Title: Proteomic Profiles of Circulating Exosomes as Biomarkers for Disease Detection in Canine Osteosarcoma

Abstract:
Osteosarcoma (OSA) is the most common primary skeletal tumor in dogs accounting for 85% of bone tumors and nearly 6% of all canine neoplasms. They are highly aggressive tumors that carry a poor prognosis despite intensive treatment, highlighting the need for more effective diagnostic and therapeutic tools. Recent research in human medicine has underlined the importance of vesicular transport via exosomes, particularly related to their role in the progression of cancer and drug resistance. Exosomes are small vesicles with a phospholipid bilayer membrane that are secreted by many cells in the body, and have been shown to transfer oncogenic proteins and nucleic acids that play influential roles in tumorigenesis, metastasis, and response to treatment. More recently, exosome-associated proteins in biofluids have been studied for their potential as minimally invasive biomarkers for a variety of human neoplasms, including OSA. The objectives of our study were twofold. First, to determine if serum-derived exosomes contain a unique protein signature that can be ascribed to canine OSA patients, which could serve as a biomarker for disease and predict response to therapy. Second, we sought to evaluate if exosomes are capable of transferring chemotherapeutic resistance to susceptible osteoblasts, with the hypothesis that acquisition of resistance will correspond with a distinct proteomic profile.
Exosomes that were isolated from sera of canine patients were measured using light scatter technology and determined to be of appropriate size. Proteomic analysis via mass spectrometry exhibited unique protein profiles for dogs with OSA, traumatic bone fracture, and normal animals. Additionally, 10 proteins were identified that could differentiate OSA from control patients (normal or traumatic fracture) with 85% accuracy. Select discriminating proteins were validated with western blot. Furthermore, unique exosomal protein signatures were evident for dogs with OSA at different disease stages (diagnosis, 2-weeks post-amputation, and detection of metastatic disease), with 2 proteins identified that could distinguish between the disease stages with 75% accuracy.

A carboplatin-resistant cell line (HMPOS-R) was created by repetitive incubations of the cell line with increased concentrations of carboplatin and expansion of the survived cells. A viability assay demonstrated that HMPOS-R had considerably improved viability compared with its naïve equivalent (HMPOS-S). Exosomes isolated from HMPOS-R were incubated with HMPOS-S and subsequent viability assay showed increased viability of resistant exosome-treated cells (HMPOS-EX), suggesting exosomal transfer of resistance. Proteins contained in exosomal preparations had a distinctive profile in comparison to cell lysates. Additionally, proteomic profiling of exosomes and cell lysates at various stages in acquiring resistance demonstrated a unique change in protein expression that may contribute to chemotherapeutic resistance.

In summary, our findings demonstrate the potential for circulating exosomes as powerful tools for discovery of novel biomarkers and potential therapeutic targets as a unique protein signature is ascribed to exosomal cargo from serum of dogs with OSA compared with control animals, and throughout various stages of disease. Our research also demonstrates the capability of exosomes to confer chemotherapeutic resistance on susceptible cells, similar to what has been demonstrated in many human malignancies. Further investigation into the biomarker potential and functional significances of exosomal cargo in veterinary medicine is warranted.
Proteomic Profiles of Circulating Exosomes as Biomarkers for Disease Detection in Canine Osteosarcoma

by
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Dean of the Graduate School

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Jacqueline V. Brady, Author
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I would like to thank my family and friends; particularly my husband, Sean, for supporting me throughout my residency and graduate school journey.

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Proteomic Profiles of Circulating Exosomes as Biomarkers for Disease Detection in Canine Osteosarcoma
1. INTRODUCTION

Osteosarcoma (OSA) is the most common primary skeletal tumor in dogs accounting for 85% of bone tumors and nearly 6% of all canine neoplasms. They are highly aggressive tumors that carry a poor prognosis, despite intensive treatment. Additionally, there is great variability in patient survival time even with proper diagnosis and an optimal treatment regimen. Although few canine patients with OSA have radiographically detectable metastases at the time of presentation, the majority die of metastatic disease within one year of diagnosis, highlighting the need for more effective diagnostic and therapeutic tools. Canine OSA bears a remarkable resemblance to pediatric OSA in humans, and research in the field of veterinary cancer can acquire valuable insight from their human counterpart.

Research over the last decade in the realm of human medicine has underlined the importance of vesicular transport via exosomes, particularly related to their role in the progression of cancer and drug resistance. Exosomes are small (30-150 nm) vesicles containing a phospholipid bilayer membrane that are secreted by a wide variety of cell types; however, neoplastic cells have been shown to secrete greater amounts than normal cells. Exosomes contain numerous intracytoplasmic molecular constituents such as proteins, RNA, and microRNA (miRNA), and serve as key molecules for trafficking biological material. These vesicles have been shown to transfer oncogenic proteins and nucleic acids that play influential roles in tumorigenesis, cancer progression, metastasis, and response to treatment. Additionally, exosome-associated proteins in biofluids have been suggested as minimally invasive biomarkers for a variety of human neoplasms. Recent investigations in human OSA have demonstrated significant differences in serum exosomal protein content, which appears to play a substantial role in progression of disease. These differentially expressed proteins may be capable of serving as biomarkers of metastasis and response to therapy.
As canine and human (pediatric) OSA are characterized by similar genetic and clinicopathologic features, we hypothesized that canine serum-derived exosomes will contain a unique protein signature that can be ascribed to OSA patients, analogous to what has been demonstrated in pediatric OSA. Furthermore, we hypothesized that exosomes derived from sera of OSA patients will have distinct protein signatures throughout different stages of disease, including at time of diagnosis, 2 weeks post-amputation, and at the onset of lung metastasis. These signatures will have the potential to serve as non-invasive biomarkers for disease, and may be useful for staging and prognostication. An additional hypothesis is that exosomes will be functionally capable of transferring chemotherapeutic resistance to susceptible osteoblasts and as resistance is acquired, a signature exosomal-protein profile will be observed. Presence of distinct proteins may serve to ultimately predict patient response to chemotherapy.

To date, there has been minimal work performed in the field of veterinary oncology in evaluating exosomal cargo derived from biofluids as biomarkers or drivers of chemotherapeutic resistance. Our hope is that the results from this investigation will move the field forward with the potential to lead to the development of novel biomarkers and therapeutic targets.
2. LITERATURE REVIEW

2.1 Extracellular vesicles

Cellular crosstalk is essential for the development and functioning of tissues. Mechanisms of intercellular communication were historically thought to be primarily confined to direct physical contact, such as cell junctions or adhesion molecules, or via secretion of soluble molecules that can travel in a paracrine or endocrine fashion. However, the importance of intercellular communication via release of extracellular vesicles (EVs) has become increasingly appreciated in the last two decades. Many mammalian cells secrete EVs, which are small spherical sacs surrounded by a phospholipid-bilayer membrane, similar to the cell plasma membrane, that contain a variety of materials depending on their cell of origin. They can be constitutively produced by cells, or produced as a result of activation from stressors, including oxidative stress or hypoxia. EVs, consisting of exosomes, microvesicles (also commonly referred to as microparticles and ectosomes), and apoptotic bodies, are categorized according to composition, size, and function. Exosomes are the smallest vesicles, ranging from approximately 40-150 nm, and are formed intracellularly via inward budding of the endosomal membrane into a multivesicular body, which then fuses with the plasma membrane for exosomal release. Microvesicles are larger in size (100 nm-1 μm) and are formed by outward budding and fission of the plasma membrane. Apoptotic bodies have well defined characteristics and have been historically extensively studied. These are formed in similar fashion to microvesicles, but are the largest in size (2-4 μm) and originate from cells in the process of programmed cell death.

