1-1-2017

Platelet-Cancer Cell Interactions: Insights from the Canine Model

Shauna Ashtin Fuhrmann

Platelet-cancer cell interactions: Insights from the canine model

By

Shauna Ashtin Fuhrmann

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Research
in the College of Veterinary Medicine

Mississippi State, Mississippi

August 2017

Platelet-cancer cell interactions: Insights from the canine model

By

Shauna A. Fuhrmann

Approved:

_____

Camilo Bulla
(Major Professor)

_____

Andrew J. Mackin
(Co-Major Professor)

_____

Kari V. Lunsford
(Committee Member)

_____

Larry Hanson
(Committee Member)

_____

Bindu Nanduri
(Committee Member)

_____

R. Hartford Bailey
(Graduate Coordinator)

_____

Mark L. Lawrence
Associate Dean for Research and Graduate Studies
College of Veterinary Medicine

Name: Shauna Ashtin Fuhrmann

Date of Degree: August 11, 2017

Institution: Mississippi State University

Major Field: Veterinary Medical Research

Major Professors: Dr. Andrew Mackin & Dr. Camilo Bulla

Title of Study: Platelet-cancer cell interactions: Insights from the canine model

Pages in Study:179

Candidate for Degree of Doctor of Philosophy

Animal models have been recognized for the valuable roles they serve in both animal and human medicine. Dogs share many of the same naturally occurring tumors as humans including osteosarcoma, lymphoma, and mammary tumors. In addition, dogs share the same environment as humans, have a shorter lifespan, and often have a quicker progression of disease, making them an attractive model of human disease. Platelets are small anucleate cell fragments that have essential roles in hemostasis, angiogenesis, and wound healing, and, more recently recognized, roles in development, survival, growth, and metastasis of various cancers. Their roles in angiogenesis has proven to be both directly and indirectly linked to tumor growth due to the angiogenic roles they play in the development of tumor blood supply.

Being able to study the interactions and mechanisms between platelets and tumor cells at the protein level, through proteomics, would allow great insight into the effects of platelets on tumor cell behavior as well as potential biomarker identification and therapeutic development. The objective of this research is to integrate the roles of canine platelet proteins into a better understanding of the effects and interactions that platelets have with different tumor cells while utilizing the canine model of neoplasms commonly

affecting their human counterparts. The first study in this research describes an efficient technique developed for the purification of canine platelets from clinically relevant volumes of whole blood with high platelet recovery and minimal contamination from other blood cells. The second study describes a non-electrophoretic detergent fractionation technique used for the digestion of canine platelet samples for proteomic analysis as well as description of the proteomic findings for the normal canine platelet. Lastly, the third study describes the proteomic analysis of proteins differentially expressed by canine osteosarcoma and mammary tumor cells following incubation with canine platelet lysate in vitro. Overall, findings of this research support the canine model of human cancers and provide comprehensive information regarding canine platelet proteomics as well as novel efficient techniques that aid the future of canine platelet-tumor cell interaction research

DEDICATION

For my mom, Shelly (aka Mummsie) - going above and beyond for you since 1988.

Thank you so much for your constant support and inspiration throughout my entire life.

You have always instilled in me that no goal is too high and I can conquer anything!

ACKNOWLEDGEMENTS

A special warm hearted thanks to my husband, Adam, for his continued love and support throughout this entire experience of PhD/DVM veterinary school life! We have shared a lifetime's worth of memories over the past years while I have been in Starkville – from meeting, to traveling the country, to getting married – all while I have been achieving my dream of graduating with both a PhD and a DVM. I love you to the moon and back.

I would also like to say a special thank you to Dr. Bulla for being an amazing mentor throughout the past six years – guiding me along the way while still letting me learn things on my own. Thank you for keeping me focused on the end goal and helping me to see the big picture at times when I was overwhelmed! Thank you to Sandra (other Dr. Bulla) for being such a wonderful fellow student, mentor, motivator, and special friend! Thank you to my committee members: Dr. Mackin, Dr. Nanduri, Dr. Lunsford, and Dr. Hanson for helping me along this process and offering valuable and knowledgeable insight along the way!

A special thank you is due to my dear friends and fellow PhD/DVM classmates since 2011 – Caitlin Riggs and Courtney Hunter – we started this journey together six years ago and are finishing it together as well! I cannot believe how far we have come in our education, careers, and especially our friendship. I am so proud of all of us and cannot wait to see what the future holds. I would not have been able to make it through

without you both. Thank you to Dr. Lawrence and Tia Perkins for always helping me keep up with what to do with the dual degree program along the way and also for keeping a positive light amidst my intermittent negativity. Thank you, again, to my mom, Shelly, for her constant support for my entire life. I conquered all the obstacles because she said I could. Thank you to my grandfather, Aubrey (aka Papaw), who listened to incredibly in depth scientific discussions for hours and never batted an eye! Thanks for being such a father to me. I have to thank Dr. Roberts at UT Martin, last but not least, for being the mentor that saw a confused college student that was being looked over and being able to inspire her to follow her veterinary and scientific dreams. Thank you to all my other friends, family, and colleagues for making this journey amazing as well as everyone at Mississippi State University's College of Veterinary Medicine.

TABLE OF CONTENTS

APPENDIX

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Much of the current research into human disease relies heavily on animal models, with some of the most notable and most recent models used for cancer research. These animal model systems utilize the animal as well as cancer cell lines for studying biochemical pathways, progression and advancement of diseases, as well as development and response to various therapeutic modalities, both *in vitro* as well as *in vivo* [1]. In more recent years, the canine model for human disease has been recognized as a valuable model because dogs share many of the same naturally-occurring neoplasias as well as treatment responses, in comparison to the commonly used mouse and rat models in which tumors are often induced using genetically altered subjects. These rat and mouse models often fall short in their ability to translate from the bench top to clinical application. Due to their shorter lifespan, trends for more rapid disease progression, as well as sharing the same environment as humans, the dog offers a multitude of benefits to the animal model system beyond having similar neoplasias to people [2, 3, 4]. This year, more than 1.6 million people are expected to be newly diagnosed with cancer in the USA, along with roughly 600,000 cancer-related deaths [5]. With these incidences in mind, early detection modalities, as well as tumor-specific treatments, are essential in the future of cancer research.

Annually, roughly 6 million dogs are diagnosed with cancer, a majority of which are similar to those cancers affecting their human companions [6]. Canine lymphoma, osteosarcoma, and mammary tumors are three naturally-occurring cancers that share a multitude of similarities with their human equivalents, ranging from clinical presentation, tumor metastasis, diagnostic findings, histopathologic assessment, treatment protocols available, as well as prognosis [7, 8, 9, 4]. Canine mammary tumors have long been utilized as a valuable model in studies related to breast cancer in women due to their similarities clinically as well as molecularly [10, 11, 4, 12].

Cancer itself is a multifactorial disease involving a number of dysregulations in cell function and cell signaling. Research has only scratched the surface in identifying and understanding the pathophysiology and underlying mechanisms involved in each different tumor type's development, survival, and proliferation, as well as modalities to effectively treat and prevent tumors from occurring. One of the most recently recognized cells discovered to have potentially valuable involvement with cancers is the platelet. The platelet is an anucleate small cell fragment produced from megakaryocytes, mostly at the level of the bone marrow, that has been recognized to have novel roles in the body, and functions in different diseases beyond just hemostasis and wound healing. Research findings have linked platelets to vital roles in angiogenesis and immune surveillance. Interestingly, platelets have been suggested to play a role in aiding tumor cell 'cloaking' from the immune cells within the vasculature to aid in tumor metastasis at distant sites [13]. Additionally, a subpopulation of proteins stored within platelet granules termed 'angiogenic factors' are utilized in angiogenesis, the process of generating new blood vessels [14]. Angiogenic factors have been shown to have multiple roles in cancer cell

survival and tumor growth, as studies have shown that additional blood supply is needed for tumor growth greater than 3mm in diameter [15]. Studies on the effects of angiogenic factors in cancer have produced conflicting results at times [16]. Many studies have shown pro-tumorigenic effects with upregulation of proangiogenic factors and down-regulation of antiangiogenic factors that are present or induced [17, 18, 19, 20]. In addition to angiogenic protein effects on cancer, platelets and their products have been shown to have varying effects on cancer cell migration, survival, proliferation, and metastasis depending on the cancer type and species being studied [21, 17, 22, 23].

One of the most important aspects of researching platelets and their various effects in different circumstances and disease processes is the purity of the sample obtained, whether the platelets are derived from human or canine subjects. In human platelet studies, leukocytes were shown to have 65-fold more protein and 12,500-fold more mRNA than platelets [24, 25]. Eliminating contaminating leukocytes from platelet samples is important for obtaining results certain to be caused by or due to platelets themselves. This is most critical in research dealing with any of the 'omics' technologies, including proteomics. For proteome studies, which is the study of a set of proteins encoded for by a given genome, samples must contain only the cell or subject of interest in order to avoid erroneous results from contaminating cells and their protein products. Protein numbers far exceed the number of protein-coding genes, therefore proteomics provides a valuable means of analyzing proteins involved in various cellular mechanisms and interactions as well as those involved in disease pathology and potential therapeutic strategies. Using proteomics in cancer research will allow the description of tumor-specific profiles at the level of the protein which could then allow the identification of

potential biomarkers for use in future disease detection, relapse, and therapeutic responses [26]. Research into the interactions and effects of platelets with various cancer types along with the protein profiles and information obtained from highly detailed proteomic studies will offer valuable information into cellular mechanisms, disease behavior, diagnostic characteristics, potential therapeutic options, as well as prognostic indicators specifically tailored to the tumor type in question.

Accordingly, the objective of this research is to employ and justify the dog as a valuable model of human disease while highlighting some of the significant effects and roles platelets play in cancers common to both canines and humans.

References

1. Masters JR, HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer, 2002. **2**(4): p. 315–319.

2. Knapp DW, Waters DJ, Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. Mol Med Today, 1997. **3**(1): p. 8-11.

3. Spugnini EP, Porello A, Citro G, Baldi A, COX-2 overexpression in canine tumors: potential therapeutic targets in oncology. Histol Histopathol, 2005. **20**(4): p. 1309-1312.

4. MacEwen EG, Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Rev, 1990. **9**(2): p. 125-136.

5. American Cancer Society. Cancer facts and figures 2017. Available from: http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-048738.pdf. Accessed January 17, 2017.

6. Mazcko C, Comparative Oncology Program, 2012. Available from: https://ccrod.cancer.gov/confluence/display/CCRCOPWeb/Home. Accessed January 17, 2017.

7. Rowell JL, McCarthy DO, Alvarez CE, Dog models of naturally occurring cancer. Trends Mol Med, 2011. **17**(7): p. 380-388.

8. Marconato L, Gelain ME, Comazzi S, The dog as a possible animal model for human non-Hodgkin lymphoma: a review. Hematol Oncol, 2013. **31**(1): p. 1-9.

9. Messerschmitt PJ, Garcia RM, Abdul-Karim FW, Greenfield EM, Getty PJ, Osteosarcoma. J Am Acad Orthop Surg, 2009. **17**(8): p. 515–527.

10. Chrisp CE, Spangler WL, The malignant canine tumour as a model for the study of human breast cancer. In: Shifrine M, Wilson FD (eds). The Canine as a biomedical research model: Immunological, hematological and oncological aspects. Washington DC: National Technical Information Center, Department of Energy; 1980: p. 331-349.

11. Owen LN, Morgan DR, Bostock DE, Flemans RJ, Tissue culture and transplantation studies on canine mammary carcinoma. Eur J Cancer, 1977. **13**(12): p. 1445-1449.

12. Strandberg JD, Goodman DG, Animal model of human disease: Canine mammary neoplasia. Am J Pathol, 1974. **75**(1): p. 225-228.

13. Xu XR, Zhang D, Oswald BE, Carrim N, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci, 2016. **53**(6): p. 409-30.

14. Walsh TG, Metharom P, Berndt MC, The functional role of platelets in the regulation of angiogenesis. Platelets, 2015. **26**(3): p. 199–211.

15. Folkman J, Tumor angiogenesis: therapeutic implications. N Engl J Med, 1971. **285**(21): p. 1182–1186.

16. Ho-Tin-Noe B, Goerge T, Wagner DD, Platelets: guardians of the tumor vasculature. Cancer Res, 2009. **69**: p. 5623-6.

17. Labelle M, Begum S, Hynes RO, Direct signaling between platelets and cancer cells induces an epithelial—mesenchymal-like transition and promotes metastasis. Cancer Cell, 2011. **20**: p. 576-90.

18. Caine GJ, Lip GY, Blann AD, Platelet-derived VEGF, Flt-1, angiopoietin-1 and P-selectin in breast and prostate cancer: Further evidence for a role of platelets in tumour angiogenesis. Ann Med, 2004. **36**: p. 273–277.

19. Salven P, Orpana A, Joensuu H, Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. Clin Cancer Res, 1999. **5**: p. 487–491.

20. Gonzalez FJ, Rueda A, Sevilla I, Alonso L, Villarreal V, Torres E, Alba E, Shift in the balance between circulating thrombospondin-1 and vascular endothelial growth factor in cancer patients: Relationship to platelet alpha-granule content and primary activation. Int J Biol Markers, 2004. **19**: p. 221–228.

21. Bulla SC, Badial PR, Silva RC, Lunsford K, Bulla C, Platelets inhibit migration of canine osteosarcoma cells. J Comp Path, 2017. **156**(1): p.3-13.

22. Labelle M, Begum S, Hynes RO, Platelets guide to the formation of early metastatic niches. Proc Natl Acad Sci USA, 2014. **111**(30): E3053-61.

23. Shumacher D, Strilic B, Sivaraj KK, Wettschureck N, Offermanns S, Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell, 2013. **24**(1): p. 130-7.

24. Fink L, Holschermann H, Kwapiszewska G, et al, Thromb Haemost, 2003. **90**: p. 749–756.

25. Birschmann I, Mietner S, Dittrich M, Pfrang J, Dandekar T, Walter U, Use of functional highly purified human platelets for the identification of new proteins of the IPP signaling pathway. Thromb Res, 2008. **122**(1): p. 59-68.

26. Yang M, Zhong J, Zhao M, Wang J, Gu Y, Yuan X, Sang J, Huang C, Overexpression of nuclear apoptosis-inducing factor 1 altered the proteomic profile of human gastric cancer cell MKN45 and induced cell cycle arrest at G1/S phase. PLoS One, 2014. **9**: p. e100216.

CHAPTER II

LITERATURE REVIEW

Animal Models of Cancer

Despite the ever-increasing number of technological advancements in these modern times, slow progress is being made to uncover the genetic basis of complex human diseases. This is primarily attributed to the diverse amount of genetic heterogeneity that exists [1]. According to the American Cancer Society, cancer is the second most common cause of death in people in the United States. More than 1.6 million people are expected to be newly diagnosed with cancer across the USA in 2017, along with approximately 600,000 cancer-caused deaths predicted to occur [2]. Earlier detection capabilities along with more effective treatment modalities are necessary to combat this deadly disease. Ongoing research into tumor-specific molecular targeting have allowed for progress to be made in this direction. Most current research in human diseases genetics, most notably in the area of cancer research, relies on animals as models for disease by utilizing animal and cancer cell lines *in vitro* and *in vivo* to study biochemical pathways associated with different cancer cells [3].

In vitro models provide many advantages for disease research in their ability to provide conditions that can be strictly controlled, homogeneity, and ease of reproducibility for determining understandings of genetic mechanisms. Beyond that, though, *in vitro* models quickly reach limits including adaptation of cell lines to the

conditions *in vitro*, mutation development in cell lines from continued cell splitting and preservation, and a lack of the naturally occurring tumor environment [4]. Some research into the production of three-dimensional models for *in vitro* use has been performed [5].

The use of *in vivo* animal models for cancer research allows for rapid translation of therapeutic investigations and clinical trials to take place from the lab bench to the patient. The most common of these animal models is the mouse, which has allowed great progress to be made in the understanding of molecular mechanisms and pathways as well as the function of numerous genes and proteins *in vivo* [6]. Mice provide many advantages to modeling human disease due their short gestation length and small size, both of which allow for quick turnover and minimal expense and space required for studies to be conducted. Since mice have been used extensively in animal model studies for years, there are many technologies developed to allow alteration and manipulation of specific genes of the animal or a subset of cells [7]. The most common forms of rodent models include xenografts or those with genetically-induced cancers [8]. Xenograft models are genetically immunocompromised (athymic nude mice or SCID mice most commonly) rodents that are later transplanted with cells from a human or animal cancer under the skin or into a particular organ [8, 9, 10].

While boasting advantages such as low expense and the ability to aid the development of cancer therapeutics and imaging modalities, rodent models quickly reach their limitations on their ability to easily translate to clinical applications in human cancer [11, 12]. The most notable cause for this disadvantage is the fact that laboratory-bred mice are deficient in aspects of their immune system, therefore not allowing accurate representation of the normal physiology seen in the cases of naturally occurring cancers

that are seen in humans or other species like the dog [13]. Genetically engineered mice have provided good models of human cancer for the development of novel therapeutics and pre-clinical studies through the selective suppression of tumor suppressor genes and specific oncogene induction, but have limitations due to an inability to control the overall level and pattern of gene expression [14, 12]. In addition, in comparison to the genetic alterations able to be made in mice, human diseases are almost always polygenic in nature. Genetic manipulation in the mouse may only involve a handful of genes or often involve alterations to the environmental conditions to cause differential gene expression in specific inbred strains. As can be expected, these alterations and genetic changes are quite different than those occurring in naturally occurring human diseases [1]. Overall, mice can serve as good models for initial human disease research, but the differences in genetic makeup and interactions create a need for a model that is more comparable to natural human disease.

Although rodents offer advantages in their use as animal models by their small size, short lifespan, short gestation length, and inexpensive maintenance, the successful translation rate for research originating in rodent models to clinical cancer trials in humans is less than 8% [15]. Companion animals (dogs and cats) offer many distinct advantages as animal models in their similarities to various cancers shared with humans, including a relatively high incidence of naturally occurring cancers, similar biological behaviors of cancers, the similar response to therapies administered, and similar responses to cytotoxic agents as those that occur in humans. The fact that companion animals have a shorter lifespan and more rapid progression of disease than humans are additional factors to support the companion animal model [16, 17, 18]. In addition to this,

clinical trials and information gained from the use of companion animal models in oncology benefits the diagnostics and therapeutics to be used in both human and veterinary medicine. Nearly 1.7 million people are predicted to be newly diagnosed with cancer in the USA in 2017 [2]. In veterinary medicine, about 6 million dogs are diagnosed annually with cancer, providing ample opportunity for the study of naturally occurring tumors similar to those that occur in humans [19].

Canine Model of Cancer

Dogs develop tumors at twice the rate of their human counterparts. Tumors that naturally occur in dogs have been proven to have greater clinical and biological similarities to human cancer compared to other animal models [18, 20]. Dogs share many similarities with humans in the cancers they develop naturally including the age of onset, rates of reoccurrence and metastasis, response to similar therapies utilized, and histologic appearance [21]. Paoloni et al also described that the same alterations in tumor oncogenes and tumor suppressor genes occur for both humans and dogs [21]. The similarities shared between canines and humans extend all the way to the genetic level. Researchers have illustrated that some of the gene families and their associated cancers had a closer relationship between humans and canines than those for any other species [22]. Molecular cytogenetic analysis of canine hematologic malignancies revealed evidence of ancestral aberrations at the level of the chromosome between humans and canines [23, 24]. Cancers that commonly affect dogs that would make excellent models for similar human cancer include lymphoma, head and neck carcinomas, bladder cancers, osteosarcoma, mammary cancers, prostate cancer, lung cancer, and melanoma [21, 25]. Recently, a number of similarities were described between canine and human

pheochromocytomas and paragangliomas, and suggested the dog as an excellent model for pathways and comparative studies [26]. Researching and testing treatments in naturally occurring cancers (as well as other diseases) in dogs does not create the troublesome ethical issues commonly encountered with animal models in which disease is experimentally induced. Alternatively, dogs have also been suggested for the valuable role they could play in the link between mechanistic studies in mice and clinical disease applications in humans [27]. Overall, the canine model is more robust and ethical when compared to mouse and rat models.

