell therapy

for osteosarcoma and other cancers in dogs more feasible and informative. However, these studies and trials are only just now beginning.

The first reported clinical trial of adoptive NK cell immunotherapy in dogs with appendicular osteosarcoma evaluated autologous *ex vivo* expanded NK cells administered intra-tumorally following completion of a hypo-fractionated palliative radiation protocol (56). In this study, NK cells were isolated and expanded from the canine patient using an expansion technique similar to that described above (56). Two injections of $7.5 \times 10^6$ NK cells/kg were co-injected with 250,000 IU/kg of rhIL-2. Ten dogs were treated in this study. Overall, there was limited systemic toxicity with this protocol. One dog had a grade 3 reaction of fever, chills, excessive salivation, and dehydration consistent with IL-2 toxicity. Three dogs had local infection/tissue breakdown at the NK cell injection site. Persistence of labeled viable NK cells could be demonstrated in tumor biopsies performed 1 week after intra-tumoral injection. Interestingly, analysis of PBMCs pre- and post-treatment demonstrated a significant increase in circulating granzyme B+ CD45+ cells (56).

Our group recently opened a phase I clinical trial of intravenously administered allogeneic TGFβ-resistant (imprinted) NK cells combined with adjuvant carboplatin chemotherapy in dogs with appendicular osteosarcoma receiving limb amputation (54). In this trial, dogs receive a single dose of NK cells 24 h prior to amputation to evaluate NK cell trafficking to the primary tumor. Three additional doses are administered during the subsequent 48 h post-amputation period. Dogs then receive standard adjuvant carboplatin chemotherapy every 3 weeks, with additional NK cell doses administered on the weeks they are not receiving chemotherapy. In total, dogs receive a total of twelve doses of adoptive NK cells—significantly more doses than in most human NK cell immunotherapy trials, to date. The use of allogeneic NK cells greatly increases the yield and potential cell doses, reduces the cost of therapy, and simplifies the logistics for delivery. Although this approach requires cryopreservation of the product which may impact NK cell viability and function, we have successfully used cryopreserved NK cells for several human studies (57, 58).

One of the major strengths of clinical trials in dogs with spontaneously occurring cancers is the ability to do intensive longitudinal patient biospecimen sampling and clinical assessments, often more intensively than is possible in a comparable clinical trial in human patients. This is well-illustrated in the afore described first-in-dog clinical trial, where pre- and post-treatment serum cytokine concentrations were assessed by ELISA, tumor gene expression profiles by qRT-PCR, circulating immune cell phenotypes by flow cytometry, and intra-tumoral immune cell phenotypes by immunohistochemistry and qRT-PCR (59). Gradually increasing availability of new canine-specific reagents and application of new technologies to the dog, will further increase the number and power of the correlative studies that can be done and their translational relevance.

Understanding of the pharmacokinetics and trafficking of adoptively transferred NK cells and consequent effects on systemic and tumor immune cell phenotypes and responses to therapy are important biological correlates for assessing adoptive NK cell strategies and in principle can be addressed in this model using a variety of approaches. Assessment of circulating NK cell numbers and phenotypes in blood can be assessed by flow cytometry; however, distinguishing donor from patient cells is problematic. Optimization of variable number tandem repeat PCR assays as is used to assess tissue chimerism in human transplant patients (60) and experimental canine bone marrow transplant models (61–63), may be useful for assessing the relative circulating donor NK cell component. Sex chromosome (XX/XY) FISH chimerism testing may be another method that could be applied when there is a sex-mismatched donor (64). Novel cell labeling agents have been developed and tested in rodent and non-human primate models and could be useful for evaluating NK cell kinetics and trafficking in the canine osteosarcoma model. *Ex vivo*-expanded human NK cells labeled with the non-radioactive isotope fluorine 19 ($^{19}$F) can be detected in rodent tissues by NMR and imaged with $^{19}$F-MRI (65, 66). Similarly, expanded NK cells from rhesus macques were labeled with $^{89}$zirconium-oxine ($^{89}$Zr-oxine) cell labeling and quantitated and imaged with positron emission tomography (PET)/CT (67).

## FUTURE APPLICATIONS

While these early studies of adoptive NK cell therapy in dogs are demonstrating the feasibility, tolerability, and safety of the approach, the model is well-suited for investigating many ongoing and new important questions in the field. As one of the mechanisms by which NK cells kill cancer cells is ADCC, combining adoptive NK cell therapy with therapeutic antibodies is of interest. Studying ADCC in spontaneous canine cancers may be feasible in some cases with murine, chimeric, or humanized antibodies, as canine Fc gamma receptors bind to dog, human, and mouse IgGs. However, caninized therapeutic antibodies may be preferred, as species differences in affinity may result in significant differences in activity, and eventual alloimmunization and neutralization by the host may significantly alter the antibody half-life of non-canine antibodies (68, 69). Of great interest is the genetic modification of NK cells to express chimeric antigen receptors (CAR) to target and enhance their killing (70, 71). Clinical trials investigating this approach are in their early stages in people. However, investigation of new CAR-NK constructs in dogs with osteosarcoma could address questions of toxicity, tumor targeting, immunologic response and anti-tumor activity. An important hurdle to genetic modification of NK cells has been their relative resistance to lentiviral and retroviral transduction (24, 72). Our group recently developed a method for genome editing of human primary and expanded NK cells using Cas9 ribonucleoprotein complexes (Cas9/RNPs) that allows for efficient knockout of genes in NK cells, thus opening the door for novel and innovative genetic modification strategies, including modifications that would affect tumor targeting and NK cell activation state, *in vivo* proliferative

capacity, and cytotoxicity (73, 74). As the use of genetically modified cells in humans has even more significant regulatory hurdles to overcome compared to similar trials in dogs, clinical trials in dogs can speed the evaluation of novel approaches, identify those that are more promising, and provide additional useful safety data to inform subsequent human trials. As noted above, because of the ability to easily acquire patient biospecimens, including tumor biopsies, and the relatively comparable size to humans, the model is ideal for investigating effects on tumor targeting achieved with different CAR-NK constructs and for studying novel NK cell labeling and imaging techniques (65–67).

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

WK and DL contributed equally to the conception of this perspective and wrote sections of the manuscript. WK wrote the first draft of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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# Immunohistochemical Characterisation of GLUT1, MMP3 and NRF2 in Osteosarcoma

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Osteosarcoma (OSA) is an aggressive bone malignancy. Unlike many other malignancies, OSA outcomes have not improved in recent decades. One challenge to the development of better diagnostic and therapeutic methods for OSA has been the lack of well characterized experimental model systems. Spontaneous OSA in dogs provides a good model for the disease seen in people and also remains an important veterinary clinical challenge. We recently used RNA sequencing and qRT-PCR to provide a detailed molecular characterization of OSA relative to non-malignant bone in dogs. We identified differential mRNA expression of the solute carrier family 2 member 1 (SLC2A1/GLUT1), matrix metallopeptidase 3 (MMP3) and nuclear factor erythroid 2–related factor 2 (NFE2L2/NRF2) genes in canine OSA tissue in comparison to paired non-tumor tissue. Our present work characterizes protein expression of GLUT1, MMP3 and NRF2 using immunohistochemistry. As these proteins affect key processes such as Wnt activation, heme biosynthesis, glucose transport, understanding their expression and the enriched pathways and gene ontologies enables us to further understand the potential molecular pathways and mechanisms involved in OSA. This study further supports spontaneous OSA in dogs as a model system to inform the development of new methods to diagnose and treat OSA in both dogs and people.

**Keywords: osteosarcoma, canine, solute carrier family 2 member 1, matrix metallopeptidase 3, nuclear factor erythroid 2-related factor 2, pathology, cancer identification**

## INTRODUCTION

Canine osteosarcoma (OSA) presents a significant veterinary clinical challenge with an estimated incidence rate of between 13.9–27.2/100,000 dogs, considerably higher than the rate in people, 1–3 cases/annum/1,000,000 people (1–4). It shares many clinical and molecular features with human OSA (5–8). The current management of choice for canine OSA is surgery followed by chemotherapy; the one year survival rarely exceeds 45% even for patients receiving treatment (5, 9–14). In contrast to human OSA, canine OSA is most common in middle aged dogs and a degree of heritability has been observed (1, 15, 16). Canine OSA presents a promising model for determining

the underlying mechanisms of OSA carcinogenesis and cancer progression, and also provides an opportunity for the development of drugs targeting OSA-specific pathways. Multidrug resistance is a critical limitation to the current success of chemotherapy and, therefore, additional therapeutic approaches are needed that could reduce the metastatic rate and recurrence of OSA (17).

To support the development of more effective therapies, there is a need to understand the underlying mechanisms of OSA etiology and progression. OSA predominantly affects large and giant breed dogs, particularly Irish Wolfhounds, Rottweilers, Deerhounds, St Bernards, and Great Danes, with association made with male dogs and increased height and weight (1, 18). This is comparable to human OSA where male sex and height are associated with higher incidence rates peaking at puberty (6, 19, 20). These findings in humans and canines support the potential role of developmental factors and increased cell proliferation in OSA etiology. Previous studies have implicated ezrin, a membrane cytoskeleton linking protein, in poor prognosis and metastasis (21–24). Evidence also supports a role of epigenetics in the development of OSA, however, this is not yet well understood (25, 26). Well-characterized oncogenes and tumor suppressors, including MYC, EGFR, AKT2, TP53, CDKN2A/B, RB1, BCL2 and PTEN, have also been implicated in canine OSA (15, 25, 27). Karyotypic instability, associated with mutations of *TP53*, is characteristic of OSA (28).

More recently our group identified several genes significantly differentially expressed between canine OSA and non-tumor bone tissue (16). Consistent with the association with bone growth and development, multiple gene ontologies of the differentially expressed genes related to cellular differentiation, morphogenesis, development, cellular proliferation, and metabolism (16). Intracellular signaling, calcium homeostasis and heme synthesis were also implicated. Analysis showed that *MMP3* and *SLC2A1* expression were significantly higher in OSA tissue compared to non-tumor tissue and protein expression in OSA was confirmed by immunohistochemistry. This study expands on the initial analysis (16) by investigating the levels of MMP3, GLUT-1 (protein expressed by *SLC2A1*) and NRF2 (transcription factor encoded by *NFE2L2*), which are known to play a role in human OSA, in an OSA canine cohort.

# MATERIALS AND METHODS

## Specimen Preparation

All animal tissue work in this study was approved by the Ethics committee at the University of Nottingham School of Veterinary Medicine and Science and complied with national ethics procedures (permission number - UG 20331). Patients were euthanised under normal veterinary practice under circumstances unrelated to research. Diagnosis of OSA was confirmed by a board certified histopathologist.

## Immunohistochemistry and Microscopy

Proteins of interest were identified following gene expression analysis (RNA sequencing), validated by qRT-PCR (16). Immunohistochemistry was performed to show positive protein expression of GLUT1, MMP3 and NRF2. Rottweiler post-mortem OSA tissue ($n = 15$) was obtained from Bridge Pathology, UK in the form of formalin fixed, paraffin embedded OSA tissue. The OSA samples were all excised from Rottweilers, 9/15 female, 5/15 male and 1/15 not specified. The females ages ranged between 7-9 years old and 2/9 were entire, and the males were between 6-10 years old and 3/5 were entire. OSA location was 10/15 appendicular, 3/15 axial, 1/15 mixed appendicular/axial, 1/15 not specified. A range of morphologic types were studied including 10/15 osteoblastic; 3/15 chondroblastic or mixed osteoblastic/chondroblastic; and 2/15 suspected giant cell rich. In addition a range of mitotic activity values [as previously defined (29)] were included: 3/15 value 1; 8/15 value 2; 4/15 value 4. All of these higher mitotic values were observed in females, in addition the two cases with highest mitotic activity overall were females and had large amounts of osteoid. Given the deliberately mixed nature of the OSA samples, statistics were not carried out on location, morphologic type, sex or mitotic activity.

Tissue was post-fixed in 4% paraformaldehyde for 2 hours, dehydrated through an ethanol series, embedded into paraffin blocks, and sectioned at 7 μm. Immunohistochemistry was carried out u Proteins of interest were identified following gene expression analysis (RNA sequencing), validated by qRT-PCR (16). Immunohistochemistry was performed to show positive protein expression of GLUT1, MMP3 and NRF2. Rottweiler post-mortem OSA tissue ($n = 15$) was obtained from Bridge Pathology, UK in the form of formalin fixed, paraffin embedded OSA tissue. OSA samples from a variety of bones including the humerus X2, scapula, femur X3, mandible X2, temporomandibular joint, tibia, maxilla, stifle, carpus and 2 unknown locations were excised from male and female Rottweilers between the ages of 6-11 years old. Tissue was post-fixed in 4% paraformaldehyde for 2 hours, dehydrated through an ethanol series, embedded into paraffin blocks, and sectioned at 7 μm. Immunohistochemistry was carried out using a Leica Novolink Polymer Detection Kit (Leica, Wetzlar, Germany) according to manufacturer's protocols with primary antibodies diluted in fetal calf serum 1:100; anti-SLC2A1(GLUT1) polyclonal unconjugated rabbit antibody (100732-TOB-SIB; Stratech, Ely, UK), anti-MMP3 polyclonal unconjugated rabbit antibody (GTX74514; GeneTex, Irvine, CA, USA), anti-NRF2 (*NFE2L2*) polyclonal unconjugated rabbit antibody (ab31163; Abcam, Cambridge, UK) were used to stain proteins of interest. Microscopy was carried out to confirm positive staining cytoplasmic and/or nuclear staining (Leica, Wetzlar, GermanyUK) and systematic random sampling employed to take photomicrographs for H-scoring. Negative controls received no primary antibody and were incubated in fetal calf serum only. Kidney sections from one of the patients were used as positive controls, as the target markers were known to be expressed in the kidney (30–32).

## H-Scoring

H-scoring, a well-established semi-quantitative technique for protein expression was used to analyse the samples. It is often considered as one of the "gold standards" for

immunohistochemistry evaluation (33–35). H-scores were undertaken by one double-blinded researcher who established a scoring definition and then undertook the scoring within a two week period to ensure interpretation consistency. Two additional researchers scored 10% of the samples, chosen randomly to ensure concordance. Staining intensity was designated into scores of 0, 1+, 2+, or 3+ (none, weak, moderate, strong staining signal) for each antibody. The percentage of cells/tissue containing positive staining (to the nearest 5%) of either cytoplasmic and/or nuclear staining were calculated independently for a fixed field of n = 4-5 photomicrographs per sample ($n$ = 10-13 OSA samples) for each antibody. H-scores were calculated using the formula: H-score = [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] for both cytoplasmic and nuclear staining separately. H-scores based on the resulting 0–300 scale were calculated for each specimen and each protein. The mean, standard error of the mean, minimum, maximum and range of H-scores were calculated. Data was plotted to demonstrate both score distributions and staining intensities. In addition, representative staining scores (based on these samples only) were created as benchmarks to discuss results. H-scores were also classified as low 0–45, moderate 45-90 or high 90+ average scores in order to describe the overall scores. Statistical analysis between cytoplasmic and nuclear H-scores was conducted using paired $T$-test (SPSS v26). Comparisons between the number of slides with 0, 1, 2, and 3 H-score staining categories in the cytoplasm and nucleus were conducted using chi-square.

Qualitative data was also recorded in order to describe general immunohistochemical staining patterns. Qualitative data was described for both neoplastic areas and, where possible, adjoining areas where no tumor was present. In addition to describing the cell/structure types present and the immunostaining observed, general staining was identified for each sample (diffuse, multifocal, focal), both cytoplasmic and nuclear staining were described (absent, weak, moderate, strong) and the predominant staining location was identified (cytoplasmic, nuclear or equal).

# RESULTS

## GLUT1 H-Score and Expression in OSA

GLUT1 staining ($n$ = 47 sections from 13 patients) showed H-score variations between the different patients, however all specimens showed positive immunostaining. Only 1 of the 13 patients showed both low cytoplasmic and low nuclear average scores (7.7%), while 2 of the 13 patients showed both high cytoplasmic and high nuclear average scores (15.4%). Only one patient had a low average GLUT-1 cytoplasmic score, 7 patients had moderate cytoplasmic scores (58.3%) and 5 had high cytoplasmic scores (38.5%). Nuclear scores showed 4/13 patients with low H scores (30.8%), 4/13 at moderate (30.8%) and 5/13 at high (38.5%), overall these were not significantly different to the cytoplasmic staining scores ($P > 0.05$). The 0-300 cytoplasmic H-scores were slightly higher than the nuclear scores, however the same range for both locations was observed and no significant differences were present ($P > 0.05$, **Table 1**, **Figures 1A,D**).

**TABLE 1 |** H-scores from GLUT1, MMP3 and NRF2 immunostained canine OSA specimens showing inter case variation.

| Protein (number of cases) | Cellular location | H-Score | |
|---|---|---|---|
| | | Mean ± SEM | Range (min-max) |
| GLUT1 ($n$ = 13) | Cytoplasmic | 74.89 ± 11.11 | 180 (5–185) |
| | Nuclear | 67.15 ± 11.38 | 180 (5–185) |
| MMP3 ($n$ = 12) | Cytoplasmic | 69.88 ± 4.60 | 95 (25–120) |
| | Nuclear | 38.58 ± 8.61 | 135 (0–135) |
| NRF2 ($n$ = 10) | Cytoplasmic | 71.89 ± 6.42 | 130 (10–140) |
| | Nuclear | 74.17 ± 13.08 | 200 (0–200) |

*H-Score indicates average scores calculated from several slides for each patient. N = number of cases. Low was classified as 0–45, moderate 45–90 or high was a score of 90+.*

The histopathology general report indicated that all specimens showed diffuse staining distribution, with cytoplasmic staining classified as mostly weak or weak to moderate (**Table 2**). Nuclear stain intensity ranged from absent to weak – moderate, and the majority of samples showed predominantly cytoplasmic staining, but in some samples the predominant stain was nuclear whereas in others the cytoplasm and nucleus were equally stained (**Table 2**).

Despite GLUT1 staining being observed in every OSA specimen, only half of the specimens stained with GLUT1 antibodies showed individual staining intensity scores of 3, and it was notable that blood vessels frequently exhibited H-score 3 nuclear staining in the tunica intima, whereas generally nuclei in the tunica media exhibited lower H-scores (**Figure 1**). Cytoplasmic staining produced higher H-scores in the tunica intima in comparison to the tunica media whereas the osteoid matrix did not exhibit immunopositive staining (**Figure 2**). Generally staining was less pronounced in neoplastic cells in comparison to the endothelium.

## MMP3 H-Score and Expression in OSA

For MMP3 H-scoring ($n$ = 51 sections from 12 patients), all specimens showed positive immunostaining and less variation was calculated between patients on the 0–300 score in comparison to GLUT1 and NRF2 H-scoring. In total 8/12 (66.7%) patients had a low average nuclear score, while only 1/12 (8.3%) had a high nuclear score. In addition, 50% (6/12) of the patients had a combination of moderate average cytoplasmic scores and low average nuclear scores. Only 1 (8.3%) patient had a low average cytoplasmic score. Low scores were more likely to be observed in the nucleus, whereas moderate and high scores were more frequently observed in the cytoplasm ($P < 0.0001$). Overall, on the 0–300 scale the cytoplasmic H-scores were higher than those observed in nuclei ($P = 0.016$, **Table 1**, **Figures 1B,D**).

The histopathology report showed that staining was diffuse in all cases, with predominantly weak cytoplasmic staining, with some cases showing weak-moderate or moderate cytoplasmic staining (**Table 2**). The MMP3 nuclear staining was reported as absent or absent-weak in the majority of cases, with some weak

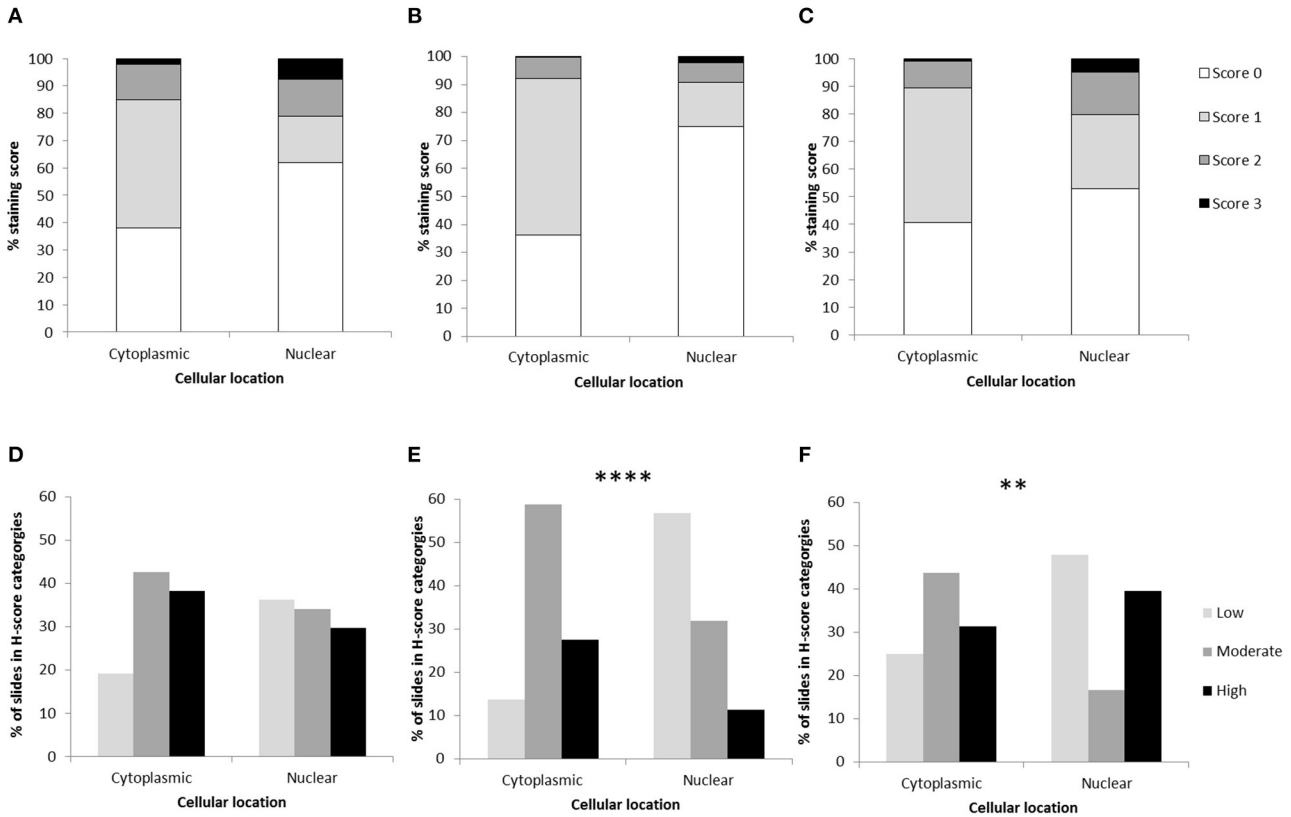


**FIGURE 1 |** Osteosarcoma H-scores in the cytoplasm and nucleus following immunohistochemical staining. Average H-scores for **(A)** GLUT1, **(B)** MMP3, and **(C)** NRF2. H-score distributions across samples for **(D)** GLUT1 ($P > 0.05$), **(E)** MMP3 (****$P < 0.0001$), and **(F)** NRF2 (**$P = 0.008$). Differences between nuclear and cytoplasmic staining were assessed using chi-square.

**TABLE 2 |** Overall blinded histopathology assessment for each OSA case.

| Protein | Staining distribution | Cytoplasmic staining intensity (% of cases) | | | | | | Nuclear staining intensity (% of cases) | | | | | | Predominant staining (% of cases) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein | Diffuse/Multifocal/Focal | Absent | Absent-Weak | Weak | Weak-Moderate | Moderate | Strong | Absent | Absent-Weak | Weak | Weak-Moderate | Moderate | Strong | Cytoplasmic | Nuclear | Equal |
| GLUT1 | 100% diffuse | - | 61.54 | - | 38.46 | - | - | 23.08 | 30.77 | 15.38 | 30.77 | - | - | 61.54 | 23.08 | 15.38 |
| MMP3 | 100% diffuse | - | - | 75.00 | 16.67 | 8.33 | - | 58.33 | 16.67 | 16.67 | 8.33 | - | - | 91.66 | - | 8.33 |
| NRF2 | 100% diffuse | 12.50 | 12.50 | 62.50 | 12.50 | - | - | 37.50 | 25.00 | 37.50 | - | - | - | 62.50 | 12.50 | 25.00 |

and weak-moderate nuclear staining in the remaining cases. In 11 out of the 12 cases, staining was predominant in the cytoplasm, whereas the remaining sample had staining distributed equally between the cytoplasm and nucleus (**Table 2**).