As previously mentioned, numerous cell types secrete EVs into the extracellular space, both in physiologic and pathologic states. Therefore, vesicles can be found in many types of body fluids, including serum, urine, amniotic fluid, ascites fluid, and breast milk. EVs can contain numerous intracytoplasmic molecular constituents, such as proteins, RNA, and
microRNA (miRNA), and serve as key molecules for trafficking of biological material.\textsuperscript{3} As a consequence, EVs can ultimately modify the functions of target cells.\textsuperscript{7}

**Figure 2.1.1. Main classes of extracellular vesicles.** Exosomes form via inward budding of endosomal membranes, forming multivesicular bodies (MVBs), which fuse with the plasma membrane and release their contents into the extracellular space. Microvesicles are created by outward budding of the plasma membrane. Apoptotic bodies are formed from cells undergoing controlled cell death via blebbing of the plasma membrane. Apoptotic bodies also contain nuclear contents. Figure from Kanada \textit{et al.}\textsuperscript{11}

Because microvesicles can be detected in biological fluids, they have been extensively evaluated, particularly in human medicine, for their potential to serve as non-invasive biomarkers for many disease processes.\textsuperscript{5, 13}

**Exosomes**

Exosomes were identified many years ago as vesicular structures that were located within multivesicular bodies (MVBs), and were thought to represent a late step in the maturation from endosomes to lysosomes (degradative MVBs).\textsuperscript{14} Only within the last two decades has it been shown that MVBs also participate in exocytic fusion with the plasma membrane (exocytic MVBs) where its contents, the intraluminal vesicles (ILVs), are released into the extracellular space (Figure 2.1.2).\textsuperscript{15} ILVs that are released by exocytosis are called exosomes. The biogenesis of MVBs, formation of ILVs, and processes that govern the
exocytic MVB pathway and release of ILVs are not fully understood.\textsuperscript{15} However, a proposed mechanism involves endosomal sorting complexes required for transport (ESCRTs), which is a complex, multimolecular system that is involved in the sorting of MVB vesicles.\textsuperscript{14}

**Figure 2.1.2. Generation of exosomes.** Endosomes created at the plasma membrane fuse to form endocytic cisternae, which are converted into multivesicular bodies (MVBs) by vesicle accumulation and sorting processes. Two types of MVBs form: degradative MVBs, which evolve into lysosomes, and exocytic MVBs, which fuse their membrane with the plasma membrane for release of exosomes into the extracellular space. The arrows indicate the direction of membrane traffic; the arrowheads indicate the directions of vesicle fusion and generation. Figure from Cocucci \textit{et al.}\textsuperscript{14}

Exosomes are very rigid structures and appear to be resistant to enzymatic degradation in blood and other body fluids.\textsuperscript{16,17} This is likely due to protection via the lipid bilayer membrane, which also serves as a transport vehicle for luminal cargo.\textsuperscript{15,17} Exosomes are capable of fusing with the plasma membrane of recipient cells to deliver their contents, but also contain a variety of surface molecules, which provides a mechanism to engage cell receptors to exchange material and induce intracellular signaling (\textbf{Figure 2.1.3}).\textsuperscript{18} These exosomal surface proteins may include integrins, molecules for antigen presentation, and tetraspanins, among many others. The luminal contents of exosomes generally reflect that
of the donor cell. However, as exosomes originate from endosomes, proteins that are involved in the endocytic pathway are frequently detected regardless of cell type of origin. Common proteins include those involved in MVB formation (e.g. Alix), heat shock proteins (e.g. HSP90), or cytoskeletal proteins. In addition to proteins, exosomes contain nucleic acids such as mRNA and miRNA, which represents a unique form of intercellular communication wherein the donor cell can regulate gene expression of the recipient cell.

Figure 2.1.3. Protein composition of exosomes. Exosome content varies depending on cell origin; however, they contain a number of common protein components, as demonstrated in the image. Figure from Schorey et al.

Currently there is no gold standard for exosome isolation from fluids. The most common method utilizes an ultracentrifugation technique; however, this tends to be time-consuming, relatively low-yield, and inefficient at separating out the EV subtypes unless
filtering or differential centrifugation is also employed. Additionally, commercial kits are available that are robust and use very little sample, which make them ideal for biomarker studies. Further analysis of isolated EVs often includes imaging, such as electron microscopy, nanoparticle tracking analysis or immunoblotting.

2.2 Role of exosomes in cancer

Tumor cells have been shown to secrete greater numbers of exosomes compared with normal cells. These exosomes are often enriched with proteins and genetic information (e.g. mRNA, miRNAs, and oncogenes) that may be transferred to the surrounding cells which could facilitate creation of an environment that is favorable for tumor survival and growth. Additionally, exosomes released by a given cell type may be able to act at a distance to set up a pre-metastatic niche. Exosome contents have been shown to accomplish this through a variety of methods, including (but not limited to) modulation of the immune response, stromal cell alteration, activation of cell migration, and angiogenesis.

A vast amount of research has demonstrated intercellular cross talk via exosomes between tumor cells and cells that regulate the immune response. In human nasopharyngeal carcinoma (NPC), a small chemokine carried by NPC-derived exosomes appears to play a crucial role in regulatory T lymphocyte (T_{reg}) recruitment. Furthermore, these exosomes are capable of inducing T_{reg} expansion and promoting conversion of CD4+CD25- T cells into CD4+CD25+FoxP3+ T cells (T_{regs}). A study by Szajnik et al. also demonstrated that vesicles isolated from tumor cells were capable of promoting conversion and proliferation of T_{regs} in peripheral blood and ascites fluid from cancer patients. Additionally, flow cytometry analyses demonstrated that vesicles expressed transforming growth factor beta (TGF-β) and interleukin (IL)-10, suggesting that their presence attenuates the antitumor response. Andreola et al. described exosome-like vesicles secreted by melanoma cells that express the Fas ligand (FasL) and subsequently induce apoptosis of Fas-positive T lymphocytes, also impairing the antitumor immune response.
Tumor-derived EVs have been linked with stromal cell alterations that favor tumor survival, expansion, and dissemination.\textsuperscript{7, 22-24} Recent research has demonstrated a role for vesicles in communication between tumor cells and fibroblasts. For example, a study by Castellana \textit{et al.} showed that vesicles derived from prostate cancer cells deliver matrix metalloproteinases (MMP) and extracellular MMP inducer to neighboring cells, enhancing extracellular matrix degeneration and promoting invasion of tumor cells.\textsuperscript{22} Additionally, Cho \textit{et al.} discovered that adipose tissue-derived mesenchymal stem cells, when treated with purified exosomes from ovarian cancer cell lines, exhibited characteristics of tumor-associated myofibroblasts.\textsuperscript{23} These included increased expression of α-smooth muscle actin (α-SMA), with increased expression of tumor-promoting factors, stromal cell-derived factor 1 (SDF-1) and TGF-β, which are involved in neovascularization and immunosuppression, respectively.\textsuperscript{23} A study by Skog \textit{et al.} demonstrated that glioblastoma-derived exosomes were capable of transferring functional mRNA and miRNA to peritumoral microvascular endothelial cells, such as epidermal growth factor receptor (EGFR), which were capable of inducing cell proliferation and angiogenesis.\textsuperscript{24}

Due to their presence and stability in biofluids, tumor-derived exosomes have demonstrated superb potential to serve as a “liquid biopsy” tool for a variety of human neoplasms (Table 2.2.1).\textsuperscript{25} One study evaluated circulating EVs in patients with breast cancer and found that levels of developmental endothelial locus-1 (Del-1), a protein involved in embryonic vascular development, were significantly higher than those in samples from healthy control patients.\textsuperscript{26} Del-1 levels did not significantly correlate with tumor size or receptor status. However, Del-1 in plasma EVs was dramatically reduced (to normal levels) after removal of the tumor.\textsuperscript{26} Similarly, a study by Khan \textit{et al.} revealed that although plasma-derived exosomal survivin was present in prostatic tissue of all subjects tested (normal, benign hyperplasia, and carcinoma), it was at significantly higher levels in patients with prostatic carcinoma, even at an early stage.\textsuperscript{27} Therefore, circulating exosomal survivin may be useful as a tool for early detection of prostatic neoplasia in men.\textsuperscript{27} In addition to proteins, many studies have characterized nucleic acids isolated from
exosomes, particularly miRNA, due to their stability against RNase-dependent degradation.\textsuperscript{25} Several miRNAs have shown their potential to serve as diagnostic markers for a variety of neoplasms, including lung adenocarcinoma and esophageal squamous cell carcinoma.\textsuperscript{25, 28, 29}

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Table 2.1.1. Exosomal protein biomarkers from body fluids of human patients in pre-clinical and clinical studies. Table adapted from Soung et al.\textsuperscript{25}

Overall, the cargo delivered by cancer-derived exosomes has received much attention in the research sector over the last decade, given that they appear to contribute broadly to the survival and spread of neoplastic disease. Additionally, exosome-associated proteins and nucleic acids represent promising biomarkers for diagnosis, progression, and monitoring of many human cancers.\textsuperscript{25}
2.3 Exosomes in chemotherapeutic resistance

Drug resistance is a major obstacle in the treatment of cancer as it changes the efficacy of chemotherapeutic agents that would otherwise be successful. Extrinsic resistance is the result of the tumor microenvironment shielding the tumor cells from treatment that would result in their demise. Recent research has shown that tumor-derived exosomes play a role in promoting chemotherapeutic resistance through a variety of mechanisms, including transfer of multi-drug resistance (MDR)-associated proteins and miRNAs. In a study performed by Ji et al., exosomes derived from mesenchymal cells were shown to confer drug resistance in gastric cancer via increased expression of MDR-associated genes and proteins, and through enhanced functionality of P-glycoprotein (P-gp)/MDR pump allowing for more efficient drug efflux. Additionally, Ning et al. demonstrated transfer of chemoresistance phenotype to Adriamycin-sensitive human breast cancer cells via exosomes derived from their Adriamycin-resistant equivalent. Exosomes carried increased levels of ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and P-gp proteins.