One of the most common neoplasias affecting dogs, lymphoma, makes up approximately 20% of all canine cancer diagnoses and 80% of canine hematopoietic cancers [28]. Arising from unregulated lymphocyte proliferation in a multitude of organs associated with the lymphatic system (spleen, bone marrow, lymph nodes, intestines), lymphomas are notorious for their tendency to be highly metastatic, with a high rate of recurrence regardless of treatment. Similarities between canine and human lymphoma include their clinical presentation, genetic mutations, tumor biology, and tumor behavior, which makes the dog an excellent model for disease progression and therapeutic modalities [20, 29]. Current treatments utilizing combined chemotherapeutic agent protocols including vincristine, cyclophosphamide, doxorubicin, and prednisone are used for lymphoma treatment in both human and canine patients [30], and recurrence is common and resistance is becoming an increasing concern. Response rates to these drug combinations are reported to be 86-91% [31, 32], although most dogs will eventually die due to the recurrence of a drug-resistant form of lymphoma. The canine model has

allowed for the development of new treatment modalities like asparaginase [33], a commonly used 'rescue' drug in veterinary medicine after lymphoma relapse occurs.

Environmental causes of cancer, e.g. tobacco smoke, have also been a point of similarity discovered between dogs and humans, most notably in cancers of the head and neck as well as lung cancers [34]. Head and neck cancers make up approximately 20% of the oral malignancies seen in dogs [35]. In humans, these squamous cell malignancies are highly aggressive and invasive with limited treatment efficacy [36]. Although the prevalence of lung cancer in canines is low (1%) [18], while the incidence represents approximately 27% of all cancer diagnoses in humans [2], various studies have linked an increased in respiratory malignancies in dogs to exposure to cigarette smoke [37]. Dogs can serve as a valuable model for human cancers such as these to study the effect on environmental factors like tobacco smoke in carcinogenesis as well as for diagnostic modalities and treatments.

Canine Osteosarcoma

Background

Osteosarcoma (OSA) is the most common primary bone tumor occurring in dogs [38], as well as the most common bone tumor affecting children and adolescents [2]. The incidence of OSA is significantly greater in dogs than humans. Roughly 10,000 new cases of OSA are identified in dogs each year [39]. In humans, more than 2,500 new cases of primary bone cancer are diagnosed annually [20]. Occurring much more frequently than in humans, the estimated incidence of canine OSA is approximately 13.9 per 100,000 [40] compared to the human incidence of 1.02 per 100,000 [41]. Making up 85-98% of all bone tumors found in dogs, OSA is usually found in giant and large breed

dogs such as Great Danes, Rottweilers, German Shepherds, Dobermans, and Greyhounds [42]. These large and giant breeds have been reported to have a 61-fold greater risk of developing OSA compared to other breeds [43]. A bimodal age distribution is shared in both canine and human OSA [20]. OSA most commonly affects dogs 6-10 years old but has also been reported in very young dogs [44]. Along with a greater occurrence, canine OSA is much more aggressive than human OSA. Regardless, the same treatment modalities are utilized in both humans and dogs [45]. A predilection for greater occurrence in males has also been reported in the literature for both humans and dogs [46, 47, 48, 49, 50, 51], along with an increased prevalence in dogs that have had gonadectomies performed [52].

As is seen with their human companions, the most common site for OSA in dogs is in the metaphyseal region of long bones (75%) [53]. In dogs, OSA occurs more commonly in the front limbs than in the hind limbs. The distal radius and the proximal humerus are the two most common locations where OSA lesions are found in dogs [54, 55]. In human patients, the most common sites for OSA lesions include the distal femur and proximal tibia [55]. The exact etiology behind OSA in either species is still unknown, but mutagenic effects including radiation, trauma, and genetics are all potential risk factors in dogs [56, 57, 58].

Clinical Signs & Diagnostics

Upon presentation, the majority of canine OSA patients initially present with general signs of lameness that may or may not include the presence of pain at the time of examination, along with localized swelling at the affected site. Due to a common location of tumors occurring at the level of the knee, signs are often attributed to sports injury

initially. Based on the stage of disease and activity level of the patient, pathologic fractures may result in a sudden worsening of the lameness and instability at the fracture site may be noted.

Radiographs of the affected limb can aid in diagnosis but, ultimately, histopathology is necessary for definitive diagnosis via biopsy of the tumor site for histopathologic assessment [59, 60]. On radiographs, an aggressive and destructive metaphyseal lesion is often present, and can be lytic and/or blastic in nature [61]. There is often evidence of a characteristic lesion at the tumor edge termed the 'Codman's Triangle' that occurs due to the production of new subperiosteal bone and periosteal lifting in aggressive bone lesions [62]. Histologically, canine and human OSA is a malignant mesenchymal tumor that produces an osteoid matrix throughout.

Once a tentative or definitive diagnosis of OSA is made in either canine or human patients, chest radiographs are recommended to assess for the presence of pulmonary metastasis, as the presence of visual metastatic sites within the thorax can greatly impact staging, prognosis, and treatment options. Only 15% or less of dogs with OSA have pulmonary metastases detectable on radiographs, although studies have shown that occult metastatic disease is present in approximately 90% of dogs at the time of presentation [63]. Advanced imaging such as CT or MRI is now being recommended and utilized for surveillance of metastatic disease because of their ability to identify smaller foci that may be undetectable via radiographs [64]. In canines, staging offers important prognostic determination as well as response to treatments. Stage I OSA describes a patient with a low-grade lesion with no evidence of metastasis. Stage II OSA consists of high-grade lesions without evidence of metastasis, while Stage III OSA has metastatic disease

present at the time of diagnosis. Compartmentalization of staging exists for Stage I and II OSA consisting of intracompartmental (A, tumor limited to the bone) and extracompartmental (B, tumor extended into nearby structures). The majority of canine OSA patients are diagnosed as having Stage IIB OSA at the time of presentation [65].

Pathophysiology

OSA most commonly (75%) occurs in the appendicular skeleton in both human and dog patients [53]. Canine OSA occurs more often (64%) in the front limbs than the hind limbs, with the most common locations including the distal radius, proximal humerus, distal femur, and proximal tibia. In humans, the distal femur and proximal tibia are the most common locations for OSA lesions [55]. Although less common, the axial skeleton (skull and ribs) as well as extra-skeletal sites (muscles, adrenal gland, eye, gastric ligament, ileum, kidney, liver, spleen, testicle, vagina) have also been affected in various canine cases reports [66, 67, 68].

The majority of dogs (90%) and humans (80%) with OSA have been shown to have pulmonary metastasis at the time of diagnosis, whether or not those lesions are visible on imaging at the time [63, 55]. The pulmonary parenchyma is the most common site of metastasis for both canine and human OSA [53, 38, 55]. Although less common, metastatic lesions have also been reported to occur in the bone or soft tissue structures including regional lymph nodes or internal organs like the spleen and/or liver [55, 69]. Dogs that have metastases present in the lymph nodes have much shorter life spans (48 days) in comparison to those that do not (318 days) [70], and humans with regional lymph node metastasis have a very poor prognosis [55].

Genetically, human and canine OSA have a 75% aneuploid DNA index with multiple shared genetic alterations [64]. Many genes associated with human OSA have been linked to canine OSA as well, including ezrin (EZR), met proto-oncogene (MET, hepatocyte growth factor), phosphatase and tensin homolog (PTEN), retinoblastoma 1 (RB1), tumor protein 53 (TP53), and v-erb-b2- erythroblastic leukemia viral oncogene homolog 2 (ERBB2) [71]. Similar alterations to the common tumor suppressor gene, p53, have been found in both human and canine OSA [72]. Similarities are also shared at the level of OSA DNA structural changes between humans and dogs. Studies have described the OSA found in both humans and dogs to have 'highly complex and chaotic karyotypes' due to the various imbalances of genes for oncogenes and tumor suppressor genes (e.g. MYC and KIT genes) [73]. Aside from genes, elevated levels of vascular endothelial growth factor (VEGF), a well-studied proangiogenic factor, present in the plasma of human cancer patients correlates with a negative prognosis [74, 75]. In angiogenic studies with canine OSA, plasma levels of VEGF negatively correlated with the disease-free interval [76].

Treatment

In both humans and canines, treatment for this highly malignant neoplasia includes limb amputation in combination with chemotherapy. Standard chemotherapy protocols in dogs consist of 3 to 6 cycles of a platinum- or doxorubicin-based regimen [77, 78, 46, 79, 80, 81, 82]. In humans, chemotherapeutic protocols can take place for up to one year [55]. Using treatment programs regarded as standard of care, the survival rate for canine patients at one year is less than 50%, and is less than 20% at two years [63].

The five-year survival rate in human OSA patients is less than 20% [83], and more than 30% are reported to be unresponsive to chemotherapy [84].

Three of the most commonly used cytostatic drugs used to treat OSA in dogs are carboplatin, cisplatin, and doxorubicin. In the case of cisplatin, interesting results were observed by Straw et al 1991, in which cisplatin increased the median survival time of dogs treated with the drug after amputation compared to those that only had amputation alone as the treatment, but that the drug had no effect on inhibition of metastasis [82]. In a study by Moore et al in 2007, 303 dogs with appendicular OSA were assessed for the efficacy of doxorubicin. The study found that doxorubicin administration via multiple doses (usually five doses total, once every two weeks) slowed the rate of metastasis with a 1-, 2-, and 3-year median survival time of 35%, 17%, and 9%, respectively [85]. Similar results were found with the use of carboplatin. Although none of the drugs completely inhibit metastasis, they are successful in prolonging the life of the patient being treated. In the treatment of human OSA, the chemotherapeutics associated with the greatest and most consistent activity include cisplatin, doxorubicin, high-dose methotrexate (with leucovorin rescue), and isofosfamide (with or without etoposide) [86].

Alternative stand-alone chemotherapy treatments are available, including lobaplatin and ifosfamide, but have been shown to have limited anti-tumor effects overall [87]. Many chemotherapy protocols used today enlist the help of multiple cytostatic agents used on an alternating basis to increase the effectiveness of each drug (eg. Wisconsin-Madison protocol). Interestingly, some studies conducted on dogs with OSA showed no significant advantage to multi-drug protocols when compared to monotherapies [88, 89, 90, 91, 92]. Research by Selmic et al in 2014 showed that

combining multiple drugs (like carboplatin along with doxorubicin) did not increase the median survival time [92]. Other studies demonstrated that monotherapy with carboplatin prolonged the disease-free interval compared to treating with both carboplatin and doxorubicin together on an alternating schedule [93, 94]. One practical advantage to multi-drug therapy in dogs with OSA, though, is the prevention or minimization of negative side effects, and multi-drug therapy should be a consideration when looking at the long term effects on patient quality of life during treatment [95]. In human treatment, multi-drug protocols (usually involving three agents) are considered the standard of care, along with surgery [86]. Additionally, treatment plans for human OSA often involve the use of neoadjuvant (pre-operative) chemotherapy which has been shown to increase the disease free interval and survival of patients with non-metastatic disease [96]. This is not often performed in canine OSA patients, likely due to the advanced state of disease often present at the time of diagnosis, and surgery is often the first initial step in treatment followed by chemotherapy.

An alternative to limb amputation is limb-sparing surgery, a technique in which the dog has played a valuable role in development and research of various surgical techniques, recovery, and outcome for use in human medicine. For these procedures, the tumor-infiltrated bone is excised, while the remaining bone is reconstructed to preserve the limb. For these procedures, internal and/or external fixation via endoprotesis (metal implant), cortical allografts, or external fixators is performed. Research in dog procedures revealed that the use of bone cement placed in the marrow during procedures utilizing large allografts reduced loosening of the implant as well as fracture of the allograft, and improved healing rates [97]. According to Liptak 2006, the type of implant for fixation

has no effect on failure of the reconstruction or the risk of post-surgical infection although, in comparison to limb amputation, there is an increased risk of post-operative complications as well as the increased time under anesthesia and higher expense [98]. Most commonly, though, complications including infection, fracture of the graft or implant, and tumor recurrence occur when limb-sparing procedures are performed. In dogs that undergo limb-sparing surgery for OSA treatment, the recurrence rate is 10-20%, while the human rate is approximately 5% [55]. Aside from these complications, limb-sparing procedures do provide a good alternative for owners resistant to the idea of a limb amputation [97, 99].

Palliative care is not recommended as the primary treatment option, but may be utilized in cases when dog owners do not wish to pursue aggressive treatment due to personal or financial limitations. The goal of palliative treatment is to control the pain associated with the tumor while having no effect on the progression of the disease. For this, radiation-based therapy is an effective means for symptomatic treatment of local bone pain in both humans and dogs with OSA by its ability to reduce inflammation, induce tumor cell necrosis, stimulate replacement of fibrous tissues, and stimulate woven bone formation [100]. Analgesic regimens combining the use of opiates and NSAIDs are often utilized with moderate efficacy in the controlling of pain associated with OSA [101].

One of the biggest issues facing canine and human OSA patients are metastases that occur in the lungs before amputation of the limb, and frequently before detection is possible. In cases where limb amputation was the sole treatment, the most common cause of death in canine and human patients was diffuse pulmonary metastasis [102]. The main

purpose of additional chemotherapeutic treatment protocols is to reduce the burden of micrometastatic disease present [103].

Canine and human OSA share similarities in clinical presentation, tumor histopathology, metastatic sites, and survival rates, making the dog an excellent disease model [104]. Dogs have already made contributions to both species in studies developing and improving limb salvage procedures, techniques that are now utilized in humans and animals with severe bone lesions like the ones caused by OSA [105]. Future treatment strategies currently under investigation, some of which utilize dog models, include molecular targeting of metastasis, markers of chemoresistance, and agents to stimulate the immune system directly. Previous studies in canine OSA patients have shown that a macrophage activator, muramyl tripeptide (MTP), had a positive effect on survival, a result that was shared in subsequent clinical trials in human patients [106, 107, 108, 109].

Prognosis

For both canine and human patients, those that present with evidence of metastasis at the time of diagnosis have a worse prognosis compared to those that do not [110, 111, 112, 63]. In cases where metastatic disease has been present at the time of diagnosis, the patient's response to chemotherapy is greatly reduced or absent, showing no efficacy at improving survival [113]. Some studies have shown benefit to performing surgical removal of the pulmonary metastatic sites (pulmonary metastesectomy) [114]. Although rarely performed in dogs due to diffuse nature of pulmonary metastatic sites, removal of pulmonary metastases in humans is recommended when possible and has been shown to significantly improve survival [115].

In dogs with OSA, prognosis has also been linked directly to alkaline phosphatase (ALP) levels, and histologic grade, as well as microvascular density [116, 117, 118, 119]. Elevated ALP levels in dogs with OSA correlate with a negative prognosis and shorter survival times by roughly 50%, regardless of combined surgical and chemotherapeutic treatments [116, 117]. ALP is regarded as one of the most valuable diagnostic serum biomarkers in human OSA [120, 121], with increased levels associated with higher tumor burdens. While histologic grade subclassifications of OSA are still being studied for their significance in prognosis [53, 122], cellular pleomorphism, mitotic figures, tumor matrix, cellularity, and the percentage of necrosis present have all been linked to the metastatic behavior of OSA [123, 118]. Dogs with lower tumor grades (grade I or II) often have a longer disease-free interval and survival time than those dogs with grade III OSA. In terms of vascular density in dogs with OSA, one study revealed that higher vascular density of the primary tumor was present in dogs with metastatic disease detectable at diagnosis [119]. Evidence of tumor necrosis following chemotherapy administration has been correlated with local tumor control in dogs, although no distinct correlation has been made with its link to time to metastasis [124].

While OSA most commonly affects middle-aged to older dogs, those cases where OSA was diagnosed in young dogs often had more aggressive OSA with shorter survival times [102]. This phenomenon has also been documented in humans [125, 126, 127, 128, 129]. In addition, large tumor size, dogs weighing over 40kg, and tumors affecting the proximal humerus have been associated with a worse overall prognosis [130, 131, 122, 46].

Frequent follow-up chest radiographs are recommended for monitoring for pulmonary metastasis. CT imaging is a more sensitive method for early detection of metastasis to the lungs [132], but has a higher expense and is not as readily available to patients or clinicians. Ultimately, more *in vivo* and *in vitro* studies must be performed to assess effective ways at increasing patient survival and eliminating tumor metastasis.

Canine Mammary Tumors

Background

Tumors of the mammary tissue are the most common tumor occurring in intact female dogs (52%) [18], with over half of them being malignant [134, 135, 136, 137, 138]. Breast cancer is the second most commonly diagnosed cancer in women, with over 200,000 new cases to be diagnosed in 2016 according to the American Cancer Society [2]. For both human and canine patients, malignant mammary tumors have a tendency to recur after being surgically removed, and may metastasize to distant locations such as lymph nodes, liver, or lung [139]. Some breeds have been documented to be more commonly affected by mammary carcinomas, including dachshunds, cocker spaniels, toy poodles, German shepherds, and various mixed-breed dogs [140]. Other factors that may play a role in the location and incidence of the different tumor types include the age of the dog, sex, and geographic location [18].

Multiple similarities are shared between canine and human mammary tumors, including dependence on hormones, metastatic patterns, age of onset, and environmental causes [18]. For roughly the past forty years, dogs have been used successfully in modeling comparative studies with human breast cancer research [141, 142, 18, 143] due to these clinical as well as molecular similarities.

Clinical Signs & Diagnostics

Most canine mammary tumor patients present with a history of one or more palpable and/or visual nodule(s) or mass(es) identified along a mammary chain. Occasionally these may be an incidental finding on physical examination. With inflammatory and/or metastatic forms of disease, there may be signs of systemic illness or metastatic site-specific signs (e.g. lameness due to metastasis to bone). Depending on the extent and stage of the disease at the time of presentation, there may be multiple different nodules consisting of one or more particular tumor type. The caudal mammary glands are more often affected than the cranial glands. Reports of multiple different tumor types occurring within a single mammary gland have also occurred. Human breast cancer patients are often diagnosed much earlier in the course of disease via utilization of breast cancer screening tests (eg. mammograms, familial susceptibility testing), but detection can also occur in a manner similar to dogs, where prominent lumps or swellings are noted in the mammary tissue or local lymph nodes [144].

For either species, signs consistent with malignant mammary tumors (carcinomas) include fixation of the tumor itself to the skin or the underlying soft tissue structures, a rapid increase in size, ill-defined borders, superficial ulcerations, pain, inflammation, and edema in the area [144]. Inflammatory mammary carcinomas usually present with firm and painful swelling in the affected gland or mammary chain. In some cases, the adjacent limb may be affected or cutaneous nodules may be visualized. Regional lymph nodes may be enlarged upon palpation due to reactive hyperplasia or metastasis of the primary tumor. These lymph nodes can include the inguinal, axillary, popliteal, internal iliac, sternal, or prescapular nodes.

Although mammary cancer can be suspected based on clinical signs and physical examination findings, histopathology is required for a definitive diagnosis. Biopsy submission for histopathologic assessment can also aid in determining tumor grade, stage, and overall prognosis. Surgical biopsies can be submitted during surgical removal of the mammary tumors, and surgical excision is the treatment of choice in cases of canine mammary cancer. Lymph node aspiration and cytologic assessment has been shown to aid in diagnostic accuracy of tumor metastasis, as not all lymph nodes containing metastatic disease will be enlarged on palpation. Imaging modalities such as X-ray, ultrasound, CT, or MRI are recommended in detecting metastatic lesions in the thoracic and abdominal cavities, with CT and MRI being more sensitive options.

Pathophysiology

Approximately 45% of canine mammary tumors are positive for estrogen receptors. This ratio is 60% in human breast cancer patients. In canine mammary tumors, exposure to progesterone has been shown to be the main risk factor for hormone-dependent development of tumors [145, 146]. Along with changes seen in steroid hormone receptors like those for estrogen, canine mammary tumor research has also shown overexpression of specific markers of proliferation, epidermal growth factor, mutations of the p53 suppressor gene, cyclooxygenases, and metalloproteinases, all of which share patterns with the human form of cancer. Previous studies have shown that decreased expression of hormone receptors has a great impact on tumor progression [147, 148] while increased expression of Erb-B2, a specific gene encoding for a member of the EGF receptor family [149], is directly linked to worsening of malignant disease in breast cancer [150, 151].