Staining was observed in the endothelium in all cases and also within the fibroblastic cells present. In addition it was noted that endothelial cell staining intensity was comparable the neoplastic cell staining observed. In contrast, some focal areas vascular/perivascular cells had distinct negative staining which contrasted to neoplastic positive staining observed. In addition connective tissue, muscle and blood vessels predominantly showed weak, diffuse staining and muscle fibers were negative

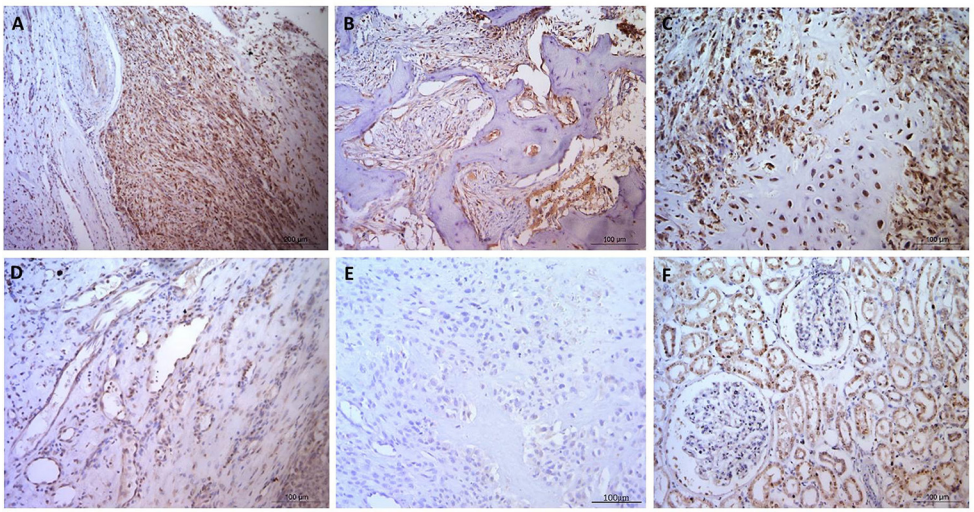


**FIGURE 2 |** Osteosarcoma GLUT1 immunohistochemical staining. **(A–C)** GLUT1 indicating positive nuclear and cytoplasmic immunostaining, immunopositive blood vessels with more pronounced cytoplasmic staining in the tunica intima in comparison to the tunica media, and negative staining in the osteoid and in **(D)** a tumor-free area. **(E)** Negative control OSA tissue showing no positive immunostaining. **(F)** Positive control canine kidney tissue staining primarily in the tubular epithelial cells. Scale bars represent 100 μm.

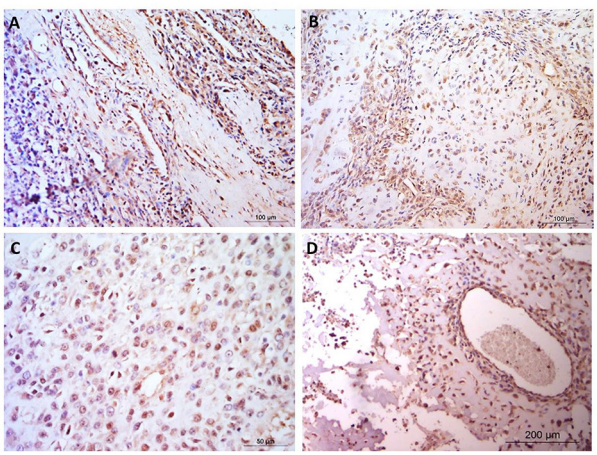


**FIGURE 3 |** Osteosarcoma immunohistochemical staining for MMP3. **(A–D)** MMP3 positive staining in blood vessels, cytoplasm and nuclei but negative staining in the osteoid, in four canine osteosarcoma samples. Scale bars represent **(A, B)** 100 μm, **(C)** 50 μm, and **(D)** 200 μm.

within the nucleus. One case showed rare, weak cytoplasmic staining in the suspected leukocytes. Four patients (33.3%) exhibited positive MMP3 immunostaining in the extracellular matrix, however osteoid staining was not present in any samples (**Figure 3**).

## NRF2 H-Score and Expression in OSA

NRF2 H-scores ($n$ = 51 sections from 10 OSA patients) showed considerable variation between patient averages on the 0-300 scale, but positive immunostaining was observed in all specimens. Some patients (20%, 2/10) demonstrated high average H-scores of both cytoplasmic and nuclear NRF2 staining, some patients demonstrated either high average nuclear staining (20%, 2/10) or high average cytoplasmic staining (10%, 1/10). The remaining 50% (5/10) demonstrated moderate levels of both nuclear and cytoplasmic NRF2 staining. In addition, low and high scores were more likely to be present in the nucleus in comparison to the moderate scores which were more frequent in the cytoplasm ($P = 0.008$). Overall the 0–300 cytoplasmic and nuclear staining H-scores were similar (within 5%, $P > 0.05$), however the range of H-scores was greater in the nuclear staining (**Table 1**, **Figures 1C,E**).

The histopathology report showed diffuse staining in 100% of the samples (**Table 2**). The majority of samples showed absent, weak or weak-moderate cytoplasmic and nuclear staining intensities. In the majority of samples, staining was predominantly observed in the cytoplasm, but in the remaining samples staining was either predominantly nuclear or equally cytoplasmic and nuclear (**Table 2**).

NRF2 immunopositive staining was observed in every blood vessel, with positive cytoplasmic staining in the tunica intima (all ten samples; 100%), while 8 patients also showed positive nuclear staining in the tunica intima (8/10, 80%; **Figure 3**). Positive staining was less frequent in the tunica media (4/10 positive cytoplasmic, 3/10 positive nuclear). Muscular tissue was present in 30 of the sections analyzed from across the patients. It was of interest that all 30 sections showed positive NRF2 immunostaining (100%), 6 slides showed heterogeneous immunostaining (20%) in terms of both distribution and stain intensity, while the remainder (80%) showed homogenous staining. Muscle, nerves and connective (adipose/fibrous) tissue presented with diffuse staining which was generally more

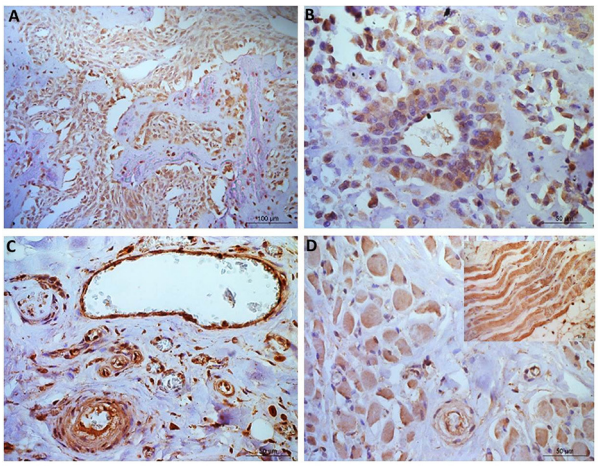


**FIGURE 4 |** Osteosarcoma immunohistochemical staining for NRF2. **(A, B)** Immunopositive staining in blood vessels, primarily nuclear, in all 10 samples within the tunica intima and in 40% of samples tunica media staining was observed. **(C)** Negative staining in the osteoid (tumor free area). [**(D)**+inset] Muscle tissue showing positive NRF2 staining. Scale bars represent **(A)** 100 μm, **(B–D)** 50 μm.

pronounced than the neoplastic cell population. Where mucosa and inflammation were present, the staining intensity was similar to that noted in neoplastic cells. The osteoid matrix remained immunonegative in all specimens (**Figure 4**).

## DISCUSSION

In recent years a detailed understanding of the transcriptional heterogeneity and mechanistic processes in human osteosarcoma has been established by the rigorous unbiased transcriptomic analysis of match tumor and non-malignant specimens (36–40). Such knowledge is driving advances in diagnostics and treatment for this disease in man.

Canine OSA remains challenging to treat and carries a poor prognosis due to the very aggressive and metastatic nature of the tumors. Given how common OSA is in dogs and likely genetic contribution to OSA in large breeds, we (16) and others (25, 41–43) have sought to extend understanding of the molecular determinants of OSA in dogs and to compare these results to those obtain from OSA from people.

Here we investigated three cancer promoting proteins that have been shown to be up regulated at the gene level in canine OSA compared to normal bone tissue (16). Indeed the importance of GLUT-1 (37, 44), MMP3 (36, 37) and NRF2 (45) is well established in human OSA. More recently a study used single cell RNAseq to investigate the cellular heterogeneity within human osteosarcoma specimens and identified *MMP-3* as one of the top differentially expressed genes in OSA specimens (40). However little is known about the expression of these in canine OSA tissue.

While glucose is an essential part of cellular metabolism, glucose metabolism is enhanced in malignant cells (46). Glucose transporter member 1 (GLUT1, also known as SLC2A1) is a cell membrane glycoprotein responsible for glucose transport that is widely expressed across cell types and is overexpressed in many cancers (46, 47). The transcription factor hypoxia-inducible factor 1-alpha (HIF-1α) was found to induce GLUT1 thus increasing survival in hypoxic conditions by allowing increased anaerobic glycolysis (48). Additionally, increased GLUT1 expression allows cancer cells to survive low glucose conditions (49), and hypoxic tumor cells are resistant to conventional therapeutics, highlighting the potential of glycolytic inhibitors in osteosarcomas (50). In human OSA, higher expression of *SLCA1I* correlated with a poor prognosis, shorter disease-free interval and increased angiogenesis (47). GLUT1 staining was previously identified in 74% of human OSA specimens and linked with increased tumor volume and metastatic potential, as well as increased recurrence rate (44).

In canine OSA, a study of 44 canine osteosarcoma specimens showed 61% positive GLUT1 staining but no significant correlation was identified between GLUT1 and disease-free interval (51). Interestingly, Petty and colleagues also showed a subset of canine OSA with no or low GLUT1 staining as was seen in human OSA (44). Our findings of GLUT1 in OSA indicated that every specimen had some degree of positive immunostaining, however this varied between patients. Investigations into whether H-scoring differs between tumor grades, type of bone affected/location, sex or other factors still need to be conducted.

GLUT1 staining was observed in every OSA specimen in the present study, and notably blood vessels frequently exhibited H-score 3+ nuclear staining in the tunica intima, and lower H-scores in tunica media nuclei, and cytoplasmic staining in both structures (higher in the tunica intima). GLUT1 immunostaining has previously shown an abundance of the protein in blood vessels within the diaphysis of normal long bones, but not in the metaphysis (52). It has been suggested that osteoblast differentiation is a high-energy demand process, met by upregulation of GLUT1 in bone blood vessels (53). Additionally the importance of GLUT1 in blood vessels has been shown in relation to blood brain barrier function, where the energy demand of the brain during childhood is greater due to the rapidly developing nature of the brain (54). Expressed in both luminal and abluminal endothelial cells within the blood brain barrier (55), haploinsufficiency of SLC2A1 causes GLUT1 deficiency syndrome resulting in delayed development, movement disorders, and seizures (56). These links with high-energy processes could not only explain expression in OSA tissue, but may also highlight GLUT1 as a therapeutic target. The potential of GLUT1 as a therapeutic target has also been demonstrated in human OSA cells, where glycolytic inhibitor sensitized hypoxic cells to chemotherapy (50). Furthermore, increased *SLC1A1* in human OSA microarray datasets has been associated with metastatic tumors and a worse prognostic effect (37). Our findings show that GLUT1 is expressed in canine OSA and confirm the need to investigate the potential of glycolytic inhibitors to increase therapeutic efficacy in both canine and human OSA.

In aggressive tumors, cell invasion and metastases require breakdown of the extracellular matrix [ECM; (57)]. Matrix metalloproteinases (MMPs) are enzymes that degrade the ECM and changes in concentrations of MMPs are important in invasion and metastasis of OSA (58, 59). Metastases in OSA are critical for disease progression and are associated with poor prognosis (60). High expression of MMP3 in many cancers has been associated with poor prognosis (61). In human OSA, MMP3 is highly expressed in OSA tissue in comparison with normal bone (62), and has been shown to be regulated by tumor suppressing microRNAs which are down-regulated in OSA (62, 63). MMP3 may also be associated with OSA metastasis, indeed survival outcomes were improved in patients expressing lower levels of *MMP3* in microarray datasets (37). Complex pathways such as estrogen receptor alpha (ERα) signaling induces FasL transcription in osteoblasts leading to MMP3 expression in these cells, resulting in sFasL production and osteoclast apoptosis (64). Additionally, MMPs are synthesized in stromal cells adjacent to tumor cells (65). Studies in people have also highlighted differentially expressed genes in OSA tissue, including the MMPs and genes which interact with the matrix metalloproteases (36, 59). In the present study, MMP3 staining varied between the canine OSA, including in stromal cells consistent with results in studies of human cancers (65). MMP3 in canine OSA could be used as marker of more invasive and metastatic tumors. Of interest, MMP3 is a druggable target, with a selective inhibitor of MMP3 available (UK370106), but this has not been tested in cancer cells (66). A generic MMP inhibitor Marimastat, showed little promise in clinical trials (67, 68), but has not been tested in OSA patients. More recently, sulfonamide-based inhibitors of MMP3 have also been developed (69). Additionally, MMP3 has been found in extracellular vesicles that were protumorigenic and highly transmissive (70), highlighting another function of MMP3 in metastases and emphasizing it as a potential key therapeutic target in canine OSA.

A feature of OSA and other cancers is chemoresistance. Chemoresistance arises via up-regulation of mechanisms that protect the cell from the impact of chemotherapy. Chemotherapy increases reactive oxygen species (ROS) in cells, which then trigger DNA damage which leads to apoptosis (71, 72). The concentrations of ROS in normal cells are maintained by inducible antioxidants which are regulated by the transcription factor, NRF2 (73). Oncogene- induced NRF2 has been shown to promote ROS detoxification (74) and play a role in tumor progression, invasion, and metastases in many cancers (75). In mice, deletion of NRF2 led to lower bone mineral density and weaker long bones (76). NRF2 has also been implicated in osteoclast activity as when NRF2 was depleted, increased intracellular ROS was observed alongside increased osteoclast numbers, suggesting increased osteoclastic activity with decreased NRF2 (77).

Nuclear staining of NRF2 has been shown in bone metastases of people with OSA (78), and expression of this protein has been associated with poor outcome in OSA patients (45). In our study we observed NRF2 staining in 100% of the canine OSA, however variation in staining intensity was observed between the different patients. This suggests that it has potential as both a prognostic marker and therapeutic target. Knock down of *NFE2L2* in human cancer cells was effective in altering the NFE2L2/NRF2 pathway and improving chemosensitivity (79). Oridonin, a drug isolated from a medicinal herb, has shown potent anti-tumor effects in OSA, by reducing NRF2 and an antioxidant pathway, leading to apoptosis (80). Tanshinone 11A also inhibited OSA growth by targeting AMPk-NRF2 pathway, knockdown of both NFE2L2/NRF2 and AMPK showed same effects as the drug (81). A liposome-based siRNA targeting *NFE2L2*, given in conjunction with cisplatin, improved treatment of OSA (82). These recent developments in pharmacological drugs and RNA interference-based therapies holds promise for treating canine OSA.

Canine OSA is divided into several morphologic subclasses: osteoblastic, fibroblastic, chondroblastic, and teleangectatic (83), however these subclassifications have not yielded significant differences in the prognosis of either human or canine OSA (83–85). In contrast, histologic grading of human tumors, serves as a good indicator for prognosis (85), but is not been widely used as a prognosticator in canine OSA and has failed to be a significant indicator for decreased survival in flat and irregular bones, including the mandible (86–88). However, a mandibular OSA seemed to have a distinctly better clinical outcome than does OSA of other locations (86). Another problematic feature in grading canine OSA is that there are several published histologic grading systems, none of which are universally accepted (89). These difficulties providing a prognosis, make finding suitable markers even more important.

Our results have shown that GLUT1, MMP3 and NRF2 are all present in canine OSA from a number of different anatomical locations including the humerus, scapula, femur, tibia, stifle, carpus and the mandible, maxilla and temporomandibular joint. Previous canine OSA studies have shown that tumor location and mitotic index can be correlated with survival time and disease-free interval (87, 89), therefore understanding expression in the differing locations and mitotic index could be informative. The H-scores of the three proteins varied greatly between individuals in the present study. Although tumor size was not a factor quantified in our clinical samples, it is potentially an area of interest for future work. As larger tumors tend to show more hypoxia and mutagenesis (90), and tumor hypoxia indicated increased expression of GLUT1 in cervical carcinomas (91), this could be an interesting factor to investigate. Higher tumor grades have also been linked to both higher levels of necrosis, and primary lesion location (with appendicular regions often scoring at higher grades) (29); both of these factors are of interest. Therefore larger studies considering multiple factors, such as OSA grade and anatomical location, need to be undertaken and compared to non-tumor tissue, in order to contextualize the complex expression patterns of GLUT1, MMP3 and NRF2. In conclusion, GLUT1, MMP3 and NRF2 are expressed in canine OSA, are good potential candidates for prognostication in OSA and therapeutic targets, and clinical trials using drugs which already target these proteins are encouraged. In addition to understanding canine OSA further, this study also supports spontaneous OSA in dogs as a model system to inform the development of new methods to diagnose and treat OSA in both dogs and people.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Nottingham School of Veterinary Medicine and Science. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

CR and NM: conceptualization. CR, JC, SS, AA, CB, SB, and NM: data curation. CR, JC, JL-R, SB, and NM: formal analysis. CR, SB, and NM: funding acquisition. CR, JC, JL-R, AH, JJ, SS, AA, CB, AB-R, AR, MD, SB, and NM: investigation. CR, JL-R, CB, SB, and NM: methodology. CR, MD, and NM: project administration. CR, MD, SB, and NM: resources. CR, JR, and NM: supervision. CR, AA, and JL-R: validation. CR, JC, AA, CB, and SB: visualization. CR, JC, AH, JJ, CB, AR, and NM: writing—original draft. CR, JC, JL-R, AH, JJ, SS, AA, CB, AB-R, AR, MD, SB, and NM: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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# An Update on Molecular Pathways Regulating Vasculogenic Mimicry in Human Osteosarcoma and Their Role in Canine Oncology

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Canine tumors are valuable comparative models for human counterparts, especially to explore novel biomarkers and to understand pathways and processes involved in metastasis. Vasculogenic mimicry (VM) is a unique property of malignant cancer cells which promote metastasis. Thus, it represents an opportunity to investigate both the molecular mechanisms and the therapeutic targets of a crucial phenotypic malignant switch. Although this biological process has been largely investigated in different human cancer types, including osteosarcoma, it is still largely unknown in veterinary pathology, where it has been mainly explored in canine mammary tumors. The presence of VM in human osteosarcoma is associated with poor clinical outcome, reduced patient survival, and increased risk of metastasis and it shares the main pathways involved in other type of human tumors. This review illustrates the main findings concerning the VM process in human osteosarcoma, search for the related current knowledge in canine pathology and oncology, and potential involvement of multiple pathways in VM formation, in order to provide a basis for future investigations on VM in canine tumors.

**Keywords: comparative oncology, dog, osteosarcoma, vasculogenic mimicry, molecular pathways**

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## INTRODUCTION

Vasculogenic mimicry (VM) is a unique ability of malignant cancer cells to create their own fluid-conducting microvascular channels without the involvement of endothelial cells. It was firstly described in human uveal melanomas as periodic acid–Schiff (PAS)-positive microvascular channel networks (1). Since then, VM has been observed in a variety of human malignant tumors, including osteosarcoma (OSA), glioblastoma and gallbladder, ovarian, prostate, lung, gastric, hepatocellular, and breast cancer (2, 3). In addition, the presence of VM has been associated with high tumor grade, invasion, metastasis, and poor prognosis in cancer patients (4, 5). Thus, VM has emerged as a potential target for anti-tumor therapy (2, 3, 6).

In veterinary pathology, the VM process has been demonstrated in canine inflammatory mammary carcinomas and in a palpebral melanocytoma (7, 8). Rasotto et al. explored the presence of VM in primary canine mammary tumors, revealing no relation with lymphatic infiltration (9). As well, primary cell lines from canine mammary tumors, showing ability to form VM *in vitro* and *in vivo*, have been recently established and characterized (10–12). Moreover, canine inflammatory mammary carcinomas were analyzed for the presence of VM by transmission and

scanning electron microscopy (13). In addition, as far as canine OSA is concerned, the presence of vessel-like structures in a long-term canine D17 OSA cell cultured on type I collagen has been recently described (14). As well, treatment with the heat shock protein 90 (Hsp90) inhibitor 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) inhibited the migration of D17 OSA cells, also decreasing VM markers *in vitro* and inducing a reduction of hypoxia-inducible factor 1α (HIF1α) transcript and protein expression (14). Notwithstanding this, information regarding VM formation, molecular features, and prognostic implications in canine oncology is still limited.

Since VM has been known from a relatively short time, the molecular mechanisms involved in this process remain largely unknown. Aggressive tumor cells capable of VM display a varied gene profile which includes that of fibroblasts and epithelial and endothelial cells (15). Hypoxia, epithelial-mesenchymal transition (EMT) and in particular epithelial-endothelial transition (EET), response to extracellular matrix (ECM), and the presence of cancer stem cells (CSCs) are considered the key regulators of VM (16, 17). Various signaling pathways, promoting tumor migration and invasion, have been reported to participate in VM formation, including those involved in vasculogenesis such as vascular endothelial (VE)-cadherin, vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) and platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) axis, and HIF1α (3). VM progression is also mediated by pathways involved in ECM adhesion and cell migration, such as focal adhesion kinase (FAK) and migration inducting gene 7 (*Mig7*) encoding for breast cancer anti-estrogen resistance protein 3 (BCARP 3), matrix metalloproteinases (MMPs), integrins and erythropoietin-producing hepatocellular receptorA2 (EphA2), as well as multiple signaling pathways including mechanistic target of rapamycin (mTOR) and Rho-associated coiled-coil kinase (RhoA/ROCK) (3). Finally, increasing evidence showed that VM can be affected by microRNA (miRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA) (18).

Thus, the aim of this review is to illustrate the main findings concerning the VM process in human OSA (**Figures 1**, **2**), as well as the current knowledge on the molecular pathways potentially involved in VM formation in canine pathology and oncology (**Supplementary Table 1**), in order to provide a basis for establishing further investigations on VM in canine tumors in the future.

## CSCs MARKERS: CD133 AND ALDEHYDE DEHYDROGENASE 1 (ALDH1)

CSCs represent an important feature of VM progression for their ability to differentiate in endothelial cells forming new microvessels (19). Stemness and differentiation potential of CSCs are enhanced under hypoxic microenvironments, through hypoxia-induced EET and ECM remodeling, thus determining the formation of the specific features of VM (17). Bao et al. (30) described a positive correlation between CD133 expression and presence of VM in OSA, which was, in turn, positively associated with ALDH1 expression. CD133, also called prominin-1, is a common biomarker of CSCs, which encodes a 120-kDa five-transmembrane domain glycoprotein. Its dysregulation has been considered as a CSC biomarker in various human cancers including OSA (20, 21), and it is correlated with VM, presence of metastasis, and poor prognosis in different tumors (21, 22). Little is still known about the mechanisms used by CSCs for promoting angiogenesis and VM (23). In this respect, the ability of CD133 to activate the Wingless-related integration site (Wnt) signaling pathway, thus increasing the expression of VEGFα and interleukin-8 (IL-8) (24), and its mechanistic link with cell motility (25), may be involved in the VM process.

ALDH1 is another common biomarker of dysregulated CSCs in a variety of human cancers (26, 27), the inhibition of which could represent a target in OSA therapy (28, 29). In the study of Bao et al. (30) multivariate analysis data showed that the expression of CD133, ALDH1, and VM; grade of differentiation; recurrence; as well as Enneking stages were independent prognostic factors for OSA patients. Despite the identified correlation with prognosis, the presence of CSC markers lining VM-dependent vessels has not been demonstrated in OSA tissues, even though CD133+ stem-like cell accumulation has been observed in the melanoma perivascular niche (30, 31).