Other mechanisms by which exosomes impede drug therapy include the expression of surface proteins that sequester the chemotherapeutic agent away from the target cell. For example, Ciravolo et al. showed that exosomes secreted by HER2-overexpressing breast carcinoma cell lines express a full-length HER2 molecule which can bind to the chemotherapeutic agent, Trastuzumab. The HER2-positive exosomes inhibited the activity of Trastuzumab on the breast cancer cells, allowing them to continue to proliferate. Additionally, exosomes transferred from chemo-resistant to chemo-susceptible human breast cancer cells have been shown to confer chemoresistance via affecting cell cycle distribution (increased G1 and G2 phase) and inhibiting drug-induced apoptosis, likely via transfer of specific miRNAs.

2.4 Canine osteosarcoma

Osteosarcoma is a malignant neoplasm that arises from primitive mesenchymal cells that exhibit osteoblastic differentiation. In both human and veterinary medicine, this neoplasm
is the most common primary tumor of bone. Of the veterinary species, OSA occurs most frequently in the dog. Cats are the second most frequently affected and cases have been reported in many other domestic species. In the United States, the incidence of OSA in humans is estimated at 600 cases annually. In canine patients >8,000 cases are diagnosed annually. Spontaneous tumors in dogs may serve as a model for human disease, as they share many molecular, biological, and clinical similarities.

Canine OSA accounts for 85-98% of bone tumors and nearly 6% of all canine neoplasms. These tumors occur most frequently in middle-aged to older dogs with a mean age of 8 years. However, there is a small peak of incidence in young dogs that are less than 3 years of age. This is in contrast to human OSA in which the peak age of incidence is in the second decade of life (adolescence). Osteosarcoma most commonly is seen in large and giant breed dogs, with an apparent strong breed disposition. Breeds with the highest risk include Saint Bernard, Great Dane, Rottweiler, Irish wolfhound, greyhound, German shepherd, golden retriever, and Doberman pinscher. Reportedly, only 5% of appendicular OSA cases occur in dogs weighing less than 15 kg. A slight male predilection for appendicular OSA has been reported, although this is not widely agreed upon in the literature. Additionally, one study demonstrated a two-fold risk for OSA in neutered dogs compared to sexually intact dogs. Similarly, a large-scale study of Rottweilers that underwent gonadectomy at less than 1 year of age demonstrated a strong inverse relationship between sex hormones and incidence of OSA.

The primary tumor is located in the appendicular skeleton in approximately 75% of canine patients with OSA. The axial skeleton is primarily affected in 24% of cases. The remaining 1% represents primary disease in the soft tissues. Lesions of the appendicular skeleton typically arise from the metaphyseal regions of the long bones and are most commonly observed in the forelimb, particularly in the distal radius and proximal humerus. Lesions in the hind limb are most frequently observed in the distal femur and proximal and distal tibia. Axial OSA most commonly arise in the mandibular, maxillary, nasal, and
cranial flat bones of the skull, and less frequently in the spine and ribs. In contrast to large and giant breed dogs, one study demonstrated that in small breed dogs weighing less than 15 kg, the majority of tumors are of axial origin (59%). Although primary extraosseous OSA is rare, reports have demonstrated tumors in the spleen, kidney, testicle, vagina, lung, and adrenal gland. Tumors arising from surgical items, such as gauze or surgical implants, have also been reported.

![Figure 2.4.1. Site of origin in 1215 primary canine osteosarcomas.](image)

Canine patients with OSA generally present with clinical signs that reflect pathology from the primary tumor, most commonly single limb lameness, that may be intermittent or mild initially, but becomes persistent and progresses in severity. Additionally, patients are often painful on palpation of the affected site and pathologic fractures of the bone may be seen. On presentation, approximately 5-10% of patients have detectable metastasis; however, the vast majority of dogs have undetectable pulmonary micrometastases at this time. Most patients with appendicular OSA will ultimately develop detectable lung masses with 72-100% of dogs having pulmonary involvement at necropsy. A lower rate of
pulmonary metastases are observed with primary axial disease (35-46%).\textsuperscript{53} Metastatic spread is typically via the hematogenous route with the lungs representing the most common metastatic site (85%). Fewer patients experience metastases to the bone (10%), lymph node (4.4-9%), or other tissues, including visceral organs, muscle, skin, and the central nervous system.\textsuperscript{3}

Osteosarcoma is often suspected based on patient signalment, history, and clinical presentation. After localization of the lesion, radiographs are often performed and may provide support to the diagnosis of OSA. Radiographic findings will depend on severity of disease but typically include monostotic lysis of cortical or medullary bone, sclerosis, and/or periosteal proliferation.\textsuperscript{54} Fine needle aspiration (FNA) biopsy may be performed to provide a preliminary diagnosis of OSA; however, definitive diagnosis frequently requires histopathology of the lesion.\textsuperscript{55} FNA offers many advantages over histological tissue biopsy, including rapid results, minimal patient discomfort, shorter procedure time, and negligible tissue disruption.\textsuperscript{55, 56} In recent study of canine destructive bone lesions, cytology was 83\% accurate for diagnosis of malignancy, correctly identifying malignancy in 44 out of 53 cases.\textsuperscript{55} All 9 cases that were unidentified had inadequate cellularity, which is a common pitfall for cytological diagnoses.\textsuperscript{57} Additionally, although cytology can accurately predict malignancy, identification of tumor type can be difficult without corresponding tissue architecture. Application of an alkaline phosphatase substrate to cytological preparations has been shown to be a highly sensitive and fairly specific marker for diagnosis of OSA in comparison with other vimentin-positive tumors (e.g. fibrosarcoma or chondrosarcoma).\textsuperscript{58}

On histopathology, the defining feature of OSA is formation of osteoid matrix or immature bone by malignant mesenchymal cells.\textsuperscript{52, 57} The amount and quality of the matrix can vary dramatically between and even within tumors.\textsuperscript{52} Canine OSA shows various morphologies and is commonly divided into 6 histological subtypes, with individual tumors frequently containing a mix of more than one subtype: osteoblastic, fibroblastic, chondroblastic, telangiectatic, giant cell-rich, and poorly-differentiated.\textsuperscript{59} Of the subtypes, osteoblastic is
the most common seen in appendicular OSA. Histological classifications may be somewhat arbitrary and the literature is lacking in significant correlation between subtype and prognosis. However, prognosis with the telangiectatic subtype has been shown to be very poor. Grading systems have been proposed for canine OSA, with an updated system presented in 2002 by Kirpensteijn et al. evaluating the following features: cellular pleomorphism, mitotic count, amount of tumor matrix, tumor necrosis, tumor cell density, and vascular invasion (Table 2.1.1). In 166 dogs with OSA, 125 (75%) had grade III tumors (high grade). High grade tumors were associated with a significantly shorter survival time and disease-free interval.

<table>
<thead>
<tr>
<th>Tumor Grade</th>
<th>Pleomorphism</th>
<th>Mitoses</th>
<th>Tumor Matrix</th>
<th>Tumors Cells</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0-1 (&lt;25%)</td>
<td>&lt;10</td>
<td>1 (&gt;50%)</td>
<td>1 (&lt;25%)</td>
<td>0-1 (&lt;25%)</td>
</tr>
<tr>
<td>II</td>
<td>2 (25-50%)</td>
<td>10-20</td>
<td>2 (25-50%)</td>
<td>2 (25-50%)</td>
<td>2 (25-50%)</td>
</tr>
<tr>
<td>III</td>
<td>3-4 (&gt;50%)</td>
<td>&gt;21</td>
<td>3 (&lt;25%)</td>
<td>3-4 (&gt;50%)</td>
<td>3-4 (&gt;50%)</td>
</tr>
</tbody>
</table>

Table 2.4.1. Classification for canine osteosarcoma tumor grade determination. Histologic tumor grade is determined by degree of nuclear pleomorphism, mitotic count, amount of tumor occupied by matrix, density of tumor cells, and percentage of tumor necrosis. If neoplastic cells are present within vessels or lymph nodes, tumors are automatically qualified as grade III. Table adapted from Kirpensteijn et al.