As a result of tumor development being linked to steroid sex hormones, early spaying (ovariohysterectomy) in dogs prevents development of mammary tumors altogether [152, 153]. In dogs spayed (sterilized) before their first estrus occurs, the probability of mammary tumor development is only 0.05%. These chances increase to as much as 26% if reproductive alteration is not performed until after the fourth estrus cycle [154]. In women, early removal of the ovaries (ovariectomy) as well as early pregnancy resulted in a lower incidence of breast cancer due to the similar effects on estrogen and progesterone receptor expression [155, 156].

Mammary carcinomas tend to be heterogenous in morphology and biologic behavior [157], and often have many different components involved, including bone, cartilage, and fat. Mammary tumors are classified as malignant epithelial tumors, special type epithelial tumors (SCC, adenosquamous carcinoma, mucinous carcinoma, secretory carcinoma, spindle cell carcinomas, and inflammatory carcinomas), malignant mesenchymal tumors, carcinosarcomas, benign tumors, or mixed-type tumor cells [158, 159]. Overall, complex adenomas and benign mixed-type tumors are the histological type most commonly encountered, while carcinoma forms are the most malignant [159]. Histologic evaluation of tumors based on nuclear differentiation produces three potential classifications – poor, moderate, and well differentiated. When tumors fall into the 'less differentiated' classification during development, those tumors have the tendency to lose their receptors for sex hormones like estrogen and progesterone, and tend to form more aggressive tumors [155]. This phenomenon is shared with human breast cancer, and is yet another representation of the valuable role dogs play as models for these tumors based on similar molecular and biological mechanisms.

An inflammatory subtype of mammary tumors has been identified in both canine (inflammatory mammary cancer) and humans (inflammatory breast cancer), representing one of the most aggressive forms [160]. This particular subtype has been shown to be highly angiogenic and invasive [161], with primary effects on blockage of lymphatic drainage due to invasion of dermal lymphatic vasculature [161, 162, 163] thereby resulting in local edema.

Further investigation into classification of mammary tumors includes work showing increased expression of vimentin, a marker for mesenchymal cells, that is present in myoepithelial cells [164], in aggressive forms of breast cancer [165, 166]. Metalloproteinases, a common marker of malignancy in human breast cancer, have been shown to be actively involved in tumor proliferation, angiogenesis, basement membrane invasion, and metastasis in canine mammary tumors [167]. COX-2, an enzyme involved in prostaglandin synthesis, has been shown to be overexpressed in both canine and human mammary tumors [168, 169, 170]. Prostaglandins, lipid mediators, have been linked to involvement in tumor development and progression. Specific targeting of COX-2 could provide a means of mammary cancer prevention and treatment development [171]. This idea is the focus of many current studies, including those on specific mammary tumor cell lines such as CF33, CF41, CMT9, CMT12, CF35, and CMT28 [172].

Treatment

The most widely used and effective treatment for canine mammary tumors is surgical resection of the tumor. The type of surgical excision performed (e.g. nodulectomy/lumpectomy, regional mastectomy, radical mastectomy) depends on the site, location, and number of tumors that are present. The type of surgery performed has

not been shown to have an impact on patient survival as long as there are clean margins on histology. Radical chain mastectomy procedures performed unilaterally have been shown to decrease the chances of tumor development in remaining mammary tissue. In previous studies, as much as 58% of dogs that underwent a regional mastectomy developed a tumor in the mammary gland chain ipsilateral after surgery [144]. Removal of draining lymph nodes (inguinal lymph node in caudal mammary gland tumors, axillary lymph node in cranial mammary gland tumors) with submission for histopathologic analysis in patients where metastasis is suspected is recommended. In the case of inflammatory mammary tumors, surgery is only viewed as a palliative treatment option, as these tumor types are considered non-resectable due to their diffuse nature and high metastatic rates.

Chemotherapy is rarely utilized in dogs with mammary cancer [18], as chemotherapeutic agents have not been shown to be consistently effective in treatment. Some studies showed partial responses of mammary tumors to doxorubicin [173, 174]. Analgesics including NSAIDs should be considered for palliative treatment in advanced stage tumors or inflammatory mammary carcinomas.

Prognosis

Overall, dogs with benign mammary tumors are often cured following complete surgical excision. Factors that have been shown to have an impact on the prognosis of dogs with mammary tumors include classification of the histological subtype, biological grade, degree of invasion, nuclear differentiation, lymphatic involvement, size of the tumor, and expression of hormone receptors [157, 175, 176, 137]. In terms of tumor size, tumors with a diameter greater than 3 cm have been reported to have a worse prognosis.

High grade tumors that are invasive with stromal, lymphatic, or vascular involvement often have a worse prognosis than low-grade well-differentiated and non-invasive tumors. Mammary tumor staging consists of stages I through V. Tumors carrying a higher stage classification include large tumors (II-V), tumors with lymph node involvement (IV-V), and tumors with metastatic lesions (V). Inflammatory carcinomas have a grave prognosis [144].

Location of the tumor itself as well as surgical removal technique used or number of total tumors present do not influence overall prognosis [177, 178]. Assessment of the tumor via histology alone is not sufficient for accurately determining prognosis, as many apparently benign tumors may end up metastasizing despite original classification as an adenoma or mixed-type tumor. Tumors that appear benign biologically may show histomorphological evidence of malignancy in some cases [179]. The stage and grade of the tumor also has a big impact on the prognosis [176].

Platelet Background

With the initial observations of platelets dating back to the late 1600s, these blood 'cells' have had a long list of names given to them along the way, including globules, corpuscles, fragments, and even the term 'besides' [180] used to describe them. There has been a lot of controversy as to the proper classification of these 1.5 – 3 μm diameter [181] megakaryocyte (MK)-derived anucleate cell fragments, regarding whether or not they are even 'cells' at all. Regardless of their classification or the name given to them, these discoid cells play vital roles in the body, with an ever expanding list of functions and duties ranging from their well-known roles in blood hemostasis, thrombosis, wound

healing, and inflammation as well as their more recently recognized interactions with tumor cells and metastasis [182].

Platelets are produced from polyploidal hematopoietic MKs in the bone marrow during MK development and maturation [183]. Even though it is known that platelets, in fact, originate from the MK, the mechanism by which this occurs is still controversial. Three main models of platelet formation exist: cytoplasmic fragmentation, platelet budding, and pro-platelet formation [183, 184, 185]. The mechanism in highest current popularity suggests that MK extend their long branching pro-platelet extensions into the sinusoidal blood vessels, releasing pro-platelets into the circulation, where further maturation occurs [183, 186]. This pro-platelet release occurs at the level of the bone marrow and, most recently discovered, within the pulmonary vasculature [187, 188, 189].

Regardless of the actual mechanism occurring, the end result of platelet development results in platelets that contain MK-derived cytoplasm complete with mitochondria, granules, and mRNA. Even though platelets lack a nucleus, and therefore DNA, they still have the ability to produce proteins courtesy of the multitude of coding messenger RNA and translational machinery from their parent cell [190, 191, 192, 193, 194]. Platelet and MK production is primarily regulated by the protein thrombopoietin (TPO) [195, 196], although recent evidence has shown that this process can also be influenced by interleukin-1 (IL-1) and potentially other interleukin molecules when there is presence of infection, inflammation, or excessive blood loss [197]. TPO stimulates MK to increase in their size and ploidy, which thereby stimulates production of proplatelet processes that later become individual platelets [195]. MK production primarily occurs in the liver during fetal development in humans, but later occurs primarily at the level of the

bone marrow in adults. Production of TPO, though, takes place in the liver throughout the life of humans [198]. Gene expression for TPO production has been illustrated to take place in various other organs (eg. kidney, bone marrow, lung, spleen, CNS) in humans, mice, and rats [198, 199, 200, 201, 202]. Recently, Figueiredo et al discovered that dogs also have the highest level of TPO transcription occurring in the liver, followed by the bone marrow, spleen, lung, and kidney [203]. In contrast to studies in humans, though, dogs showed less TPO gene expression in the kidney. This finding is contrary to results seen in mice and rats, in which the kidney had the highest level of TPO gene expression [199, 204]. The lung was found to be a potentially important site for TPO expression in dogs as it had notably higher levels of expression than the kidney, the tissue with the second highest TPO expression in humans [203].

The proteins found within platelets have three potential sources from which they originate. These proteins can come from the MK, be taken up by surrounding fluids and plasma through a sponge-like mechanism (endocytosis), or they can be produced by the platelet itself through use of a retrotranscription RNA process and splicosome (termed *de novo* protein synthesis) [180]. Proteins that have been proven to be synthesized by platelets include IL-1, tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), cyclooxygenase-1 (COX-1), and P-selectin [205, 206, 207, 208, 209]. Most of the proteins utilized by platelets are stored in granules. Platelet granules consist of three different types: α-granules, dense granules, and lysosomes. These granules have each been shown to contain different subpopulations of proteins and other components.

The platelet's α-granules are the most abundant of these intracellular granules (approximately 50 to 80 granules per cell in humans) and contain proteins that function

within the membrane (e.g. αIIbβ3 integrin, P-selectin, CD36), cell adhesion (fibrinogen, von Willebrand factor (vWf), vitronectin, multimerin I), and coagulation (factors V, IX, XIII) [210]. More specifically, platelets are now known for their ability to capture and concentrate molecules involved in the modulation of angiogenesis within distinct populations of α-granules [211, 212, 213], which will be further discussed later in this chapter. The exact mechanism by which this heterogenous population of α-granules is controlled for differential release is not clearly understood yet, but is currently and actively being studied [214, 215, 216]. Platelet α-granules have also been shown to contain chemokines which aid in the host response to pathogens due to their anti-microbial properties [217, 218, 219], as well as various pro-inflammatory and immunomodulatory factors that are involved in inflammation and have been studied with regards to atherosclerosis [220, 221, 222].

Platelet's less abundant dense granules (approximately 3-8 per platelet in humans) contain components including cations (Ca2+), phosphates, and compounds active in hemostasis (adenosine diphosphate (ADP) and adenosine triphosphate (ATP)) [210]. The majority of dense granule contents have been found to be non-protein in nature. Platelet lysosomes are known to play key roles in the endocytic and secretory pathways. Defects in lysosomes have been linked to pathologies including albinism, immunodeficiency, and a disease in humans named Hermansky-Pudlak syndrome [223, 224].

A unique and intricate network of channels within the plasma membrane of platelets, called the open canalicular system (OCS), allows for platelet's plasma components to be taken into the cell (endocytosed) or specific granular contents to be released out of the cell (exocytosed) [216, 225]. Upon activation, platelet granules

migrate to the cell periphery and bind with the plasma membrane – either utilizing this OCS to release granular content or by directly exocytosing the contents out into the extracellular space [216]. Even more recently, this OCS has been shown to potentially play a vital and important role in the regulation of platelet adhesion [226].

Platelets have been the subject of a multitude of various research studies aimed at elucidating causes and mechanisms behind many different disease processes in various different species, ranging from inflammatory conditions, autoimmune diseases, and even neoplasia. Platelet transcriptomic analysis in humans has been used to demonstrate differential RNA expression profiles in platelets in several different conditions. Research by Kondar et al illustrated significant differences between platelets with hyper- and hypo-reactive aggregation responses to agonists [227]. They also demonstrated differences between platelets from patients with essential thrombocythemia and reactive thrombocytosis compared to those of healthy individuals [228, 229, 230]. Similar studies also compared platelets from patients with sickle cell anemia or systemic lupus erythematous [231, 232]. Other applications have been in the identification of mRNA patterns associated with platelet reactivity, body mass index, and cardiovascular disease [227, 233, 234], as well as a comprehensive comparison of mouse and human platelet transcriptomes [235].

Platelets in Hemostasis

Initially noted by early physicians to be located at the site of wounds over 150 years ago, the most well-known function of platelets is their important role in the cessation of hemorrhage following trauma and/or injury to the vasculature and surrounding tissues [236, 237]. Under normal physiological conditions, platelets circulate

the vasculature in a dormant quiescent state due to the presence of healthy intact endothelium lining the vessel walls. The platelet surface is riddled with various receptors utilized in hemostasis and adhesion including GPIb-IX-V complex, GPVI, integrins (αIIbβ3, α2β1, α5β1, α6β1), protease activated receptors (PARs), ADP receptors (P2Y1, P2Y12, P2YX), thromboxane receptors, platelet endothelial cell adhesion molecule (PECAM-1), and carcinoembryonic antigen-related cell adhesion molecule (CEACAM-1) [238, 239, 240, 241, 242, 243]. Due to their small size, platelets circulate along the outer edge of the blood stream. This allows detection and fast responses by the platelets to any vascular damage that may occur [210]. An intact endothelial layer acts as an anti-platelet barrier protecting against any unnecessary thrombosis and through the endothelium's release of thrombotic mediators such as nitric oxide (NO) and prostacyclin [244]. When the endothelium becomes disrupted, platelets are triggered to transform into an activated state due to the exposure of the subendothelial matrix which results in the exposure of proteins such as vWf, collagen, fibrinogen, fibronectin, laminin, and thrombospondin-1 (TSP-1). Once activated, the platelet's function is dependent on hundreds of bioactive proteins that are then released [182].

The structure of the platelet itself has the ability to change due to rapid cytoskeleton remodeling and the presence of an asymmetric plasma membrane. The internal surface of the plasma membrane is lined with anionic phospholipids such as phosphatidylserine (PS). In a resting state, the outer surface of the platelet membrane is covered with neutral phospholipids. Upon activation of the platelet, negatively-charged PS molecules are flipped to the external surface for roles involved in coagulation [245, 246, 247].

Upon vessel injury, initial loose platelet tethering to the vessel wall is achieved by an interaction between platelet GPIb-IX-V complex and vWf in the subendothelial layer at the site of injury [248, 249]. Following tethering, GPVI and a2β1 interact with exposed collagen to mediate activation and adhesion of platelets. Formation of a complex between GPVI and FcR-y leads to an increase in cystolic Ca2+ which may stimulate platelet shape change, secretion of granules, and activation of additional integrins [248]. Stable collagen binding is then secured by α2β1 integrin [250, 251] as well as through subsequent binding of integrin αIIbβ3-vWf, fibronectin, fibrinogen/fibrin, GPVI-collagen, α2β1-collagen, α6β1-laminin, and α5β1-fibronectin [252]. The entire process is then amplified through intracellular fluctuations of Ca2+, stimulating the generation of thromboxane-A2 (TXA2), granule secretion, and further αIIbβ3 activation [189, 253, 254]. Binding of αIIbβ3 to vWf or fibrinogen allows crosslinking of platelets to occur which ultimately leads to platelet aggregation and formation of a primary platelet plug (primary hemostasis) [252, 255].

Following formation of the platelet plug, additional platelets aggregate and trap other circulating blood cells, thereby contributing to growth of the thrombus. Through activation of either the intrinsic or extrinsic pathways of the coagulation cascade, a stable fibrin clot is formed by the actions of thrombin (factor IIa) converting fibrinogen (factor I) into fibrin (factor Ia) (secondary hemostasis) [256, 257, 258]. Although primarily derived from hepatic production, thrombin is also partially derived from platelet-derived prothrombin (factor II) [259]. Platelets are actively involved in the process of coagulation, in addition to their roles in initiating hemostasis. Flipping of PS onto the outer surface following platelet activation facilitates the pro-thrombinase complex

assembly as well as promotes the generation of thrombin itself [245, 246]. The negatively charged PS leads to the binding of calcium with subsequent attachment of vitamin K modified factors. This chain of events ultimately leads to fibrin formation on the primary platelet plug. Recent studies have also shown that platelets have a role in initiating the intrinsic pathway of the coagulation cascade by activation of factor XII via dense granule release of PolyP [260]. Overall, platelets and the coagulation cascade reinforce each other through a positive feedback loop of activation throughout hemostasis.

Beyond Hemostasis

Beyond the roles platelets play in coagulation and primary plug formation, platelets are also intimately involved with many other biological events including recruitment of immune cells, inflammation, wound healing, angiogenesis, and vascular remodeling.

Immune Function

Platelets are not only innate and inflammatory cells themselves, but they can also assist, depending on the circumstances, adaptive immunity. They do not only assist immunity, as has long been known, but have been shown to be active immune cells [180]. One example of this is the evidence provided by Yang et al illustrating the dependency of expression and amount of P-Selectin found within platelets on plasma fibrinogen levels, as well as fibrinogen-β3 integrin outside-in signaling [209]. This finding is not only important in the role of platelets in hemostasis and thrombosis, but also in the interactions of the platelet with other cells including leukocytes, stem cells, and even tumor cells, which leads to implications and roles in immune response and tumor growth and

metastasis [210]. P-selectin is a transmembrane protein that is stored in platelet granule membranes and, upon activation, translocates to the platelet membrane for roles in platelet aggregation, interactions between platelets and leukocytes, as well as interactions between platelets and endothelial cells [261, 262, 263, 264]. These findings have only been reported in human and/or murine studies. There is very little to no canine specific research present in the literature with regards to platelet-specific roles in immune surveillance and function.

Angiogenesis

The formation of new blood vessels from the pre-existing vasculature is termed angiogenesis. This multistep process involves the sprouting and configuration of new microvessels [265], and is vitally important in normal physiologic functions including early fetal development, wound healing, and neovascularization of ischemic tissue [266]. A variety of different cell types have been proven to release angiogenic-regulating factors, including endothelial cells, stromal cells, leukocytes (neutrophils, macrophages, natural killer cells), platelets, and tumor cells [267, 268]. Platelet research over the years has led to the discovery of direct links between platelets and angiogenesis, and more recent studies have illustrated the ability of the platelet to both potentiate and inhibit angiogenesis [265]. This suggests that platelets have value as a future target of many potential therapeutic studies for use in different pathologic diseases including atherosclerosis [269], rheumatoid arthritis [270], diabetic retinopathy [271], psoriasis [272], and neoplasia [273]. While most research on the role of platelets in angiogenesis has taken place in human, rat, and mouse studies, some studies have taken place in

canines, including those looking specifically at platelet granule localization and contents [274].

Physiologically, angiogenesis only occurs during times of development (in the placenta during pregnancy), within the cycling ovary, or during wound healing after acute vascular complications or trauma [275]. During these times, angiogenesis is active for days to weeks, and later inhibited. In the case of various disease states such as cancer, angiogenesis is instead continually activated, a process that is referred to as an 'angiogenic switch'. This overactive angiogenesis can create a hallmark of various disease states [267]. Although platelet effects in angiogenesis have been the subject of many research studies, many of which have contradicted or contrasted others in nature, the overall and well-accepted opinion is that the activation of platelets has a net stimulatory effect on angiogenesis [276, 277].

As previously described, platelets have distinct types of intracellular granules (α, dense, and lysosomes) that have the ability to secrete their contents upon different external stimuli (platelet activation). Of the multitude of granular contents, various pro-angiogenic factors, including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), and angiopoietin-1, as well as antiangiogenic factors such as endostatin, angiostatin, platelet factor 4 (PF4), thrombospondin-1 (TSP1), and PAI-1, have all been proven to be localized into different α-granules and preferentially released from the platelet [278, 279]. Additional pro- and anti-angiogenic factors found in and released from activated platelets are listed in Table 2.1.

As in human and murine platelets, differential packaging of molecules in α-granules has also discovered for canine platelets by Curotto et al [274]. In this study, canine platelets were shown to distinctively house vWf and fibrinogen in separate alpha granules, with very minimal co-localization. This information was in agreeance with findings in human and mouse platelet studies, suggesting that platelets selectively package hemostatic proteins in different subsets of alpha granules [214, 280, 281]. Little is known about the selective packaging and release of canine platelet angiogenic factors, although research is currently ongoing as the dog progressively reveals itself as a valuable model for human disease.