## CD133 and ALDH1 as Canine CSC Markers and Their Expression in Madin–Darby Canine Kidney Cells

The general structure of prominin-1, including its membrane topology, has been conserved throughout the animal kingdom (32). Non-tumor canine cells, in particular MDCK cells, have been widely used for understanding the mechanisms on the basis of cell motility. In fact, considering that prominin-1 is associated with plasma membrane protrusions, the overexpression of *Prom1* gene increased the number of MDCK microvilli, while the overexpression of a dominant-negative mutant variant significantly decreased ciliary length (25). The involvement of CD133 in cell motility was also demonstrated by Liu et al., showing the ability of isolated canine CD133+ epithelial cells to form a tubular-like structure when cultured on Matrigel (33). Likewise, endothelial progenitor cells isolated from canine bone marrow CD133+ are capable of forming a capillary structure on Matrigel after 24 h of culture and can be transplanted in ischemic injured tissues to enable neovascularization (34, 35).

In cancer, CD133 staining, together with functional properties including ALDH enzyme activity and spheroids formation *in vitro*, is commonly used to characterize potential CSCs in canine OSA and others types of spontaneous canine cancer, not only those deriving from a hematopoietic lineage (36–40). Immunohistochemical investigations revealed that CD133 was expressed in all grades of OSA, glioma, melanoma, hepatocellular carcinoma, B-cell lymphoma, and granular cell tumor, with a higher proportion of positive cells in high-grade tumors (41–45).

Although a direct association between CD133 and VM has not been investigated in canine tumors, CD133+ cancer cells showed different features linked to VM both *in vivo* and *in vitro*. Highly invasive and tumorigenic canine insulinoma CSC-like cells and canine prostate cancer cellsCD133+ showed

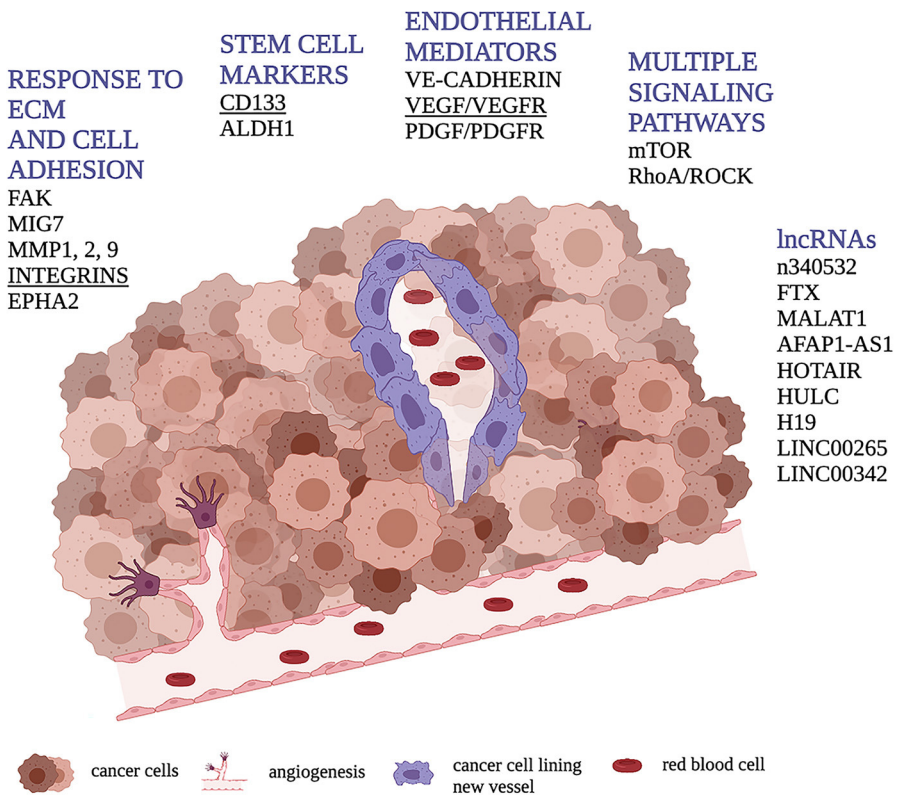


**FIGURE 1 |** Schematic representation of VM through cancer cells (in purple) forming a vessel containing red blood cells. Figure shows the main molecular pathways involved in the VM process in human osteosarcoma highlighting, in underlined bold, those found to be related with VM presence or tubular/vessel-like formation *in vitro* in dog.

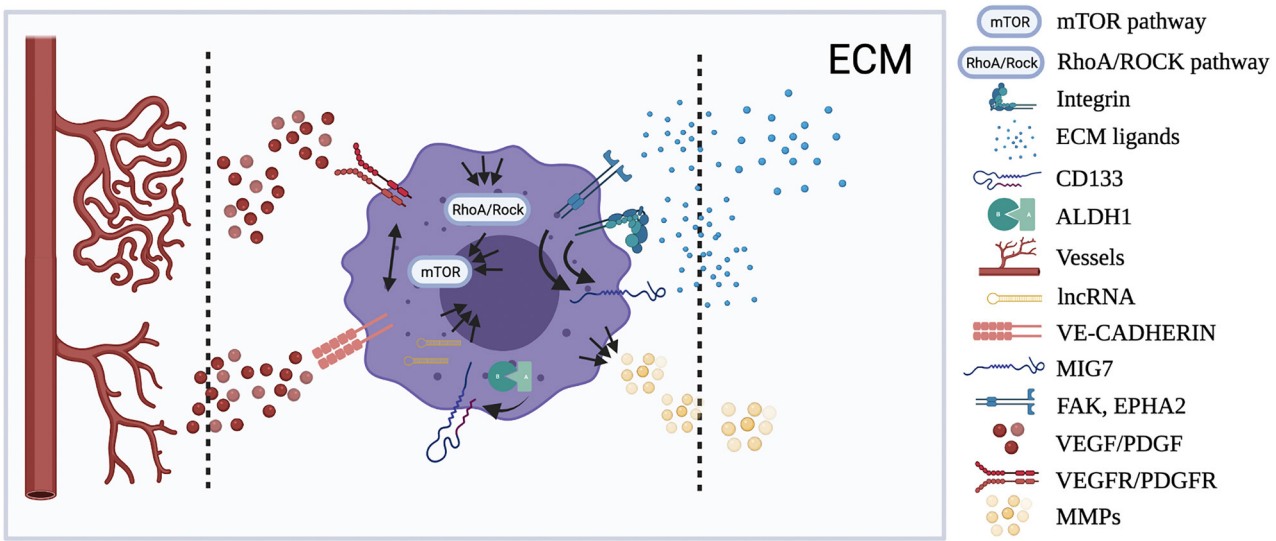


**FIGURE 2 |** Localization of the principal molecular pathways involved in VM. Figure shows the cellular and tumor microenvironmental distribution of the human OSA pathways resumed in the review showing, when known, the possible interactions (black arrow) between them. Multiple arrows show multiple interactions between pathways.

an invasive and tumorigenic phenotype *in vivo*, similar to hepatocellular carcinoma and lung adenocarcinoma cell lines that were also capable of forming spheroids in culture (46–49). CD133+ hemangiosarcoma cell lines cultured under normal and sphere-forming conditions generated three distinct tumor subtypes *in vitro*, associated with angiogenesis, inflammation, and adipogenesis (50). CD133+ canine cell lines derived from OSA, melanoma, transitional cell carcinoma, and lung adenocarcinoma resulted to be significantly resistant against X-ray irradiation (38). Gatti et al. observed that canine OSA primary cultures containing CD133+ CSCs exhibited distinctive sensitivity to anticancer agents (51), as well as spheroids derived from canine mammary gland adenocarcinoma (52).

As far as ALDH1 is concerned, despite ALDH enzymatic activity being also considered a cancer marker in canine samples (37, 53, 54), to the best of our knowledge, its protein and gene expression has not been directly investigated in canine tumors, as well as in normal tissues or in other canine pathological conditions (55–57).

## ENDOTHELIAL MEDIATORS

VM takes place independently of angiogenesis or endothelial cell proliferation, although it is often associated with endothelial marker expression (15). Gene expression analysis showed that aggressive tumor cells capable of VM display a diversified gene profile, expressing genes from multiple cell types including those of endothelial cells (58). In fact, the concept of "embryonic-like and vascular phenotype in the absence of endothelial markers," referred to as the first histological definition of VM (1), is controversial. In this respect, it has been demonstrated that primary and established sarcoma cell lines, after prolonged stimulation with post-surgery fluids from a cohort of patients affected by giant cell tumors of bone, transdifferentiated into VE-Cadherin+ and CD31+tubular-like structures (59). For this reason, the term "endothelial mediators" (and not endothelial markers) is preferred, to avoid controversy concerning the attribution of specific endothelial markers to highly aggressive cells that undergo VM. In several tumors, especially melanoma, an important group of endothelial mediators has been found in association with VM, including VE-cadherin (60–62) and VEGFR1 (63).

In MG63 OSA cells, the inhibition of *Cdh5* gene encoding for VE-cadherin with small interfering RNA (siRNA) reduced the ability of cells to form endothelial-like networks when cultured on type I collagen or Matrigel (64), and the same phenomenon has been observed in silencing the *Vegf* gene (65). In fact, autocrine VEGF/VEGFR1 signaling, associated with increased tumor growth and tumor vascularity, may possibly confer the capacity to develop vasculogenic properties to OSA cells (66). In recent studies, differentially expressed genes (DEGs) were investigated between different OSA cells cultured on Matrigel for profiling the molecular patterns involved in VM phenotypes. Results from these studies showed that the endothelial mediators PDGFRα and PDGFRβ were correlated with malignancy and tubular-like structure formation *in vitro* (67, 68).

### VE-Cadherin as Regulator of EMT and Vascular Integrity in Canine Pathology

VE-cadherin is an endothelial cell-specific cadherin that functions to stabilize cell structure because of its involvement in calcium-dependent intercellular adhesion (69). In dog, it has not been linked with VM, nor investigated in MDCK cells, although its role in EMT, a process closely related to VM and vascular integrity, has been explored (70, 71). In fact, VE-cadherin gene expression and immunohistochemical staining was evaluated in canine myxomatous mitral valve disease to investigate the role of EMT in chronic valvulopathies, showing a significant *cdh5* gene dysregulation (72).

In cancer, VE-cadherin protein expression was observed at intercellular junctions in both normal canine tissue-derived cells (NECs) and in canine tumor-derived cells (TECs), isolated from thyroid carcinoma and perianal gland epithelioma. The observed zigzag pattern in TECs, with respect to the linear in NECs, may be indicative of VE-cadherin dysfunction and increased vascular permeability, probably dependent on the high concentration of VEGF in the tumor microenvironment *in vivo*. In fact, an abnormal VE-cadherin expression pattern was observed in 100% confluent NECs, following culture in a tumor-conditioned medium containing excessive VEGF (73). Moreover, this study showed that Combretastatin A-4 phosphate (CA4P) has selective effects on TEC morphology and NECs in tumor culture conditions, also disrupting vasculature in canine OSA xenografted into mice (74). Furthermore, genome-wide methylation analysis performed in canine mammary tumors showed a significant hypermethylation at the PAX5 (paired box protein 5) motifs in the intron regions of *cdh5* gene and a consequent gene down-regulation (75).

### VEGF/VEGFR Axis in Relation to VM in Canine Osteosarcoma and Mammary Tumors

As far as VEGF/VEGFR axis in veterinary oncology is concerned, VEGF family members were identified in several canine cancers (76), as well as OSA tissue, serum, and cultured cells (77–79). A relation between VM and VERGFR was found in D17 canine OSA cells cultured on type I collagen where malignant cancer cells with endothelial morphology express VEGFR1 (14). Correlation between VM and VEGF axis has been firstly investigated in dogs with mammary tumors (7). VEGFα, VEGFγ, and VEGFR3 were expressed in spontaneous canine mammary tumor and xenograft models (80), showing increased expression in the inflammatory mammary carcinoma (IMC) model compared to non-IMC and mammary OSA (80, 81). VM has been shown to occur more frequently in IC compared with other types of canine mammary tumors (7). Furthermore, overexpression of VEGFα, VEGFγ, and VEGFR3 was observed in canine malignant non-IMC, and it was correlated with cyclooxygenase 2 (COX2) immunoexpression, which is particularly related to VM progression (82, 83).

Another indirect relation to VM can be found in the study of Cam et al. in which VEGFα expression in different OSA cell lines and its correlation with ΔNp63 and cell migration on Matrigel

was described, demonstrating that ΔNp63 exerts its angiogenesis and invasion property through VEGFα (84). It has been also demonstrated that VEGFα is the direct target of miR34a, which is less expressed in OSA cell lines with respect to normal osteoblast; OSA cells that have been induced to overexpressing miR34a show decreased motility and invasion ability on Matrigel and increased levels of VEGFα (85). On the other hand, a correlation between VEGFα transcript and chemical hypoxia was not observed (77).

As far as OSA *ex vivo* samples are concerned, literature data regarding VEGF axis and VM are lacking. Moreover, no correlation was observed between VEGF expression and clinicopathological parameters or hypoxia markers, which are often related to VM (77). On the contrary, its serum concentration has been previously correlated with poor prognosis in canine OSA (86). Increased levels of VEGF in serum or in cell supernatant were also observed after treatment of canine OSA with tyrosine kinase inhibitors such as Toceranib, Erlotinib, and Masitinib mesylate, probably due to a mechanism of feedback response to VEGFR2 inhibition (87–90).

## PDGF/PDGFR Axis in Osteosarcoma and Other Canine Tumors

An extensive knowledge concerning PDGF/PDGFR axis is available in veterinary literature. This molecular axis has been investigated as endothelial marker (91), in wound healing (92), spontaneous canine astrocytoma (93), fibrosarcoma (94), squamous cell carcinoma (95), lymphoma (96), prostate cancer (97), hemangioma and hemangiosarcoma (98), melanoma (99), mast cell tumors (100), hepatocellular carcinoma (101), mammary tumors (102), and nervous system tumors (103, 104).

PDGFs and PDGFRs were also found to be coexpressed and overexpressed in canine OSA, suggesting an autocrine and/or paracrine loop. In particular, in the study of Maniscalco et al. (105) all evaluated canine OSA cell lines overexpressed PDGFRα, while 6/7 overexpressed PDGFRβ, when compared to a normal osteoblastic cell line (106). The involvement of an autocrine loop of PDGF signaling pathway in the pathogenesis of canine OSA was confirmed in other studies, showing the overexpression of *cis*, the coding gene of PDGFRβ, in a OSA cell line (CO8), and the ability of its supernatant to induce tyrosine phosphorylation and therefore the activation of the PDGFRα and PDGFRβ on murine 3T3 cells (107, 108). Meyer et al. demonstrated that, in addition to tumor cells, giant cells and osteoblasts in canine OSA were positive for PDGFBB immunostaining, composed of two subunits β, also showing the detection of its mRNA in all study cases (109). Finally, the dysregulation of the expression levels of PDGFRβ in canine OSA has been attributed to the strong demethylation of CpG sites within the promoter (110).

No evidence currently exists concerning a relationship between VM and PDGF/PDGFR axis in canine oncology. Furthermore, no significant correlation was observed between the expression of these molecules and survival or histological grading in canine OSA (105). Despite this, the significant relation of this axis with malignant features of canine OSA has been observed both *in vivo* and *in vitro* (111). In fact, treating OSA cells with Toracenib, a potent inhibitor of PDGFRs, has been shown to induce a decrease in cell growth, migration, motility, and colony formation, as well as a significant blunting of tumor growth and proliferation index in an orthotopic xenograft model (111). These findings suggest that PDGF/PDGFR axis can represent a target therapy more than a diagnostic tool. With the coming of new technologies linked to miRNA, miR34a was tested on OSA cell lines and xenograft mouse models, showing PDGFRα reduction, together with decrease in cell proliferation and migration *in vitro* and tumor growth *in vivo* (112).

# RESPONSE TO ECM ENVIRONMENT AND CELL ADHESION

Among the myriad of microenvironmental factors affecting cancer cell resistance, cell adhesion to the ECM has been recently identified as a key determinant (113). FAK is a non-receptor tyrosine kinase that mediates signaling events downstream of integrin engagement of the ECM, regulating cell survival, proliferation, and migration and supporting neovascularization and maintenance of CSCs (114). FAK is expressed in different cancer types, where it is involved in the progression of tumor aggressiveness. Small molecule FAK inhibitors in clinical phase trials demonstrated to be effective in cancer by inducing tumor cell apoptosis in addition to reducing metastasis and angiogenesis (115). Association between FAK and VM or invasive behavior has been observed in different cancer types, including OSA. Ren et al. showed FAK staining in the cytoplasm of OSA tissue cells with high intensity around VM vessels (116). Similarly, *Mig7* gene was expressed in the cytoplasm with higher percentage of positivity in the VM with respect to non-VM group, suggesting an association between Mig7expression and VM formation and identifying in VM a prognostic marker of OSA (116). *Mig7* protein is enriched in embryonic cytotrophoblast cells during placental development and in more than 80% of tumors compared to normal tissue samples and blood from normal subjects (117). It was found to colocalize with VE-cadherin in cells lining VM structures in a lymph node metastasis (118) and to initiate a signaling cascade that results in tumor VM (119, 120). Moreover, *Mig7* knockdown inhibited tubular-like vessel formation and invasion of MG63 and 143B OSA cells cultured on Matrigel, as well as growth and metastasis of OSA cells in a mouse model (121). *Parispolyphylla*, from traditional Chinese medicine, inhibited cell migration, invasion, and VM formation *in vitro* and *in vivo* by reducing expression of FAK, Mig7, MMP2 (gelatinase A), and MMP9 (gelatinase B) (122). MMP1 (interstitial collagenase) also resulted to be the first upregulated gene among the DEGs of the abovementioned studies performed on OSA cells cultured on Matrigel (67, 68).

Among the plethora of membrane proteins interacting with the ECM, integrin-α2 (ITGA2) has acquired an important role for its involvement in tumor cell proliferation, invasion, metastasis, and angiogenesis. In fact, its abnormal expression correlates with unfavorable prognosis in multiple types of cancer (123). *Itga2* gene overexpression has been reported to be related to increased OSA metastasis and invasion (124) and was upregulated in malignant OSA cells *in vitro* (68). In the

study of Yao et al., gene signal transduction networks (Signalnet) were performed to identify the key genes involved in VM formation in OSA and the top-ranked ones resulted to be *Itga2*, integrin subunit alpha 1 (*Itga1*) and integrin subunit alpha 6 (*Itga6*) together with protein kinase cAMP-activated catalytic subunit beta (*Prkacb*), actinin alpha 1(*Actn1*), actinin alpha 4 (*Actn4*), phospholipase C beta 4 (*Plcb4*), gap junction protein alpha 1(*Gja1*), and the already mentioned gene encoding for PDGFRβ and PDGFα. Finally, this study demonstrated that *Itga1* knockdown inhibited VM formation by 143B cells *in vitro* and *in vivo* (68).

In addition, the tyrosine kinase EphA2, which belongs to the family of Eph tyrosine kinase receptors, is highly expressed in tumors, while it has been found at relatively low levels in most normal adult tissues, indicating its potential application in cancer treatment (125). Recent evidence suggests that VM occurrence is positively correlated with high expression of EphA2 and that its gene silencing inhibits VM formation (126). Interesting is also the correlation with Epstein–Barr virus (EBV) infection that stimulates plasticity in epithelial cells to express an endothelial phenotype (127). As well, Zhang et al. demonstrated that *Epha2* gene silencing inhibited VM formation in MG63 OSA cells (128).

## FAK Protein in Canine Tumor Progression

Interactions between tumor cells and tumor microenvironment are considered critical in carcinogenesis, tumor invasion, and metastasis (129). The involvement of adhesion proteins in canine OSA has been demonstrated through an expression profiling comparison between dogs with disease-free intervals (DFI) of $<100$ and $>300$ days (130).

The study of Brachelente et al. exploring the differential expression between melanomas and melanocytomas, identified differentially expressed gene clusters including nine genes belonging to the focal adhesion family (129). As far as FAK protein in humans is concerned, it is well-established that FAK serves as a scaffold for multiple protein signaling complexes, and its scaffolding function is very important for tumor progression (131). In canine oncology, interesting results were shown by Rizzo et al., demonstrating that the treatment of highly invasive D17 cells and other two OSA cell lines with Sulforaphane significantly decreased the phosphorylated state of FAK, also diminishing the invasion ability of cells cultured on Matrigel (132). These findings indirectly suggest a correlation between FAK activity and VM, considering that the inhibition of D17 OSA cell invasiveness corresponds to a decrease of VM features *in vitro* (14). Moreover, inhibition of FAK phosphorylation improved migration of canine hemangiosarcoma cells (133). FAK-mediated signaling was induced by numerous microenvironmental inputs and plays a central role in tumor-associated EMT and epithelial cells extrusion, migration, and response to the transforming growth factorβ (TGFβ) and the hepatocyte growth factor (HGF), as often demonstrated on MDCK cells (134–140). The use of these cells has also allowed understanding the involvement of FAK in the EMT induced by latent membrane protein 1 (LMP1) of EBV (141). Finally, the FAK inhibitor Masitinib mesylate (AB1010) has been the first anticancer therapy approved in veterinary medicine for the treatment of unresectable canine mast cell tumors (142).

## MMPs in Canine Tumors

In veterinary literature, current knowledge on the activity and function of proteases and stroma and their relationship with canine cancer malignancy is still limited (143), despite the fact that MMPs have been widely explored in several human cancers and are strictly related to the VM process (144, 145). Inhibition of extracellular proteolysis, in particular of collagenases MMP1, MMP2, and MMP9, is recognized as a valid approach to canine cancer therapy including OSA (146). In fact, Doxycycline at doses $>5\,\mu g/ml$ significantly decreased OSA cell proliferation and MMP1 activity *in vitro* (147).

*Mmp1* is the most significantly downregulated gene in Hsp70 knockdown canine OSA cells, and increased expression of *mmp2* and *mmp9* was linked to increased invasive capability in canine OSA (78, 148, 149).

Furthermore, MMP2 and MMP9 enzyme activity was found by means of zymography in three high malignant OSA cell lines (150).

The association between collagenase expression and activity and histological grade has also been demonstrated in canine mast cell tumor and lymphoma, together with VEGF dysregulation (151, 152), in mammary tumors, in relation to E-cadherin (153, 154), and in chondrosarcoma (153, 155–160). No differences in MMP9 expression were observed between IMC and non-IMC, although its expression was associated with higher nuclear grade in IMC tumors (161). As well, MMP2 and MMP9 dysregulation was found in canine oronasal tumors, hemangiosarcomas, and meningiomas, not always in association with malignant morphological patterns (143).

## Integrin Signaling in MDCK Cells and Canine Cancers

Integrin subunits may combine each other to affect the characteristics of cancer cells and the progression of tumors, both binding with proteins that directly regulate the actin cytoskeleton of cells and by phosphorylating the relative kinases, including FAKs (162). It is well-known that integrin complexes bind ECM components to promote cell adhesion and invasion, also mediating tissue tropism (163, 164). MDCK cells were used to demonstrate that α2β1 integrin mediates adhesion to types I and IV collagen in an $Mg^{2+}$-dependent manner, thus improving cell survival, EMT, cell spreading, and brunching morphogenesis. Furthermore, overexpression of Galectin8, which activates selective β1-integrins involved in EMT, promotes oncogenic-like transformation of MDCK cells (134, 154, 165, 166).

In veterinary oncology, a deregulation of integrin pathway, together with Wnt and chemokine/cytokine signaling, has been found in relation to short survival in canine OSA (167). The expressions of β1 integrin and α5β1 complex were immunohistochemically evaluated in a series of normal, dysplastic, and neoplastic canine mammary glands, and in lymph node metastases (168, 169), while β2 integrin was found in canine cutaneous histiocytoma (170). Finally, canine hemangiosarcoma cell lines expressing several endothelial

mediators including VEGF and αvβ3 integrin recapitulate features of mitotically activated endothelia and stimulate robust angiogenic responses in mice, forming tumor masses composed of aberrant vascular channels. Furthermore, they showed anchorage-independent growth and were motile and invasive, forming vessel-like structures when cultured on a basement membrane matrix (171, 172).