Treatment for canine OSA depends on whether palliative- or curative-intent therapy is pursued. Both approaches typically involve surgical excision of the primary tumor, as the discomfort associated with its presence contributes to a poor quality of life. For the majority of patients with appendicular OSA, removal of the primary tumor entails limb amputation, which is the current standard of care for local disease. Amputation can be contraindicated in patients that suffer from obesity, neurologic disease, or severe osteoarthritis in the contralateral limb. Limb-sparing surgery is an alternative option for removal of local disease and has become the standard of care in humans with primary bone tumors. The surgery involves resection of the tumor and restoration of the bony column, commonly using cortical bone allografts. Either of these surgical procedures alone is
considered palliative therapy; mean survival times are reported to range from 103-175 days, with a 1-year survival of 11-20%.³

For canine patients with appendicular OSA, the gold standard for curative intent treatment involves surgical removal of the tumor in conjunction with chemotherapy for control of metastatic disease.⁶⁰ There appear to be no survival differences between amputation and limb-sparing surgeries when systemic chemotherapy is given⁶¹; however, of interest, infection of the surgical site after a limb-sparing procedure has been shown to increase the median survival time compared with patients that did not acquire an infection.³ This phenomenon has been also reported in human medicine.⁶² Proposed possible mechanisms for the beneficial effect are stimulation of cytotoxic cell-mediated tumor suppression, cytokine stimulation and resultant lysis of tumor cells, and prevention of neovascularization.⁶² Adjuvant chemotherapy improves survival time and many single- and multiple-agent protocols involving carboplatin, cisplatin, and doxorubicin, have been studied. In general, median survival times with chemotherapy (in conjunction with surgery and/or radiation therapy) range from 262-450 days, with a 1- and 2-year survival of 31-48% and 10-26%, respectively.³ Bisphosphonates are drugs that inhibit osteoclastic bone resorption, and can be used as an adjunctive therapy for OSA. Although a placebo-controlled study found that there was no difference in pain relief (via force plate analysis) between patients given bisphosphonates versus a placebo, it is still often believed that they can provide some degree of analgesic effect.⁴⁸,⁶³

Radiation therapy can be performed on OSA patients with palliative or curative intent. Palliative intent radiotherapy is most commonly used in patients that are not ideal candidates for surgery or have tumors in inoperable sites. It is performed to improve quality of life, by relieving pain and reducing lameness.⁶⁴ Pain relief has been demonstrated in 50-93% of dogs within two weeks of receiving palliative radiotherapy. Duration of pain relief is variable, ranging from 53-180 days.³,⁶⁴ Reported doses range from 3-22 Gy given over multiple fractions; however, this can be unfeasible for many owners as it involves
multiple anesthetic episodes and prolonged hospitalization. Radiation therapy with curative intent has been shown to achieve good local tumor control; unfortunately, these are commonly associated with radiation-induced complications, including pathologic bone fractures. Stereotactic radiosurgery (gamma knife therapy) was performed in a small number of dogs as a limb-sparing alternative with a median survival time of 363 days. This method of radiotherapy involves precisely-targeted radiation in an effort to minimize damage to surrounding tissues. Limiting factors for this method of treatment are tumor size and condition of the bone at time of therapy. Additionally, this technique involves equipment that is not readily available to veterinarians.

Regardless of therapy pursued, the prognosis for long-term survival in canine OSA remains poor. As such, many investigational immunotherapeutic strategies have been explored, including use of monoclonal antibodies that can be targeted toward tumor cells or the microenvironment, cytokine therapies and therapeutic vaccination to enhance immune responses, among others. In a study by Dow et al., 20 canine patients with chemotherapy-resistant OSA and lung metastases were given intravenous cationic liposome-DNA complexes containing canine IL-2 cDNA, with the intent to induce IL-2 expression and modulation within the lung. Ultimately, 7 out of 20 (35%) patients had complete or partial regression of lung metastases or stable disease, and the overall survival time was significantly increased, suggesting that this therapy could elicit antitumor activity.

Several prognostic factors have been identified in canine OSA, including those associated with signalment, anatomic location, clinicopathologic features, and molecular and genetic factors. Young dogs (<5 years of age) tend to have a shorter survival time compared with older dogs. Additionally, poor outcomes have been associated with increased tumor size due to the higher incidence of pulmonary metastases at presentation, as well as tumors located in the proximal humerus compared with other anatomic sites. Regarding clinicopathologic features, serum alkaline phosphatase (ALP) activity has strong prognostic significance, with increased total ALP or bone isoenzyme of ALP associated with a
decreased survival time. A study by Sotnik et al. found that higher numbers of circulating monocytes and lymphocytes were associated with a significantly decreased disease-free interval. However, these findings have not been substantiated. In a study by Biller et al., canine OSA patients had significantly fewer circulating CD8+ T cells and more Tregs when compared with healthy control animals. Additionally, the CD8+/Treg ratio was significantly lower in OSA patients, which corresponded with a significantly shorter survival time. As mentioned above, high tumor grade is strongly associated with a shorter survival time and shorter disease-free interval. Increased expression of molecular proteins, such as vascular endothelial growth factor (VEGF), survivin, p53, total and pro-MMP-2 and MMP-9, and cyclooxygenase-2 (COX-2), are associated with higher grade tumors, increased risk of metastasis, or decreased survival time. Mullin et al. evaluated COX-2 expression in appendicular OSA and found that intensity of COX-2 expression, in conjunction with percentage of positive cells was associated with decreased survival time.

2.5 Exosomes in osteosarcoma
Few studies have evaluated the role of EVs in OSA. A study performed by Jerez et al. evaluated exosomal proteins derived from human OSA cell lines and compared them with proteins in exosome-free conditioned media. Osteosarcoma exosomes were shown to be enriched with proteins associated with angiogenesis, cell adhesion, and cell migration. Additionally, Shen et al. evaluated serum-derived exosomes and found that they uniquely expressed proteins associated with tumor metabolism. Along with differential protein expression, studies have shown that serum-derived exosomes of human OSA patients have differential miRNA expression, which corresponds with chemotherapeutic response. Exosomes also appear to confer chemotherapeutic resistance in OSA. A recent study demonstrated exosomal transfer of MDR-1 mRNA as well as P-gp from a doxorubicin-resistant OSA cell line, which was able to induce a doxorubicin-resistant phenotype on previously sensitive cells.
3. EXPERIMENTAL DESIGN AND METHODOLOGY

3.1 Experimental design
To evaluate differential expression of protein cargo from serum-derived exosomes in patients with OSA, we designed an *in vivo* study using serum from patients with appendicular OSA along with healthy, adult, size-matched controls and patients with non-neoplastic traumatic bone fracture. From the serum samples, we collected exosomes by utilizing a commercially available kit, and demonstrated successful isolation via nanoparticle tracking analysis and fluorescent labeling techniques. Exosomal protein content was examined with mass spectrometry (LC-MS/MS) and select, differentially expressed proteins were identified by immunoblot. Additionally, we evaluated serum exosomes collected longitudinally from patients with OSA at three distinct time points: diagnosis of disease, 2 weeks post-amputation (prior to initiation of chemotherapy), and at first signs of lung metastasis; the ultimate goal being to evaluate changes in protein expression that could ultimately provide therapeutic targets or serve as biomarkers for disease progression. To assess the ability of exosomes to confer chemotherapeutic resistance onto neighboring drug-sensitive tumor cells, we created a carboplatin-resistant OSA cell line using sub-toxic drug doses at incremental levels. Exosomes were isolated from the drug-resistant cells once adequate resistance was acquired, as determined by cell viability assay, and subsequently applied to naïve cells. Additionally, cell lysates were collected and exosomes were isolated from cell culture media at various levels of drug resistance, and protein content was examined to determine changes in protein expression in the cells and exosomes throughout the course of acquiring resistance.