VEGF has been described as indispensable to vessel formation in previous research studies on angiogenesis [277]. More information about VEGF functions is detailed in the 'tumor growth & metastasis' section. One of the angiogenic inhibitors contained within platelet granules in greatest abundance is thrombospondin (TSP-1), which has been shown to inhibit the proliferation of endothelial cells, induce apoptosis of cells [282], and counteract the pro-angiogenic factors released from platelets [283]. Selective release of pro- and anti-angiogenic factors was demonstrated by Ma et al via their study using human platelets as well as *in vitro* work with rats which showed that stimulation of the thrombin receptor, PAR-1, induced platelet release of VEGF while PAR-4 stimulation caused the release of endostatin, inhibiting VEGF release [211]. Additional research then demonstrated that some angiogenic factors, VEGF and endostatin, for example, are packaged into separate α-granules, which allows the platelet to selectively release factors after activation and stimulation [281]. In slight contrast to this, additional research showed that instead of distinct and separate α-granule

populations, there was a spatial separation of cargo within each α-granule [284]. Further studies are needed to elucidate the exact arrangement and organization of these organelles and their cargo, but the overall function of platelet angiogenic factors and their granule vessels is clear in their ability to respond and be secreted following platelet activation to various stimuli and/or disease states. Again, canine specific research mirroring these findings has not been completed in the literature to our current knowledge, illustrating gaps in the links between canine and human platelet research.

In addition to platelet granules and their contents, another area of increasing platelet research is on platelet microparticles (PMPs) or 'platelet dust'. These platelet plasma membrane and granule fragments are shed from platelets after activation, and have been shown to contain a unique set of platelet proteins and lipids. Of all the microparticles present within the bloodstream, platelet microparticles account for 70-90% in the healthy human [285, 286]. Involved in many physiologic roles including hemostasis and coagulation, PMPs have also been associated with many similar disease states previously mentioned including atherosclerosis, rheumatoid arthritis, and cancer [287]. PMPs have the ability to promote endothelial survival, proliferation, migration, and tube formation due to their contents including, but not limited to, VEGF and bFGF [288]. Additional studies have demonstrated that PMPs could induce angiogenesis after thrombin activation [289]. Platelet microparticle research for canines is limited, but has been initiated through studies looking at collection techniques and their effects on the thrombin generating ability of PMPs [290], although detailed effects in different disease processes in canines have not been studied in depth to date.

Tumor Growth and Metastasis

Platelets play a significant role in tumor progression by influencing angiogenesis, immune evasion, and metastasis [291]. Bi-directional signaling between platelets and tumor cells affects the tumor microenvironment and impacts angiogenic and metastatic potential. Under normal circumstances in the healthy body, angiogenesis is overall dormant, with only 0.01% of endothelial cells dividing [275], along with tight regulation during times of activity. In pathologic cases such as cancers that involve active angiogenesis, a specific angiogenic 'switch' takes place allowing excessive vascular proliferation with the involvement and participation of platelets. Angiogenesis has been implicated to have involvement in over 70 disease conditions [275]. Of particular note is the discovery of the involvement of platelets and angiogenesis in tumor regulation, a theory which was first proposed over 50 years ago [292]. Several studies have established that platelets play an important role in both angiogenesis within primary tumors and tumor capacity for metastasis mediated by specific adhesion molecules. There is additional evidence supporting the role of the platelet releaseate favoring either the pro- or anti-angiogenic state [293, 294].

Tumors can actively sustain their survival at a size less than 3 mm diameter through simple diffusion of essential oxygen and nutrients [292]. After this point, the tumor cells require additional sources of nutrient and oxygen delivery, therefore requiring an independent blood supply. The vascular network established within tumors allows the malignant cells to escape from the primary tumor, enter circulation, and metastasize to distant locations in the body, making angiogenesis an important component of tumorigenesis. Since the link between platelets and angiogenesis was proposed near the

most recent turn of the century, research into the links between platelets, angiogenesis, and tumors has boomed for all species, especially those regarding animal models.

Tumor vasculature, in general, is abnormal and aberrant when compared to normally developed and maintained vasculature of the body. Tumor microvessels are heterogenous in nature with uneven diameters, hemodynamic upsets, increased permeability to large molecules, and disrupted endothelial linings [295, 296]. Studies have shown that the stabilizing effects of platelets on the tumor microvasculature are very likely dependent on the release of platelet factors after activation, although the exact mechanisms and factors critically involved have not been identified [297]. In studies where platelets were depleted, hemorrhaging occurred within the tumor stroma, suggesting that these platelet factors involved play a role in counteracting the damage caused by immune cells that migrate into the stroma [297].

The ability of platelets to play a role in 'hiding' or 'cloaking' tumor cells circulating in the vasculature is well documented in the literature. This function provides a way for tumor cells (that would normally be a target for immune cells) to evade detection and killing by immune cells such as NK cells, both through physically shielding the tumor cells from detection as well as through factors released from platelets [298, 299]. One example of this is platelet released TGF-β following interaction with tumor cells, which results in suppressed NK cell receptor activation [300]. Tumor cells have been shown to induce platelet aggregation, a response termed 'tumor cell induced platelet aggregation' (TCIPA), in which some tumor cells express platelet integrins ($\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$) to enhance metastasis [301, 302, 303]. The *in vivo* relevance of this intricate network is evident, as the metastatic potential of a tumor has been demonstrated to correlate with its

ability to aggregate platelets [304]. Other platelet-derived factors that have been shown to have key roles in tumor metastasis following gene-depletion studies include GPIbα, GPVI, P-selectin, and α2bβ3 [305, 306, 307, 294]. Thrombocytopenia, anti-platelet treatment, or blockage of specific platelet adhesion proteins with antibodies against P-selectin, GPIb-IX, or GPIIb-IIIa have all been shown to negatively impact tumor progression [308, 309, 310, 311].

Although most focus on platelet-tumor interactions have been on the effects of factors contained and released from platelet α-granules, recent research has shown that dense granule components, notably ADP and ATP, can aid tumor metastasis via disruption of the assembly of endothelial intracellular junctions. This aids metastasis in the ability for tumor cells to extravasate from the vasculature into the subendothelial space. By the mediating the effects of ADP and ATP on $P2Y_2$ receptors, new targets for therapies targeting tumor metastasis now exist that may not interfere directly with platelet function [312]. The α-granule factors mentioned above (VEGF, PDGF, TGF-β) have also been shown to play roles in altering the integrity of endothelial junctions. In addition to molecules that control angiogenesis, activated platelets release factors that modulate hemostasis, inflammation, and cell proliferation. These processes are all necessary for the switch from dormancy to progression to take place. The injection of tumor cells that were previously incubated with washed platelets resulted in an increase of pulmonary metastasis in mice. This indicates a role of tumor cell priming by platelets for metastasis to take place [301]. Similarly, exposing tumor cells to PMPs also resulted in an increased metastatic potential, as shown in other studies [313]. Platelets also have a role in 'priming' future sites of metastasis, as evidenced by studies looking at bone tumors. This

pre-metastatic method of communication between platelets and distant sites (bone in this particular study) was made possible through the sequestering of tumor-derived proteins by the platelets [314]. This suggests a more complex role of platelets in metastasis of tumors beyond direct interactions and effects.

In general, thrombocytosis is a condition that is often observed in patients with cancer. When present, this elevation in platelet number is usually associated with an advanced stage of disease and a poor prognosis [315, 316]. In many cases, human cancer patient platelet α-granules contain elevated amounts of VEGF and angiopoietin as well as decreased levels of TSP-1 [317, 318, 319]. Various therapies have been tested and implemented targeting platelet function and/or angiogenic molecule blockade throughout the years, since as early as the 1970s [292]. One of the most well studied angiogenic targets for therapy development has been the VEGF signaling pathway. VEGF is a key player in both early and late angiogenesis, and has been shown to be produced by tumor cells and stroma, and its receptors are found on tumor cells and stroma as well [320]. VEGF has been linked to poor prognosis in human cancers including colon cancers, breast cancers, gastric carcinomas, prostrate cancers, lung cancers, various sarcomas, as well as melanoma [320], and has therefore been the target of current anti-angiogenic therapeutics. Specific platelet function blockade through therapies including aspirin has been shown to have positive effects on increasing survival and decreased recurrence in some studies [321, 322], although additional research is needed to identify more effective therapies and therapeutic targets as results have been somewhat beneficial but overall inconsistent across species, type of neoplasia, and stage of disease.

Of the thousands of molecules thought to play a role in angiogenesis and/or tumor progression, about one hundred have entered human clinical testing for their roles in anti-angiogenic therapy. Of these, only approximately ten have been approved for clinical use [323]. The first to be FDA approved was the anti-VEGF antibody therapeutic bevacizumab (Avastin), which was shown to improve the survival of human patients with colorectal cancer, non-small cell lung cancer, breast cancer, glioblastoma, and renal cell carcinoma [324, 325, 326, 327, 328]. While many studies and trials utilizing these anti-angiogenic therapies have shown reduced tumor growth, recent studies have suggested that these therapeutic approaches may actually select for a more invasive phenotype in the end [329, 330]. This occurrence can potentially be attributed to the development of an adaptive/evasive resistant population of tumor cells to anti-angiogenic therapies due to selection for more aggressive cells capable of surviving [331]. This theory of therapy resistance is seen with various disease processes in medicine, including antibiotic-resistant strains of bacteria and resistant populations of parasites in veterinary medicine. As detailed above, future integration and use of canine models for various human cancers will aid the progression of research into therapies through increasing efficient translation from the bench top to clinical use.

In the case of canine tumors and their interactions with platelets, studies are limited. Interestingly, in a recent study by Bulla et al, canine platelets and their molecular contents were shown to actually inhibit the migration of three canine OSA cell lines (OSA-8, OSA-40, OSA-78) *in vitro* [332]. In addition, effects of platelets on tumor cell migration varied between different cell lines, suggesting that variation among different tumor cell lines can effect the magnitude of platelet effects that take place. Overall, these

findings contrast similar studies in humans and other species, that have shown platelets to have a prometastatic effect on tumor cell lines. For those studies, though, the tumor cell lines have mostly consisted of epithelial cancers (eg. carcinomas, adenocarcinomas) [301, 312, 333], which contrasts to the OSA cells lines in this canine study. There is very little evidence of the effects of platelets on the migratory or metastatic effects of mesenchymal tumors. One study in mice illustrated that induction of thrombocytopenia resulted in a decrease in pulmonary metastasis in fibrosarcoma, which fits with platelets having positive effects on tumor growth and metastasis [334]. Further investigation by Bulla et al revealed a downregulation of epithelial-mesenchymal transition (EMT)-related genes in the canine OSA study, which could explain the inhibitory effects observed [332].

Wound Healing

Through the formation of a platelet plug and later a blood clot during hemostasis, platelets also provide a scaffold system by which wound healing can take place [335]. Initially, the release of various platelet cytokines and growth factors (greater than 60 substances active in wound healing) at the site of a wound elicits influx of inflammatory cells as well as endothelial cells and fibroblasts [336, 337, 338]. Subsequently, relaxation of the vascular smooth muscle and vasodilation occurs as a result of the calcium mobilization and production of NO after binding of VEGF [339], which aids in the delivery of blood flow to the wound. While later stages of wound healing (proliferative and remodeling phases) take place without direct platelet interaction, studies have shown the vital role that platelet secreted factors play in allowing these complex events to take place [338].

Modern therapies now include the use of platelet-rich plasma (PRP) gel placement in wounds to promote proper healing. Examples of PRP use in wound healing therapy in humans include diabetic digit ulcerations, orthopedic surgical procedures, muscle and tendon repair, ophthalmic procedures, and cardiac surgeries [340, 341, 342, 343]. In veterinary medicine, PRP has been studied for use in various wound healing circumstances. For example, recent work from Canapp et al revealed significant improvement in dogs with partial cruciate ligament tears after intra-articular injections of PRP [344]. In horses, PRP and platelet concentrates have been used in the treatment and enhanced healing of various tendon and ligament injuries for many years [345, 346, 347].

Platelet Purification

Due to the small amount of protein and RNA present in a platelet relative to their nucleated counterparts, appropriate sample collection and preparation is key for obtaining reliable results in proteomic or transcriptomics analyses of platelets. [348, 349]. Significant challenges arise when working with platelet protein and mRNA, as samples are easily contaminated by proteins and RNA derived from other blood cells in much higher proportion including, most notably, leukocytes. In human platelet studies, platelets have been shown to contain 65-fold less protein and 12,500-fold less mRNA when compared to leukocytes [350, 351]. Exact numbers comparable to this study are not available for the canine species, although the proportions are likely similar. Regardless of species, it is essential to develop platelet isolation techniques and to characterize the purity of these samples, not only on the basis of cell count, but also by volume and protein and mRNA content, to ensure a pure and valid representation of the platelet component being studied. Additionally, clinical samples obtained for use in platelet

research tend to be of low total volume. Due to this, sample purification techniques must produce high yields when performing proteomic and transcriptomic studies for the use in biomarker discovery and identification.

Throughout the years, platelet purification techniques have greatly evolved. In human medicine, platelet purification technology has evolved via the use of platelet apheresis. With this technique, platelets are directly collected from a patient while other blood components are replaced back into the body during collection. This allows large platelet samples to be collected from a single patient, but the drawback is the need for expensive and specific equipment to accomplish this methodology. Alternatively, human medicine relies on platelet pooling from whole blood utilizing large volumes of blood collected from multiple patients. Specialized filters and centrifugation techniques are used to ensure highly pure platelet yields. The downside of these techniques, though, is that platelet losses are high. In veterinary research and medicine, smaller patient size and lack of sophisticated and expensive equipment greatly limits the volume of blood and platelet samples that can be obtained from each patient.

In addition to issues with total platelet volume and purity, the platelet proteome, and even transcriptome, can be greatly altered by the activation of platelets within the sample. Great care must be taken in the techniques used to collect and isolate platelets to prevent platelet activation during sample collection and also minimize sample handling and prevent activation *in vitro*. The volume of the sample obtained, the target sample purity to be reached, and the potential for platelet activation ultimately determine the techniques that can be successfully be used to obtain a reliable and representative platelet sample suitable for proteomic and/or transcriptomic studies in all species.

Proteomics

Background

Proteins, made up of amino acids, are the building blocks of all living organisms. Proteins can vary greatly in their structure and function and are the product of the genes encoding them. While many species' genomic sequences have been described, many limitations in genomics exists with regard to understanding the underlying function and implications of each gene. A 'proteome' is the total set of the protein products resulting from the protein-coding genes within a particular cell type or the genome of an entire organism. The study of 'proteomics' involves analysis of the entire protein composition of a particular cell or an entire organism [352]. Using proteomics, the structure, interactions, and functions of proteins can be understood along with the biological mechanisms they are involved in within cells and tissues including those in different diseased states as compared to normal states. Ultimately, the information gathered from proteomic studies can be applied to the development of biomarkers of diseases, including cancer, to aid scientists as well as clinicians in early disease detection, disease pathogenesis, and development of novel therapeutic modalities [353].

Protein numbers far exceed the number of protein-coding genes. The human genome contains greater than 30,000 genes. From these genes, as many as 100,000 proteins could result. This discovery led to a shift in the focus of research from that of single gene or protein studies to more broad based studies looking at entire protein sets within a cell or organism. Aside from not only providing protein identification, mass spectrometry-based approaches like proteomics can elucidate all of the various aspects of protein functions and interaction within a cell including different modifications made to

proteins, their isoforms, and their interactions with different proteins and involvement in biological pathways [355]. The development and utilization of proteomics provides a valuable tool to expose the links between cells and disease processes at the level of the protein. This information cannot be obtained through genetic studies or through looking at RNA, as poor correlation exists between those molecules and their corresponding protein levels as, for example, many mRNA are non-coding and/or undergo alternative splicing [356].

An important initial step in proteomics is to separate out proteins within the sample itself to decrease its complexity. Multiple methodologies have evolved over time beginning with the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and later 2-DE (two-dimensional gel electrophoresis), the latter of which is the most commonly used gel-based technique today. In these electrophoretic methodologies, currently the most commonly used method for protein separation, samples are denatured and separated based on their isoelectric points as well as their molecular weights. Stained protein spots within the gel are removed and digested into fragments for analysis via mass spectrometry [357].

Mass spectrometry allows extremely high sensitivity and specificity when identifying, characterizing, and quantifying proteins present in a sample. Variations in techniques (e.g. top-down and bottom-up approaches) may or may not involve the use of a protein digestion step for the generation of peptides. For bottom-up proteomic studies following protein digestion, peptides are ionized (via electrospray ionization or matrix-assisted laser desorption ionization) and passed through a mass analyzer for determination of their mass-to-charge ratio (*m/z*). Five different mass analyzers may be

used in proteomics including the time-of-flight (TOF), the ion trap (IT), the quadrupole (Q), the Fourrier transform-ion cyclotron (FT-ICR), and the Orbitrap. The MS spectra is then used to identify proteins by a number of bioinformatic methods that rely on database searching for the species of interest. Various different areas of research in proteomics utilize mass spectrometry, including those looking at protein-protein interactions, post-translational modifications, structural proteomics, quantification or modifications of proteins, and those with protein identification [358].

One limitation and obstacle faced when performing proteomic studies was the loss of many large, basic, or hydrophobic proteins when utilizing 2-DE gel separation of proteins. This problem has been addressed in the years following by creation and utilization of various gel-free methods. Some of these novel gel-free techniques include COFRADIC (combined fractional diagonal chromatography) [359] and MudPIT (multidimensional protein identification technology) [360]. Combining several different proteomic techniques has proven to provide the greatest collection of protein data overall [361, 362].

Additional analysis of protein data can be completed through the use of a quantitative step. Quantitative analysis of a proteome is an efficient study comparing two or more conditions that may have different phenotypes or environments, also known as expression proteomics. With these quantitative analyses, one main focus is on identification of differentially expressed proteins between samples, highlighting those abundant in various disease states, such as those abundant in cancer tissues. The differentially expressed proteins can then be used as biomarkers in diagnosis and

determining the prognosis. These can be performed via gel or gel-free methodologies (using the mass spectrometer) as well.

Proteomics and Cancer

Cancer is a disease that is multifactorial in nature, and is the result of dysregulation of cellular functions and signaling used to normally control cell behavior, including proliferation and apoptosis. These changes in function are due to mutations and alterations at the genetic, genomic, and/or epigenetic level of the cell [362]. Proteins are the key players in directing the proliferation and advancement of cancers from survival of tumor cells themselves, growth of tumors in size, invasion of the vasculature and neighboring tissues, metastasis at distant locations in the body, and the response formed to the therapies administered [364]. Revealing and understanding these protein-fueled pathways and biological mechanisms will help researchers and clinicians understand cancer development, progression, metastasis, and ultimately ways by which cancer can be detected earlier and treated efficiently.

Proteomics is a highly efficient and accurate technique that is used for identifying changes in protein patterns in many diseases, including cancers [365]. Use of proteomics in different cancer studies allows the creation of tumor-specific profiles to be created on the protein level. This will then allow the detection of a multitude of novel tumor specific biomarkers and allow more accurate prognostic predictions to be made. Various studies have examined the different cancer cell proteomes in recent years. One example of this is with Yang et al in their efforts at gastric cancer biomarker identification [366]. Including other similar studies, comparative proteomes conducted on gastric cancer samples identified more than five protein differences (eg. SLC3A2, MET proto-oncogene receptor

tyrosine kinase, ephrin type A receptor 2, fibroblast growth factor receptor 2, integrin beta 4) for potential future use as biomarkers [367].

Aside from detection and prognosis, though, protein mapping will allow detailed insight into the tumor-specific pathogenesis for each type of cancer [368], as well as the potential to offer individual-based assessments. Until recently, proteomics use in canine cancer research has been greatly limited. Slow advancements in interest and progress has been initiated, though, as more and more evidence linking canine cancer to similarities in human cancer through their benefits to medicine as animal models has been shown. While limited in case numbers, proteomic profiling has been utilized in analyzing gene expression as well as differentially expressed proteins in some comparative studies on various tumor types (lymphoma, mammary tumors, prostatic tumors, mast cell tumors) [369, 370, 371, 372, 373, 374, 375]. Initial steps have been made in utilizing genomic and proteomic analyses in identification of key gene and protein changes that could later lead to use in diagnostics [376].

Platelet Proteomics

The proteome of a cell can be highly dynamic and offers unprecedented opportunities to unlock the mystery of biologic processes and to discover new diagnostics and therapeutics for human diseases. This is particularly true for platelets, which are readily available and have a limited repertoire of proteins, and where the proteome is largely independent of changes in gene expression [377].