## EphA2 Inhibition in Canine Tumor Therapy and Its Mechanisms of Action

Targeting EphA2 represents an important goal in the development of recent anti-cancer drugs also in veterinary medicine, as shown by the attempt to evaluate the mechanism of Desanitib in the treatment of canine histiocytic sarcoma and the development of a cytotoxic compound that targets EphA2, EphA3, EphAB2, and interleukin 31 receptor A2 (IL31RA2) in canine high-grade gliomas (173, 174). The inhibition of EphA2 and IL31RA activity reduced up to 94% of tumor volume in 50% of dogs in the cohort (175). Furthermore, dogs were used to test the performance of a nanotherapeutic encapsulating a hydrolytically sensitive Docetaxel prodrug and conjugated to an antibody specific for EphA2, demonstrating an improvement in tumor penetration and antitumor activity (174). In *ex vivo* specimens, EphA2 resulted to be highly overexpressed in neoplastic cells of canine appendicular OSA, together with EphA3 (176). *In vitro*, *ephA2* expression was increased by up to 60-fold in canine prostate carcinoma lines derived from lung or bone metastases (177). MDCK cells were used to demonstrate the role of EphA2 in the epithelial morphogenesis in 3D culture and in the apical extrusion of transformed epithelial cells as a protective event. MDCK cells were also used to investigate EphA2 role in the decreased integration of claudin4 into sites of cell–cell contact as tumorigenic trigger and in the anoikis resistance process (178–181).

# mTOR AND RhoA/ROCK PATHWAYS

DEP domain-containing mTOR-interacting protein (DEPTOR) is an important modulator of mTOR, a kinase at the center of two important protein complexes named mTORC1 and mTORC2 (182). DEPTOR is able to interact with mTOR, thus inhibiting its kinase activity. It is involved in several molecular pathways controlling cellular homeostasis and it can behave either as an oncogene or oncosuppressor, depending on the cell or tissue type (183). It has been demonstrated that DEPTOR knockdown significantly decreased the number of tube-like structures and the invasion ability of the methylnitronitrosoguanidine transformed human OSA cells (MNNG/HOS) (184).

RhoA/ROCK pathway is a versatile regulator of multiple cellular processes, and it is dysregulated in several cancers. Recently, ROCK has attracted attention for its crucial role in angiogenesis, in regulating permeability, migration, proliferation, and tubulogenesis of endothelial cells (185). RhoA/ROCK stabilizes HIF1α during hypoxia inducing VM in hepatocellular carcinoma (186). Moreover, RhoA/ROCK expression was found to be higher in human OSA tissues and in the human OSA cell line U2OS with respect to control. Inhibition of RhoA/ROCK signaling pathway by the pharmacological inhibitor Fasudil reduced vascular-like channels in U2OS and melanoma cells cultured on Matrigel, decreasing cell plasticity and motility, both of which play key roles in VM formation (187, 188).

## Role of mTOR Pathway in Canine MDCK Cells and Cancers

mTOR pathway belongs to the series of conserved pathways that impact upon longevity and aging-related diseases such as cancer (189). Phosphatidyl inositol 3-kinase (PI3K)-AKT-mTOR was identified as one of the most relevant pathways involved in OSA progression both in humans and canines (190). The screening of protein kinase inhibitor compounds, particularly against PI3K-AKT-mTOR activity, represents an important topic of canine OSA therapy (191–193). Although the effect of the aberrant PI3K-AKT-mTOR signaling on tumor cell proliferation and apoptosis is well-known in canine OSA, the relation between mTOR and migration, invasion, and angiogenesis properties has been better explored in other types of canine cancer including hemangiosarcoma (194), prostate cancer (195), mammary tumors (196, 197), melanoma (198), and mast cell tumors (199).

Of relevance, MDCK cell model was used to demonstrate that mTOR signaling plays important roles in the regulation of epithelial tubule formation on Matrigel. It was observed that PI3-kinase regulates early epithelial remodeling stages, while mTOR modulates latter stages of tubule development (200), suggesting a possible involvement of mTOR pathway in VM progression. To the best of our knowledge, there are no studies investigating mTOR modulation mediated by the DEPTOR domain in dog.

## RhoA/ROCK in Canine MDCK Cells

Considering that cell migration plays crucial roles in cancer cell invasion, the study of mechanisms of junction and cytoskeletal organization mediated by guanosine triphosphatases (GTPases) of the Rho family has acquired great importance (201, 202). RhoA/ROCK pathway has been widely investigated in MDCK cells as a model of cell migration, cell-cell interaction and adhesion, EMT promotion, and virus entry (201–204).

In Moloney sarcoma virus-(MDCK)-invasive (MSV-MDCK-INV) variant tumor cells, it has been observed that Rho/ROCK activation may affect tumor cell migration and metastasis by stimulating the pseudopodal translocation of mRNAs and thereby regulating the expression of local signaling tumorigenic cascades (205, 206). RhoA hyperactivation can also influence normal MDCK cell polarity (Yu et al., 2008). The inhibition of RhoA pathway leads to a decrease of anchorage-independent growth of MDCK cells *in vitro* and in syngeneic mice, also downregulating *Cox2*gene (207, 208).

## LncRNAs

Non-coding RNAs, especially miRNAs and lncRNAs, have been widely investigated due to their roles as key players in regulating various biological and pathological processes involved in OSA progression, including cancer cell migration, invasion,

angiogenesis, and metastasis (209, 210). LncRNAs are non-coding transcripts >than 200 bp in length, and different studies demonstrated the influence of these molecules in gene expression at the epigenetic, transcriptional, and post-transcriptional levels. One of the most classical mechanisms through which lncRNAs regulate gene expression involves their association with chromatin modeling complexes and transcription factors, influencing transcriptional repression and activation of gene promoters (211).

Ren et al. profiled the expression of lncRNAs in highly aggressive OSA cell line 143B in comparison with its parental poorly aggressive cell line HOS, both plated on Matrigel. The top five upregulated lncRNAs were n337322, n333984, n381586, n338209, and TCONS_l2_00028738-XLOC_l2_014777, while the five downregulated lncRNAs were n334144, n342556,n410003, n335665, and ENST00000442174, also indicating that the top-ranked hub lncRNA that had the highest connections with the majority of the others in the network was n340532 (67). Through VM assay, this study also showed that VM ability of 143B cells strongly decreased following n340532 knockdown, as well as the number of metastatic nodules after injection of 143B cells stably transfected with sh-n340532 into nude mice. Tumor tissues collected from the sh-n340532 group exhibited a decreased number of VM channels compared to the control group (67). FTX and $MALAT_1$ were also strongly upregulated in this study. As far as FTX is concerned, its involvement in migration and metastasis was also previously demonstrated, as well as the induction of VM by $MALAT_1$ (16, 212).

Among others, lncRNA AFAP1-AS1 was found to be aberrantly expressed in OSA together with HOTAIR, HULC, and H19 that were upregulated in human OSA tissues and cell lines. Shi et al. also performed an in-depth investigation to explore the role and the mechanism of AFAP1-AS1 in OSA progression, demonstrating that the stable transfection of different OSA cell lines with siRNA AFAP1-AS1 strongly reduced their ability to form tube-like structures *in vitro*. In the same work, a concomitant decrease of EMT and RhoC/ROCK1/p38MAPK/Twist1 signaling pathway was also observed (213).

Moreover, differences between non-VM and VM cells compared in a microarray highlighted the significant overexpression of the lncRNAs LINC00265 and LINC00342 in the VMOSA cell line with respect to control. The study also confirmed that both LINC00265 and LINC00342 were upregulated in OSA tissues and that the high expression of LINC00265 was positively correlated with Spermine N1-Acetyltransferase 1 (*Sat1*) and Vav Guanine Nucleotide Exchange Factor 3 (*Vav3*) gene expression, as well as with poor prognosis. LINC00265 was also demonstrated to promote proliferation, migration, invasion, and tube formation via miR3825p targeting *Sat1* and *Vav3* genes in OSA cells cultured on Matrigel. SAT1 is a polyamine acetyltransferase that has a controversial role among different tumors, although it has been demonstrated to promote proliferation and metastasis of OSA cells both *in vitro* and *in vivo* (214). VAV3 is an important factor regulating angiogenesis and regulates the Rho/Rac family of GTPases involved in cell growth and motility (214).

## LncRNA in Dogs

Among the multiple epigenetic mechanisms found in canine cancer, DNA methylation and histone modification have been identified on the basis of OSA progression (211). Le Beguec et al. characterized the expression profiles of 10.444 canine lncRNAs in 26 distinct tissue types. Their study showed that lncRNA expression is mainly clustered by tissue type, highlighting that 44% of canine lncRNAs are expressed in a tissue-specific manner and also identifying more than 900 conserved dog-human lncRNAs (215). An alignment-free program that accurately annotates lncRNAs (FEELnc) was used on a real data set of 20 RNA-Seq from 16 different canine tissues, produced by the European LUPA consortium to expand the canine genome annotation, including 10.374 novel lncRNAs and 58.640 mRNAs transcripts (216). This work allowed identifying three new cancer susceptibility candidate lncRNAs in dogs, which are well-described in human cancer, including $MALAT_1$, that is associated with human VM and metastasis (16, 217). Other studies observed more than 900 dog-human conserved lncRNAs using comparative genomics, confirming the presence of well-studied lncRNAs in dogs, such as HOTAIR and $MALAT_1$ in canine B cell lymphoma and identifying lncRNAs differential expression as a prognostic tool (218–220). Of relevance, 417 differentially expressed lncRNAs were identified in canine oral melanomas in comparison with control samples, including the well-studied lncRNA ZEB2-AS, a lncRNA involved in the regulation of the transcription factor Zinc Finger E-Box Binding Homeobox 2 (*Zeb2*) during EMT in human colon, pancreatic, and breast cancer cell lines, as well as SOX21 Antisense Divergent Transcript 1(*Sox21-as1*) and Cancer Susceptibility 15(*Casc15)* (211, 221, 222). Finally, long non-coding transcripts from telomeres, called telomeric repeat-containing RNA (TERRA), were identified as blocking telomerase activity in canine tumor cell lines originated from soft tissue sarcomas (223). MDCK cells were also tested for the presence of tumorigenic lncRNAs, with the aim of preparing a safer and more reliable non-neoplastic MDCK cell line for vaccine production, founding several tumor-associated lncRNAs (224). Furthermore, a highly upregulated lncRNA in liver cancer was demonstrated to be a promoter during the epithelial and smooth-muscle-like differentiation of adipose-derived stem cells (ADSCs) via the bone morphogenetic protein 9(BMP9)/Wnt/β-catenin/Notch network (225). Genome-wide association studies (GWAS) identified a set of variants within the intron of a lncRNA upstream of the adrenoceptor beta 1(*Adrb1*) gene which is strongly associated with coat color. Two variants were found at high frequency in single-coated dogs and are rare in wolves (226).

# THERAPEUTIC POTENTIAL AND CURRENT LIMITATIONS

Both western and traditional Chinese medicines were used to evaluate a potential VM inhibition. Current anti-angiogenic drugs are often useless in the dampening of VM, inhibiting directly endothelial cell proliferation. At the same time, the consequent vascular density decrease can cause hypoxia in the tissue triggering VM as a compensatory stimulus (2).

The combination of drugs targeting VM and classical tumor angiogenesis can definitively reduce the blood and nutrient supply of tumors (227). Furthermore, in the era of chimeric antigen receptor (CAR)-T cell therapy, it is increasingly urgent to find specific markers for cancer management, and VM can represent an opportunity to find a cancer selective therapeutic target. In fact, in the VM process, multipotent tumor cells with CSC-like phenotype can transdifferentiate, generating ECM-rich, CD31-negative, and PAS-positive vascular networks, but CD31+, PAS-negative tubular-like structures have also been observed (6, 59). This evidence demonstrates that the mechanism of endothelial transdifferentiation of cancer cells within the tumor is still unclear, and this issue complicates the identification of specific cancer biomarkers. Recently, increasingly advanced *in vitro* models have been developed for the deeper investigation of this relative new process.

## CONCLUSION AND PERSPECTIVES

A growing body of evidence indicates that VM plays fundamental roles in tumor invasion, metastasis, and poor prognosis in human patients with malignant tumors, including OSA. Thus, VM may represent a potential novel target of anti-tumor therapy, even though the cellular mechanisms and molecular pathways by which VM is promoted have not been fully clarified. Endothelial mediators have been especially explored in human OSA and in veterinary oncology, together with the presence of CSC markers and the pathways involved in ECM interaction and cell adhesion. The molecular pathways involving VEGF/VEGFR and integrins have been found to be related to VM and vessel-like formation *in vitro* in canine oncology, while CD133 resulted to be determinant for tubular-like structure formation *in vitro* of canine normal cells (**Supplementary Table 1**). Information concerning the VM process and its biological implications in cancer is still limited in veterinary literature, despite the importance of canine tumor models in comparative oncology. The current knowledge concerning VM findings in human OSA, summarized in the present review, may provide a basis for stimulating future studies investigating VM in canine oncology as a possible target with great promise in cancer therapy.

## AUTHOR CONTRIBUTIONS

MM, MR, and LDS conceived and designed the review. MM and MR wrote the review. LDS and RDM supervised and guided the entire project. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

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# Natural Killer and T Cell Infiltration in Canine Osteosarcoma: Clinical Implications and Translational Relevance

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Metastatic osteosarcoma has a bleak prognosis in both humans and dogs, and there have been minimal therapeutic advances in recent decades to improve outcomes. Naturally occurring osteosarcoma in dogs is shown to be a highly suitable model for human osteosarcoma, and limited data suggest the similarities between species extend into immune responses to cancer. Studies show that immune infiltrates in canine osteosarcoma resemble those of human osteosarcoma, and the analysis of tumor immune constituents as predictors of therapeutic response is a promising direction for future research. Additionally, clinical studies in dogs have piloted the use of NK transfer to treat osteosarcoma and can serve as valuable precursors to clinical trials in humans. Cytotoxic lymphocytes in dogs and humans with osteosarcoma have increased activation and exhaustion markers within tumors compared with blood. Accordingly, NK and T cells have complex interactions among cancer cells and other immune cells, which can lead to changes in pathways that work both for and against the tumor. Studies focused on NK and T cell interactions within the tumor microenvironment can open the door to targeted therapies, such as checkpoint inhibitors. Specifically, PD-1/PD-L1 checkpoint expression is conserved across tumors in both species, but further characterization of PD-L1 in canine osteosarcoma is needed to assess its prognostic significance compared with humans. Ultimately, a comparative understanding of T and NK cells in the osteosarcoma tumor microenvironment in both dogs and humans can be a platform for translational studies that improve outcomes in both dogs and humans with this frequently aggressive disease.

**Keywords: osteosarcoma, NK cells, T cells, tumor microenvironment (TME), immunotherapy, canine model**

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## INTRODUCTION

Osteosarcoma (OSA) is an aggressive cancer of the skeleton in both dogs and humans with high rates of metastasis. Untreated, 90% of dogs with OSA develop metastasis within 1 year, and 85–90% of humans do so within 2 years (1). When gross metastatic disease develops, survival is dismal, and fewer than 20% of human patients survive 5 years and fewer than 5% of dogs survive 2 years with disseminated disease (2, 3). In the past few decades, there has been limited advancement of OSA

therapies, and outcomes for patients with metastatic disease have remained stagnant (4, 5). Canine OSA (cOSA) occurs spontaneously and shares notable genomic profiles, clinical presentations, and progression patterns with human OSA (hOSA) (1, 6–8). The intact immune system of dogs with naturally occurring cancer along with the relatively high incidence of cOSA and extensive similarities between cOSA and hOSA make companion dogs an ideal platform for translational oncology, especially in the investigation of novel immunotherapies (9, 10).

NK cells are innate immune cells with cytokine-producing and cytotoxic effector capabilities that have been identified in the OSA tumor microenvironment (TME) along with cytotoxic and helper T cells (11, 12). Both NK and CD8+ T cells have the capability to kill cancer cells using their cytotoxic functions, but their potential cooperation is complex. The downregulation of MHC-I by certain cancer cells effectively circumvents recognition by CD8+ T cells but simultaneously increases activation of NK cells by removing a major inhibitory signal (13). Additionally, IFN-γ secreted by NK cells stimulates CD4+ T cell activation and is required for proliferation of CD8+ T cell precursors (13). In many cancers, such as melanoma, gastric cancer, and myeloma, among others, secretion of IFN-γ is also shown to induce PD-L1 expression in tumor cells (14). IFN-γ-induced upregulation of PD-L1 expression on immune and tumor target cells is recognized as a conserved mechanism of adaptive immune resistance and tolerance as a response to chronic antigen stimulation, which is observed in both cancers and chronic pathogen exposure (15–17). These cooperative antitumor properties of NK and both CD4+ and CD8+ T cells are contrasted by studies showing that NK cells kill activated T cells to protect against virus-induced immunopathology (18, 19). Even among tumor-infiltrating T cells, tumor and immune cells expressing PD-L1 can inhibit neighboring PD-1+ T cells through the PD-1/PD-L1 axis, an immune checkpoint that cancer cells can exploit to inhibit antitumor immune responses (20). In humans, NK and T cells also show increased exhaustion markers in the solid TME, making reversal of the resulting immunosuppression a key aim of emerging immunotherapies (21). Veterinary studies also identify features of immune exhaustion in dogs with cancer (22, 23), but focused studies are needed to answer lingering questions of the consistency of these markers and how to target them. Analyses establishing the extent to which cOSA infiltrating NK and T cells are comparable to hOSA support a deeper understanding of the OSA TME and advance bench-to-bedside studies to speed the translation of novel immunotherapies. This review focuses on the recent literature characterizing NK and T cell infiltrates in OSA tumors and their prognostic significance in humans and dogs.

## BLOOD VS. TUMOR

The TME is made up of tumor cells, healthy stromal and nonimmune cells, and immune cells, all of which are communicating in dynamic interactions that work both for and against the tumor (24). These interactions occur in the context of a systemic immune response, including immune cell activity within the peripheral circulation, which, interestingly, does not inherently parallel activity in the TME (25–29).

In healthy dogs, CD4+ and CD8+ T cells comprise approximately 49 and 22% of lymphocytes, respectively, in peripheral blood, and T regulatory cells (Tregs) account for 4.5% of CD4+ T cells (25). Walter et al. (12) looked at peripheral immune responses in dogs prior to and following chemotherapy and found that dogs with osteosarcoma have fewer pretreatment CD4+ and CD8+ T cells in the blood than healthy dogs. Canine Tregs have also been identified and found to be higher in blood from dogs with OSA compared with healthy dogs (25, 30, 31). Later, the same working group established the clinical relevance of circulating lymphocytes in cOSA. For example, Sottnik et al. (32) observed that dogs with lower monocyte counts and lymphopenia prior to treatment with amputation and adjuvant chemotherapy had an increased disease-free interval (DFI). The authors call attention to the fact that this contrasts with human studies in which lymphopenia is associated with worse outcomes in sarcomas and other cancers (33). However, recent hOSA studies largely focus on lymphocytes in the context of other blood parameters, such as high neutrophil-to-lymphocyte ratios (NLRs) or low lymphocyte-to-monocyte ratios (LMRs), which are both associated with poor prognosis (34, 35). The necessity of lymphocyte ratios could be explained by the importance of other immune cell populations and the conflicting functions of different lymphocyte subsets, such as Tregs. For example, Biller et al. (25) analyzed CD4+ T, CD8+ T, and Treg (defined as CD4+FOXP3+) cells by flow cytometry in cOSA and found that low circulating CD8/Treg ratios were associated with shorter survival time. Investigation of NLR and LMR within cOSA are needed for an accurate comparison of the prognostic significance of circulating lymphocytes in dogs.

Although circulating CD8/Treg ratios were associated with a significantly worse prognosis, this was not seen in cOSA tumor-infiltrating lymphocytes (TIL), an indication of the differing immune populations between blood and tumors (25). This discordance is further substantiated with evidence from the same study that Tregs are highest in cOSA tumors, making up 21% of lymphocytes in the TME, compared with Tregs in the lymph nodes and circulation (25). The pattern stays consistent in mouse and human OSA, where, compared with blood, tumors have a higher concentration of Tregs as well as more activated Tregs based on cellular proliferation and increased expression of activation markers (26). The similarities extend to other immune cell subsets. A recent comparative study by Judge et al. (27) observed that proportions of T and NK cells (using CD3, CD8, and NKG2D by PCR as readouts) were significantly higher in peripheral blood compared with the TME in both cOSA and human sarcomas. The authors also found that, though tumors have low infiltration of lymphocytes, activation and exhaustion markers of infiltrating CD8+ T and NK cells are higher than those found in circulation (28). In another study, CD3+ T cells in hOSA similarly had significantly higher expression of exhaustion markers than those in peripheral blood (29).

Based on the current literature, both human and dog OSA tumors contain CD3+ T, CD8+ T, and NK cells, and the

activation and exhaustion of these immune cell subsets varies significantly between the tumor and circulation. The immune landscape of both the TME and peripheral circulation is important in identifying novel immunotherapies and patients most likely to respond to them (36). However, immunotherapies targeting immune cells in the TME, such as PD-1/PD-L1 inhibitors, have the added benefit of eliciting targeted antitumor responses, sometimes with minimal side effects (37). As a critical window into the mechanism of immune cell and solid tumor interaction, summarized in **Figure 1**, the remainder of this review focuses on the OSA TME specifically and characteristics of infiltrating T and NK cells.

## T CELLS

Recent evaluation of cOSA tumors from our group using immunohistochemistry (IHC) confirmed minimal CD3 infiltration compared with normal lymph nodes (27). There was varied cOSA intra-tumoral CD3 and CD8 gene expression after radiotherapy (RT) plus NK transfer, which did not correlate significantly with survival, acknowledging that sample size was a limiting factor (27). However, these results suggest that an immune "cold" cOSA tumor could be transformed into a "hot" tumor with immunotherapy (27). This hypothesis stems from increasing studies of lymphocyte infiltration, or immune score, in human cancers with higher levels indicating hot tumors and those with low infiltration being cold tumors, which may be more accurate in predicting survival than the tumor-node-metastasis staging system (38). The ability to increase immune scores therapeutically is demonstrated by Modiano et al. (39), who found that the percentage of CD3+ T cells in cOSA jumps from 8 to 17% after fas-ligand gene therapy. The increase in TILs also correlates with survival because dogs with greater lymphocyte infiltration after treatment had longer survival times than dogs with lower infiltration (39). Similarly, in hOSA, CD8+ cells were observed in the majority of tumors but only made up 1% of intra-tumor cells (40). Even with low CD8+ staining within hOSA tumors, CD8+ cells were still significantly associated with improved prognosis and also favorably predicted survival posttreatment with zoledronic acid (40). These results together provide evidence of OSA being an immunologically cold tumor that can be treated to increase immune cell activity and improve survival.

On the other hand, some studies show cOSA to have varying patterns of TILs. Biller et al. (25) were among the first to evaluate tumor infiltrates of cOSA, finding that tumors were relatively highly infiltrated, made up of 19.2% CD4+ and 8.6% CD8+ T cells, but TILs were not associated with survival. The discrepancy may be due to varying techniques as this study determined percentage of cells by flow cytometric analysis of strained tumor samples rather than IHC evaluation. But Withers et al. (41) later also showed evidence of varying degrees of infiltration using IHC with CD3+ cells ranging from 4.6 to 607.6 cells/mm$^2$ in cOSA tumors. Although CD3+ infiltrates alone were not prognostic, increased infiltration of CD204+ macrophages was associated with increased DFI, leading the authors to suggest that cOSA is an immunogenic tumor (41). In a second study, Withers et al. (42) further examined heterogeneity of infiltrates by comparing infiltrates within matched primary and metastatic cOSA tumors. They reported that overall immune infiltrates of the primary tumor correlated with a patient's metastatic lesions, but importantly, they also found that CD3+ and CD204+ macrophages were significantly higher in metastatic lung lesions compared with their primary tumor (42). The range of TILs in cOSA and inconsistent associations with survival, rather than conflicting each other, may point to intra-tumoral heterogeneity within cOSA and complicate the idea of cOSA being uniformly cold. Cascio et al. (43) found cOSA to have virtually no infiltration of CD3+ and CD8+ T cells within the tumors but found both subsets in much higher concentrations in the peri-tumor areas. This aligns well with the definition of "altered" or "excluded" tumors, an intermediate between hot and cold, that have T cells present in tumor margins that are excluded from entering the tumor (38). The presence of distinct immune subtypes with low, intermediate, and high immune infiltrate has already been described in hOSA and is shown to affect response to immunotherapy treatments (44). Each tumor type—cold, altered, or hot—has distinct features that make them more or less likely to respond to a specific treatment, such as checkpoint inhibitors or adoptive cell therapy (38, 44, 45). Based on the available literature, cOSA recapitulates the heterogeneity of immune infiltrates and distinct immune score subtypes seen in hOSA. Still, choosing therapeutics based on levels of immune infiltrates has not yet been explored expressly in cOSA, and further studies are needed to corroborate the use of immune score to predict response to treatment and survival as seen in humans.