3.2 Methodology

*Cell lines and culture conditions*

The previously characterized canine OSA cell lines COS, C4, POS, and HMPOS were utilized in our study. Additionally, SAOS, a previously characterized human OSA cell line, was
Cells were maintained in RPMI 1640 medium (Thermo Fischer Scientific, Waltham, MA) supplemented with 100 units/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine with 10% fetal bovine serum (FBS; Thermo Fischer Scientific). All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Creation of a carboplatin-resistant cell line**

To create a cell line that would acquire resistance to carboplatin, cell lines COS, C4, POS, and HMPOS were evaluated for carboplatin sensitivity using a cell viability assay (as described in the “cell viability” methods section). The most carboplatin-sensitive cell line, HMPOS, was utilized for this portion of the study. In addition to cell culture media, cells were treated with carboplatin at an initial dose of 0.5 µM for 72 hours. The remaining viable cells were then expanded and treated for 72 hours at increasing carboplatin dosages: 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 µM. Additional cells were placed in freezing media (8% DMSO + 10% FBS DMEM) and stored at -80°C after each stage of treatment. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Patient samples**

Patient serum samples that had been previously collected for routine biochemical analysis and stored at -20°C were used in this study, precluding the need for an Animal Care and Use Protocol (ACUP). Approximately 600 µl of serum was obtained from each of 18 adult (>1 year) medium and large breed dogs (>20 kg). Dogs were placed in the following categories: patients with appendicular OSA that was verified by histopathology (OSA; n=8), otherwise healthy animals that have experienced traumatic bone fractures (FX; n=5), and healthy, size-matched canine controls (N; n=5). Control dogs were deemed healthy based on complete blood count, chemistry panel, urinalysis, normal physical examination, and no current history of illness or treatment with prescription medication. Samples were obtained from canine OSA patients at initial diagnosis of OSA prior to standard treatment of limb amputation and carboplatin chemotherapy (270-300 mg/m² administered every 3 weeks for a total of 4 doses). Patients were excluded from the study if they had
radiographic evidence of metastasis at time of diagnosis, non-appendicular disease, or did not receive the standard chemotherapeutic protocol, specified above. Longitudinal samples were obtained from 5 additional patients with appendicular OSA at diagnosis, 2 weeks post-amputation (before initiation of chemotherapy), and upon first detection of metastatic disease.

Exosome isolation

For exosome isolation from OSA cell culture media, sub-confluent cells were incubated in media containing 10% exosome-depleted FBS (Exo-FBS™, System Biosciences, Inc., Palo Alto, CA) for 24 hours and supernatant was removed prior to cells reaching 100% confluence. Switching to exosome-depleted FBS is an essential step as normal FBS contains high levels of exosomes that may contaminate the exosomes of interest. The supernatant was subsequently centrifuged for 10 minutes at 2000 x g and the cell-free supernatant was collected. For exosome isolation from patient serum samples, the serum was also centrifuged for 10 minutes at 2000 x g to remove residual cells or debris that remained in the sample. Exosomes were isolated from the OSA cell-free culture supernatant and patient serum using the Total Exosome Isolation reagent (Invitrogen, Thermo Fisher Scientific). Five formulations exist to permit isolation from major body fluids (serum, plasma, urine, and “other bodily fluids”) and cell culture media. These reagents all contain the same core compound, which works by binding up water-soluble molecules and forcing less-soluble components, such as exosomes, out of the solution. However, the protocols for exosome isolation are slightly different, each being optimized for the specific sample type. For this study, reagents for exosome isolation from cell culture media and serum were used as directed. Briefly, the reagent was vigorously mixed with the cell-free media or serum at a ratio of 1:2 and 1:5, respectively. Exosomes were then collected after centrifugation at 10,000 x g for 60 minutes, and re-suspended in 1x phosphate-buffered saline (PBS). Protein content of exosomes was determined using a Pierce BCA kit (Thermo Fisher Scientific) on exosome aliquots lysed in 1x RIPA (Pierce RIPA Buffer, Thermo Fisher
Samples were stored at -80°C and aliquoted to minimize free/thaw cycles that could be potentially damaging to the exosomes.

**NanoSight Analyses**

Purified exosomes were suspended in 100 µl of 0.22 µm filtered PBS and analyzed using the NanoSight LM10 system (NanoSight Ltd., Amesbury, United Kingdom, NTA 3.00060 software). Analysis was performed by applying a monochromatic 404 nm laser to diluted exosomal preparation and measuring the Brownian movements of each particle. The Nanoparticle Tracking Analysis software version 2.3 was used to analyze 60 second videos of data collection to give mean, median, and mode of vesicle size and concentration.

**Culture of canine PBMCs with exosomes**

Blood from a normal healthy donor dog was obtained by venipuncture. Peripheral blood mononuclear cells (PBMCs) were purified from blood using density gradient separation (Histopaque 1077; Sigma Aldrich, St. Louis, MO). Isolated PBMCs were washed twice with PBS and incubated at 37°C for 15 minutes in 1 mL PBS with 1 mM Violet Proliferation Dye 450 (BD Biosciences, San Jose, CA). Stained PBMCs were washed with PBS, washed with RPMI medium, and then counted using a hemocytometer. Cells were placed in 96-well plates with 200,000 cells per well in 200 µL exosome-depleted media (described above) also containing 0.075% sodium bicarbonate (Thermo Fisher Scientific), MEM non-essential amino acids (Thermo Fisher Scientific), 25 ng/mL recombinant human IL-2 (PeproTech, Rocky Hill, NJ), and 0.5 µg/mL concanavalin A (Sigma Aldrich). Exosomes were added to a concentration of 50 µg/mL (10 µg per well) and cells were incubated for 5 days at 37°C in a humidified incubator with 5% CO₂.

**Fluorescent labeling of exosomes and tracing of exosomal uptake by recipient cells**

Exosomes were labeled with SYTO RNASelect green fluorescent stain (Thermo Fisher Scientific) by adding 1 µL of a 1 mM stock solution in DMSO to 100 µL of exosomes in PBS. The samples were incubated at 37°C for 20 minutes and subsequently passed through
Exosome Spin Columns MW 3000 (Thermo Fisher Scientific) to remove any unincorporated dye from the labeled exosomes. For evaluation of exosome uptake, peripheral blood mononuclear cells from a healthy dog were co-cultured with the labeled exosomes and incubated at 37°C for 2 hours. Samples were rinsed twice, re-suspended in 200 μL PBS, and then concentrated via cytocentrifugation (Shandon Cytospin 4, Thermo Electron, Pittsburgh, PA) onto a positively-charged glass slide. Samples were fixed by placing 3-4 drops of 4% paraformaldehyde over the cytocentrifuged pellets (20 minutes at room temperature) and permeabilized with 0.1% Triton X-100 (5 minutes at room temperature). The stained cells were mounted in Prolong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) and evaluated using a Nikon Eclipse Ti fluorescent microscope with NIS-Elements imaging software v. 3.22.11 (Nikon, Tokyo, Japan).

**Cell viability**

To assess the effect of carboplatin, proliferation experiments were performed on HMPOS, POS, COS, C4, and SAOS using the CellTiter96 Aqueous One Solution MTS Assay (Promega, Fitchburg, WI). Experiments were performed in three biological replicates, with three technical replicates per experiment. Cells were seeded into 96-well culture plates at a density of 2,000-3,000 cells per well and incubated for 24 hours. For assays evaluating exosomal transfer of resistance, exosomes derived from untreated and carboplatin resistant HMPOS were added to wells at a concentration of 50 µg/mL and allowed to incubate for 48 hours using media with exosome-depleted FBS. The cells were then incubated in fresh media containing carboplatin (CARBOplatin Injection, 10 mg/ml, Hospira, Inc., Lake Forest, IL) at concentrations of 0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µM. Wells containing only media were used as a “no cell” control. The MTS assay was performed according to the manufacturer’s protocol. Absorbance was measured at 490 nm on the microplate reader Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific).
Proteomic analysis

Aliquots of isolated exosomes from all 18 canine serum samples along with exosomes isolated from the serum of 5 distinct canine OSA patients at three distinct time points in their disease (diagnosis, 2 weeks post-amputation, and initial detection of lung metastases) were evaluated by mass spectrometry (LC-MS/MS) at the Oregon State University Mass Spectrometry Center. Additionally, exosome preparations that were isolated from culture media of carboplatin-resistant cell lines (as described in the “exosome isolation” methods section) and protein lysates from carboplatin-resistant cells were submitted for proteomic analysis. Mass spectrometry-grade trypsin was used to digest protein following the protocol provided by Promega. Peptide analysis was achieved using an Orbitrap Fusion Lumos mass spectrometer with a Nano ESI source (Thermo Fischer Scientific) coupled with a Waters nanoAcquity UPLC system (Waters, Milford, MA). The proteolytic products were desalted and loaded on a nanoAcquity UPLC 2G Trap Column (180 μm x 20 mm, 5 μm) for 5 minutes with solvent 0.1% formic acid in 3% ACN at a flow rate of 5 μL/min. The NanoLC separation used an nanoAcquity UPLC Peptide BEH C18 column (100 μm x 100 mm, 1.7 μm) and applied a 120-minute gradient consisting of 0.1% formic acid in H2O (mobile phase A) and 0.1% formic acid in ACN (mobile phase B), where B was increased from 3-10% over 3 minutes, from 10-30% over 102 minutes, from 30-90% over 3 minutes and held 4 minutes, and from 90-3% over 1 minute and held 7 minutes. A flow rate of 500 nL/min was used. Mass spectral data were acquired in the positive ion mode. The spray voltage was 2400 V and the ion transfer tube temperature was 300°C. MS spectra were acquired using the Orbitrap analyzer at resolution settings of 120K at m/z 200. MS/MS spectra were acquired using an Ion Trap collision induced dissociation MS2 fragmentation mode with top speed method. Automatic gain control target was set to 4.0 x 105 for precursor ions and 104 for product ions. All raw data files were analyzed with Thermo Scientific Proteome Discoverer 2.1 software and searched against the Uniprot Homo sapiens protein database using Sequest HT as search engine. Mass tolerances were set at ±10 ppm for precursor ions and 0.6 Da for fragment ions. A maximum of two missed cleavage sites was allowed. Carbamidomethylation of cysteine and oxidation of methionine
were specified as static modification and dynamic modification, respectively. The overall false discovery rate (FDR) at the protein level was less than 1%.