The most important step of proteomic analysis, especially in the case of platelets, is preparation of the sample to be studied. Electrophoresis, liquid chromatography, and mass spectrometry are all extremely sensitive to sample contamination. In the study of

the platelet proteome, other blood cells are the main source of contamination concern. Platelet isolation methodologies must be sure not to alter the structure or function of the platelets themselves, as well as make sure to eliminate all or most other blood cells and their proteins from the samples to be analyzed in order to obtain a proteomic profile representative of the platelet itself. Some of these important factors to be considered include the use of drugs that alter platelet function (aspirin, non-steroidal anti-inflammatories), vessel trauma during venipuncture, rapid platelet isolation techniques, temperature of platelet sample storage, and length of platelet sample storage before use [378, 379, 380]. Efforts are made to prevent iatrogenic platelet activation and/or lysis. Platelet samples are ideally collected using citrate-containing anticoagulants and stored at room temperature or 37C. Some of the very first studies of the platelet proteome utilized 2-DE gel in 1979 [381]. The first platelet proteome analysis with mass spectrometry was performed in 2000 by Marcus et al using MALDI-TOF after 2-DE was performed [382]. Nearly 190 proteins were identified in their study.

As previously mentioned, the platelet, albeit small in size and without a nucleus, is complex in the packaging and volume contained within its intracellular granules (alpha, dense, lysosomes). One of the most important functions of platelets following their aggregation is the release of their granule contents. Previous studies consisting of proteomic analysis of platelets in rats bearing actively growing tumors revealed that levels of VEGF, bFGF and PGDF tended to be higher and the levels of the angiogenesis inhibitor endostatin tended to be lower. This proportion was persistent throughout the experiment (120 days) and reflects a pro-angiogenic tumor environment [383].

Platelet granules - their contents of particular known interest for various roles in hemostasis, wound healing, inflammation, and even cancer – have also been the subject of component-specific proteome studies by isolation of granules themselves. Special attention must be paid to fraction purity when studying particular cell organelles as contamination from neighboring organelles (eg. mitochondria) can greatly skew results. Steps including sucrose gradients followed by validation methods such as electron microscopy and Western blot assays allow isolation of platelet alpha granules for use in proteomic studies [259]. Dense granules present greater difficulty to study due to their low abundance (only 3-9 per platelet approx.) and high amounts of non-protein components. Studies have been successful, though, such as those by Hernandez-Ruiz et al, which identified roughly 40 dense granule proteins after validation using electron microscopy and Western blot [384]. Limited studies have been conducted on platelet lysosome proteomics, although their importance in diseases such as Hermansky-Pudlak syndrome suggest a valuable use for these studies in the future [224].

Proteomic studies of platelets extend beyond those conducted on the entire cell. Specific platelet compartments have been studied by the use of various platelet agonists. Among various platelet agonists, thrombin is one of the most important and most efficient agonists in triggering platelet activation and subsequent granule release [385]. Specific protease-activated receptors (PAR-1 and PAR-4) are required for this activation process to take place along with GPIba [286]. Agonists like thrombin have been utilized in various studies looking at activation cascades at the protein level. Maguire et al performed studies on the platelet phosphoproteome after thrombin activation, overall detecting 67 phosphorylated protein spots on 2-DE gel [387]. Another study used

thrombin to activate platelets to study the platelet releaseate proteome in atherosclerosis, identifying more than 300 proteins overall using MudPIT, with 51 of them having no previous evidence of being found in platelet secretion [388]. Platelet agonists can also be used to compare proteins involved in platelet signaling pathways. Garcia et al used thrombin receptor-activating peptide (TRAP), a molecule that promotes platelet secretion, to compare PAR-1 signaling proteomes between activated and resting platelets [389]. Other studies such as those by Senis et al have been used to explore the signaling pathway of alphaIIb-beta3, utilizing fibrinogen activation of platelets to identify 27 proteins involved [390].

Platelet membrane proteins, important factors involved in platelet activation, adhesion, and metabolism, are often underrepresented in many proteomic studies due to their low numbers, heterogenous structure, and hydrophobicity [391]. These properties make them difficult to use in many of the steps involved in proteomics, including electrophoresis, protein digestion, and MS analysis. Due to these limitations, modifications and novel techniques have been utilized to allow more accurate studies to be conducted on platelet membrane proteins. Moebius et al was the first study conducted on MS analysis of the platelet membrane. Membrane fractions were prepared with additional cleaning steps along with specific hydrophobic extraction steps within the protocol [392]. Protein separation was conducted using a 16-BAC/SDS PAGE technique which allows greater resolution of membrane protein separation as compared to traditionally used SDS methods. Overall, an increase in membrane protein products of six-fold was obtained. Other studies have utilized glycosylation trapping techniques to isolate and enrich membrane protein products, since most surface and secreted proteins

are glycosylated [393]. More recently, Lewandrowski et al identified over 1,200 membrane proteins by combining SDS-PAGE, MudPIT, and COFRADIC [394].

Platelet proteomics involving either the entire cell or their sub-cellular compartments is a valuable method to be used in studying a wide variety of diseases using descriptive, comparative, and quantitative analyses. Insight into the roles platelets play in the pathogenesis of various diseases, cellular signaling, and biologic mechanisms will greatly impact the development of future diagnostics as well as treatment modalities and modes of evaluative treatment responses in all patients, both human and animal.

Summary

Growing evidence has linked platelets to critical roles in cancer survival, pathogenesis, and metastasis, and has pioneered the development of novel treatment strategies and targets. The dog has proven to be an extremely valuable model for human diseases, most notably for various forms of naturally occurring cancers shared with their human counterparts. Through investigations into ways to efficiently and accurately evaluate canine platelets along with their roles in various cancers that affect dogs as well as humans, progress can be made in understanding complex biologic signaling pathways and chemical mechanisms as well as molecular targets identified for use in developing specific chemotherapeutic agents.

Platelets' roles in the development, survival, proliferation, and metastasis of cancer through growth factor secretion, angiogenesis, and escape from immune surveillance are primarily governed through the actions of proteins involved in all of those processes. The recent advances in mass spectrometry and the study of proteomics has allowed deepening exploration of the hundreds, and now thousands, of proteins

present in platelets and their functions throughout various platelet functions and cellular interactions, including those of disease states like cancer. The surface has just been scratched on determining details behind platelet roles in cancer, especially those in valuable animal models such as the canine, providing an open landscape for future discovery, mechanism understanding, and therapeutic development.

Table 2.1 List of angiogenic factors within platelet granules

Pro-angiogenic Factors	**Anti-angiogenic Factors**
Vascular endothelial growth factor (VEGF)	Endostatin
Platelet derived growth factor (PDGF)	Angiostatin
Insulin-like growth factor (IGF)	Platelet factor 4 (PF4)
Angiopoietin-1	Thrombospondin 1 (TSP1)
Matrix metalloproteinases (MMP) 1, 2, 9	Plasminogen activator inhibitor 1 (PAI1)
Interleukin 8 (IL8)	Tissue inhibitor metalloproteinases (TIMPS)
Epidermal growth factor (EGF)	CXCL4L1 (non-allelic variant of PF4)
Basic fibroblast growth factor (bFGF)	
Stromal cell derived factor (SDF) 1	

References

1. Karlsson EK, Lindblad-Toh K, Leader of the pack: gene mapping in dogs and other model organisms. Nat Rev Genet, 2008. **9**(9): p. 713–725.

2. American Cancer Society. Cancer facts and figures 2017. Available from: http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-048738.pdf. Accessed January 17, 2017.

3. Masters JR, HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer, 2002. **2**(4): p. 315–319.

4. Cekanova M, Rathore K, Animal models and therapeutic molecular targets of cancer: utility and limitations. Drug Design Development and Therapy, 2014. **8**: p. 1911-1922.

5. Wang H, Bang KW, Blanchette VS, et al, Phosphatidylserine exposure, microparticle formation and mitochondrial depolarization in Glanzmann thrombasthenia platelets. Thromb Haemost, 2014. **111**: p. 1184–1186.

6. Cheon DJ, Orsulic S, Mouse models of cancer. Annu Rev Pathol, 2011. **6**: p. 95-119.

7. Gondo Y, Fukumura R, Murata T, Makino S, Next-generation gene targeting in the mouse for functional genomics. BMB Rep, 2009. **42**(6): p. 315–323.

8. Ruggeri BA, Camp F, Miknyoczki S, Animal models of disease: pre-clinical animal models of cancer and their applications and utility in drug discovery. Biochem Pharmacol, 2014. **87**(1): p. 150-161.

9. Huynh AS, Abrahams DF, Torres MS, Baldwin MK, Gillies RJ, Morse DL, Development of an orthotopic human pancreatic cancer xenograft model using ultrasound guided injection of cells. PLoS One, 2011. **6**(5): e20330.

10. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. Eur J Cancer, 2004. **40**(6): p. 852-857.

11. Sharpless NE, Depinho RA, The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov, 2006. **5**(9): p. 741-754.

12. Cekanova M, Uddin MJ, Bartges JW, et al, Molecular imaging of cyclooxygenase-2 in canine transitional cell carcinomas in vitro and in vivo. Cancer Prev Res, 2013. **6**(5): p. 466-476.

13. Frese KK, Tuveson DA, Maximizing mouse cancer models. Nat Rev Cancer, 2007. **7**(9): p. 645-658.

14. Cekanova M, Lee SH, Donnell RL, et al, Nonsteroidal anti-inflammatory drug-activated gene-1 expression inhibits urethane-induced pulmonary tumorigenesis in transgenic mice. Cancer Prev Res, 2009. **2**(5): p. 450-458.

15. Mak IW, Evaniew N, Ghert M, Lost in translation: animal models and clinical trials in cancer treatment. Am J Transl Res, 2014. **6**(2): p. 114-118.

16. Knapp DW, Waters DJ, Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. Mol Med Today, 1997. **3**(1): p. 8-11.

17. Spugnini EP, Porello A, Citro G, Baldi A, COX-2 overexpression in canine tumors: potential therapeutic targets in oncology. Histol Histopathol, 2005. **20**(4): p. 1309-1312.

18. MacEwen EG. Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer and Metastasis Rev, 1990. **9**: p. 125–136.

19. Mazcko C. Comparative Oncology Program, 2012. Available from: https://ccrod.cancer.gov/confluence/display/CCRCOPWeb/Home. Accessed January 17, 2017.

20. Rowell JL, McCarthy DO, Alvarez CE, Dog models of naturally occurring cancer. Trends Mol Med, 2011. **17**(7): p. 380-388.

21. Paoloni M, Khanna C, Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer, 2008. **8**(2): p. 147–156.

22. Breen M, Modiano JF, Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans—man and his best friend share more than companionship. Chromosome Research, 2008. **16**(1): p. 145-154.

23. Thomas R, Smith KC, Ostrander EA, Galibert F, Breen M, Chromosome aberrations in canine multicentric lymphomas detected with comparative genomic hybridization and a panel of single locus probes. British Journal of Cancer, 2003. **89**(8): p. 1530-1537.

24. Rungsipipat A, Tateyama S, Yamaguchi R, Uchida K, Miyoshi N, Hayashi T, Immunohistochemical analysis of c-yes and c-erbB-2 oncogene products and p53 tumor suppressor protein in canine mammary tumors. Journal of Veterinary Medical Science, 1999. **61**(1): p. 27-32.

25. Porrello A, Cardelli P, Spugnini EP, Oncology of companion animals as a model for humans, an overview of tumor histotypes. J Exp Clin Cancer Res, 2006. **25**(1): p. 97-105.

26. Galac S, Korpershoek E, Pheochromocytomas and paragangliomas in humans and dogs. Veterinary and Comparative Oncology, 2017. [Epub ahead of print].

27. Park JS, Withers SS, Modiano JF et al, Canine cancer immunotherapy studies: linking mouse and humans. Journal for Immunotherapy of Cancer, 2016. **4**: p. 97.

28. Advances in Canine Cancer: Lymphoma. Canine Health Foundation. American Kennel Club. 2015. <http://www.akcchf.org/canine-health/top-health-concerns/canine-cancer/advances-in-canine-cancer-lymphoma.html>.

29. Marconato L, Gelain ME, Comazzi S, The dog as a possible animal model for human non-Hodgkin lymphoma: a review. Hematol Oncol, 2013. **31**(1): p. 1-9.

30. Hansen K, Khanna C, Spontaneous and genetically engineered animal models; use in preclinical cancer drug development. Eur J Cancer, 2004. **40**(6): p. 858-880.

31. Keller ET, MacEwen EG, Rosenthal RC, et al, Evaluation of prognostic factors and sequential combination chemotherapy for canine lymphoma. J Am Vet Med Assoc, 1992. **7**: p. 289-295.

32. Vail DM, Recent advances in chemotherapy for lymphoma of dogs and cats. Compend Cont Educ Pract Vet, 1993. **15**: p. 1031-1037.

33. MacEwen EG, Rosenthal RC, Fox LE, Loar AS, Kurzman ID, Evaluation of L-asparaginase: polyethylene glycol conjugate versus native L-asparaginase combined with chemotherapy. A randomized double-blind study in canine lymphoma. J Vet Intern Med, 1992. **6**(4): p. 230-234.

34. Reif JS, Bruns C, Lower KS, Cancer of the nasal cavity and paranasal sinuses and exposure to environmental tobacco smoke in pet dogs. Am J Epidemiol, 1998. **147**(5): p. 488-492.

35. Boria PA, Murry DJ, Bennett PF, et al, Evaluation of cisplatin combined with piroxicam for the treatment of oral malignant melanoma and oral squamous cell carcinoma in dogs. J Am Vet Med Assoc, 2004. **224**(3): p. 388-394.

36. Raju B, Ibrahim SO, Pathophysiology of oral cancer in experimental animal models: a review with focus on the role of sympathetic nerves. J Oral Pathol Med, 2011. **40**(1): p. 1-9.

37. Coggins CR, A minireview of chronic animal inhalation studies with mainstream cigarette smoke. Inhal Toxicol, 2002. **14**(10): p. 991-1002.

38. Brodey RS, Riser WH, Canine osteosarcoma – a clinicopathologic study of 194 cases. Clin Orthop Relat Res, 1969. **62**: 54-64.

39. O'Donoghue LE, Ptitsyn AA, Kamstock DA, Siebert J, Thomas RS, Duval DL, Expression profiling in canine osteosarcoma: identification of biomarkers and pathways associated with outcome. BMC Cancer, 2010. **10**: p. 506.

40. U.S. pet ownership & demographics sourcebook. AVMA, 2007. v.:]. Available from: http://www.avma.org/reference/marketstats/sourcebook.asp

41. Mirabello L, Troisi RJ, Savage SA, Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer, 2009. **115**(7): p. 1531–1543.

42. Ru G, Terracini B, Glickman LT, Host related risk factors for canine osteosarcoma. Vet J, 1998. **156**(1): p. 31-39.

43. Shearin AL, Ostrander EA, Leading the way: canine models of genomics and disease. Dis Model Mech, 2010. **3**(1-2): p. 27–34.

44. Brodey RS, The use of naturally occurring cancer in domestic animals for research into human cancer: general considerations and a review of canine skeletal osteosarcoma. Yale J Biol Med, 1979. **52**: p. 345–361.

45. Messerschmitt PJ, et al, Osteosarcoma. J Am Acad Orthop Surg, 2009. **17**(8): p. 515–527.

46. Bergman PJ, MacEwen EG, Kurzman ID et al, Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). J Vet Intern Med, 1996. **10**(2): p. 76–81.

47. Brodey RS, Abt DA, Results of surgical treatment in 65 dogs with osteosarcoma. J Am Vet Med Assoc, 1976. **168**(11): p. 1032–1035.

48. Jongeward SJ, Primary bone tumors. Vet Clin N Am Small Anim Pract, 1985. **15**: p. 609–641.

49. Pool RR, Tumors of bone and cartilage. In: Tumors in Domestic Animals, JE Moulton (Ed.), University of California press, Berkeley, 1990. 3rd Ed: p.157–230.

50. Selvarajah GT, Kirpensteijn J, Prognostic and predictive biomarkers of canine osteosarcoma. Vet J, 2010. **185**(1): p. 28-35.

51. Withrow S, Powers B, Straw R, et al, Comparative aspects of osteosarcoma. Dog versus man. Clin Orthop, 1991. **270**: p. 159-168.

52. Cooley DM, Beranek BC, Schlittler DL et al, Endogenous gonadal hormone exposure and bone sarcoma risk. Cancer Epidemiol Biomarkers Prev, 2002. **11**(11): p. 1434–1440.

53. Misdorp W, Hart AA, Some prognostic and epidemiologic factors in canine osteosarcoma. J Natl Cancer Inst, 1979. **62**: p. 537-545.

54. Knecht CD, Priester WA, Musculoskeletal tumors in dogs. J Am Vet Med Assoc, 1978. **172**: p. 72-74.

55. Withrow SJ, Wilkins RM, Cross talk from pets to people: translational osteosarcoma treatments. ILAR J, 2010. **51**(3): p. 208-213.

56. Gillette SM, Gillette EL, Powers BE, Withrow SJ, Radiation-induced osteosarcoma in dogs after external beam or intraoperative radiation therapy. Cancer Res, 1990. **50**: p. 54-57.

57. Bennett D, Campbell JR, Brown P, Osteosarcoma associated with healed fractures. J Small Anim Pract, 1979. **20**: p. 13-18.

58. Johnson AS, Couto CG, Weghorst CM, Mutation of the p53 tumor suppressor gene in spontaneously occurring osteosarcomas of the dog. Carcinogenesis, 1998. **19**: p. 213-217.

59. Mehl ML, Withrow SJ, Seguin B et al, Spontaneous regression of osteosarcoma in four dogs. J Am Vet Med Assoc, 2001. **219**(5): p. 614–617.

60. Thompson RR, Pool KG, Tumors of bones. In: Meuten DJ (ed) Tumors in domestic animals, Iowa State Press, Ames, 2002. 4th ed: p. 245-318.

61. Wrigley RH, Malignant versus nonmalignant bone disease. Vet Clin North Am Small Anim Pract, 2000. **30**: p. 315-347.

62. Kundu ZS, Classification, imaging, biopsy and staging of osteosarcoma. Indian J Orthop, 2014. **48**(3): p. 238-246.

63. Dernell WS, Straw RC, Withrow SJ, Tumors of the skeletal system. In: Small Animal Clinical Oncology. Withrow SJ, McEwen EG (eds.). Philadelphia, WB Saunders, 2001. p. 378-417.

64. Mueller F, Fuchs B, Kaser-Hotz B, Comparative biology of human and canine osteosarcoma. Anticancer Research, 2007. **27**: 155-164.

65. Withrow SJ, Vail DM, Page RL, Withrow and MacEven's small animal clinical oncology. Mosby Elsevier, 2013. 5th ed.

66. Calvacanti MG, Santos DT, Perrella A, Vannier MW, CT-based analysis of malignant tumor volume and localization. A preliminary study. Braz Oral Res, 2004. **18**(4): p. 338-344.

67. Trost ME, Kommers GD, Brown CC et al, Primary bone neoplasms in dogs: 90 cases. Pesq Vet Bras, 2012. **32**(12): p. 1329–1335.

68. Langenbach A, Anderson MA, Dambach DM et al, Extraskeletal osteosarcomas in dogs: a retrospective study of 169 cases (1986–1996). J Am Anim Hosp Assoc, 1998. **34**(2): p. 113–120.

69. Ogilvie GK, Straw RC, Jameson VJ et al, Evaluation of single-agent chemotherapy for treatment of clinically evident osteosarcoma metastasis in dogs: 45 cases (1987–1991). J Am Vet Med Assoc, 1993. **202**(2): p. 304–306.

70. Hillers KR, Dernell WS, Lafferty MH, Withrow SJ, Lana SE, Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986–2003). J Am Vet Med Assoc, 2005. **226**(8): p. 1364–1367.

71. Kirpensteijn J, Kik M, Teske E, Rutteman GR, TP53 gene mutations in canine osteosarcoma. Vet Surg, 2008. **37**(5): p. 454–460.