## CHECKPOINT INHIBITORS: PD-1/PD-L1

Although beyond the scope of this review and reviewed in detail elsewhere (46, 47), an understanding of the PD-1/PD-L1 pathway is critical to understanding the interactions of T cells with tumor cells as well as other immune cells. PD-L1 is frequently upregulated on tumor cells, and its interaction with PD-1 on immune cells induces tumor tolerance and allows for immune evasion (46). PD-L1 is also found to be expressed on T cells in mouse models with PD-1+ T cells exhibiting multiform interactions that lead to protumor effects (20). Both anticanine PD-1 and PD-L1 therapeutic antibodies have been developed and proven to possess antitumor activity in dogs with cancer (48, 49).

The first study to look at PD-L1 in cOSA did not find expression in samples using IHC, although the study only had three cOSA samples (50). Subsequent studies have found that the majority or all cOSA samples evaluated by IHC express PD-L1 (51, 52). PD-L1 expression in cOSA tumors was likewise consistently found by Cascio et al. (43), whose results show that expression of PD-L1 is associated with resistance to T cell infiltration from the peri-tumor environment to within the tumor, but the study did not evaluate prognostic significance. Although the expression of PD-L1 varies in hOSA, it is consistently associated with TILs. Studies found that PD-L1

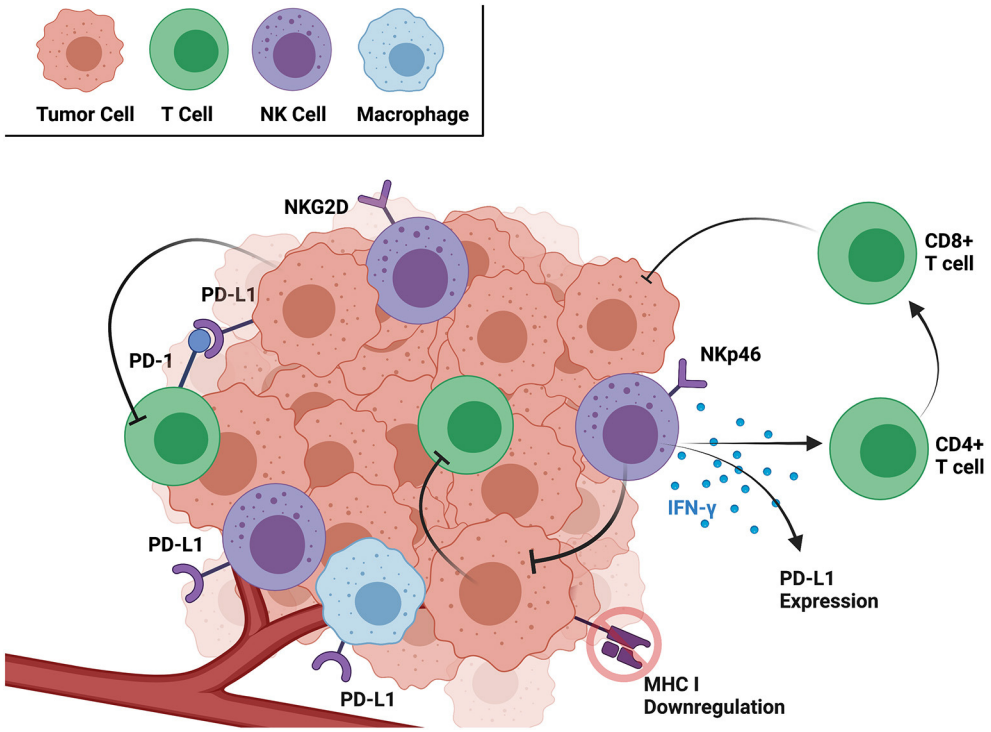


**FIGURE 1 |** Interactions between cancer and immune cells within the TME as well as relevant receptors and soluble factors. Created with BioRender.com.

is expressed in up to 25% of hOSA tumors and correlates with increased infiltration of PD-1+, CD3+, and CD56+ cells; however, there is no significant correlation to survival (53). A later study found that more than 43% of hOSA harbor PD-L1+ tumor cells with positive correlations to TILs (54). Similar to overall levels of immune infiltration in OSA, the impact of PD-L1 expression in hOSA is conflicting because PD-L1 expression is associated with a negative prognosis secondary to immune dysfunction and also better event-free survival and overall survival because of greater density of TILs and other immune cells (54). Additionally, an increase in PD-L1-expressing tumor-infiltrating immune cells is significantly associated with response to humanized anti-PD-L1 antibody (55), though the specific indications of these biomarkers for response to treatment varies within different cancer types (56). Consequently, further characterization of PD-L1 expressing cells in cOSA is needed for accurate comparison to human studies and investigation of cOSA's sensitivity to PD-1/PD-L1 blockade.

## NK CELLS

Even in scenarios in which T cells are present in the TME, cancer cells can suppress MHC-I expression, which is necessary for CD8+ T cells to recognize a target and enact their cytotoxic functions. NK cells, on the other hand, recognize "missing-self" or the lack of MHC-I molecules and can exert their cytotoxic functions in situations in which CD8+ T cells cannot, forming a basis of reasoning for their use in immunotherapies (13). This is seen specifically in hOSA, in which the majority of tumors showed diminished expression of MHC-I, and its downregulation is associated with a worse prognosis (57). NK cells are proven to be capable of lysing hOSA cells (58), and adoptive transfer of NK cells serves as a mechanism to increase the numbers of cytotoxic cells capable of targeting OSA cells *in vivo*. Canine and human NKp46+ NK cells show impressive similarities in expression of natural cytotoxicity receptors and secretion of factors, such as IFN-γ and TNF-α (59). In addition, NKp46+ is not expressed uniformly across NK cells, and its absence correlates with decreased cytotoxicity across species (59). The similarities in both NK cells and OSA in general make dogs an ideal candidate for comparative studies of NK cell infiltrates in OSA.

Mouse models of osteomyelitis with concurrent OSA were early implications of the role of innate immune cells, including NK cells, in the OSA antitumor response (60). Through NK cell depletion, NK cells were found to be critical in OSA tumor growth inhibition (60). One mechanism by which tumors continue to grow in the presence of NK cells may be through overexpression of TGF-β, a potent inhibitor of NK cells. Canine OSA tumors consistently stain positive for TGFβRI and TGFβRII (61), providing a rational for the expansion and transfer of expanded and TGF-β-imprinted NK cells in cOSA therapy (62, 63). Imprinting of NK cells involves prolonged coculture with IL-2 and TGF-β to produce NK cells that are desensitized to the inhibitory effects of TGF-β and thereby capable of prolonged hyperfunctionality with increased

cytotoxicity, cytokine production, and longevity. This approach has the potential for novel use in NK immunotherapies (63). In their phase I trial using hypofractionated RT and autologous intratumoral NK cell transfer in dogs with naturally occurring OSA, Canter et al. (64) demonstrate increased progression-free survival in dogs with OSA compared with historical controls. The same group collected tumor specimens from patients in this first-in-dog clinical trial and found that pre- to post-treatment immune-related gene transcript changes varied considerably between dogs (27). NK gene transcripts have significantly less expression of both CD3+ and CD8+ cells in untreated cOSA tumor samples, but there were no patterns of expression that significantly correlated with survival at six months post-treatment in paired samples (27). Intra-tumoral changes in expression of IL-6, a gene linked to cytotoxic lymphocytes, was higher in dogs with prolonged survival though statistical significance may have been limited by the sample size (27). Future clinical trials with increased sample sizes are needed to better evaluate the prognostic value of cOSA tumor-infiltrating NK cells and the therapeutic benefit of NK cell immunotherapy. It should be noted that the full characterization of canine NK cells and their surface markers is still in progress compared with human NK cells and could provide critical information in their use for NK immunotherapies (65). The use of NK cell transfer has not been explored extensively in hOSA, likely due to limiting factors in the sourcing and expansion of NK cells (66, 67), but early successes seen in cOSA can potentially drive translation of NK immunotherapy to clinical trials in humans.

## CONCLUSION

Osteosarcoma is an aggressive disease for which novel therapeutics are needed, and dogs with spontaneously occurring cancer are a useful model for hOSA studies. Both cOSA and hOSA share extensive similarities, including the frequency and phenotype of immune cells within the TME and peripheral circulation. The OSA TME constitutes a complex web of interactions, especially among NK and T cells, that can be targeted with immunotherapies. OSA tumors in both humans and dogs fall on a spectrum of immune infiltrate levels that correlate with prognosis, express PD-L1 with association to increased TILs, and show sensitivity to NK cell cytotoxicity. The parallels between cOSA and hOSA can be best put to used after filling the gaps in current knowledge regarding the characterization of the cOSA TME and immunotherapies to target it. Future studies in cOSA are needed to characterize NK cells and the expression of PD-1/PD-L1 in TILs as well as to validate the use of immune infiltrates to predict immune response to therapeutics. Increased understanding of intra-tumoral NK and T cells will influence clinical applications of TIL-targeting treatments in both dogs and humans, ultimately leading to better outcomes for patients with OSA.

## AUTHOR CONTRIBUTIONS

AR formulated the research topic, performed literature review and critical analysis, wrote, and edited the manuscript and figure. SJ, AG, SC, WC, MK, and RR provided comparative oncology expertise and critical review of the manuscript. RC formulated the research topic, provided comparative oncology and immunology expertise, and provided critical analysis and review of the manuscript. All authors contributed to the article and approved the submitted version.

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# A Pilot Study of Cancer-Induced Bone Pain Using Validated Owner Questionnaires, Serum N-Telopeptide Concentration, Kinetic Analysis, and PET/CT

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Cancer-induced bone pain, despite its frequency and severity, is a poorly understood phenomenon in people and animals. Despite excitement regarding translational osteosarcoma studies, there is a lack of attention toward examining cancer pain in dogs. In this pilot study, we used a multimodal pain assessment methodology to evaluate pain relief after therapeutic intervention in dogs with primary bone cancer. We hypothesized that intervention would cause objective evidence of pain relief. Evaluations of 8 dogs with primary bone cancer included $^{18}$F-FDG PET/CT scans, kinetic analysis, validated owner questionnaires (Canine Brief Pain Inventory, canine BPI), and serum N-telopeptide (NTx) concentration. Dogs were routinely staged and had $^{18}$F-FDG PET/CT scans prior to treatment with day 0, 7, 14, and 28 canine BPI, serum NTx, orthopedic exam, and kinetic analysis. Dogs treated with zoledronate and radiation underwent day 28 $^{18}$F-FDG PET scans. All clinical trial work was approved by the University of Missouri IACUC. Four dogs underwent amputation (AMP) for their appendicular bone tumors; four received neoadjuvant zoledronate and hypofractionated radiation therapy (ZOL+RT). Canine BPI revealed significant improvements in pain severity and pain interference scores compared to baseline for all dogs. Positive changes in peak vertical force (+16.7%) and vertical impulse (+29.1%) were noted at day 28 in ZOL+RT dogs. Dogs receiving ZOL+RT had a significant (at least 30%) reduction in serum NTx from baseline compared to amputated dogs ($p = 0.029$). $SUV_{max}$ ($p = 0.11$) and intensity ($p = 0.013$) values from PET scans decreased while tumor uniformity ($p = 0.017$) significantly increased in ZOL+RT-treated tumors; gross tumor volume did not change ($p = 0.78$). Owner questionnaires, kinetic analysis, and $^{18}$F-FDG PET/CT scans showed improved pain relief in dogs receiving ZOL+RT. Serum NTx levels likely do not directly measure pain, but rather the degree of systemic osteoclastic activity. Larger, prospective studies are warranted to identify the ideal objective indicator of pain relief; however, use of multiple assessors is presumably best. With improved assessment of pain severity and relief in dogs with cancer, we can

better evaluate the efficacy of our interventions. This could directly benefit people with cancer pain, potentially decreasing the amount of subtherapeutic novel drugs entering human clinical trials.

**Keywords: osteosarcoma, cancer-induced bone pain, comparative oncology, cancer pain, PET imaging**

## INTRODUCTION

The translational value of naturally occurring animal models of pain is understudied and underutilized (1). Cancer-induced bone pain (CIBP), despite its frequency and severity, is a poorly understood phenomenon in humans and animals. Bone is not only a common metastatic site in people; it is one of the most frequent initiators of cancer pain (2–5). The prevalence of metastatic to bone neoplasia and CIBP in veterinary patients is lacking. However, primary bone tumors in dogs, specifically osteosarcoma (OS), have a 10–50-fold increased incidence compared to their human counterparts (6). The similarities between human and canine OS are striking (7): bimodal age distribution, appendicular location, metastatic rate and route, genomic instability, and resistance to chemotherapy in the macroscopic setting. These parallels are exploited in nationwide collaborative efforts to better understand and treat this aggressive disease in dogs, with the hope of positively affecting outcome in their two-legged friends (8). Despite the significant push of comparative OS models, there is a lack of attention toward using the dog to study CIBP. A recent review of translational pain assessment focused on the gaps in currently used experimental animal models (1). The authors described a "crisis" of both translational and reproducible affect, which has contributed to drug development failure rates of over 90% once reaching human clinical phases. Benefits of using natural animal models (i.e., client-owned dogs) over experimental animal models (i.e., laboratory rodents) include the spontaneity of tumor occurrence, shared environments, long-term survival, and genetic diversity (1).

CIBP is multifactorial, consisting of both background and breakthrough pain (9). While background pain may be a dull ache that increases with disease progression, breakthrough pain is short, unpredictable, and difficult to treat with current analgesics (10). The components of CIBP are at minimum tumorigenic, inflammatory, and neurologic (11). Tumors, whether primary or metastatic, invade normal tissue and often induce osteoclasts to break down normal bone. This results in destruction of distal sensory fibers of cancellous/cortical nerves (12, 13) and a painful, acidic environment associated with osteoclastic resorption (14). Inflammatory molecules, whether released by pro- or antineoplastic cells, incite pain through nociceptive activity. Prostaglandins, endothelins, and nerve growth factor may directly activate sensory neurons or alter neurotransmitter expression (10, 11). The neurologic system responds to this inflammatory and cancerous milieu in complex ways. There is direct stimulation of both periosteal, cortical, and marrow sensory and sympathetic nerves (15). At the dorsal root ganglion (DRG), peripheral sensory nerves (whether nociceptive-specific or wide dynamic range) synapse with ascending neurons in the spinal cord (11). There, CIBP differs from simple inflammatory or neuropathic pain (16). Finally, neurochemicals, including substance P and glutamate, may further contribute to CIBP and excite the nervous system (11).

In human CIBP, the mainstay of therapy is radiation therapy (17). As previously mentioned, the complex nature of CIBP mandates multimodal therapy (10). Multiple studies have evaluated biomarkers to better quantify response of CIBP treated with radiotherapy. In a study of over 1,000 patients with bone metastases, a normalized bone-resorption marker (N-telopeptide, NTx) concentration after bisphosphonate treatment resulted in reduced risks of skeletal related events and death (18). However, a systematic review of clinical biomarkers of analgesic response to radiotherapy for CIBP showed no predictor of analgesic response (19). Because CIBP is not only caused by osteoclastic bone resorption or direct tumor destruction, better indicators of pain response are needed to modulate analgesic care.

In dogs, OS has been used as a model to study CIBP. Validation of subjective pain assessment was performed with a questionnaire based on the human Brief Pain Inventory (BPI) (20). This canine BPI was administered to owners of 100 dogs with bone cancer and reliably measured this comparative tumor model. Additionally, a group pioneering the use of bisphosphonates in dogs with CIBP evaluated the use of pamidronate, radiation therapy, and doxorubicin in canine OS. Subjective pain scores, urine NTx excretion, tumor relative bone mineral density, and pressure platform gait analysis were used to assess pain response (21). This same group also evaluated the expression of nociceptive ligands, including nerve growth factor, endothelin-1, and prostaglandin E2 in canine OS cells (22). Most recently, 13 dogs with OS were compared with control dogs to assess quantitative sensory testing, QST, in dogs receiving stepwise palliative analgesic therapy (23). While this study assessed techniques that evaluate central and peripheral sensitization, not all dogs finished the study, and disease progression may have affected the authors' ability to assess efficacy of the analgesics examined. To our knowledge, the use of a multimodal analgesic assessment involving PET scans in dogs treated with standard of care (surgical vs. non-surgical) has not been implemented. Therefore, we aimed to assess CIBP in dogs with multiple subjective and objective tools, while monitoring response during standard treatment for primary bone cancer.

## METHODS

### Trial Design

We designed a single-site, two-arm [arm 1 = amputation (AMP), arm 2 = zoledronate and radiation therapy (ZOL+RT)], pilot

study evaluating subjective and objective pain measures in client-owned dogs with malignant primary bone tumors presenting to the University of Missouri Veterinary Health Center (MU-VHC). Client-owned dogs of any age, sex, or breed and weighing >20 kg were eligible for enrollment. All dogs were diagnosed with a primary bone tumor of the appendicular skeleton *via* cytology or histopathology. Informed owner consent was collected under an approved University of Missouri Animal Care and Use Committee protocol (#9336). Dogs needed to have adequate bone marrow function as measured by complete blood count and normal organ function as measured by biochemical profile (ALT <3x ULN and normal creatinine) with the exception of ALP, an enzyme frequently elevated in dogs with OS (24). Treatment with NSAIDs and other analgesics was allowed prior to enrollment and during the trial; all dogs were receiving an NSAID before screening commenced. Dogs were not eligible for the study if they were treated with prior chemotherapy, radiation therapy, or surgery, if dogs had evidence of soft tissue or bone metastasis, or if dogs had significant comorbidities that would limit their expected lifespan. All dogs were screened for evidence of pulmonary neoplasia with thoracic radiographs, and lack of metastatic disease was confirmed on baseline whole-body PET/CT scans.

Dogs received baseline (screening) orthopedic examinations, weight/BMI, validated owner questionnaire (20), serum collection for NTx assays, kinetic analysis, and $^{18}$F-FDG PET/CT scans. Within 7 days of screening, treatment intervention was initiated. Treatments were standardized; however, dogs were not randomized. Ethically, it is inappropriate for veterinarians to mandate amputation for dogs deemed as poor amputation candidates. Client owners were given the option, based on orthopedic soundness, to pursue AMP [complete forequarter (scapulothoracic disarticulation) or hindquarter (femoroacetabular disarticulation)] or ZOL+RT for their dog. Dogs that received AMP had their malignant tumors confirmed by histopathology and could receive adjuvant chemotherapy, based on tumor confirmation and tumor grade. All ZOL+RT dogs received neoadjuvant 0.1 mg/kg zoledronic acid (Mylan Institutional LLC *via* McKesson, NDC 67457-390-54) 24 h prior to their first fraction of radiation therapy. All irradiated dogs received a total of 4 weekly fractions of 8 Gy (32 Gy total central axis dose) from a Siemens ONCOR Impression Plus linear accelerator (Siemens, Munich, Germany).

After starting treatment, dogs followed a weekly assessment of their pain control. This included a 7-, 14-, and 28-day orthopedic exam, owner questionnaire, kinetic analysis, and serum collection for NTx assay. At day 28, a repeat $^{18}$F-FDG PET/CT scan was performed for ZOL+RT dogs. Standard treatment and follow-up were continued in all dogs after cessation of the trial.

## Validated Pain Questionnaire

Client owners completed the validated canine Brief Pain Inventory (canine BPI) questionnaire, totaling 11 questions, at each visit: baseline, days 7, 14, and 28. The canine BPI tool can be found here: https://www.vet.upenn.edu/research/clinical-trials-vcic/our-services/pennchart/cbpi-tool. Treatment response assessments (positive responder or non-responder) were determined by assignment of canine BPI scores. Briefly, the canine BPI system involves assignment of scores ranging from 0 to 10 on the basis of the degree to which pain appears to interfere with 6 daily activities (Pain Interference Score or PIS; 0 = no interference and 10 = complete interference) and perceived pain severity (Pain Severity Score or PSS; 0 = no pain and 10 = severe pain). For this study, the mean PSS and PIS scores obtained for all dogs at each examination period were compared between treatments (AMP vs. ZOL+RT) and testing time points.

## Biomarker—Serum N-Telopeptide

Whole blood was collected *via* jugular venipuncture at each timepoint as previously described. Whole blood (10 ml) was allowed to clot at room temperature for ∼30–45 min. The sample was centrifuged at 2,000 × g for 15 min at room temperature to separate the serum. Leaving the clot undisturbed, serum was removed and placed in polypropylene cryovials. Samples were frozen within 1 h of collection in liquid nitrogen and stored at −80°C until the N-telopeptide assay was performed. Serum NTx concentrations were measured using a commercially available ELISA, Osteomark® NTX (Alere Scarborough, Inc., Scarborough, ME, USA). Values were expressed as normalized nanomolar (nM) bone collagen equivalents (BCE).

## Kinetic Analysis

Kinetic data were obtained at days 0 (baseline), 7, 14, and 28 by use of a pressure sensitive walkway (PSW) system (HR Walkway 4 VersaTek System, Tekscan Inc., South Boston, MA, USA). All dogs were walked on a leash by the same handler on the PSW in an isolated laboratory. Each dog was walked at a velocity of 0.9–1.2 m/s and an acceleration of ±0.5 m/s$^2$. The PSW was calibrated according to the manufacturer's specifications, and the vertical ground reaction force (GRF) data obtained from the PSW were reported and analyzed by use of designated software (I scan 5.23, Tekscan Inc, South Boston, MA, USA). Before data acquisition, each dog was weighed on an electronic scale and walked across the walkway a minimum of 3–5 times to allow habituation, or acclimation, to the environment, the PSW, handler, and leash. At least 10 trials were recorded for each dog, and data from the first 5 valid trials were analyzed. A valid trial included a straightforward walk without stopping, hesitating, trotting, or pacing; no overt head movement during the trial; and maintenance of a constant speed on the PSW within the defined velocity and acceleration ranges. For comparison, all values were reported as a percentage of body weight (%BW).

## $^{18}$F-FDG PET Scans

Dogs underwent baseline $^{18}$F-FDG PET/CT imaging with a Celesteion PET/CT system (Canon Medical Systems, Tustin, CA, USA) under general anesthesia. Dogs were administered a mean of 3.99 mCi (2.83–4.58 mCi) $^{18}$F-FDG IV 1 h prior to initiation of scan. Whole-body PET was performed, immediately followed by whole-body CT. Iodinated contrast (Omnipaque 350 at a dosage of 2 ml/kg) was administered, and 30-s and 3-min post-contrast infusion images were acquired. Image assessment

was performed by a board-certified radiologist (AZ) using MIM Software Inc. (Cleveland, OH, USA). Tumor $SUV_{Max}$, count intensity, and count uniformity were measured by region-of-interest (ROI) analysis using a combination of RayStation and 3D Slicer (Raysearch Labs, Stockholm, Sweden) (25). SUV, or standard uptake value, is a measure of isotope uptake over dose of $^{18}$F-FDG administered; it indicates metabolic activity of a specific tissue. Count intensity is a measure of the total counts of a tissue on the PET scan and is not normalized to dose administered. GTV, or gross tumor volume, is measured from the concurrent CT scan and allows one to measure tumor size. Tumor count uniformity is a measure of the metabolic (or other radioisotope) count rate heterogeneity among voxels within a tissue. For neoplastic lesions, the entire volume was contoured on MIM, and the whole tumor volume was evaluated. $SUV_{Max}$ was calculated as the highest point of activity within the tumor volume.

## Statistical Evaluation

This was a pilot trial to evaluate subjective and objective measures of pain. Intra- and inter-treatment arm comparisons were made for dogs receiving non-surgical therapy (ZOL+RT) or surgery (AMP). Data were collected at baseline and 7, 14, and 28 days. For canine BPI, the mean PSS and PIS scores obtained for all dogs at each examination period were compared with an analysis of variance and multiple comparisons were performed with Holm–Sidak's multiple-comparison test (**Figure 2**). For N-telopeptide (NTx) concentrations, data were obtained for all dogs at each examination period. For comparison, a response to therapy was defined by changes from baseline measurements.