**Immunoblots**

To confirm presence of the differentially expressed proteins indicated by mass spectrometry, Western blot was performed. Patient serum exosomes (n = 9; 5 OSA, 2 FX, and 2 N) were lysed by adding 5x RIPA buffer containing phosphatase and proteinase inhibitors to a final 1x concentration. Protein concentrations were determined with the Pierce BCA protein assay. Homogenate aliquots (containing 10 μg protein) were mixed at a volume ratio of 1:1 with Laemmli buffer and 5% β-mercaptoethanol, boiled for 10 minutes at 100 °C, and electrophoresed on a 10% SDS–PAGE gel at a constant current of 10 mA/plate. The separated proteins were transferred to a nitrocellulose membrane, blocked for one hour with Odyssey blocking buffer (LI-COR, Lincoln, NE) diluted 1:1 in TBS (20 mM Tris–HCl pH 7.4 and 150 mM NaCl), followed by overnight incubation at 4°C with rabbit anti-HSP90 antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1000 in TBST (TBS with 0.1% Tween-20). Replicate blots were probed with rabbit anti-QSOX1 antibody (ProteinTech Group, Inc., Rosemont, IL) and rabbit anti-ITIH1 antibody (Abcam, Cambridge, MA). All antibodies used were previously validated for canine samples by western blot. Proteins labeled with primary antibody were detected with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Dallas, TX) diluted 1:40,000 in TBST. Membranes were developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and scanned on an ImageQuant LAS4000 (GE Healthcare Life Sciences, Marlborough, MA).

For visual assessment of uniform protein quantity between all samples, the 10% SDS–PAGE gel was stained with LabSafe™ GEL Blue (G-Biosciences, Geno Technology Inc., St. Louis, MO). Briefly, the gel was washed three times in deionized water for 5 minutes. LabSafe™ GEL Blue was added to cover the gel and gently rocked; protein bands were visible at 5 minutes with maximum intensity at 60 minutes.
Statistical analysis

Statistical analysis on proteomic data was performed using Metaboanalyst 3.0 Software. Data were normalized and log transformed before multivariate analysis. Predictive accuracy for discriminating proteins was determined with R statistical software. Statistical significance was set at \( p < 0.05 \).

4. RESULTS

4.1 Evaluation of serum-derived exosomes

Demonstration of presence of serum-derived canine exosomes

Exosomes were isolated from the serum of canine patients with histologically-confirmed appendicular OSA, patients with traumatic bone fracture, and normal, healthy, size-matched controls. Represented breeds included Labrador retriever (n=5), mixed breed (n=4), Great Pyrenees (n=3), English mastiff (n=2), golden retriever (n=2), and one of each Great Dane, Rottweiler, American pit bull terrier, goldendoodle, Australian shepherd, Newfoundland, and Doberman pinscher. Microvesicle sizes were measured using NanoSight light scatter technology; a representative sample is shown (Figure 4.1.1). The measured mode of vesicle size was 109.1 ± 2.7 nm and mean vesicle size was 131.9 ± 1.9 nm, which is consistent with the size of exosomes measured in previous studies using similar technology.\(^{83, 84}\)
Figure 4.1.1. Characterization of exosomes isolated from canine serum. Particle size distribution of purified exosomes as determined by nanoparticle tracking analysis (NanoSight). The measured mode of vesicle size was 109.1 ± 2.7 nm and mean vesicle size was 131.9 ± 1.9 nm.

To confirm the presence of exosomes and demonstrate their cellular uptake, we incubated canine PBMCs with fluorescent-labeled exosomes and visualized the cells using fluorescent microscopy (Figure 4.1.2). Cells with lymphocyte morphology demonstrated exosome uptake indicated by strong cytoplasmic fluorescent signal compared to minimal signal for a no exosome (dye only) control.

Figure 4.1.2. Demonstration of cellular uptake of exosomes via fluorescence microscopy. Osteosarcoma (HMPOS) exosomes were labeled with SYTO RNAscope green fluorescent stain and incubated with normal canine PBMCs. Nuclei of cells were labeled with DAPI (blue) and uptake of labeled exosomes (green) into the cytoplasm of cells with lymphocyte morphology was visualized by microscopy. Cells incubated with no exosomes (stain alone) showed minimal fluorescence.
Proteomic evaluation of exosomes derived from sera of OSA, FX, and N dogs

Mass spectrometry (LC-MS/MS) on purified serum-derived exosomes revealed 177 proteins contained within the exosomes of canine osteosarcoma patients (OSA), 152 proteins in the exosomes from patients with traumatic bone fracture (FX), and 160 proteins in the normal dogs (N). All exosomes shared about 124 proteins. Serum-derived exosomes from OSA contained 19 unique proteins and shared 14 and 20 proteins with FX and N, respectively. FX and N had 10 and 12 unique proteins, respectively, and shared 4 proteins exclusively (Figure 4.1.3). A supervised partial least squares discriminate analysis (PLS-DA) was able to divide the patients into three distinct groups based on proteomic cargo isolated from circulating exosomes (Figure 4.1.3). Based on logistic regression analysis, with penalized maximum likelihood and with feature selection based on a t-test, we achieved 85% (± 0.003811) accuracy for discriminating patients with OSA from normal or traumatic fracture via evaluation of the top 10 discriminating circulating exosomal proteins. These top 10 proteins are diagrammed based on relative expression (Figure 4.1.4).
Figure 4.1.3. Exosome proteomic cargo from normal, fracture, and osteosarcoma patients determined by LC-MS/MS. [A] The number of proteins shared by exosomes derived from sera of canine patients with osteosarcoma (OSA), traumatic bone fracture (FX), and normal patients (N) are illustrated with a Venn diagram. [B] A supervised partial least squares discriminate analysis (PLS-DA) was able to divide the patients into three distinct groups based on proteomic cargo isolated from circulating exosomes.
**Figure 4.1.4. Relative expression of 10 discriminating exosomal proteins.** The 10 most significant proteins for discrimination between osteosarcoma (OSA) and fracture (FX) or normal (N) are listed along with relative protein expression (Red=high, Blue=low). OSA appears to have a distinct profile when compared with the other two groups, including primarily upregulated protein expression.

GO annotation analysis of all three groups demonstrated an exosomal protein cargo that is functionally enriched with proteins associated with cellular component organization, metabolic processes, immune system defense, and cellular processes, particularly cell-to-cell communication (**Figure 4.1.5**). Unique proteins associated with each group were evaluated; however, significant functional enrichment was not detected for any group, likely due to low numbers.
Western blot analysis of exosomal cargo confirmed the presence of select proteins, QSOX1 and ITIH1, along with a ubiquitous exosome marker protein, HSP90 (Figure 4.1.6). Visual evaluation of the blot demonstrates increased protein density in all patients where QSOX1 and ITIH1 were detected on mass spectrometry. Overall, QSOX1 and ITIH1 were detected more frequently in OSA exosomes; interestingly, FX patients did not contain these proteins in their exosomal cargo. Of note, the protein commonly carried by exosomes (HSP90) does not dramatically differ between the groups. To demonstrate equivalent protein quantity between all samples evaluated, Coomassie blue stain was performed the 10% SDS–PAGE gel following protein electrophoresis (Figure 4.1.6).
Figure 4.1.6. Immunoblots of exosomal cargo. [A] Immunoblots of exosomal cargo confirm universal expression of ubiquitous exosomal protein, HSP90. Proteins QSOX1 and ITIH1 are expressed to a greater extent in exosomes from patients with concurrent protein detection by mass spectrometry. Overall, these proteins were detected more frequently in OSA exosomes; interestingly, patients with traumatic bone fracture did not contain these proteins in their exosomal cargo. Positive (+) = protein detected by LC-MS/MS; negative (-) = protein not detected by LC-MS/MS. [B] Coomassie blue stain performed on the 10% SDS-PAGE gel following protein electrophoresis demonstrates equivalent protein quantity between all samples evaluated.