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

73. Thomas R, Wang HJ, Tsai PC, Langford CF, Fosmire SP, Jubala CM, Getzy DM, Cutter GR, Modiano JF, Breen M, Influence of genetic background on tumor karyotypes: evidence for breed- associated cytogenetic aberrations in canine appendicular osteosarcoma. Chromosome Res, 2009. **17**(3): p. 365–377.

74. Jin-no K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Endo H, Doi T, Mandai K and Ishitsuka H, Circulating platelet-derived endothelial cell growth factor increases in hepatocellular carcinoma patients. Cancer, 1998. **82**(7): p. 1260-1267.

75. Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, Jinno K, Kotani Y, Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer, 1998. **34**(13): p. 2041-2045.

76. Thamm DH, O'Brien MG, Vail DM, Serum vascular endothelial growth factor concentrations and postsurgical outcome in dogs with osteosarcoma. Vet Comp Oncol, 2008. **6**(2): p. 126-132.

77. Berg J, Weinstein MJ, Springfield DS, et al, Results of surgery and doxorubicin chemotherapy in dogs with osteosarcoma. J Am Vet Med Assoc, 1995. **206**(10): p. 1555–1560.

78. Shapiro W, Fossum TW, Kitchell BE, Couto CG, Theilen GH, Use of cisplatin for treatment of appendicular osteosarcoma in dogs. J Am Vet Med Assoc, 1988. **192**: 507-511.

79. Kraegel SA, Madewell BR, Simonson E, Gregory CR, Osteogenic sarcoma and cisplatin chemotherapy in dogs: 16 cases (1986–1989). J Am Vet Med Assoc, 1991. **199**(8): p. 1057–1059.

80. Thompson JP, Fugent MJ, Evaluation of survival times after limb amputation, with and without subsequent administration of cisplatin, for treatment of appendicular osteosarcoma in dogs: 30 cases (1979-1990). J Am Vet Med Assoc, 1992. **200**: p. 531-533.

81. Berg J, Weinstein MJ, Schelling SH, Rand WM, Treatment of dogs with osteosarcoma by administration of cisplatin after amputation or limb-sparing surgery: 22 cases (1987-1990). J Am Vet Med Assoc, 1992. **200**: p. 2005-2008.

82. Straw RC, Withrow SJ, Richter SL et al, Amputation and cisplatin for treatment of canine osteosarcoma. Vet Intern Med, 1991. **5**(4): p. 205–210.

83. Mialou V, Philip T, Kalifa C, Perol D, Gentet JC, Marec-Berard P, Pacquement H, Chastagner P, Defaschelles AS, Hartmann O, Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome--the French pediatric experience. Cancer, 2005. **104**(5): p. 1100–1109.

84. Mankin HJ, Hornicek FJ, Rosenberg AE, Harmon DC, Gebhardt MC, Survival data for 648 patients with osteosarcoma treated at one institution. Clin Orthop Relat Res, 2004. **429**: p. 286–291.

85. Moore AS, Dernell WS, Ogilvie GK et al, Doxorubicin and BAY 12–9566 for the treatment of osteosarcoma in dogs: a randomized, double-blind, placebo-controlled study. J Vet Intern Med, 2007. **21**(4): p. 783–790.

86. Lindsey BA, Markel JE, Kleinerman ES, Osteosarcoma overview. Rheumatol Ther, 2016.

87. Batschinski K, Dervisis NG, Kitchell BE, Evaluation of ifosfamide salvage therapy for metastatic canine osteosarcoma. Vet Comp Oncol, 2014. **12**(4): p. 249-257.

88. Mauldin GN, Matus RE, Withrow SJ et al, Canine osteosarcoma. Treatment by amputation versus amputation and adjuvant chemotherapy using doxorubicin and cisplatin. J Vet Intern Med, 1988. **2**(4): p. 177–180.

89. Chun R, Kurzman ID, Couto CG, Klausner J et al, Cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma: a pilot study. J Vet Intern Med, 2000. **14**(5): p. 495–498.

90. Kent MS, Strom A, London CA, Seguin B, Alternating carboplatin and doxorubicin as adjunctive chemotherapy to amputation or limb-sparing surgery in the treatment of appendicular osteosarcoma in dogs. J Vet Intern Med, 2004. **18**(4): p. 540–544.

91. Bacon NJ, Ehrhart NP, Dernell WS, Lafferty M, Withrow SJ, Use of alternating administration of carboplatin and doxorubicin in dogs with microscopic metastases after amputation for appendicular osteosarcoma: 50 cases (1999-2006). JAVMA, 2008. **232**(10): p. 1504-1510.

92. Selmic LE, Burton JH, Thamm DH et al, Comparison of carboplatin and doxorubicin-based chemotherapy protocols in 470 dogs after amputation for treatment of appendicular osteosarcoma. J Vet Intern Med, 2014. **28**(2): p. 554-563.

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

94. Skorupski KA, Uhl JM, Szivek A, Allstadt Frazier SD, Rebhun RB, Rodriguez CO Jr, Carboplatin versus alternating carboplatin and doxorubicin for the adjuvant treatment of canine appendicular osteosarcoma: a randomized, phase III trial. Vet Comp Oncol, 2016. **14**(1): p. 81-87.

95. Szewczyk M, Lechowski R, Zabielka K, What do we know about canine osteosarcoma treatment? Review. Vet Res Commun, 2015. **39**(1): 61-67.

96. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, Pritchard J, Malpas JS, Baker AR, Kirkpatrick JA, et al, The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med, 1986. **314**(25): 1600-1606.

97. Straw RC, Withrow SJ, Limb-sparing surgery versus amputation for dogs with bone tumors. Vet Clin North Am Small Anim Pract, 1996. **26**(1): p. 135–143.

98. Liptak JM, Dernell WS, Ehrhart N et al, Cortical allograft and endoprosthesis for limb-sparing surgery in dogs with distal radial osteosarcoma: a prospective clinical comparison of two different limb-sparing techniques. Vet Surg, 2006. **35**(6): p. 518–533.

99. MacDonald TL, Schiller TD, Limb-sparing surgery using tantalum metal endoprosthesis in a dog with osteosarcoma of the distal radius. Can Vet J, 2010. **51**(5): p. 497–500.

100. McEntee MC, Radiation therapy in the management of bone tumors. Vet Clin North Am Small Anim Pract, 1997. **27**: p. 131-138.

101. Lester P, Gaynor JS, Management of cancer pain. Vet Clin North Am Small Anim Pract, 2000. **30**: p. 951-966.

102. Spodnick GJ, Berg J, Rand WM et al, Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978–1988). J Am Vet Med Assoc, 1992. **200**(7): p. 995–999.

103. Weigelt B, Peterse JL, van't Veer LJ, Breast cancer metastasis: markers and models. Nat Rev Cancer, 2005. **5**: p. 591-602.

104. Morello E, Martano M, Buracco P, Biology, diagnosis and treatment of canine appendicular osteosarcoma: similarities and differences with human osteosarcoma. Vet J, 2011. **189**(3): p. 268–277.

105. LaRue SM, Withrow SJ, Powers BE, Wrigley RH, Gillette EL, Schwarz PD, Straw RC, Richter SL, Limb-sparing treatment for osteosarcoma in dogs. J Am Vet Med Assoc, 1989. **195**(12): p. 1734–1744.

106. MacEwen EG, Kurzman ID, Rosenthal RC, Smith BW, Manley PA, Roush JK, Howard PE, Therapy for osteosarcoma in dogs with intravenous injection of liposome encapsulated muramyl tripeptide. J Natl Cancer Inst, 1989. **81**: p. 935-938.

107. Kleinerman ES, Jia SF, Griffin J, Seibel NL, Benjamin RS, Jaffe N, Phase II study of liposomal muramyl tripeptide in osteosarcoma: the cytokine cascade and monocyte activation following administration. J Clin Oncol, 1992. **10**: p. 1310-1316.

108. Asano T, Kleinerman ES, Liposome-encapsulated MTPPE: a novel biologic agent for cancer therapy. J Immunother, 1993. **14**: p. 286-292.

109. Kleinerman ES, Gano JB, Johnston DA, Benjamin RS, Jaffe N, Efficacy of liposomal muramyl tripeptide (CGP19835A) in the treatment of relapsed osteosarcoma. Am J Clin Oncol, 1995. **18**: p. 93-99.

110. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jurgens H, Winkler K, Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol, 2002. **20**: p. 776-790.

111. Harris MB, Gieser P, Goorin AM, Ayala A, Shochat SJ, Ferguson WS, Holbrook T, Link MP, Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group Study. J Clin Oncol, 1998. **16**: p. 3641-3648.

112. Hillers KR, Dernell WS, Lafferty MH, Withrow SJ, Lana SE, Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986-2003). J Am Vet Med Assoc, 2005. **226**: p. 1364-1367.

113. Ogilvie GK, Straw RC, Jameson VJ, Walters LM, Lafferty MH, Powers BE, Withrow SJ, Evaluation of single-agent chemotherapy for treatment of clinically evident osteosarcoma metastases in dogs: 45 cases (1987-1991). J Am Vet Med Assoc, 1993. **202**: p. 304-306.

114. O'Brien MG, Straw RC, Withrow SJ et al, Resection of pulmonary metastases in canine osteosarcoma: 36 cases (1983–1992). Vet Surg, 1993. **22**(2): p. 105-109.

115. Briccoli A, Rocca M, Salone M, Bacci G, Ferrari S, Balladelli A, Mercuri M, Resection of recurrent pulmonary metastases in patients with osteosarcoma. Cancer, 2005. **104**(8): p. 1721–1725.

116. Ehrhart N, Dernell WS, Hoffmann WE, Weigel RM, Powers BE, Withrow SJ, Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990-1996). J Am Vet Med Assoc, 1998. **213**: p. 1002-1006.

117. Garzotto CK, Berg J, Hoffmann WE, Rand WM, Prognostic significance of serum alkaline phosphatase activity in canine appendicular osteosarcoma. J Vet Intern Med, 2000. **14**: p. 587-592.

118. Kirpensteijn J, Teske E, Kik M et al, Lobaplatin as an adjuvant chemotherapy to surgery in canine appendicular osteosarcoma: a phase II evaluation. Anticancer Res, 2002. **22**(5): p. 2765–2770.

119. Coomber BL, Denton J, Sylvestre A, Kruth S, Blood vessel density in canine osteosarcoma. Can J Vet Res, 1998. **62**: p. 199-204.

120. Liu PPL, Leung KS, Kumta SM, Lee KM, Fung KP, Bone-specific alkaline phosphatase in plasma as tumour marker for osteosarcoma. Oncology, 1996. **53**(4): p. 275–280.

121. Limmahakhun S, Pothacharoen P, Theera-Umpon N, Arpornchayanon O, Leerapun T, Luevitoonvechkij S, Pruksakorn D, Relationships between serum biomarker levels and clinical presentation of human osteosarcomas. Asian Pac J Cancer Prev, 2011. **12**(7): p. 1717–1722.

122. Hammer AS, Weeren FR, Weisbrode SE, Padgett SL, Prognostic factors in dogs with osteosarcomas of the flat or irregular bones. J Am Anim Hosp Assoc, 1995. **31**: p. 321-326.

123. Kirpensteijn J, Kik M, Rutteman GR, Teske E, Prognostic significance of a new histologic grading system for canine osteosarcoma. Vet Pathol, 2002. **39**: p. 240-246.

124. Powers BE, Withrow SJ, Thrall DE, Straw RC, LaRue SM, Page RL, Gillette EL, Percent tumor necrosis as a predictor of treatment response in canine osteosarcoma. Cancer, 1991. **67**: p. 126-134.

125. Taylor WF, Ivins JC, Pritchard DJ, Dahlin DC, Gilchrist GS, Edmonson JH, Trends and variability in survival among patients with osteosarcoma: a 7-year update. Mayo Clin Proc, 1985. **60**: p. 91-104.

126. Taylor WF, Ivins JC, Dahlin DC, Edmonson JH, Pritchard DJ, Trends and variability in survival from osteosarcoma. Mayo Clin Proc, 1978. **53**: p. 695-700.

127. Bentzen SM, Poulsen HS, Kaae S, Jensen OM, Johansen H, Mouridsen HT, Daugaard S, Arnoldi C, Prognostic factors in osteosarcomas. A regression analysis. Cancer, 1988. **62**: p. 194-202.

128. Price CH, Zhuber K, Salzer-Kuntschik M, Salzer M, Willert HG, Immenkamp M, Groh P, Matejovsky Z, Keyl W, Osteosarcoma in children. A study of 125 cases. J Bone Joint Surg Br, 1975. **57**: p. 341-345.

129. French Bone Tumor Study Group. Age and dose of chemotherapy as major prognostic factors in a trial of adjuvant therapy of osteosarcoma combining two alternating drug combinations and early prophylactic lung irradiation. Cancer, 1988. **61**: p. 1304-1311.

130. Kuntz CA, Asselin TL, Dernell WS, Powers BE, Straw RC, Withrow SJ, Limb salvage surgery for osteosarcoma of the proximal humerus: outcome in 17 dogs. Vet Surg, 1998. **27**: p. 417-422.

131. Forrest LJ, Dodge RK, Page RL, Heidner GL, McEntee MC, Novotney CA, Thrall DE, Relationship between quantitative tumor scintigraphy and time to metastasis in dogs with osteosarcoma. J Nucl Med, 1992. **33**: p. 1542-1547.

132. Armbrust LJ, Biller DS, Bamford A, Chun R, Garrett LD, Sanderson MW, Comparison of three-view thoracic radiography and computed tomography for detection of pulmonary nodules in dogs with neoplasia. J Am Vet Med Assoc, 2012. **240**(9): p. 1088-1094.

133. MacEwen EG, Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Rev, 1990. **9**(2): p. 125-136.

134. Daleck CR, Franceschini PH, Alessi AC, Santana AE, Martins MIM, Canine mammary neoplasia: clinical and surgical evolution. Cienc Rural, 1998. **28**(1): p. 95-100.

135. Oliveira LO, Oliveira RT, Loretti A, Rodrigues R, Driemeier D, Epidemiological aspects of canine mammary tumor. Act Sci Vet, 2003. **31**(2): p. 105-110.

136. Fonseca CS, Daleck CR, Canine mammary tumors: hormonal influence and effects of ovariohysterectomy as an adjuvant therapy. Ciência Rural, 2000. **30**(4): p. 731-735.

137. Gilbertson SR, Kurzman ID, Zachrau RE, Hurvitz AI, Black MM, Canine mammary epithelial neoplasms: biological implications of morphological characteristics assessed in 232 dogs. Vet Pathology, 1983. **20**: p. 127-142.

138. Sorenmo K, An update on canine mammary gland tumors. ACVIM Forum 16 1998. p. 387-388, Proceedings.

139. Owen LN. A comparative study of canine and human breast cancer. Invest Cell Pathol. 1979; **2**(4): p. 257-275.

140. Rutteman GR, Hormones and mammary tumor disease in the female dog: an update. In Vivo, 1990. **4**(1): p. 433-440.

141. Chrisp CE, Spangler WL, The malignant canine tumour as a model for the study of human breast cancer. In: Shifrine M, Wilson FD (eds). The Canine as a biomedical research model: Immunological, hematological and oncological aspects. Washington DC: National Technical Information Center, Department of Energy; 1980: p. 331-349.

142. Owen LN, Morgan DR, Bostock DE, Flemans RJ, Tissue culture and transplantation studies on canine mammary carcinoma. Eur J Cancer, 1977. **13**(12): p. 1445-1449.

143. Strandberg JD, Goodman DG, Animal model of human disease: Canine mammary neoplasia. Am J Pathol, 1974. **75**(1): p. 225-228.

144. Cote, E. Mammary Gland Neoplasia, Dog. Clinical Veterinary Advisor, Dogs and Cats. 2015. 3r Ed: p. 635-637.

145. Misdorp W, Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins. Vet Q, 1988. **10**(1): p. 26-33.

146. Schneider R, Dorn CR, Taylor DON, Factors influencing canine mammary cancer development and postsurgical survival. Journal of the National Cancer Institute, 1969. **43**: p. 1249-1261.

147. Nieto A, Peña L, Pérez-Alenza MD, Sànchez MA, Flores JM, Castanõ M, Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. Vet Pathol, 2000. **37**(3): p. 239-247.

148. Millanta F, Calandrella M, Bari G, Niccolini M, Vannozzi I, Poli A, Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. Res Vet Sci, 2005. **79**(3): p. 225-232.

149. Tan M, Yu D. Molecular Mechanisms of ErbB2-Mediated Breast Cancer Chemoresistance. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013.

150. Martin de las Mulas J, Ordás J, Millán Y, Fernández-Soria V, Ramón y Cajal S, Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study. Breast Cancer Res Treat, 2003. **80**(3): p. 363-367.

151. Ahern TE, Bird RC, Bird AE, Wolfe LG, Expression of the oncogene c-erbB-2 in canine mammary cancers and tumor-derived cell lines. Am J Vet Res, 1996. **57**(5): p. 693-696.

152. Sonnenschein EG, Glickman LT, Goldschmidt MH, McKee LJ, Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study. Am J Epidemiol, 1991. **133**(7): p. 694–703.

153. Root-Kustritz M, Determining the best age at which to spay or neuter. American Kennel Club Canine Health Foundation, 2008. Available from: http://www.akcchf.org/canine-health/your-dogs-health/determining-the-best-age-at.html. Accessed January 18, 2017.

154. Schneider R, Dorn CR, Taylor DO, Factors influencing canine mammary cancer development and postsurgical survival. J Natl Cancer Inst, 1969. **43**(6): p. 1249-1261.

155. MacEwen EG, Patnaik AK, Harvey HJ, Panko WB, Estrogen receptor in canine mammary tumors. Cancer Res, 1982. **42**(6): p. 2255-2259.

156. Martin PM, Cortard M, Mialot JP, André F, Raynaud JP, Animal models for hormone-dependent human breast cancer. Relationship between steroid receptor profiles in canine and feline mammary tumors and survival rate. Cancer Chemother Pharmacol, 1984. **12**(1): p. 13-17.

157. Misdorp W, Tumors of the mammary gland. In: Meuten DJ. (ed.), Tumors in domestic animals. Ames, USA: Iowa State Press, 2002. 4th Ed: p. 575-606.

158. Goldschmidt M, Peña L, Rasotto R, Classification and grading of canine mammary tumors. Vet Pathol, 2011. **48**: p. 117–131.

159. Hellmen E, Moller M, Blankenstein R, Andersson L, Westermark B, Expression of different phenotypes in cell lines from canine mammary spindle-cell tumours and osteosarcomas indicating a pluripotent mammary stem cell origin. Breast Cancer Res Treat, 2000. **61**: p. 197-210.

160. Peña L, Pérez-Alenza MD, Rodriguez-Bertos A, Nieto A, Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. Breast Cancer Res Treat, 2003. **78**(2): p. 141-148.

161. Clemente M, Sánchez-Archidona AR, Sardon D, Díez L, Martín-Ruiz A, Caceres S, et al, Different role of COX-2 and angiogenesis in canine inflammatory and non-inflammatory mammary cancer. Vet J, 2013. **197**(2): p. 427-432.

162. Pérez-Alenza MD, Tabanera E, Peña L, Inflammatory mammary carcinoma in dogs: 33 cases (1995-1999). J Am Vet Med Assoc, 2001. **219**: p. 1110-1114.

163. Giordano SH, Hortobagyi GN, Inflammatory breast cancer: clinical progress and the main problems that must be addressed. Breast Cancer Res, 2003. **5**(6): p. 284-288.

164. Gama A, Alves A, Gartner F, Schmitt F, p63: a novel myoepithelial cell marker in canine mammary tissues. Vet Pathol, 2003. **40**(4): p. 412-420.

165. Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, et al, Vimentin contributes to human mammary epithelial cell migration. J Cell Sci, 1999. **112**(Pt 24): p. 4615-4625.

166. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M, Vimentin and epithelial-mesenchymal transition in human breast cancer – observations in vitro and in vivo. Cells Tissues Organs, 2007. **185**: p. 191-203.

167. Gilles C, Newgreen D, Sato H, Thompson EW, Matric metalloproteinases and epithelial-to-mesenchymal transition: implications for carcinoma metastasis. In: Savagner P (ed.). Rise and fall of epithelial phenotype. Georgetown: Landes Bioscience, 2004: p. 297-315.