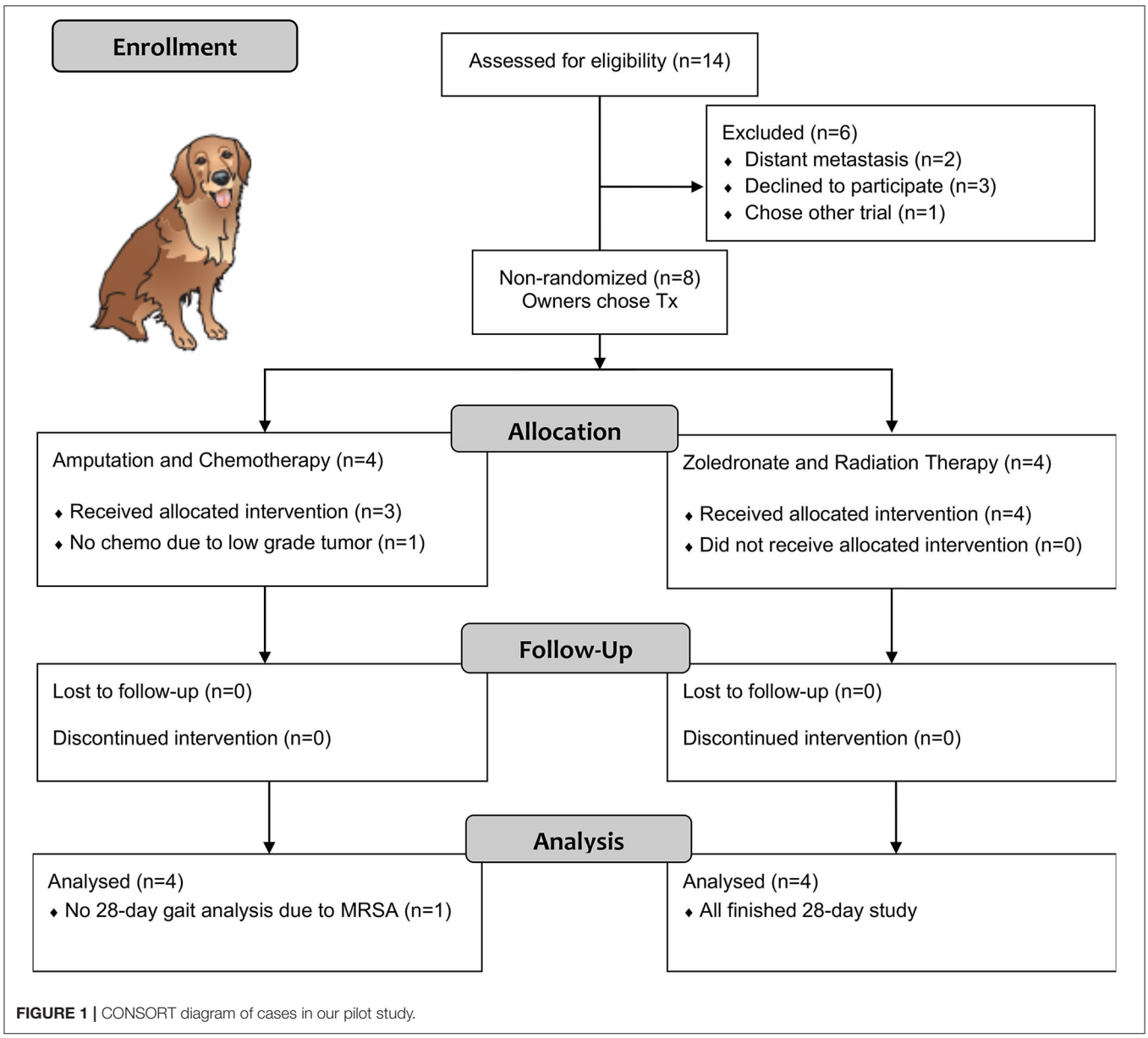


**FIGURE 1 |** CONSORT diagram of cases in our pilot study.

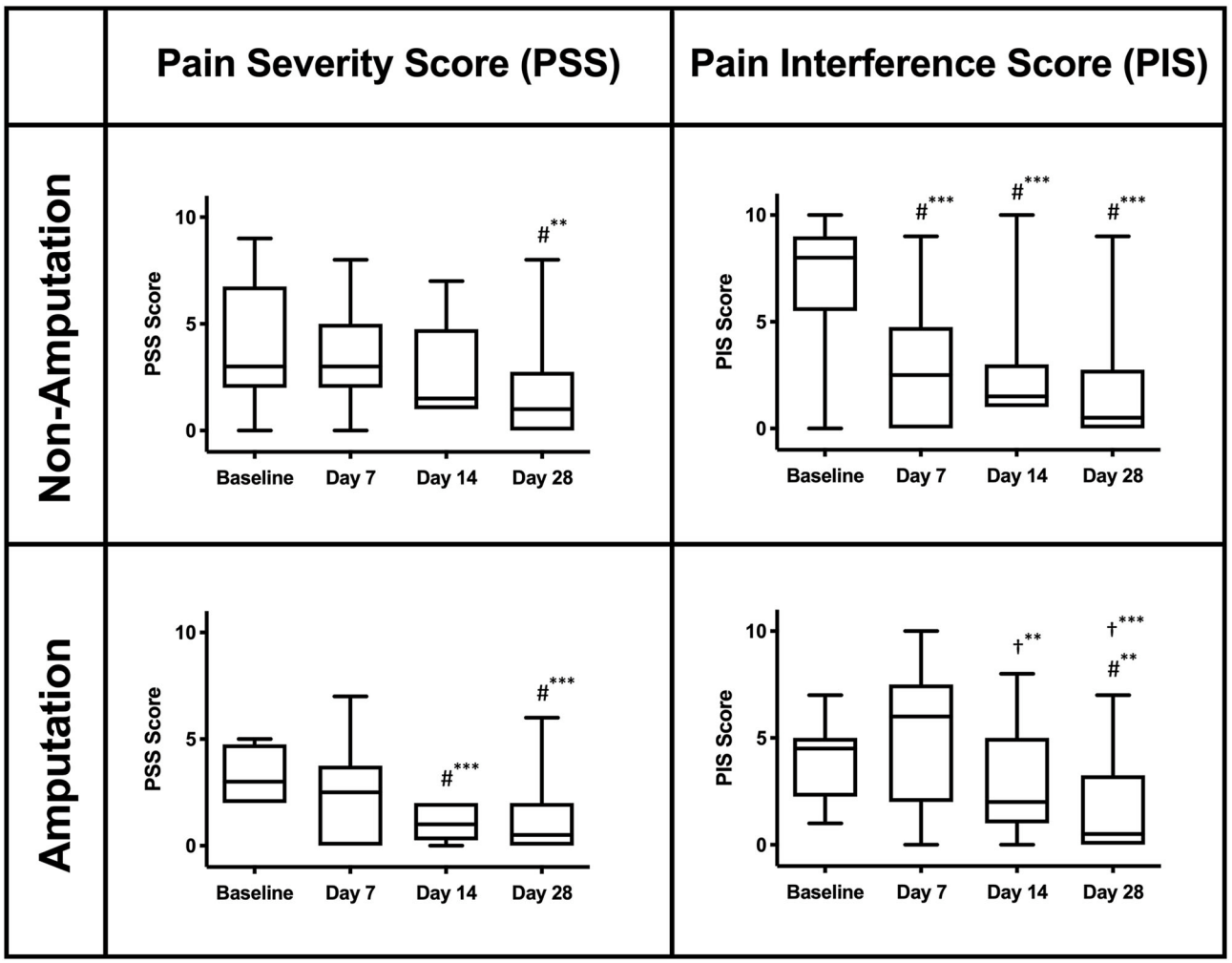


**FIGURE 2 |** Canine Brief Pain Inventory scores (PSS and PIS) for the affected limb in dogs with and without amputation. A reduction in score indicates a clinical improvement. **(#)** denotes a significant difference compared to baseline. (†) denotes a significant difference compared to day 7. **Significant difference ($p < 0.05$). ***Significant difference ($p < 0.001$).

These values were compared between and within treatment days with an analysis of variance, and multiple comparisons were performed with Holm–Sidak's multiple-comparison test (**Figure 3**). For GRF, only data from dogs in the ZOL+RT arm were available for comparison as response to therapy was defined by changes from baseline measurements. These values were compared between treatment days with an analysis of variance, and multiple comparisons were performed with Holm–Sidak's multiple comparisons test (**Figure 4**). $^{18}$F-FDG PET scan values were recorded; comparisons were performed between pre- and post-therapy values with a *t*-test, and clinical response to therapy was graphically depicted by changes from baseline measurements (**Figure 5**). All statistical comparisons were performed with the use of GraphPad Prism 6.0 h software (GraphPad Software, San Diego, CA, USA); normality testing was performed for all data, and all comparisons were two-sided with statistical significance set at $p < 0.05$.

# RESULTS

## Recruitment

Fourteen dogs with spontaneously occurring primary bone tumors were screened for the pilot study. Six screened dogs were not enrolled due to distant metastasis or client owner decision to not pursue the trial (**Figure 1**, CONSORT diagram). Therefore, 8 dogs were enrolled from July to October 2018. Four were enrolled in the AMP arm, and four were enrolled in the ZOL+RT arm. All dogs finished the 28-day study and were then followed according to their clinical treatment protocol. Dog breeds included Golden Retriever ($n = 2$), mixed breed ($n = 2$), Mastiff, Bullmastiff, Labrador Retriever, and Irish Wolfhound. The mean age was 9 years (range 5–13 years), and mean weight was 46.7 kg (range 33.5–72.4 kg), similar to previous publications (7). Tumor locations included the distal radius ($n = 3$), tibia ($n = 2$), proximal humerus ($n = 2$), and distal femur ($n =$

1). Three patients had increased ALP values, a known negative prognostic indicator in dogs (26). ALP values in the rest of the dogs were within reference intervals; ALP was not performed on one dog at screening (only renal panel performed), but ALP was normal on subsequent serum chemistries. An orthopedic examination was performed by a board-certified orthopedic surgeon at the initiation of each visit. During evaluation of study dogs, none of the patients enrolled developed novel sources of musculoskeletal disease (other than previously noted orthopedic or CIBP at baseline).

## Validated Pain Questionnaire

The canine BPI scores [Pain Severity Scores (PSS) and Pain Interference Scores (PIS)] were reported and compared between treatment and time points for dogs with and without an amputation as part of their therapy. The mean (±SD) for PSS were 4.4 (±2.7), 3.4 (±2.5), 2.8 (±2.2), and 1.8 (±2.3) for ZOL+RT dogs and 3.2 (±1.2), 2.3 (±2.1), 1.1 (±0.8), and 1.2 (±1.6) for AMP dogs at days 0, 7, 14, and 28, respectively. The mean (±SD) for PIS were 7.5 (±2.5), 2.9 (±2.7), 2.3 (±2.2), and 1.8 (±2.6) for ZOL+RT dogs and 4.0 (±1.7), 5.1 (±3.4), 2.9 (±2.5), and 1.7 (±2.4) for AMP dogs at days 0, 7, 14, and 28, respectively. There were no significant inter-treatment differences for PSS and PIS on baseline and days 7, 14, and 28. There were significant intra-treatment differences found for PSS and PIS scores compared to baseline for both arms (AMP and ZOL+RT, **Figure 2**). In all cases, a significant improvement in pain relief, as indicated by a reduction in score, was found for PSS and PIS scores as compared to baseline.

## Biomarker—Serum N-Telopeptide

N-Telopeptide (NTx) concentration is a direct biomarker of bone turnover, where osteoclasts degrade collagen and release NTx into circulation. Circulating NTx is also referred to as BCE, measured in nM/L. ZOL+RT dogs had a significant (at least 30%) reduction in serum NTx (BCE) from baseline compared to AMP dogs, $p = 0.029$ (**Figure 3**); significant differences between treatment arms and days are also noted in **Figure 3**. The mean (±SD) serum NTx (BCE) concentrations were 37.9 (±6.1), 22.7 (±8.3), 22.4 (+/10.9), and 22.8 (±5.6) for ZOL+RT dogs and 3.9

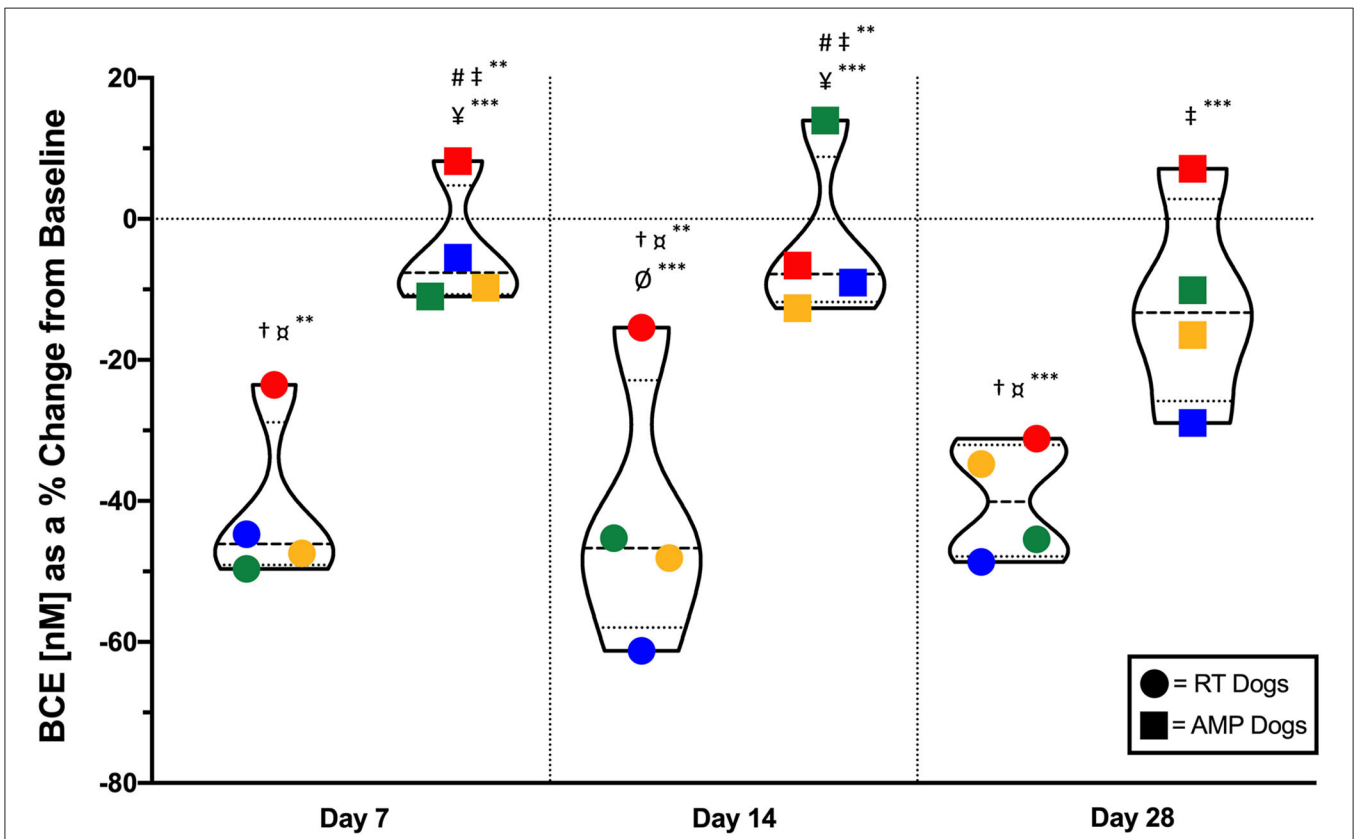


**FIGURE 3 |** Violin plot of serum N-telopeptide (NTx) concentration, or bone collagen equivalent (BCE), as a percent change from baseline in dogs receiving either zoledronate and RT or amputation for appendicular primary bone tumors. Dogs receiving zoledronate and RT had a 30% or greater percentage decrease in BCE by day 28 of the study; no dogs in the amputation arm had a decrease of 30% ($p = 0.029$). Dogs are grouped by treatment modality (zoledronate and RT, circles; amputation, squares). Individual dogs can be tracked by color in each group. Means and standard deviation are noted by thicker hash mark and thinner hash mark within the violins, respectively. #Significantly different from RT Dogs on Day 7. †Significantly different from AMP Dogs on Day 7. ‡Significantly different from RT Dogs on Day 14. ¤Significantly different from AMP Dogs on Day 14. ¥Significantly different from RT Dogs on Day 28. ∅Significantly different from AMP Dogs on Day 28. **Significant difference ($p < 0.01$). ***Significant difference ($p < 0.05$).

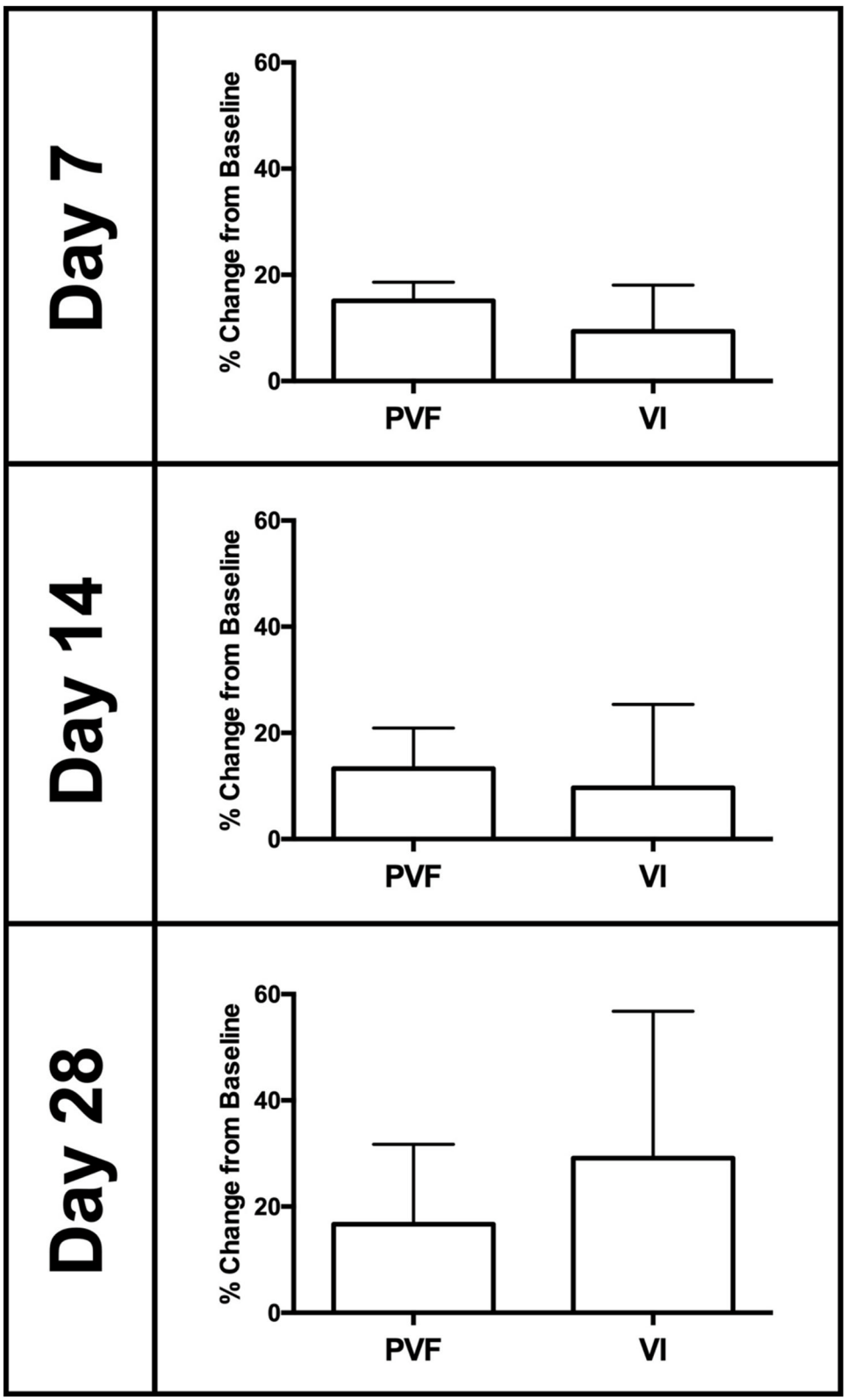


**FIGURE 4 |** Mean (±SD) ground reaction force (GRF) data (peak vertical force (PVF) and vertical impulse (VI)) presented as a percentage change from baseline (normalized as a percentage of body weight). Data represent the affected limb of dogs with non-surgical treatment (i.e., ZOL+RT). Overall, improvements in GRF measurements (PVF and VI) showed improved weight bearing in the affected limb as compared to baseline measurements, following therapy.

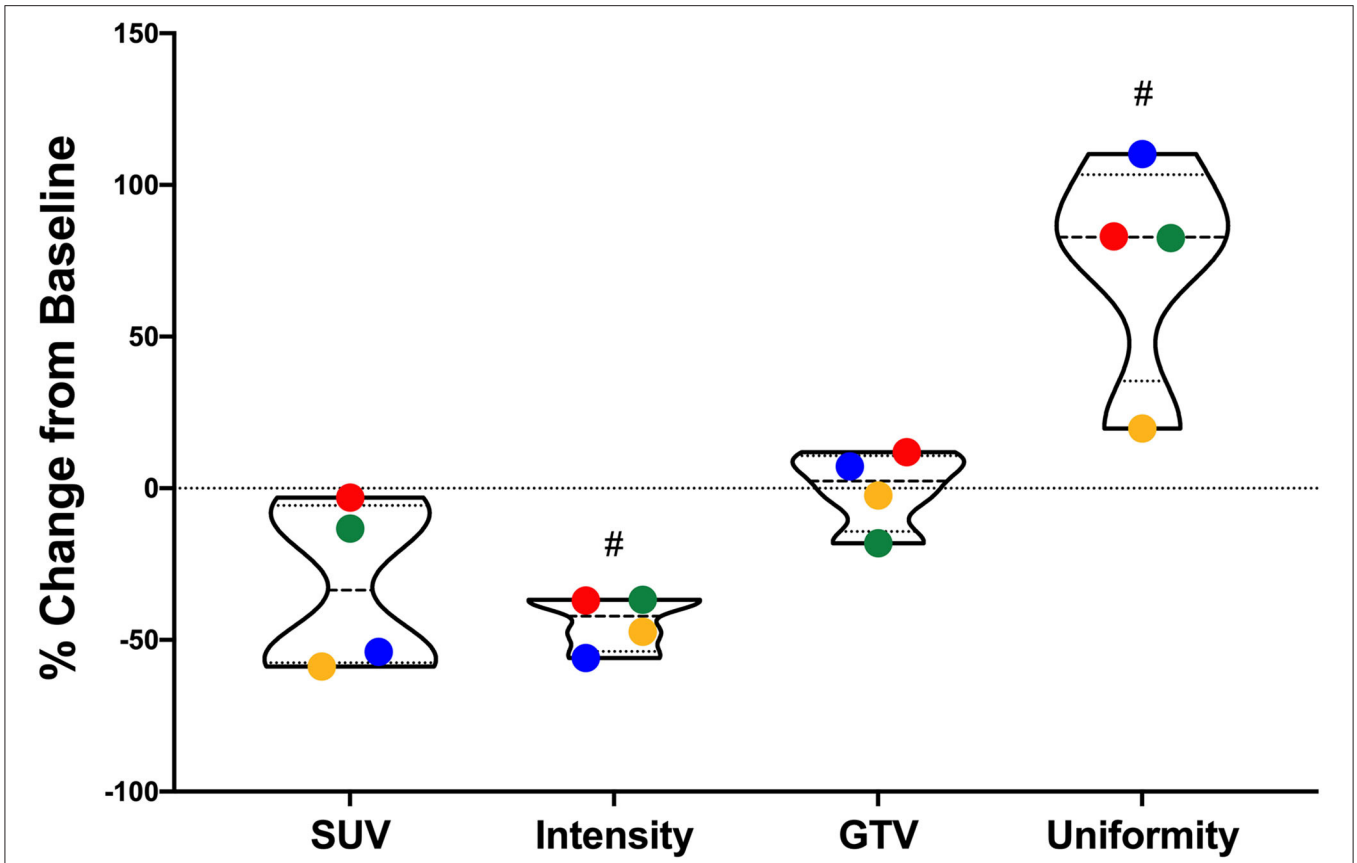


**FIGURE 5 |** $^{18}$F-FDG PET scan values from dogs receiving zoledronate and coarse fraction radiation therapy for their primary bone tumors. Percent changes from baseline for standard uptake value (SUV), count intensity, gross tumor volume (GTV), and count uniformity are shown. Maximum intensity significantly decreased in all irradiated dogs ($p = 0.013$); maximum SUV decreased, but not significantly ($p = 0.11$). GTV was not statistically different pre- and post-therapy. Tumors became more uniform after therapy ($p = 0.017$). $^{\#}$Significant difference between pre- and posttreatment measurements ($p < 0.05$).