Proteomic evaluation of longitudinally collected exosomes from sera of patients with OSA
Mass spectrometry (LC-MS/MS) on purified serum-derived exosomes revealed 82 proteins in the exosomes of canine OSA patients at diagnosis (DX), 92 proteins in the exosomes from patients 2 weeks post-amputation (PA), and 95 proteins from the same patients at time of progressive disease (PD). All exosomes shared approximately 74 proteins. Serum-derived DX exosomes contained 1 unique protein and shared 1 and 6 proteins with PA and PD, respectively. PA and PD had 8 and 6 unique proteins, respectively, and shared 9 proteins exclusively (Figure 4.1.7).
Figure 4.1.7. Exosome proteomic cargo from osteosarcoma patients at various stages of disease determined by LC-MS/MS. [A] The number of proteins shared by serum-derived exosomes of canine patients at various stages of disease, including diagnosis (DX), post-amputation (PA), and onset of lung metastasis (PD) are illustrated with a Venn diagram. [B] A supervised partial least squares discriminate analysis (PLS-DA) was able to divide the stages into three distinct groups based on proteomic cargo isolated from circulating exosomes.
Similar to the previous analysis, PLS-DA revealed distinct protein clusters by which one could discriminate between the sera of dogs from the different disease stages, demonstrating the potential of circulating exosomes as a powerful tool for discovery of novel biomarkers and potential therapeutic targets (Figure 4.1.7). Based on logistic regression analysis, we obtained 75% accuracy for distinguishing the different stages of OSA via evaluation of the top 2 discriminating circulating exosomal proteins. These 2 proteins are diagrammed based on relative expression (Figure 4.1.8).

![Figure 4.1.8.](image)

**Figure 4.1.8. Relative expression of discriminating proteins in exosomes derived sera of osteosarcoma patients at different stages of disease.** The 2 most significant proteins for discriminating between exosomes from different disease stages of OSA, including diagnosis (DX), post-amputation (PA), and progressive disease (PD) are listed along with relative protein expression (Red=high, Blue=low). Each group appears to have a relatively distinct profile.

### 4.2 Evaluation of exosomes derived from carboplatin-resistant cells

*Demonstration of chemotherapeutic resistance*

A carboplatin-resistant cell line (HMPOS-R) was created by repetitive incubations of HMPOS cell line with increased concentrations of carboplatin and expansion of the survived cells. To demonstrate acquisition of resistance, a viability assay was performed on HMPOS chemotherapy-sensitive cell line (HMPOS-S) and its carboplatin-tolerant equivalent (HMPOS-R) (Figure 4.2.1). HMPOS-R demonstrates significantly increased viability compared with HMPOS-S over carboplatin concentrations up to approximately 50 µM.
Figure 4.2.1 Viability assay comparing carboplatin-sensitive and carboplatin-resistant HMPOS. [A] Scatter plot and [B] bar graph: Carboplatin-resistant cell line (HMPOS-R) demonstrates significantly increased cell viability compared with its carboplatin tolerant equivalent (HMPOS-S) up to approximately 50.0 µM carboplatin. The red dashed line demonstrates the carboplatin concentration at which 50% of cells display viability (IC50).
*Exosomes confer carboplatin resistance*

A viability assay was performed to evaluate the effect of exosomes derived from carboplatin-resistant cells on a naïve population. Exosomes isolated from HMPOS-R were added to HMPOS-S at a dose of 50 µg/mL and allowed to incubate for 48 hours in media with exosome-depleted FBS. Viability assay demonstrates increased viability of resistant exosome-treated cells (HMPOS-EX) compared with the naïve untreated control, HMPOS-S (Figure 4.2.2). HMPOS-S exosomes were also isolated and incubated with HMPOS-S cells to serve as a control.
Figure 4.2.2 Viability assay comparing resistant-exosome treated HMPOS with carboplatin-sensitive and carboplatin-resistant HMPOS. [A] Scatter plot and [B] bar graph: Carboplatin-resistant cell line, HMPOS-R, continues to demonstrate significantly increased cell viability compared with its carboplatin tolerant equivalent, HMPOS-S, over a range of carboplatin concentrations. Naive HMPOS, when incubated with exosomes from the resistant cell line (HMPOS-EX), have increased cell viability, demonstrating transfer of resistance. The red dashed line demonstrates the carboplatin concentration at which 50% of cells display viability (IC50).
Proteomic analysis via LC-MS/MS on carboplatin-sensitive HMPOS and carboplatin-resistant HMPOS cell lysates and exosomes detected 1515 total proteins. Substantial differences were detected between the two groups, with the cell lysates containing the majority of proteins (1424), sharing 187 with the exosomes. Exosomes contained 91 exclusive proteins (Figure 4.2.3). GO annotation analysis of biological pathways demonstrated that unique proteins are quite diverse between cells and their exosomes (Figure 4.2.3). Exosomes are enriched with proteins associated with cell adhesion and inflammatory signaling. The 50 most significant proteins for distinction between exosomes and cell lysates are diagrammed according to relative protein expression (Figure 4.2.4).

Figure 4.2.3 Evaluation of proteins derived from HMPOS cell lysates and exosomes. [A] The number of proteins shared by exosomes derived from the HMPOS cell lines and their exosomes are illustrated with a Venn diagram. [B] A pie chart demonstrates relative enrichment of exosomal proteins associated with integrin signaling and inflammatory pathways.
Figure 4.2.4. Relative expression of proteins in exosomes and cell lysates. The 50 most significant proteins for discriminating between exosomes and cell lysates listed along with relative protein expression (Red=high, Blue=low).

All exosome preparations contained ubiquitous exosomal proteins, such as HSP90 and HSP70, which were not detected on proteomic analysis of cell lysates. Additionally, immunosuppressive proteins, such as TGF-β, and proteins enhancing extracellular matrix degeneration, such as MMP-2, were detected in all exosome preparations. Of interest, periostin was also expressed in exosomes.

On further evaluation of proteomic data, HMPOS-S cell lysates contained 1176 total proteins, sharing 950 with the most resistant HMPOS-R lysates, which had a total of 1101. Exosomes from sensitive and resistant HMPOS revealed 183 exosomal proteins for HMPOS-S (Exos-S) and 193 exosomal proteins for HMPOS-R (Exos-R). All exosomes shared 127
proteins, where Exos-S and Exos-R had 56 and 66 unique proteins, respectively (Figure 4.2.5). When comparing resistant cell lysates with their resistant exosomes, lysates expressed 134 unique proteins and their exosomes expressed 18; HMPOS-R and Exos-R shared 3 proteins exclusively. Of interest, one of these exclusive proteins, connexin 43, has been implicated in platinum compound resistance in a number of human malignancies. Sensitive HMPOS lysates and their exosomes had 218 and 16 unique proteins respectively, and shared 2 proteins exclusively.

Figure 4.2.5. Proteomic cargo from carboplatin-sensitive and carboplatin-resistant HMPOS cell lysates and exosomes. The number of proteins shared by carboplatin-sensitive HMPOS cells (HMPOS-S) and exosomes (Exos-S) and carboplatin-resistant HMPOS cells (HMPOS-R) and exosomes (Exos-R) are illustrated with a Venn diagram.

The 20 most significant proteins for distinction between Exos-R and Exos-S are diagrammed according to relative protein expression (Figure 4.2.6).
Figure 4.2.6. Relative expression of proteins in exosomes derived from sensitive and resistant HMPOS. The 20 most significant proteins for discriminating between exosomes from carboplatin-sensitive HMPOS (Exos-S) and exosomes from carboplatin-resistant HMPOS (Exos-R) are listed along with relative protein expression (Red=high, Blue=low).
5. DISCUSSION

Exosomes are distinguished from other extracellular vesicles based on their size and composition, being the smallest vesicles and having unique mechanism of formation and thus unique associated proteins.\textsuperscript{9,11} In our study, nanoparticle tracking analysis was performed on purified exosome preparations subsequent to isolation using a commercially available reagent. This analysis confirmed the presence of small vesicles that were consistent with reported exosomal sizes.\textsuperscript{83,84} Nanoparticle tracking analysis is recognized as a reliable method for direct, real-time visualization of exosomes, and can accurately measure and resolve different-sized particles within the same solution.\textsuperscript{84} Therefore, it can distinguish between exosomes and other larger vesicles in suspensions. Further evaluation of exosome preparations via western blot demonstrated presence of the ubiquitous exosomal protein, HSP90, using an anti-HSP90 antibody that is validated for canine samples. As such, we had confidence that our preparations contained exosomes derived from serum of canine patients; evaluation of which, to our knowledge, has not been reported in the literature.