168. Half E, Tang XM, Gwyn K, Sahin A, Wathen K, Sinicrope FA, Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. Cancer Res, 2002. **62**(6): p. 1676–1681.

169. Ristimake A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, et al, Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res, 2002. **62**(3): p. 632-635.

170. Heller DA, Clifford CA, Goldschmidt MH, Holt DE, Shofer FS, Smith A, et al, Cyclooxygenase-2 expression is associated with histologic tumor type in canine mammary carcinoma. Vet Pathol, 2005. **42**(6): p. 776-780.

171. Arun B, Goss P, The role of COX-2 inhibition in breast cancer treatment and prevention. Sem Oncol, 2004. **31**: p. 22-29.

172. Brunelle M, Sartin EA, Wolfe LG, Sirois J, Doré M, Cyclooxygenase-2 expression in normal and neoplastic canine mammary cell lines. Vet Pathol, 2006. **43**(5): p. 656-666.

173. Hahn K, Richardson R, Knapp D, Canine malignant mammary neoplasia: biologic behavior, diagnosis, and treatment alternatives. J Anim Hosp Assoc, 1992. **28**: p. 251.

174. Ogilvie G, Phase I1 evaluation of doxorubicin for treatment of various canine neoplasms. J Am Vet Med Assoc, 1989. **195**: p. 1580.

175. Ellis IO, Schmitt SJ, Sastre-Garau X, et al, Invasive breast carcinoma. In: Tavassoli FA, Deville P. (eds.), World health organization classification of tumors: Pathology and genetics. Tumors of the breast and female genital organs. France, Lyon: IARC Press 2003. p. 13-59.

176. Williams L, Predictors of tumor response. In Proceedings: North American Veterinary Conference. Orlando, USA 2003. p. 679-681.

177. Allen S, Mahaffey E, Canine mammary neoplasia: prognostic indicators and responses to surgical therapy. J Am Anim Hosp Assoc, 1990. **31**(4): p. 184.

178. Kurzman I, Gilbertson S, Prognostic factors in canine mammary tumors. Semin Vet Med Surg, 1986. **1**: p. 25-32.

179. Pereira FM, Ferreira E, Leitao DRA, et al, Double-staining immunohistochemistry of canine mammary neoplasms. Arq Bras Med Vet Zootec, 2006. **58**(4): p. 681-684.

180. Garraud O, Cognasse F, Are Platelets Cells? And If Yes, Are They Immune Cells? Front Immunol, 2015. **6**: p. 70.

181. Hartwig JH, Chapter 8: the platelet cytoskeleton. In: Michelson AD, ed. Platelets, Amsterdam: Academic Press/Elsevier, 2013. 3rd Ed: p. 145–168.

182. Nurden AT, Platelets, inflammation and tissue regeneration. Thromb Haemost, 2011. **105**(Suppl 1): S13-33.

183. Machlus KR, Thon JN, Italiano Jr JE, Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. Br J Haematol, 2014. **165**: p. 227–236.

184. Italiano JE Jr, Shivdasani RA, Megakaryocytes and beyond: the birth of platelets. J Thromb Haemost, 2003. **1**: p. 1174–1182.

185. Malara A, Balduini A, Blood platelet production and morphology. Thromb Res, 2012. **129**: p. 241–244.

186. Junt T, Schulze H, Chen Z, et al, Dynamic visualization of thrombopoiesis within bone marrow. Science, 2007. **317**: p. 1767–1770.

187. Wang Y, Hayes V, Jarocha D, et al, Comparative analysis of human ex vivo-generated platelets vs megakaryocyte-generated platelets in mice: a cautionary tale. Blood, 2015. **125**: p. 3627–3636.

188. Fuentes R, Wang Y, Hirsch J, et al, Infusion of mature megakaryocytes into mice yields functional platelets. J Clin Invest, 2010. **120**: p. 3917–3922.

189. Hou Y, Carrim N, Wang Y, et al, Platelets in hemostasis and thrombosis: novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis. J Biomed Res, 2015. **29**: p. 437–444.

190. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, et al, Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med, 2006. **203**: p. 2433–2440.

191. Zimmerman GA, Weyrich AS, Signal-dependent protein synthesis by activated platelets: new pathways to altered phenotype and function. Arterioscler Thromb Vasc Biol, 2008. **28**: p. s17–24.

192. Rowley JW, Weyrich AS, Coordinate expression of transcripts and proteins in platelets. Blood, 2013. **121**: p. 5255–5256.

193. Shi DS, Smith MC, Campbell RA, Zimmerman PW, Franks ZB, Kraemer BF, et al, Proteasome function is required for platelet production. J Clin Invest, 2014. **124**: p. 3757–3766.

194. Li Y, Miao L, Xiao Y, et al, Elevated platelets enhance cancer cell migration, promote hematogenous metastasis and associate with a poor prognosis in advanced non-small cell lung cancer cases. Asian Pac J Cancer Prev, 2014. **15**(1): p. 139-143.

195. Kaushansky K, Thrombopoiesis. Semin Hematol, 2015. **52**: p. 4–11.

196. Kuter DJ, Milestones in understanding platelet production: a historical overview. Br J Haematol, 2014. **165**: p. 248–258.

197. Nishiura S, Nagasaki M, Kunishima S, Sawaguchi, Sakata A, et al, IL-1a induces thrombopoiesis through megakaryocyte rupture in response to acute platelet needs. J Cell Biol, 2015. **209**(3): p. 453-466.

198. Sungaran R, Markovic B, Chong BH, Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. Blood, 1997. **89**(1): p. 101-107.

199. McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K, Murine thrombopoietin mRNA levels are modulated by platelet count. Blood, 1995. **86**(10): p. 3668-3675.

200. Qian S, Fu F, Li W, Chen Q, de Sauvage FJ, Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. Blood, 1998. **92**(6): p. 189-191.

201. Dame C, Wolber EM, Freitag P, Hofmann D, Bartmann P, Fandrey J, Thrombopoietin gene expression in the developing human central nervous system. Brain Res Dev Brain Res, 2003. **143**(2): p. 217-223.

202. Kazama I, Endo Y, Toyama H, et al, Compensatory thrombopoietin production from the liver and bone marrow stimulates thrombopoiesis of living rat megakaryocytes in chronic renal failure. Nephron Extra, 2011. **1**(1): p. 147-156.

203. Figueiredo AS, Bulla SC, Lunsford KV, Bulla C, Expression of the Thrombopoietin Gene in Tissues from Healthy Dogs. J Comp Path, 2013. **149**: p. 298-302.

204. Stoffel R, Wiestner A, Skoda RC, Thrombopoietin in thrombocytopenic mice: evidence against regulation at the mRNA level and for a direct regulatory role of platelets. Blood, 1996. **87**(2): p. 567-573.

205. Denis MM, Tolley ND, Bunting M, et al, Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell, 2005. **122**: p. 379–391.

206. Panes O, Matus V, Saez CG, et al, Human platelets synthesize and express functional tissue factor. Blood, 2007. **109**: p. 5242–5250.

207. Brogren H, Karlsson L, Andersson M, et al, Platelets synthesize large amounts of active plasminogen activator inhibitor 1. Blood, 2004. **104**: p. 3943–3948.

208. Evangelista V, Manarini S, Di Santo A, et al, De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin. Circ Res, 2006. **98**: p. 593–595.

209. Yang H, Lang S, Zhai Z, et al, Fibrinogen is required for maintenance of platelet intracellular and cell-surface P-selectin expression. Blood, 2009. **114**: p. 425–436.

210. Xu XR, Zhang D, Oswald BE, Carrim N, et al, Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci, 2016. **53**(6): p. 409-430.

211. Ma L, Perini R, McKnight W, Dicay M, Klein A, Hollenberg MD, Wallace JL, Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. Proc Natl Acad Sci U S A, 2005. **102**(1): p. 216-120.

212. Italiano JE Jr, Richardson JL, Patel-Hett, Battinelli, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL, Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet granules and differentially released. Blood, 2008. **111**(3): p. 1227-1233.

213. Klement GL, Yip TT, Cassiola F, Kikuchi L, Cervi D, Podust V, Italiano JE, Wheatley E, Abou-Slaybi A, Bender E, Almog N, Kieran MW, Folkman J, Platelets actively sequester angiogenesis regulators. Blood, 2009. **113**(12): p. 2835-2842.

214. Chatterjee M, Huang Z, Zhang W, et al, Distinct platelet packaging, release, and surface expression of proangiogenic and antiangiogenic factors on different platelet stimuli. Blood, 2011. **117**: p. 3907–3911.

215. White GC, Rompietti R, Platelet secretion: indiscriminately spewed forth or highly orchestrated? J Thromb Haemost, 2007. **5**: p. 2006–2008.

216. Heijnen H, van der Sluijs P, Platelet secretory behaviour: as diverse as the granules or not? J Thromb Haemost, 2015. **13**: p. 2141–2151.

217. Von Hundelshausen P, Petersen F, Brandt E, Platelet-derived chemokines in vascular biology. Thromb Haemost, 2007. **97**: p. 704–713.

218. Klinger MH, Jelkmann W, Role of blood platelets in infection and inflammation. J Interferon Cytokine Res, 2002. **22**: p. 913–922.

219. Kraemer BF, Campbell RA, Schwertz H, et al, Novel antibacterial activities of b-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. PLoS Pathog, 2011. **7**: e1002355.

220. Li C, Li J, Li Y, et al, Crosstalk between platelets and the immune system: old systems with new discoveries. Adv Hematol, 2012. **2012**: e384685.

221. Gleissner CA, von Hundelshausen P, Ley K, Platelet chemokines in vascular disease. Arterioscler Thromb Vasc Biol, 2008. **28**: p. 1920–1927.

222. Koenen RR, von Hundelshausen P, Nesmelova IV, et al, Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. Nat Med, 2009. **15**: p. 97–103.

223. Stinchcombe J, Bossi G, Griffiths GM, Linking albinism and immunity: The secrets of secretory lysosomes. Science, 2004. **305**: p. 55–59.

224. Wei ML, Hermansky–Pudlak syndrome: A disease of protein trafficking and organelle function. Pigment Cell Res, 2006. **19**: p. 19–42.

225. Choi W, Karim ZA, Whiteheart SW, Protein expression in platelets from six species that differ in their open canalicular system. Platelets, 2010. **21**: p. 167–175.

226. Mountford JK, Petitjean C, Putra HW, et al, The class II PI 3- kinase, PI3KC2a, links platelet internal membrane structure to shear-dependent adhesive function. Nat Commun, 2015. **6**: p. 6535.

227. Kondkar AA, Bray MS, Leal SM,et al, VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost, 2010. **8**(2): p. 369-378.

228. Gnatenko DV, Cupit LD, Huang EC, Dhundale A, Perrotta PL, Bahou WF, Platelets express steroidogenic 17 beta-hydroxysteroid dehydrogenases. Distinct profiles predict the essential thrombocythemic phenotype. Thromb Haemost, 2005. **94**(2): p. 412-421.

229. Nagalla S, Bray PF, Platelet RNA chips dip into thrombocytosis. Blood, 2010. **115**(1): p. 2-3.

230. Gnatenko DV, Zhu W, Xu X, et al, Class prediction models of thrombocytosis using genetic biomarkers. Blood, 2010. **115**(1): p. 7-14.

231. Raghavachari N, Xu X, Harris A, et al, Amplified expression profiling of platelet transcriptome reveals changes in arginine metabolic pathways in patients with sickle cell disease, Circulation, 2007. **115**(12): p. 1551-1562.

232. Lood C, Amisten S, Gullstrand B, et al, Platelet transcriptional profile and protein expression in patients with systemic lupus erythematosus: up-regulation of the type I interferon system is strongly associated with vascular disease. Blood, 2010. **116**(11): p. 1951-1957.

233. Freedman JE, Larson MG, Tanriverdi K, et al, Relation of platelet and leukocyte inflammatory transcripts to body mass index in the Framingham heart study. Circulation, 2010. **122**(2): p. 119-129.

234. Goodall AH, Burns P, Salles I, et al, Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. Blood, 2010. **116**(22): p. 4646-4656.

235. Rowley JW, Oler A, Tolley ND, et al, Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood, 2011. **118**(14): e101-111.

236. Ruggeri ZM, Mendolicchio GL, Adhesion mechanisms in platelet function. Circ Res, 2007. 100(12): p. 1673-1685.

237. Davi G, Patrono C, Platelet activation and atherothrombosis. N Eng J Med, 2007. **357**: p. 2482-2494.

238. Clemetson KJ, Clemetson JM, Chapter 9: platelet Receptors. In: Michelson AD, ed. Platelets. 3rd ed. Amsterdam: Academic Press/ Elsevier, 2013: p. 169–94.

239. Ni H, Freedman J, Platelets in hemostasis and thrombosis: role of integrins and their ligands. Transfus Apher Sci, 2003. **28**: p. 257–264.

240. Bergmeier W, Hynes RO, Extracellular matrix proteins in hemostasis and thrombosis. Cold Spring Harb Perspect Biol, 2012. **4**: a005132.

241. Patel S, Huang YW, Reheman A, et al, The cell motility modulator Slit2 is a potent inhibitor of platelet function. Circulation, 2012. **126**: p. 1385–1395.

242. Vendrig AC, Reheman A, Siraj MA, et al, Glucagon-like peptide 1 receptor activation attenuates platelet aggregation and thrombosis. Diabetes, 2016. **65**: p. 1714–1723.

243. Crockett J, Newman DK, Newman PJ, PECAM-1 functions as a negative regulator of laminin-induced platelet activation. J Thromb Haemost, 2010. **8**: p. 1584–1593.

244. Golebiewska EM, Poole AW, Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev, 2015. **29**(3): p. 153-162.

245. Wang C, Tang Z, Zhao Y, Yao R, Li L, Sun W, Three-dimensional in vitro cancer models: a short review. Biofabrication, 2014. **6**(2): e022001.

246. Roberts HR, Hoffman M, Monroe DM, A cell-based model of thrombin generation. Semin Thromb Hemost, 2006. **32**: p. 32–38.

247. Monroe DM, Hoffman M, Roberts HR, Platelets and thrombin generation. Arterioscler Thromb Vasc Biol, 2002. **2**(9): p. 1381-1389.

248. Ruggeri ZM, Jackson SP. Chapter 20: platelet thrombus formation in flowing blood. In: Michelson AD, ed. Platelets. 3rd ed. Amsterdam: Academic Press/Elsevier, 2013. p. 399–424.

249. Jackson SP, The growing complexity of platelet aggregation. Blood, 2007. **109**: p. 5087–5095.

250. Neiswandt B, Watson SP, Platelet-collagen interaction: is GPVI the central receptor? Blood, 2003. **102**(2): p. 449-461.

251. Varga-Szabo D, Pleines I, Nieswandt B, Cell adhesion mechanisms in platelets. Arterioscler Thromb Vasc Biol, 2008. **28**: p. 403–412.

252. Ni H, Freedman J, Platelets in hemostasis and thrombosis: role of integrins and their ligands. Transfus Apher Sci, 2003. **28**: p. 257–264.

253. Mackman N, Triggers, targets and treatments for thrombosis. Nature, 2008. **451**: p. 914–918.

254. Brass LF, Newman DK, Wannemacher KM, Stalker TJ, Chapter 19: signal transduction during platelet plug formation. In: Michelson AD, ed. Platelets. 3rd ed. Amsterdam: Academic Press/Elsevier, 2013. p. 367–398.

255. Savage B, Cattaneo M, Ruggeri ZM, Mechanisms of platelet aggregation. Curr Opin Hematol, 2001. **8**: p. 270–276.

256. Bouchard BA, Silveira JR, Tracy PB, Chapter 21: interactions between platelets and the coagulation system. In: Michelson AD, ed. Platelets. 3rd ed. Amsterdam: Academic Press/Elsevier, 2013. p. 425–452.

257. Gui T, Reheman A, Funkhouser WK, et al, In vivo response to vascular injury in the absence of factor IX: examination in factor IX knockout mice. Thromb Res, 2007. **121**: p. 225–234.

258. Gui T, Reheman A, Ni H, et al, Abnormal hemostasis in a knockin mouse carrying a variant of factor IX with impaired binding to collagen type IV. J Thromb Haemost, 2009. **7**: p. 1843–1851.

259. Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA, Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost, 2007. **5**: p. 1945–1955.

260. Mu¨ller F, Mutch NJ, Schenk WA, et al, Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell, 2009. **139**: p. 1143–1156.

261. Burger PC, Wagner DD, Platelet P-selectin facilitates atherosclerotic lesion development. Blood, 2003. **101**: p. 2661-2666.

262. Merten M, Thiagarajan P, P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation, 2000. **102**: p. 1931-1936.

263. Dole VS, Bergmeier W, Mitchell HA, Eichenberger SC, Wagner DD, Activated platelets induce Weibel-Palade-body secretion and leukocyte rolling in vivo: role of P-selectin. Blood, 2005. **106**: p. 2334-2339.

264. Cambien B, Wagner DD, A new role in hemostasis for the adhesion receptor P-selectin. Trends Mol Med, 2004. **10**: p. 179-186.

265. Walsh TG, Metharom P, Berndt MC, The functional role of platelets in the regulation of angiogenesis. Platelets, 2015. **26**: p. 199–211.

266. Yougbare I, Zdravic D, Ni H, Angiogenesis and bleeding disorders in FNAIT. Oncotarget, 2015. **6**: p. 15724–15725.

267. Carmeliet P, Jain RK, Angiogenesis in cancer and other diseases. Nature, 2000. **407**: p. 249–257.

268. Carmeliet P, Angiogenesis in health and disease. Nat Med, 2003.**9**: p. 653–660.

269. Gawaz M, Langer H, May AE, Platelets in inflammation and atherogenesis. J Clin Invest, 2005. **115**: p. 3378–3384.

270. Milovanovic M, Nilsson E, Jaremo P, Relationships between platelets and inflammatory markers in rheumatoid arthritis. Clin Chim Acta, 2004. **343**: p. 237–240.

271. Yamashiro K, Tsujikawa A, Ishida S, Usui T, Kaji Y, Honda Y, Ogura Y, Adamis AP, Platelets accumulate in the diabetic retinal vasculature following endothelial death and suppress blood-retinal barrier breakdown. Am J Pathol, 2003. **163**: p. 253–259.

272. Berrettini M, Parise P, Constantini V, Grasselli S, Nenci GG, Platelet activation in psoriasis. Thromb Haemost, 1985. **53**: p. 195-197.

273. Ho-Tin-Noe B, Goerge T, Cifuni SM, Duerschmied D, Wagner DD, Platelet granule secretion continuously prevents intratumor hemorrhage. Cancer Res, 2008. **68**: p. 6851–6858.

274. Curotto S, Lunsford K, Smith W, Thomason J, Bulla C, Evidence of selective packaging and different α-granules subtypes in canine platelets. J Comp Pathol, 2012. **147**(4): p. 499-502.

275. Carmeliet P, Angiogenesis in life, disease and medicine. Nature, 2005. **438**: p. 932–936.

276. Kisucka J, Butterfield CE, Duda DG, Eichenberger SC, Saffaripour S, Ware J, Ruggeri ZM, Jain RK, Folkman J, Wagner DD, Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. Proc Natl Acad Sci USA, 2006. **103**: p. 855–860.

277. Brill A, Elinav H, Varon D, Differential role of platelet granular mediators in angiogenesis. Cardiovasc Res, 2004. **63**: p. 226–235.

278. Hallberg H, Mattsson B, Premature deaths among men in a Swedish municipality-social characteristics prior to death. Scand J Soc Med, 1991. **19**: p. 181–186.

279. Battinelli EM, Markens BA, Italiano Jr. JE, Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis. Blood, 2011. **118**: p. 1359–1369.

280. Sehgal S, Storrie B, Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. Journal of Thrombosis and Haemostasis, 2007. **5**(10): p. 2009-2016.

281. Italiano Jr JE, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL, Angiogenesis is regulated by a novel mechanism: Pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood, 2008. **111**: p. 1227–1233.

282. Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N, Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med, 2000. **6**: p. 41–48.