(±12.0), 32.3 (±11.7), 32.9 (±12.9), and 30.7 (±13.1) for AMP dogs at days 0, 7, 14, and 28, respectively. Comparing the two groups, the mean 28-day serum NTx of ZOL+RT dogs (22.8 BCE) was significantly lower than that of AMP dogs (30.7 BCE, $p < 0.05$). All dogs receiving ZOL+RT had a 30% or greater reduction in BCE by day 28; no dogs in the AMP arm had a decrease of 30%, with only one dog reaching >20% reduction in BCE at day 28.

## Kinetic Analysis

The vertical GRF variables (PVF and VI) from the affected limb in dogs that received ZOL+RT were collected and reported as a percentage change from baseline (**Figure 4**). For peak vertical force (PVF), the mean percentage change (+/–SD) from baseline was +15.1% (±3.5), +13.3% (±7.7), and +16.7% (±15.0) on days 7, 14, and 28, respectively. For vertical impulse (VI), the mean percentage change (±SD) from baseline was +9.4% (±8.7), +9.7% (±15.7), and +29.1% (±27.6) on days 7, 14, and 28, respectively. Data (PVF and VI) were not available for 2 dogs on day 28. Additionally, comparable GRF data from the affected limb of dogs that received an amputation as a part of therapy were not available for comparison. Reduced pain and improved weight bearing following therapy are indicated by a positive percentage change from baseline. Overall, positive improvements in GRF measurements (PVF and VI) were noted on each testing day as compared to baseline measurements. However, there were no statistically significant differences in these improvements between testing days for PVF or VI.

**TABLE 1 |** 18F-FDG PET variables assessed in dogs receiving zoledronate and radiation therapy (ZOL+RT) for appendicular osteosarcoma.

| PET variable | Scan | Mean | SD | % Change | *p* |
|---|---|---|---|---|---|
| Tumor SUV max | Baseline | 9.9 | 0.2 | −32.3 | 0.107 |
| | Post-Tx | 6.7 | 2.7 | | |
| Tumor count intensity | Baseline | 8501.0 | 3012.0 | −44.3 | *0.013 |
| | Post-Tx | 4729.0 | 2005.0 | | |
| Gross tumor volume | Baseline | 43.5 | 16.6 | −0.3 | 0.781 |
| | Post-Tx | 44.4 | 21.8 | | |
| Tumor count uniformity | Baseline | 0.0048 | 0.0019 | 73.8 | *0.017 |
| | Post-Tx | 0.0078 | 0.0013 | | |

*Signifies statistical significance $p < 0.05$.

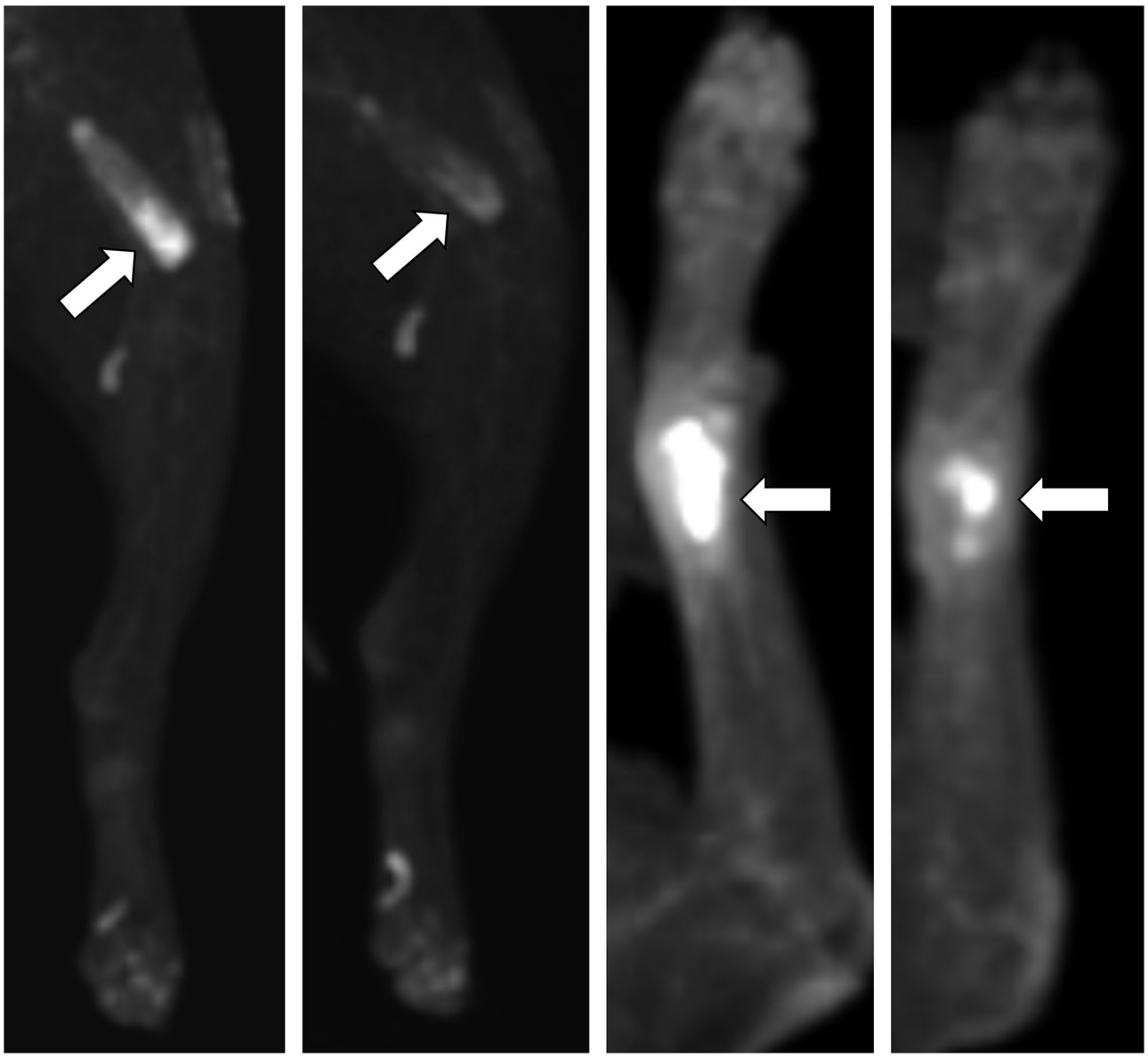

**FIGURE 6 |** Baseline and day 28 2D images of 18F-FDG PET scans in two dogs with appendicular primary bone tumors receiving ZOL+RT. Both dogs had reductions in standard uptake value ($SUV_{max}$) and tumor count intensity, with no change in gross tumor volume (GTV), and increases in tumor count uniformity. Dog #5 (two left panels) had a distal femur lesion; dog #7 (two right panels) had a distal radius lesion. Primary bone tumors with high intensity 18F-FDG uptake are noted by white arrows, with visual increase in tumor uniformity and decrease in intensity noted on 28-day scans.

## $^{18}$F-FDG PET Scans

All dogs had a screening 18F-FDG PET/CT scan. However, only dogs receiving ZOL+RT had a repeat day 28 PET scan. **Figure 5** shows percent changes from baseline for 18F-FDG PET scans. Mean, standard deviation, % change, and **p**-values are provided in **Table 1**. Maximum intensity significantly decreased in all irradiated dogs ($p = 0.013$); maximum SUV decreased, but not significantly ($p = 0.11$). Gross tumor volume was not statistically different pre- and post-therapy, meaning that tumors did not grow/shrink. Tumors became more uniform after zoledronate and RT ($p = 0.017$). Visual representations of the increase in uniformity and decrease in intensity of two dogs receiving zoledronate and RT are shown in **Figure 6**.

# DISCUSSION

We achieved our goal of assessing pain in dogs with primary bone tumors using a multimodal pain assessment methodology. However, our results do not indicate which objective/subjective marker should play the most significant role. This is in part due to the small sample size of our pilot study resulting

in the inability to perform correlative statistical analysis and to the multifactorial causes of cancer pain. A key problem of relying on one pain indicator is shown by serum NTx, which is often touted as a way to assess disease burden and/or pain in animals and people with widespread osteolysis (27, 28). While dogs in our trial receiving zoledronate and radiation therapy had significant decreases (>30%) in serum NTx at 28 days after treatment, dogs with tumors that had been completely removed (i.e., amputation) did not have corresponding reductions in serum NTx. A false conclusion would be that dogs receiving amputation for their primary bone tumors do not have resolution of their pain. More likely, especially when validated owner questionnaires are simultaneously evaluated over 4 weeks, those dogs' pain did improve after recovering from amputation (albeit delayed due to postoperative recovery time). A potential cause for static serum NTx is systemic bone remodeling to account for altered weight bearing on three limbs. This is not to say that the other methods we analyzed are perfect for monitoring pain in dogs receiving more than one therapy, as kinetic analysis and PET imaging require tumors to remain "on" the subject for valid follow-up analysis.

Pain scales are used widely in human medicine and often focus on degree of pain noted by a visual face scale and depend on the patient's or patient guardian's assessment of his/her pain (29, 30). Because questioning veterinary patients about their pain is impossible, veterinarians often rate their patients' pain retrospectively *via* owner questionnaires (31) or descriptive/numerical scales (32). Due to interobserver variability and owner bias, multidimensional pain scales (33), posture/facial expression [specifically in cats (34, 35)], and quantitative sensory testing (36, 37) have more recently been used. However, almost all of these pain assessments are used in induced-pain models or in veterinary patients undergoing surgery. While the aforementioned canine BPI (20) study was validated in dogs with primary bone tumors, comprehensive pain studies in naturally occurring, chronic pain diseases are lacking.

Kinetic analysis in dogs is an objective metric that is widely utilized as an indirect measure of pain in dogs. It is frequently used to assess treatment efficacy in chronic painful conditions such as osteoarthritis. In this study, positive improvements in GRF data were demonstrated in the ZOL+RT dogs at each time point when compared to baseline measurements. However, these improvements were static with no statistically significant changes between testing days. Overall, a >13% improvement was seen in PVF for all time points and >9% was seen in VI. These findings demonstrate that kinetic data can be collected in dogs with CIBP and can provide valuable objective assessments. However, these data are gleaned from a limited number of dogs in this pilot study and the population of dogs available for post-treatment kinetic testing was diminutive as compared to what would be expected in a larger clinical trial. It is possible that a larger population or longer follow-up testing would have detected differences between testing time points. Additionally, the impact of sources of variability on this smaller population size is not known. Kinetic data collection in dogs, especially those with chronic painful conditions, can be influenced by factors including habituation, handler variability, and extended physical exertion. Efforts were made to reduce the impact of these sources of viability in this study population. Habituation to the testing facility and laboratory environment is critically important. Previous studies have demonstrated that adequate habituation can improve kinetic data collected in dogs by reducing data variability (38). In this study, animals were allowed to habituate to the testing environment for a minimum of 3–5 min prior to data collection. Prolonged physical exertion can also impact kinetic data collection, and previous studies have demonstrated that kinetic analysis in dogs with lameness secondary to osteoarthritis can be exacerbated by exercise (39). The impact of physical exertion on trials collected later in each testing period in this study population is unknown. However, all efforts were made to facilitate timely and efficient data collection. Finally, data variability has been shown to be impacted by different handlers and can result in up to 7% of data variance (40). To address this, all dogs were walked by the same handler at all-time points.

Our study is the first to include PET imaging in the assessment of dogs with CIBP. In human medicine, several groups have evaluated $^{18}$F-FDG PET scans in patients receiving RT for painful bone metastases (41–43). Those studies have shown that $SUV_{max}$ could be used to predict the improvement in pain for people receiving palliative radiation for their metastases, as well as pre-RT pain severity. One study of 74 patients diagnosed with non-small-cell lung cancer bone metastases showed that higher pre-RT $SUV_{max}$ resulted in worse progression-free and event-free survivals (43). The relationship of $^{18}$F-FDG PET $SUV_{max}$ and pain or response to therapy is likely due to glucose metabolism's relationship to growth rate and biologic aggressiveness of a tumor (43). In another chronic pain translational model, cats with osteoarthritis-associated pain underwent $^{18}$F-FDG PET scans (44); in 7 cats with OA, significant differences in brain metabolism were noted compared to normal cats. While not directly assessing pain, another group of veterinary researchers evaluated $^{18}$F-FDG PET scans in dogs with OS (45). This group found that dogs with treatment-naïve OS with $^{18}$F-FDG PET scan $SUV_{max}$ values $\geq$7.4 had shorter survival times than dogs with $SUV_{max} < 7.4$. However, this study was retrospective and dogs were allowed to have either surgical (amputation vs. limb-spare) or stereotactic radiation, and follow-up PET scans were not performed. While our study sample size is small, patients were prospectively enrolled, treatments were standardized, and our pilot study findings warrant further investigation into the role of PET scans in assessing pain in dogs with cancer.

A weakness of many canine OS studies is lack of definitive diagnosis. In fact, because of confirmation of our cases with either cytology or histopathology by a board-certified pathologist, one of the dogs in our study was diagnosed with primary bone chondrosarcoma, instead of osteosarcoma. The dog was included in the amputation arm but did not receive chemotherapy due to the low-grade nature of its tumor. Historical pain studies in dogs with aggressive bone neoplasms have not uniformly confirmed a

diagnosis of OS (20, 23), instead relying on radiographic findings, signalment, and tumor location. The lack of definitive diagnosis could result in inclusion of primary bone tumors of non-osteoid lineage, potentially affecting outcomes such as progression-free survival and overall survival. Future prospective studies should always confirm diagnosis of OS, *via* either ALP-positive cytology or histopathology, to remove non-osteoid primary bone tumors' skewing of results.

## CONCLUSION

Validated owner questionnaires (canine BPI), kinetic analysis, and $^{18}$F-FDG PET scans showed improved pain relief in dogs with appendicular primary bone tumors receiving ZOL+RT. Serum NTx levels likely do not directly measure pain, but rather the degree of systemic osteoclastic activity. Larger, prospective studies are warranted to identify the ideal objective indicator of pain relief; however, use of multiple assessors is presumably best. Ongoing projects in our oncology/orthopedic research group at the University of Missouri include evaluating novel pain targets in dogs with PET imaging. With improved description and quantification of pain severity and relief in dogs with cancer, we can better evaluate the efficacy of our interventions. Dogs could prove to be a better cancer pain model because of their similar and shared environment with humans, genetic diversity, long-term survival compared to rodents, and naturally occurring (rather than induced) neoplasms. This could directly benefit people with CIBP, potentially decreasing the amount of subtherapeutic novel drugs entering human clinical trials.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Missouri IACUC. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

BF, BT, and KH designed the study. BF ensured all patients met the enrolment criteria and completed the study as scheduled. BF and BT assessed canine BPI. BF and HR performed NTx assays. BT and KH performed orthopedic exams and kinetic analysis. CM and AZ analyzed FDG PET scans. CM and BF were involved in treatment plans. BT performed statistical analysis with assistance from HR and BF. All authors contributed to writing and editing the manuscript and contributed to the study.

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# Canine osteosarcoma in comparative oncology: Molecular mechanisms through to treatment discovery

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Cancer is a leading cause of non-communicable morbidity and mortality throughout the world, similarly, in dogs, the most frequent cause of mortality is tumors. Some types of cancer, including osteosarcoma (OSA), occur at much higher rates in dogs than people. Dogs therefore not only require treatment themselves but can also act as an effective parallel patient population for the human disease equivalent. It should be noted that although there are many similarities between canine and human OSA, there are also key differences and it is important to research and highlight these features. Despite progress using chorioallantoic membrane models, 2D and 3D *in vitro* models, and rodent OSA models, many more insights into the molecular and cellular mechanisms, drug development, and treatment are being discovered in a variety of canine OSA patient populations.

KEYWORDS

**bone cancer, canine, genes, human, osteosarcoma, protein, treatment**

## Introduction

In both human and canine patients the predominant bone cancer diagnosis is OSA (1, 2). Sarcomas are tumors originating in tissues derived from the mesoderm, affecting bone, cartilage and connective tissue (3). Osteosarcoma produces malignant bone or osteoid tissue, but a unifying feature is that all types of OSA histologically produce tumor osteoid (4). Archetypal OSA consists of a primary tumor, usually originating within the medullary cavity and spreading to the surface of the bone, but they can be extra-osseous (5). Typically the tumor grows, proliferates, invades, and left unchecked frequently metastasises to the lungs (6). OSA subtypes include osteoblastic (bony), chondroblastic (cartilaginous), and fibroblastic (resemble atypical fibroblasts), with a range of rare types, and those not originating in the medullary cavity (5), see also a previous review (7).

## Overview of experimental OSA models

Rodent and chorioallantoic membrane (CAM) models have been utilized in OSA studies, in addition to a variety of *in vitro* methods, however each has a number of limitations. Early rodent models represent OSA well-histologically, but do not represent the true etiology of the disease (8). Immunocompromised mice inoculated with human OSA cell lines or grafts have served well for studying metastasis, drug screening, and helped toward identifying activation pathways, but have limited capacity in understanding OSA development and immune system interactions, although advances in this area are continuing (9–11). P53 and Rb mutation transgenic mouse studies have an overall relatively high cost and difficulties relating to breeding and development of non-OSA cancers, but have shown similarities to the human disease (12–15).

Although CAM models have been used for over a century, an avian environment does not always replicate the mammalian tumor environment or immune system. Although angiogenesis in this model can assist with looking at invasion, drug development, and metastasis, it has not been widely used for OSA models (16, 17). Indeed many of the models developed for OSA failed to produce a tumor and/or osteoid, a key component of OSA (18). The advancements in 3D *in vitro* models over 2D ones, represents a step forward in understanding microenvironment interactions and mechanisms, with fewer limitations than traditional culturing models (19, 20). Examples using liquid overlays (21) and ultra-low binding plates to develop spheroid formation have been used, the latter helping identify a potential role of miR-335 in OSA (21). Hanging drop methods have also been used, especially alongside 2D cell cultures to investigate VEGF expression, vital for angiogenesis (22).

Unfortunately, none of these methods perfectly recreate the tumor microenvironment, or replicate growth and development of the cancer. While these methods have improved prevention, diagnosis, and treatment of a range of diseases, OSA cure rates and survival times have not improved significantly in decades (23, 24). What is really required is a model or parallel patient population that accurately recapitulates the clinical, biological and molecular aspects of human/pediatric OSA.

## OSA in dogs and people–parallel patient populations

Given the spontaneous nature of OSA in dogs, and the clinical relevance of canine to human OSA, these natural models might be better described as parallel patient populations (7, 25). Naturally occurring parallel patient populations allow researchers access to additional cases of disease without inducing disease.

Current understanding of OSA disease processes and treatments is largely based on studying affected individuals compared to unaffected individuals, or assessing differing types of OSA, with computer simulations/bioinformatics playing an increasing role (26, 27). The development and progression of OSA is frequently influenced by a combination of environmental and genetic risk factors. Understanding the basis of disease and development of new treatments *via* animal models, particularly within naturally occurring animal populations, is crucial, however care must be taken to ensure phenotypes are representative of the disease.

The overall canine population is genetically heterogeneous, however breeds can be comparatively homogeneous which further enhances their value for comparing genetic mechanisms of disease (28). Some breeds are at increased risk of developing OSA (1), making them a valuable parallel patient population. Human diseases may progress over a number of years, and spontaneously occurring canine OSA reflects this progression in contrast to laboratory models which are often investigated over much shorter periods of time. Indeed many human disease phenotypes are closely matched to canine disease phenotypes, exhibiting similar pathologies, progression, treatment options, and prognosis (29–31), this includes OSA (7). The canine and human OSA biological and histological similarities, alongside treatment trials and comparisons have been evidenced through numerous studies across the decades. More recently, the molecular and cellular comparisons undertaken between the two species, as detailed within this review, have provided crucial steps toward understanding both the limitations and benefits of studying canine OSA as a parallel population.

## Similarities and differences in OSA incidence, risk factors and survival rates between people and dogs

Dogs naturally have a higher OSA incidence than people. Human population studies have shown there are roughly 0.89 cases of bone cancer per 100,000 people/annum (32, 33). In a population of 394,061 insured dogs, 764 (0.19%) developed a bone tumor (1), representing an incidence rate of 27.2 dogs per 100,000/annum, a much higher rate than in people. The higher incidence rate of canine OSA makes the pet dog population an ideal parallel patient population for investigating the disease in humans. In people, there is increasing evidence of variation in the incidence rate between families and different populations (2, 34, 35). Interestingly, OSA in dogs is highly influenced by breed, with Irish wolfhounds displaying the highest levels (12.3% of the population), with some other breeds mostly unaffected (1, 36).

Osteosarcoma is bimodal in people peaking in the young (<20 years old) and elderly (>60 years old) (2, 32, 33, 37, 38).

Although widely reported as a bimodal occurrence in canines, with peaks at 1.5–3 and 7–9 years, this bimodal observation has not been shown all studies (39–43). An additional important difference between the species is that OSA is more prominent in the older dog range (7–9 years), whereas in people the incidence is highest within the pediatric population. The first cross-species genomic analysis between canine and human OSA indicated that there were very strong gene expression similarities between the two species (25). Hierarchical clustering showed branching between OSA and normal tissues but showed no distinct branching of canine and human OSA. This study specifically compared pediatric tumors from children against canine (ages not stated) OSA and was able to draw the conclusion regarding the similarities between canine and pediatric OSA but did not test adult human tumors (25). Notably a later study looked at both juvenile and adult canine tumors indicated that the adult dog was a good model regarding genomic features and clinical characteristics (44). This supports the data indicating clinical presentation and diagnosis, histological presentation and treatment similarities between canine and juvenile human OSA (45, 46).

Despite a general trend of improving 5-year-event-free-survival rates across all cancer types in people (24, 47), OSA has not shown comparable improvements in mortality rates (2, 47, 48). The 5-year-event-free-survival for individuals with metastatic tumors at diagnosis was reported to be 27.4%, increasing to 70% in individuals with no metastases at diagnosis (2, 6). The 1-year survival rate for canines is typically <45% (49–51). It is worth noting that for appendicular OSA, the 1 and 2-year survival rates have been published at just 11.5 and 2%, respectively for dogs receiving amputation only as a treatment option (52). These similarities in presentation not only support the rationale for the dog as a parallel patient population for studying OSA but also highlight the urgent need to develop improved treatments and cures.

The common risk factors associated with OSA development in both humans and canines include sex, growth, puberty (2, 34, 53), in addition to population/breed and a range of molecular associations. Growth has been associated with the development of OSA in both people and dogs (1, 36). In people, age of onset frequently coincides with rapid bone growth during puberty, tumor sites are most frequently situated at the end of bones where active growth occurs (2). In canines there is not as much evidence linking to growth, given the later onset of OSA in general, however OSA predominantly occurs in weight-bearing bones and adjacent to late-closing physes (1, 36). Large dog breeds make up the majority of canine OSA cases reflecting the human population where affected individuals are more likely to be taller than average (1, 34, 54).

In the canine population, as with the human population, there appears to be a skewed sex ratio with males typically more affected by OSA, and at younger ages, than females (1, 2, 32, 34, 55). Additionally, neutering status, although less relevant in the human context, appears to contribute to risk with neutered dogs more likely to develop OSA than non-neutered counterparts (36). The neutering effect, combined with the association with puberty, indicates that sex hormone signaling may play complex roles in OSA.