The results of our study reveal the unique protein cargo that is expressed by circulating exosomes in canine patients with OSA. This cargo, when evaluated via mass spectrometry, contained >170 proteins, which were largely associated with proteolysis, activation of the immune response, activation of stress response, and regulation of metabolic processes. The top 10 discriminating proteins were utilized for logistic regression analysis and demonstrated that tumor-bearing patients could be differentiated from control animals (healthy and traumatic fracture) with impressive accuracy. Additionally, circulating exosomes collected longitudinally from 5 OSA patients demonstrated distinct protein clustering based on disease stage. Logistic regression analysis demonstrated that various disease stages in OSA could be relatively accurately differentiated based on 2 discriminating exosomal proteins.
With this information, we were able to confirm our initial hypothesis that serum-derived exosomes contain a unique protein signature that can be ascribed to canine OSA patients. Additionally, this signature appears to evolve throughout the course of disease. Evaluation for these discriminating serum exosomal proteins could be potentially utilized as a non-invasive “liquid biopsy” for canine patients with appendicular OSA, aiding in diagnosis and providing information about disease progression, without the need for invasive procedures, similar to what has been reported in many human malignancies.25

Exosome protein cargo from canine OSA cell line, HMPOS, was evaluated via mass spectrometry in this study. Of note, comparison of serum-derived exosomes from OSA patients with cell line exosomes demonstrates very little overlap in protein content, which is an unsurprising finding. The differences in protein cargo are expected to reflect the fact that serum exosomes originate from a multitude of cells in the body as opposed to purely the neoplastic population; as a result, serum exosomes provide information about the body’s cumulative and systemic response to presence of cancer. Additionally, serum samples represent responses to tumor in vivo, which likely exhibits significant variation from in vitro conditions.

In this study, we compared proteomic profiles of cell lysates with their exosomes and also noted a relatively small amount of overlap. For one, lysates contain much larger numbers of proteins in comparison with the exosomes. Many proteins of interest were detected in the exosomal preparations that were not noted from the cell lysates, including TGF-β and MMP-2. Both of these proteins have been identified as key players in exosomally-derived progression of neoplastic disease due to their ability to create a favorable environment for tumor growth and spread.22, 23 Of interest, periostin was also observed in all exosomal preparations. Periostin is a protein secreted by fibroblasts and is a major component of the extracellular matrix; it has been shown to promote malignancy by enhancing invasiveness of tumor cells via interaction with integrins.85 Recent research also demonstrated that periostin was enriched in exosomes secreted by metastatic breast cancer cells.85
Clinically, OSA is considered one of the most chemotherapy-resistant tumors in both humans and dogs, leading to variable treatment response, high metastatic rate, and poor survival time. Therefore, in addition to evaluation of exosomes for biomarker potential, we also wanted to assess the capacity for exosomes to confer chemotherapeutic resistance, which has been demonstrated as a mechanism for extrinsic drug resistance in a variety of human cancers.\textsuperscript{39-43}

In our study, we show successful \textit{in vitro} development of a carboplatin-resistant canine OSA cell line, HMPOS-R. Resistance was created over the course of approximately 7 months via incubation of HMPOS with carboplatin using a pulsed treatment strategy.\textsuperscript{86} Cells were incubated with low doses of drug initially, and doses were steadily increased throughout the course of treatment. HMPOS-R demonstrates clinically relevant resistance, demonstrated by an approximately two-fold increase in the IC\textsubscript{50} (drug concentration causing 50\% growth inhibition).\textsuperscript{86} After obtaining low-level resistance, exosomes from HMPOS-R were incubated with the naïve HMPOS cells (HMPOS-EX). Subsequent viability assay revealed increased viability of HMPOS-EX when compared with the naïve untreated control, HMPOS-S. These results suggest exosomal transfer of chemotherapeutic resistance to chemo-sensitive cells.

Proteomic analysis of cell lysates from HMPOS-R, along with the exosomes from HMPOS-R (Exos-R) revealed a number of proteins that had increased expression in the resistant populations. However, only three upregulated proteins were shared by the resistant cells (lysates) and their exosomes. One of these proteins, connexin 43 (Cx43; also known as gap junction alpha-1 protein) has been implicated in platinum compound resistance (e.g. carboplatin, cisplatin) in a range of human cancers. A study by Li \textit{et al.} showed that a cisplatin-resistant ovarian cancer cell line had markedly upregulated expression of \textit{GJA1}, which encodes Cx43.\textsuperscript{87} Functional analyses demonstrated the importance of Cx43 in drug
resistance. Further analysis is required to determine the role of Cx43 in exosomal transfer of chemotherapeutic resistance in canine OSA.
6. CONCLUSION

Exosomes have proven to be a vital component in intracellular communication via the transfer of proteins and nucleic acids to target cells. This transaction is particularly important in neoplastic diseases, where exosomal contents have a fundamental role in creating an environment that favors tumor growth and dissemination, particularly through immune modulation, stromal cell alteration, increased angiogenesis, and activation of cell migration. Furthermore, exosome-associated proteins and miRNAs have emerged as promising biomarkers for early detection, monitoring, and progression of a number of human malignancies due to their robustness in biofluids.

Osteosarcoma is an aggressive primary bone tumor that affects human and canine patients alike. The prognosis for OSA is poor in both species as a result of rapid onset of metastasis in spite of aggressive treatment that often entails removal of the primary tumor and adjuvant systemic chemotherapy. Studies assessing the role of exosomes in OSA have demonstrated potential protein and miRNA biomarkers, which may correspond with disease progression and development of chemotherapeutic resistance. To date, research on exosomes and their significance in disease, including OSA, is lacking in the field of veterinary oncology.

In our research, we sought to evaluate the profile of exosome-associated proteins derived from sera of canine OSA patients. Our hypothesis was that patients with OSA would have a distinct protein signature compared with control dogs that could be used as a biomarker for disease detection. Additionally, we hypothesized that samples collected in a longitudinal fashion over the course of disease would show a changed protein profile so that discrimination between disease stages would be possible, serving as a marker for prognostication. In a similar but distinct investigation, we wanted to determine if exosomes derived from OSA cell lines would be capable of transferring chemotherapeutic resistance to susceptible osteoblasts, with a hypothesis that, as resistance is acquired, a
distinct exosomal-associated protein profile will be observed. Presence of distinct proteins may serve to ultimately predict patient response to chemotherapy.

Overall, we observed that canine sera contain small vesicles consistent with exosomes, and that these exosomes indeed contain protein cargo that has an altered expression profile depending on disease state. OSA could be reliably detected based on a 10-protein signature when compared with serum-derived exosomal proteins from normal patients and those with traumatic (non-cancerous) bone disease. A 2-protein signature may be used to determine stage of disease in OSA patients. This study highlights the potential use for exosomal proteins as biomarkers in biofluids of canine patients. Additionally, after creation of a carboplatin-resistant OSA cell line, we were able to demonstrate exosomal transfer of chemotherapeutic resistance to susceptible cells. Unique proteins associated with resistant exosomes, such as Cx43, may be an indicator for development of chemotherapeutic resistance.

This research reveals for the first time the presence and potential utility of serum-derived exosome proteins in canine patients with appendicular OSA. In the future, discriminating proteins may be used as a liquid biopsy to aid in disease detection and provide useful information about metastatic spread and chemotherapeutic susceptibility. As this study was performed using a small number of samples, further research is warranted to validate and refine the protein signature for OSA patients. Also, functional studies to determine the effect (if any) of upregulated exosomal-associated proteins, and to elucidate the mechanism by which exosomes confer resistance, are necessary. Our desire is that this research will significantly contribute to our understanding of OSA pathobiology, and that further elaboration will aid in the development of biomarkers and novel therapeutic targets.
7. BIBLIOGRAPHY


80. Product information: Total exosome isolation (from cell culture media). Invitrogen by Life Technologies. Revision date: 28 June 2012.