Presently there are over 544 canine "potential models for human traits" listed in OMIA (Online Mendelian Inheritance in Animals), more than any other species (29, 30). Dogs are typically treated as family members and so inhabit the same environment as their owners, alongside many of the environmental and other risk factors impacting disease risk, initiation and progression. Pet dogs also frequently benefit from high quality medical care, such that illnesses are detected and treated promptly, similar in a way to people (56). This also means that the amount of information being collected by veterinary clinics, researchers, and insurance companies expands the data available. These canine population characteristics represent a valuable resource for modeling human disease. Although understanding diseases and developing novel treatments in companion animals exhibiting occurring disease is less contentious than inducing disease in experimental animals, ethical concerns regarding treatment of individuals and gaining informed consent from owners remain (57).

## Similarities and differences between OSA molecular mechanisms in people and dogs

Developing new treatments is expensive and time consuming. Only 4.1% of potential new compounds progress from preclinical discovery to patient use, taking on average 13.5 years (58, 59). In order to create targeted pharmaceuticals in shorter time frames, understanding the genetic mechanisms behind diseases are critical (60). Indeed, parallel animal patient populations of disease, including OSA, play crucial roles in identifying genetic loci associations and biomarkers, which may lead to target identification, to help determine appropriate drugs, leading through to target validation (61–63). Much of the molecular work, underpinning early drug development and repurposing, is facilitated by the conservation of many fundamental biological pathways between species (64–66).

Pedigree breeds in dogs are generally fairly closed populations, ancestry can often be traced for many generations, and even back to the breed's founding members (28, 67, 68). Although this restricts genetic diversity within breeds, it facilitates understanding the mode of inheritance of traits and diseases (67). Both the founder effects and later inbreeding within canine pedigree breeds have led to divergent allele frequencies between breeds, resulting in some breeds exhibiting higher disease frequencies (28, 69). As a result, differing breeds

have homologs of numerous human conditions, making them ideal for identifying potential genetic loci associated with disease for both canine and human benefit.

Some cases of human OSA have been associated with heritable cancer syndromes, and the genetic basis of these has been established (70–72). Despite this, most human OSA cases are not considered to be heritable. Some somatic mutations in tumor suppressor genes have been identified in individuals with heritable cancer syndromes such as Li-Fraumeni syndrome, and other mutations have been identified in OSA tissue compared to normal tissue (70, 73–75). Interesting, to date, only two somatic genetic mutations have been specifically associated with OSA (53). Despite the lack of heritability and somatic genetic mutations, over 900 genes are associated with human OSA (76). These associations have been identified due to differences in expression, or identification of mutations, that have arisen in the tumor compared to the non-tumor tissue (77–79). Mutations within OSA tumor tissue may exist as a cause, or as a result, of the tumor. Differential expression, and mutations, may also exist *via* genomic and chromosomal instability, which in itself is a reported factor in many types of cancer progression (80, 81). Osteosarcoma in people has been shown to display chromosomal instability associated with mutations in the *TP53* gene (82). Aneuploidy can occur as a consequence of chromosomal instability, which can lead to the gene overexpression in affected malignant cells, causing disruption to the normal cell processes (83). Although mutations in *TP53* look likely to be associated with chromosomal instability, the gene itself is not over expressed following aneuploidy (82, 83). TP53 has also been implicated in canine OSA with whole genome sequencing and whole exome sequencing (WES) indicating frequent *TP53* mutations in canine OSA tumors, at rates of up to 83%, specific mutation rates were variable between breeds (44, 84, 85). *TP53* mutations have featured heavily in many of canine OSA studies, however findings still differ between these studies overall. For example it was found that *TP53* missense mutations in dogs who had amputation followed by chemotherapy were associated with a longer DFI than wild type of null tumor samples investigated (85). Although similar results have not yet been observed in human OSA, other cancer types and mutant cell lines have shown improved treatment responses (86, 87).

Gene expression following treatment has also highlighted key similarities between people and dogs. Studies identifying gene expression in canine patients responding, and not responding, to chemotherapy treatment, were later found to be similar in people, indicating the value of the dog as a parallel patient population for human OSA (88). It should also be noted that gene expression variations have been observed on some occasions, despite the often-high similarities in many other studies (25), thus indicating a potential limitation of canine comparisons with human OSA.

In canine OSA patients, 33 loci have been associated with the disease across three breeds, and an additional single locus is associated in Deerhounds (89, 90). None of these loci are consistently associated across breeds, suggesting there may be a difference between breeds regarding genetic predisposition to developing OSA (89, 90). In addition to the 34 genetic loci identified, genes have been identified as differentially expressed in canine OSA compared to non-tumor tissue, many of which have implications for growth and metastasis, and are potential drug targets (55, 91–96). These genes have been identified utilizing canine OSA tumor tissue, and/or canine OSA cell lines. Some proteins of interest have also had histological work undertaken to start identifying their presence and relevance in OSA (55, 95). There has also been variation in the expression of genes within tumors associated with survival time in canine OSA (97–100).

## Shared genes and proteins of interest for development of future treatments

In both humans and dogs, effective treatment for OSA involves surgery to remove primary tumors 27, 32], often combined with neoadjuvant and/or adjuvant radiotherapy and chemotherapy [33, 34]. The type of surgery rarely has an impact on survival for most human tumors [27], more important prognostic factors are how the tumor responds to chemotherapy and the presence of metastases prior to surgery [27]. In order to advance the treatments available, genomics and drug discovery are providing potential new treatments. Increasingly, comparisons between results from human and canine OSA studies are showing shared genes of interest between the two species. Many of these studies also highlight the need for further testing in relation to potential therapeutic agents.

Comparative transcriptional profiling of dogs and human OSAs has highlighted the similarities between the tumor tissues in the two species. One example was OSA tissue cluster analysis undertaken on 265 orthologous transcripts on pediatric human OSA compared to canine (age not stated) OSA (25). The conclusion was that it was not possible to differentiate between canine and pediatric human OSA tissues yet normal tissues from both species did branch (25). Similar outcome predictions for specific genes in both humans and dogs were also observed. Examples of these include interleukin-8 (IL-8) and solute carrier family 1 (glial high affinity glutamate transporter), member 3 (SLC1A3). Increased expression levels of IL-8 and SLC1A3 predicted poor clinical outcomes in tissues from both species, a result initially identified in canine samples, then followed up and confirmed using human OSA data and both human and canine OSA cell lines (25). Interestingly, increased expression of *SLC2A1* (GLUT1) within tumors also resulted in poorer prognosis and a shorter disease free interval in people (101). *SLC2A1*/GLUT1 (see Figure 1) levels were also significantly increased in naturally occurring canine OSA tissue compared

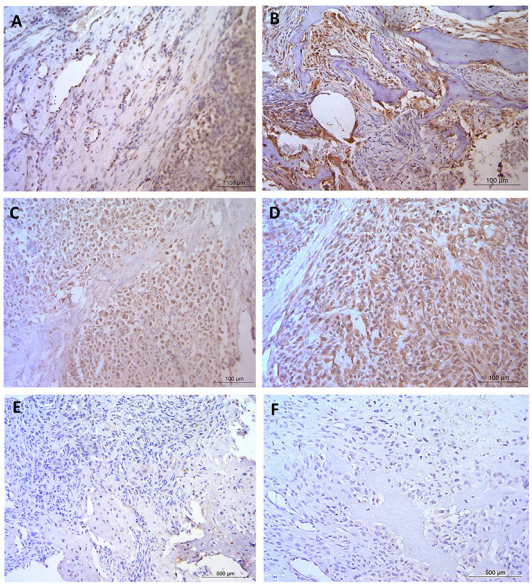


FIGURE 1
Immunohistochemical staining in naturally occurring canine osteosarcoma tissue. **(A,B)** GLUT1 and **(C,D)** MMP3, both staining patterns expressing positive nuclear, cytoplasmic and vascular tissue with negative staining observed in the osteoid. **(E,F)** Negative control. Scale bars represent **(A–D)** 100 μm, **(E,F)** 500 μm. Staining was conducted with ethics, techniques and tissues as previously published (95).

to normal bone tissue (55, 95). Inhibition of SLC2A1 and cellular glucose transport has been achieved by a number of pharmaceuticals, however, as with the MMP3 inhibitors, these have yet to be utilized in OSA trials in either people or dogs (102–105). Monoclonal IL-8 antibody therapy could also be of interest given the importance of this chemo-attractant angiogenic factor, which has been implicated in a number of cancers (106, 107). Although not yet tested in OSA, clinical trials using IL-8 monoclonal antibodies in other cancers types are ongoing (108) and provide an interesting target given the links between increased IL-8 expression and doxorubicin resistance (109, 110).

Another good example of comparative OSA highlighted the role of MMP3, with increased expression linked with a poor prognosis in OSA, and to formation of metastases (23, 111). Tsai et al. (112) and Huang et al. (113) identified higher expression of *MMP3* in human OSA compared to normal bone. Additionally Adiguzel et al. (114) reported on MMP3 polymorphisms associated with OSA in people. Naturally occurring OSA was also associated with increased *MMP3* levels in canine patients (55), and work was later undertaken to show expression patterns (Figure 1) of the protein in tissue (95). Despite the increasing evidence regarding MMP3, neither the selective inhibitor of MMP3 (UK370106) (115) or the generic MMP inhibitor (marimastat) (116) have been assessed in relation to restricting primary tumors or metastatic tumor growth in canine or human OSA, despite some trials in other tumor types.

The Dickkopf proteins are differentially expressed in a number of cancers, and inhibit Wnt signaling which, in turn, is aberrant in many cancers (117–119). Reduced *DKK3* expression in human breast, endometrial, and cervical cancer, has implicated it as a tumor suppressor (120–123). *DKK3* expression within OSA has resulted in conflicting reports. In human OSA cell lines, and in xenograft mice, *DKK3* expression was reduced, however subsequent restoration of *DKK3* expression resulted in reduced tumor and metastatic growth (124). In contrast, *DKK3* was more highly expressed in human OSA cells overexpressing *NKD2* and in tumor tissue (125), and also in tumor tissue compared to non-affected bone in naturally occurring canine OSA (55). Despite differences compared to some cancers, this outcome agreed with *DKK3* knockdown in cells overexpressing *NKD2* which exhibited increased proliferation, indicating a possible mechanism of *NKD2* induced metastasis, although the authors noted more work into the mechanisms was required (125). With a lack of drugs available acting on *DKK3*, development in this direction could prove useful for OSA in both people and dogs. Although these examples represent just a small number of the genes and proteins of interest in both human and canine OSA, it helps show the benefits of using the dog as a parallel patient population for this cancer, especially in relation to drug development. Table 1 provides a summary of the genes, proteins and pathways detailed in this review.

Whole genome and exome sequencing have also discovered not only where mutations within pathways such as PI3K and MAPK are similar between human and canine OSA, but have also identified novel aberrations in canines, such as those in *SETD* and *DMD*, which have not yet been reported in people (44). Although these aberrations have not yet been found in human OSA cases, despite the high sequence homology between the two species, it is known that dysregulation of SETD2 has been implied in human OSA (25, 133). Although the *DMD* gene encoding dystrophin is more commonly associated with Duchene and Becker muscular dystrophy in both species, other studies have shown somatic *DMD* variants in human OSA patients (129). Comparative canine and human transcriptomic studies have also identified annotations and pathways unique to particular cancers. For example, annotations unique to bone material synthesis, including *COL5A2*, *COL6A3*, and *COL12A*, were discovered in OSA in both species but were not present in melanoma, pulmonary carcinoma, or B- and T-cell lymphoma (130). Considerable insights into possible pathways and biomarkers can be provided by such studies. Often potential biomarkers, or targets that have known chemistries presently available, including examples such as *COL16A1* and *KDELR2* (130), are highlighted as areas needing more research.

TABLE 1 Summary of key comparative genes, proteins and pathways in human and canine osteosarcoma.

| Gene/Protein/Pathway | Human | Canine |
|---|---|---|
| *TP53* | Chromosomal instability associated with mutations in the TP53 gene (82). Chromosomal abnormalities leading to aneuploidy, resulting in overexpression, disrupted cell processes (83). | Frequent TP53 mutations in canine OSA (44, 84, 85). |
| *TP53, RB1, MYC, PTEN, RUNX2, CDKN2A, CDKN2B* | Copy number aberrations (126–128). | Copy number aberrations (126–128). |
| *DMD* | Somatic DMD variants found in 5/8 patients (44). | *DMD* aberrations, including canine specific mutations, in 50% specimens (44). |
| *SETD* | Dysregulation of SETD2 implied in human OSA (25, 101). | Putatively inactivating somatic *SETD2* in 42% of specimens including some canine specific compared to human (44). |
| IL-8 and SLC1A3 | Increased expression levels of IL-8 and SLC1A3 predicted poor clinical outcomes (25). Increased expression of *SLC2A1* (GLUT1) within tumors resulted in poorer prognosis and a shorter disease free interval (104). | Increased expression levels of IL-8 and SLC1A3 predicted poor clinical outcomes (25). Increased *SLC2A1*/GLUT1 levels in OSA tissue compared to normal bone tissue (55, 95). |
| MMP3 | Higher expression of *MMP3* in OSA tissue compared to normal bone (115, 116). MMP3 polymorphisms associated with OSA (117). Increased expression linked with a poor prognosis and to formation of metastases (23, 114). | Increased *MMP3* levels associated with OSA (55), proteins expression shown in OSA tissue (95). |
| DKK3 | *DKK3* expression reduced in OSA cell lines, but subsequent restoration of *DKK3* expression resulted in reduced tumor and metastatic growth (129). In contrast, *DKK3* was more highly expressed in OSA cells overexpressing *NKD2* and in tumor tissue (130), | *DKK3* expression increased in OSA tissue compared to non-affected bone (55). |
| PI3K, P13K-Akt, P13K/mTOR and MAPK pathway mutations | PI3K/mTOR shared vulnerability for both species (131) | Mutations in PI3K in 37% of the samples and 17% for MAPK (44). Dysregulation of P13K-Akt pathway and *COL6A3, COL5A2, TNC, and ITGB5* activation (103, 132), and PI3K/mTOR (131) |

## Shared MicroRNAs in comparative studies

Recent reviews outlining the potential comparative values for investigating vasculogenic mimicry molecular pathways and microRNAs (miRNA) in the dog, highlight how little work has been conducted in this species compared to humans (134, 135). They contain detailed discussions around miRNAs and lncRNAs and provide interesting reading around these areas, especially in relation to vasculogenic mimicry in canines in comparison to people, which is not therefore covered in the present review. In both dogs and people decreased expression of miR-1, miR-133b and miR-196A have been shown to be involved in proliferation-invasion, miR-34 with proliferation and 14q32 locus (including mir-544, miR-396-3p, miR134 and miR-382) with proliferation-apoptosis in OSA (135). Additionally increased miR-9 has been associated with invasion and increases in miR-106b cluster have been associated with proliferation (135). Comparative examples such as the dysregulation of the 14q32 miRNA cluster in both dogs and people, not only identified a potentially conserved mechanism related to the aggressive and invasive biological behavior of OSA in both species, but yet again emphasize the similarities between the two species (136). Another example is the discovery of miR-1 and miR-133b which showed lower expression levels in canine OSA compared to normal tissue, yet increased expression of their targets MET and MCL1 (137). Interestingly a previous study had shown that both miR-1 and miR-133b were differentially expressed in human OSA affected tissue compared to non-OSA bone (138). MiR-34a looks especially promising given its links to both human and canine OSA, its anti-proliferation and metastasis inhibition activities and the research relating to a genetically engineered pre-microRNA-34a prodrug (139–141). Work published after the recent review (135) compared 19 miRNA candidates expressing differential expression in OSA samples compared to non-affected tissue in both people and dogs were also assessed (142). This research showed that expression miR-223 increases and in let-7b and miR-130a decreases were associated with increased risk and a shorter disease free interval. These were highlighted as potential targets and/or biomarkers for OSA.

## Translational drug development studies

In addition to the molecular studies highlighting potential targets of interest, a number of canine OSA trials have assessed treatment regimens which were primarily designed to increase survival times. Early evidence showed that while amputation alone increased canine survival by around 2.5 months, addition of liposome-encapsulated muramyl tripeptide (L-MTP-PE) administration following amputation prolonged survival by an additional 5 months, primarily by reducing OSA metastasis development (143). Later randomized canine trials showed the outcomes of combining differing protocols of L-MTP-PE and cisplatin chemotherapy treatment. L-MTP-PE exhibited antimetastatic activity when administered post amputation, increasing survival times to around 14.4 months when cisplatin was administered after L-MTP-PE (144). The survival advantages observed following L-MTP-PE alone were not observed when cisplatin and L-MTP-PE were administered concurrently rather than sequentially, indicating that treatment timing is crucial. Trials of this drug in children with OSA revealed an 8% improvement in survival (145), but anti-tumor effects and increased survival times were also noted when treating human OSA patients with L-MTP-PE, especially when chemotherapy was administered (146–148). For example, a 24-week treatment with L-MTP-PE increased median time to relapse from 4.5 months for the control group to 9 months for the treatment group (146, 147). It was also noted that plasma levels of cytokines including IL-8, TNF-α and IL-6 reduced following treatment, all of which may play roles in monocyte-mediated tumor cell death (146, 147). This work followed the smaller phase II trial indicating histological changes to pulmonary metastases in OSA patients (148).

HER2/neu, a tyrosine kinase receptor within the epidermal growth factor receptor family, is expressed in osteosarcoma stem cells (149). Expression has been found in 40% of pediatric and canine osteosarcoma, and associated with higher metastatic rates, reduced response to neoadjuvant chemotherapy, and reduced survival times (150–152). A chimeric human HER2/neu fusion protein (ADXS31-164, also now known as ADXS-HER2 and OST-HER2) was tested in dogs with a histopathological and immunohistochemical diagnosis of HER2/neu OSA, following amputation/limb sparing surgery and treatment with carboplatin (153). Disease-free interval (DFI) following the intervention was 615 days, median survival time (MST) was 956 days, and overall survival rates at 1, 2, and 3 years were 77.8, 67, and 56%, respectively. The authors noted significant outcome improvements compared to matched historical control group rates showing a DFI of 123–257 days, a MST of 207–321 days, and overall survival rates of 35.4% (1 year) and 10–15% (2 years). Additionally, this study showed only mild side-effects of ADXS31-164 when administered to canine patients. This therapy specifically induced HER2-specific immunity, targeting the cells expressing HER2/neu, broke peripheral tolerance to HER2/neu and mediated cytotoxic T-cell–dependent tumor regression (153). In 2016, ADXS-HER2 was granted orphan-drug designation, then rare pediatric disease designation in 2021, from the FDA and EMA, for the treatment of OSA. In 2021 ADVAXIS Immunotherapies, in collaboration with the Children's Oncology Group, reported that the first human OSA patient had received doses in the Phase IIb trial of this drug (154). The outcomes from this trial, including any clinical results and mechanistic studies will be of great interest regarding not only human and canine OSA, but in relation to other cancer types which also express HER2 including mammary carcinoma (126).

The angiotensin-receptor blocker losartan, when used in combination with the kinase inhibitor toceranib, has also shown promising results in canine OSA patients (127). By blocking OSA-elicited monocyte recruitment *via* the action of losartan inhibiting the CCL2–CCR2 axis, clinical benefits including tumor stabilization and/or regression were observed in half of the dogs. Notably, both human and canine OSA cells secrete CCL2, resulting in monocyte migration. By interrupting the CCR2–CCL2 axis and by blocking monocyte migration, these trials have provided more insights into the tumor microenvironment and indicated a direct mechanism by which these therapeutic agents could work in human OSA patients. Owing to the success of this canine OSA trial published in 2021, a phase I clinical trial (NCT03900793) was initiated in pediatric and young adult OSA patients with lung metastases.

## Limitations of canine OSA models

One of the limitations of this area of research is that frequently the research concentrates on either dogs or people with relatively few comparisons of the two using the same analysis and techniques. Although this individual species specific research is required, the number of directly comparative studies is much lower and makes comparative conclusions more complex. This also complicates matters with regards to potential differences observed between breeds and age of onset, as highlighted in this review. For example although the differences between breeds are often presented, in many cases the comparisons between each of the breeds and human OSA are not frequently investigated. Canines are often referred to as a good model for juvenile human OSA but in addition to the published comparisons between general canine OSA and juvenile human OSA, it must be highlighted that many studies do draw any conclusions regarding juvenile or later onset OSA specifically in either species. In some OSA studies, particularly the canine studies, the ages of the patients are not presented or the juvenile/later onset differences, if any, are not specifically referred to or investigated. Additionally, particularly when thinking about juvenile OSA, matters such as whether

growth is a risk factor is not as easy to justify in dogs compared to people.

With molecular differences between OSA samples differing between individuals within the same species, it is natural to expect differences between the species and between the ages of the individuals. When comparing canine OSA to human pediatric OSA in particular, mutational burden must be considered. Generally pediatric human cancers present with fewer mutational burdens, compared with geriatric tumors [as reviewed previously (128)], therefore this must be considered when comparing against older canines with pediatric OSA. There are concomitant arguments for using the dog as a model of aging (also presenting with limitations and differences) (131). Although similarities between canine and human DNA repair machinery have been shown, such as in lymphoma, mammary tumors and even OSA (132, 155, 156), not every mechanism may be similar, for example base excision repair and nucleotide excision repair have both been shown to be lower in canines (157). Unfortunately little is known about DNA repair in canine OSA (158) even though it may play significant roles when comparing geriatric with juvenile/pediatric OSA. This further accentuates the need for vigilance when researching OSA in general including both OSA specific molecular mechanisms and pathways, and those related to more general factors such as aging.

## Discussion

Canine OSA occurs naturally within the population, reflecting the development of human OSA (7, 159). In contrast animal models of OSA rely on chemical induction, xeno/allografts, and genetically engineered animals which are unlikely to reflect many aspects of naturally occurring disease (159–162). Canine OSA has several features that can accelerate the understanding of the molecular basis of OSA, potentially facilitating more rapid development of novel diagnostic and therapeutic targets relevant to both people and dogs. The advantages of canine OSA parallel patient populations include a shared environment with people, natural disease progression, higher incidence rates, alongside shorter lifespans resulting in a quicker clinical course. Arguments have also been put forward that in addition to the dog being a good parallel patient population for OSA in people, the reverse is also technically true. It has also been indicated that canine OSA may represent a more accelerated biology than human OSA and that novel metastasis-associated tumor targets may be more readily identifiable in canine tissues (25). Whilst this review has concentrated on some of the shared molecular observations and mechanisms, there are many examples presented where canines do not exactly mirror human OSA. Canine OSA parallel patient populations can therefore give valuable insights, advancing knowledge about disease progression and development, cellular and molecular mechanisms, and therapeutic and treatment strategies, in both people and dogs.

## Author contributions

CR developed the concept, designed and supervised the manuscript, and created Figure 1. SS and CR drafted the manuscript. CR and AR received funding. All authors revised the manuscript critically, contributed intellectually, and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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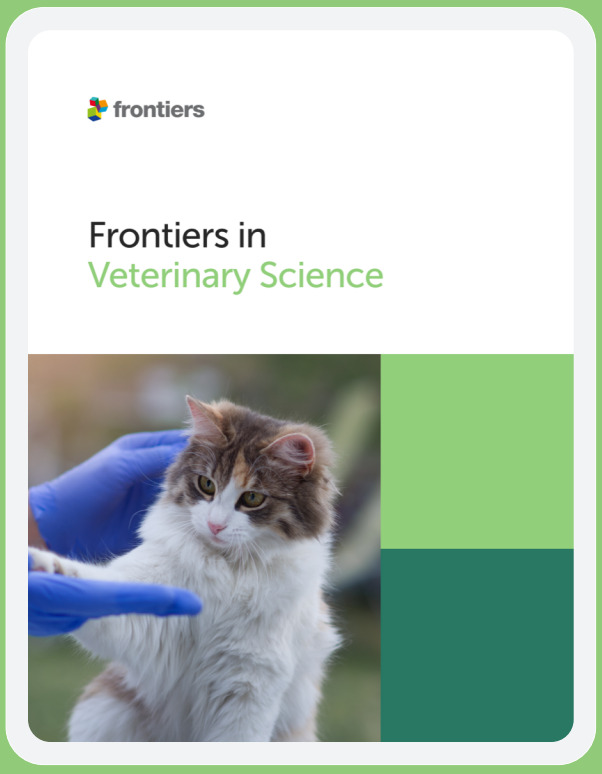


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