283. Feng W, Madajka M, Kerr BA, Mahabeleshwar GH, Whiteheart SW, Byzova TV, A novel role for platelet secretion in angiogenesis: Mediating bone marrow-derived cell mobilization and homing. Blood, 2011. **117**: p. 3893–3902.

284. Kamykowski J, Carlton P, Sehgal S, Storrie B, Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet alpha-granules. Blood, 2011. **118**: p. 1370–1373.

285. Horstman LL, Ahn YS, Platelet microparticles: A wide-angle perspective. Crit Rev Oncol Hematol, 1999. **30**: p. 111–142.

286. Berckmans RJ, Nieuwland R, Boing AN, Romijn FP, Hack CE, Sturk A, Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost, 2001. **85**: p. 639–646.

287. Italiano Jr JE, Mairuhu AT, Flaumenhaft R, Clinical relevance of microparticles from platelets and megakaryocytes. Curr Opin Hematol, 2010. **17**: p. 578–584.

288. Kim HK, Song KS, Chung JH, Lee KR, Lee SN, Platelet microparticles induce angiogenesis in vitro. Br J Haematol, 2004. **124**: p. 376–384.

289. Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D, Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. Cardiovasc Res, 2005. **67**: p. 30–38.

290. Helmond SE, Catalfamo JL, Brooks MB, Flow cytometric detection and procoagulant activity of circulating canine platelet-derived microparticles. AJVR, 2013. **74**(2): p. 207-215.

291. Gay LJ, Felding-Habermann B, Contribution of platelets to tumour metastasis. Nat Rev Cancer, 2011. **11**: p. 123-134.

292. Folkman J, Tumor angiogenesis: Therapeutic implications. N Engl J Med, 1971. **285**: p. 1182–1186.

293. Pinedo HM, Verheul HM, D'Amato RJ, Folkman J, Involvement of platelets in tumour angiogenesis? Lancet, 1998. **352**(9142): 1775-1777.

294. Karpatkin S, Ambrogio C, Pearlstein E, The role of tumor-induced platelet aggregation, platelet adhesion and adhesive proteins in tumor metastasis. Prog Clin Biol Res, 1988. **283**: p. 585–606.

295. Aird WC, Molecular heterogeneity of tumor endothelium. Cell Tissue Res, 2009. **335**: p. 271–281.

296. Jain RK, Determinants of tumor blood flow: A review. Cancer Res, 1988. **48**: p. 2641–2658.

297. Ho-Tin-Noe B, Goerge T, Wagner DD, Platelets: guardians of the tumor vasculature. Cancer Res, 2009. **69**: p. 5623-5626.

298. Nieswandt B, Hafner M, Echtenacher B, Mannel DN, Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res, 1999. **59**: p. 1295-1300.

299. Palumbo JS, Talmage KE, Massari JV, et al, Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood, 2005. **105**: p. 178-185.

300. Kopp HG, Placke T, Salih HR, Platelet-derived transforming growth factor-beta down-regulates NKG2D thereby inhibiting natural killer cell antitumor reactivity. Cancer Res, 2009. **69**: p. 7775–7783.

301. Labelle M, Begum S, Hynes RO, Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell, 2011. **20**(5): p. 576-590.

302. Hall CL, Dubyk CW, Riesenberger TA, Shein D, Keller ET, van Golen KL, Type I collagen receptor (alpha2beta1) signaling promotes prostate cancer invasion through RhoC GTPase. Neoplasia, 2008. **10**: p. 797–803.

303. Chen YQ, Trikha M, Gao X, Bazaz R, Porter AT, Timar J, Honn KV, Ectopic expression of platelet integrin alphaIIb beta3 in tumor cells from various species and histological origin. Int J Cancer, 1997. **72**: p. 642–648.

304. Gasic GJ, Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metastasis Rev, 1984. **3**(2): p. 99-114.

305. Jain S, Zuka M, Liu J, Russell S, Dent J, Guerrero JA, Forsyth J, Maruszak B, Gartner TK, Felding-Habermann B, Ware J, Platelet glycoprotein Ib alpha supports experimental lung metastasis. Proc Natl Acad Sci USA, 2007. **104**: p. 9024–9028.

306. Jain S, Russell S, Ware J, Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models. J Thromb Haemost, 2009. **7**: p. 1713–1717.

307. Ludwig RJ, Boehme B, Podda M, Henschler R, Jager E, Tandi C, Boehncke WH, Zollner TM, Kaufmann R, Gille J, Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res, 2004. **64**: p. 2743–2750.

308. Pearlstein E, Ambrogio C, Karpatkin S, Effect of antiplatelet antibody on the development of pulmonary metastases following injection of CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma tumor cells into mice. Cancer Res, 1984. **44**(9): p. 3884-3887.

309. Trikha M, Zhou Z, Raso E, et al, Multiple roles for platelet GPIIb/IIIa and alphavbeta3 integrins in tumor growth, angiogenesis, and metastasis. Cancer Res, 2002. **62**(10): p. 2824-2833.

310. Jain S, Zuka M, Liu J, et al, Platelet glycoprotein Ib alpha supports experimental lung metastasis. Proc Natl Acad Sci USA, 2007. **104**(21): p. 9024-9028.

311. Garcia J, Callewaert N, Borsig L. P-selectin mediates metastatic progression through binding to sulfatides on tumor cells. Glycobiology, 2007. **17**(2): p. 185-196.

312. Schumacher D, Strilic B, Sivaraj KK, Wettschureck N, Offermanns S, Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell, 2013. **24**: p. 130–137.

313. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, et al, Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer, 2005. **113**(5): p. 752-760.

314. Kerr BA, McCabe NP, Feng W, Byzova TV, Platelets govern premetastatic tumor communication to bone. Oncogene, 2013. **32**: p. 4319–4324.

315. Stone RL, Nick AM, McNeish IA, Balkwill F, Han HD, Bottsford-Miller J, Rupairmoole R, Armaiz-Pena GN, Pecot CV, Coward J, et al, Paraneoplastic thrombocytosis in ovarian cancer. N Engl J Med, 2012. **366**: p. 610–618.

316. Salgado R, Vermeulen PB, Benoy I, Weytjens R, Huget P, Van Marck E, Dirix LY, Platelet number and interleukin-6 correlate with VEGF but not with bFGF serum levels of advanced cancer patients. Br J Cancer, 1999. **80**: p. 892–897.

317. Caine GJ, Lip GY, Blann AD, Platelet-derived VEGF, Flt-1, angiopoietin-1 and P-selectin in breast and prostate cancer: Further evidence for a role of platelets in tumour angiogenesis. Ann Med, 2004. **36**: p. 273–277.

318. Salven P, Orpana A, Joensuu H, Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. Clin Cancer Res, 1999. **5**: p. 487–491.

319. Gonzalez FJ, Rueda A, Sevilla I, Alonso L, Villarreal V, Torres E, Alba E, Shift in the balance between circulating thrombospondin-1 and vascular endothelial growth factor in cancer patients: Relationship to platelet alpha-granule content and primary activation. Int J Biol Markers, 2004. **19**: p. 221–228.

320. Schneider BP, Radovich M, Miller KD, The role of vascular endothelial growth factor genetic variability in cancer. Clin Cancer Res, 2009. **15**: p. 5297–5302.

321. Fontaine E, McShane J, Page R, Shackcloth M, Mediratta N, Carr M, Soorae A, Poullis M, Aspirin and non-small cell lung cancer resections: Effect on long-term survival. Eur J Cardiothorac Surg, 2010. **38**: p. 21–26.

322. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE, Aspirin intake and survival after breast cancer. J Clin Oncol, 2010. **28**: p. 1467–1472.

323. Kerbel R, Folkman J, Clinical translation of angiogenesis inhibitors. Nat Rev Cancer, 2002. **2**: p. 727–739.

324. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med, 2004. **350**: p. 2335–2342.

325. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, et al, Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med, 2007. **357**: p. 2666–2676.

326. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al, Paclitaxel–carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med, 2006. **355**: p. 2542–2550.

327. Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, et al, Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet, 2007. 370: p. 2103–2111.

328. Friedman HS, Prados MD, Wen PY, Mikkelsen T, Schiff D, Abrey LE, et al, Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. J Clin Oncol, 2009. **27**: p. 4733–4740.

329. Norden AD, Young GS, Setayesh K, Muzikansky A, Klufas R, Ross GL, et al, Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence. Neurology, 2008. **70**: p. 779–787.

330. Narayana A, Kelly P, Golfinos J, Parker E, Johnson G, Knopp E, et al, Antiangiogenic therapy using bevacizumab in recurrent high-grade glioma: impact on local control and patient survival. J Neurosurg, 2009. **110**: p. 173–180.

331. Bridges EM, Harris AL, The angiogenic process as a therapeutic target in cancer. Biochemical Pharmacology, 2011. **81**: p. 1183-1191.

332. Bulla SC, Badial PR, Silva RC, Lunsford K, Bulla C. Platelets inhibit migration of canine osteosarcoma cells. J Comp Path, 2017, **156**(1): p.3-13.

333. Labelle M, Begum S, Hynes RO, Platelets guide to the formation of early metastatic niches. Proc Natl Acad Sci USA, 2014. **111**(30): E3053-61.

334. Gasic GJ, Gasic TB, Galanti N, Johnson T, Murphy S, Platelet-tumor-cell interactions in mice. The role of platelets in the spread of malignant disease. Int J Cancer, 1973. **11**(3): p. 704-18.

335. Laurens N, Koolwijk P, de Maat MP, Fibrin structure and wound healing. J Thromb Haemost, 2006. **4**: p. 932–939.

336. Werner S, Grose R, Regulation of wound healing by growth factors and cytokines. Physiol Rev, 2003. **83**: p. 835–870.

337. Singer AJ, Clark RA, Cutaneous wound healing. N Engl J Med, 1999. **341**: p. 738–746.

338. Rozman P, Bolta Z, Use of platelet growth factors in treating wounds and soft-tissue injuries. Acta Dermatovenerol Alp Panonica Adriat, 2007. **16**: p. 156–165.

339. Pandya NM, Dhalla NS, Santani DD, Angiogenesis–A new target for future therapy. Vascul Pharmacol, 2006. **44**: p. 265–274.

340. Margolis DJ, Kantor J, Santanna J, Strom BL, Berlin JA, Effectiveness of platelet releasate for the treatment of diabetic neuropathic foot ulcers. Diabetes Care, 2001. **24**: p. 483–488.

341. Bhanot S, Alex JC, Current applications of platelet gels in facial plastic surgery. Facial Plast Surg, 2002. **18**: p. 27–33.

342. Sanchez M, Anitua E, Azofra J, Andia I, Padilla S, Mujika I, Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. Am J Sports Medm 2007. **35**: p. 245–251.

343. Nurden AT, Nurden P, Sanchez M, Andia I, Anitua E, Platelets and wound healing. Front Biosci, 2008. **13**: p. 3532–3548.

344. Canapp SO Jr, Canapp DA, Ibrahim V, Carr BJ, Cox C, Barrett JG, The use of adipose-derived progenitor cells and platelet-rich plasma combination for the treatment of supraspinatus tendinopathy in 55 dogs: a retrospective study. Front Vet Sci, 2016. **3**: p. 61.

345. Waselau M, Sutter WW, Genovese RL, Bertone AL, Intralesional injection of platelet-rich plasma followed by controlled exercise for treatment of midbody suspensory ligament desmitis in Standardbred racehorses. J Am Vet Med Assoc, 2008. **232**(10): p. 1515-1520.

346. Arguelles D, Carmona JU, Climent F, Munoz E, Prades M, Autologous platelet concentrates as a treatment for musculoskeletal lesions in five horses. Vet Rec, 2008. **162**(7): p. 208-211.

347. Brossi PM, Moreira JJ, Machado TS, Baccarin RY, Platelet-rich plasma in orthopedic therapy: a comparative systematic review of clinical and experimental data in equine and human musculoskeletal lesions. BMC Vet Res, 2015. **11**: p. 98.

348. Macaulay IC, Carr P, Farrugia R, Watkins NA, Analyzing the platelet transcriptome. Vox Sang, 2004. **87**(Suppl 2): p. 42-46.

349. Zellner M, Winkler W, Hayden H, et al, Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets. Electrophoresis, 2005. **26**(12): p. 2481-2489.

350. Fink L, Holschermann H, Kwapiszewska G, et al, Characterization of platelet-specific mRNA by real-time PCR after laser-assisted microdissection. Thromb Haemost, 2003. **90**: p. 749–756.

351. Birschmann I, Mietner S, Dittrich M,et al, Use of functional highly purified human platelets for the identification of new proteins of the IPP signaling pathway. Thromb Res, 2008. **122**(1): p. 59-68.

352. Shruthi BS, Vinodhkumar P, Selvamani, Proteomics: A new perspective for cancer. Adv Biomed Res, 2016. **5**: p. 67.

353. Posadas EM, Simpkins F, Liotta LA, MacDonald C, Kohn EC, Proteomic analysis for the early detection and rational treatment of cancer: Realistic hope? Ann Oncol, 2005. 16: p. 16–22.

354. Patricelli MP, Activity-based probes for functional proteomics. Brief Funct Genomic Proteomic, 2002. **1**: p. 151–158.

355. Tyers M, Mann M, From genomics to proteomics. Nature, 2003. **422**: p. 193–197.

356. Paradanani A, Wieben ED, Spelsberg TC, Tefferi A, Primer on medical genomics. Part IV: Expression proteomics. Mayo Clin Proc, 2002. **77**(11): p. 1185-1196.

357. Mattow J, Schmidt F, Höhenwarter W, Siejak F, Schaible UE, Kaufmann SH, Protein identification and tracking in two-dimensional electrophoretic gels by minimal protein identifiers. Proteomics, 2004. **4**: p. 2927–2941.

358. Stults JT, Arnott D, Proteomics. Methods Enzymol, 2005. **402**: p. 245–289.

359. Gevaert K, Van Damme J, Goethals M, Thomas GR, Hoorelbeke B, Demol H, Martens L, Puype M, Staes A, Vandekerckhove J, Chromatographic isolation of methionine-containing peptides for gel-free proteome analysis: Identification of more than 800 Escherichia coli proteins. Mol Cell Proteomics, 2002. **1**: p. 896–903.

360. Kislinger T, Gramolini AO, MacLennan DH, Emili A, Multidimensional protein identification technology (MudPIT): Technical overview of a profiling method optimized for the comprehensive proteomic investigation of normal and diseased heart tissue. J Am Soc Mass Spectrom, 2005. **16**: p. 1207–1220.

361. Qureshi AH, Chaoji V, Maiguel D, Faridi MH, Barth CJ, Salem SM, Singhal M, Stoub D, Krastins B, Ogihara M, Zaki MJ, Gupta V, Proteomic and phospho-proteomic profile of human platelets in basal, resting state: Insights into integrin signaling. PLoS ONE, 2009. **4**: e7627.

362. Lewandrowski U, Wortelkamp S, Lohrig K, Zahedi RP, Wolters DA, Walter U, Sickmann A, Platelet membrane proteomics: A novel repository for functional research. Blood, 2009. **114**: e10–e19.

363. Zhang DY, Ye F, Gao L, Liu X, Zhao X, Che Y, et al, Proteomics, pathway array and signaling network-based medicine in cancer. Cell Div, 2009. **4**: p. 20.

364. Yarbrough WG, Slebos RJ, Liebler D, Proteomics: Clinical applications for head and neck squamous cell carcinoma. Head Neck, 2006. **28**: p. 549–558.

365. McCaw DL, Chan AS, Stegner AL, Mooney B, Bryan JN, Turnquist SE, et al, Proteomics of canine lymphoma identifies potential cancer-specific protein markers. Clin Cancer Res, 2007. **13**(8): p. 2496-2503.

366. Yang M, Zhong J, Zhao M, Wang J, Gu Y, Yuan X, Sang J, Huang C, Overexpression of nuclear apoptosis-inducing factor 1 altered the proteomic profile of human gastric cancer cell MKN45 and induced cell cycle arrest at G1/S phase. PLoS One, 2014. **9**: e100216.

367. Guo T, Fan L, Ng WH, Zhu Y, Ho M, Wan WK, Lim KH, Ong WS, Lee SS, Huang S, Kon OL, Sze SK, Multidimensional identification of tissue biomarkers of gastric cancer. J Proteome Res, 2012. **11**: p. 3405-3413.

368. Srinivas PR, Verma M, Zhao Y, Srivastava S, Proteomics for cancer biomarker discovery. Clin Chem, 2002. **48**(8): p. 1160-1169.

369. McCaw DL, Chan AS, Stegner AL et al, Proteomics of canine lymphoma identifies potential cancer-specific protein markers. Clinical Cancer Research, 2007. **13**: p. 2496-2503.

370. Gaines PJ, Powell TD, Walmsley SJ et al, Identification of serum biomarkers for canine B-cell lymphoma by use of surface-enhanced laser desorption-ionization time-of-flight mass spectrometry. American Journal of Veterinary Research, 2007. **68**: p. 405-410.

371. Klopfleisch R, Klose P, Weise C et al, Proteome of metastatic canine mammary carcinomas: Similarities to and differences from human breast cancer. Journal of Proteome Research, 2010. **9**: p. 6380-6391.

372. Klose P, Weise C, Bondzio A et al, Is there a malignant progression associated with a linear change in protein expression levels from normal canine mammary gland to metastatic mammary tumors? Journal of Proteome Research, 2011. **10**: p. 4405-4415.

373. Zamani-Ahmadmahmudi M, Nassiri SM, Rahbarghazi R, Serological proteome analysis of dogs with breast cancer unveils common serum biomarkers with human counterparts. Electrophoresis, 2014. **35**: p. 901-910.

374. LeRoy B, Painter A, Sheppard H et al, Protein expression profiling of normal and neoplastic canine prostate and bladder tissue. Veterinary Comparative Oncology. 2007, **5**: p. 119-130.

375. Schlieben P, Meyer A, Weise C et al, Differences in the proteome of high-grade versus low-grade canine cutaneous mast cell tumours. The Veterinary Journal, 2012. **194**: p. 210-214.

376. Morris JS, Genomic and proteomic profiling for cancer diagnosis in dogs. The Veterinary Journal, 2016. **215**: p. 101-109.

377. Maguire PB, Fitzgerald DJ, Platelet proteomics. J Thromb Haemost, 2003. **1**(7): p. 1593-1601.

378. Patrono C, Rocca B. Aspirin, 110 years later. J Thromb Haemost, 2009. **7**(Suppl. 1): p. 258–261.

379. Thon JN, Schubert P, Devine DV, Platelet storage lesion: A new understanding from a proteomic perspective. Transfus Med Rev, 2008. **22**: p. 268–279.

380. Egidi MG, D'Alessandro A, Mandarello G, Zolla L, Troubleshooting in platelet storage temperature and new perspectives through proteomics. Blood Transfus, 2010. **8**: s73–s81.

381. Clemetson KJ, Capitanio A, Luscher EF, High resolution two-dimensional gel electrophoresis of the proteins and glycoproteins of human blood platelets and platelet membranes. Biochim Biophys Acta, 1979. **553**(1): p. 11-24.

382. Marcus K, Immler D, Sternberger J, Meyer HE, Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. Electrophoresis, 2000. **21**: p. 2622–2636.

383. Klement GL, Yip TT, Cassiola F, et al, Platelets actively sequester angiogenesis regulators. Blood, 2009. **113**(12): p. 2835-2842.

384. Hernandez-Ruiz L, Valverde F, Jimenez-Nunez MD, Ocana E, Saez- Benito A, Rodriguez-Martorell J, Bohorquez JC, Serrano A, Ruiz FA, Organellar proteomics of human platelet dense granules reveals that 14-3-3zeta is a granule protein related to atherosclerosis. J Proteome Res, 2007. **6**: p. 4449–4457.

385. Smyth SS, Woulfe DS, Weitz JI, Gachet C, Conley PB, Goodman SG, Roe MT, Kuliopulos A, Moliterno DJ, French PA, Steinhubl SR, Becker RC, G-protein-coupled receptors as signaling targets for antiplatelet therapy. Arterioscler Thromb Vasc Biol, 2009. **29**: p. 449–457.

386. Sadler JE, Structural biology. A menage a trois in two configurations. Science, 2003. **301**: p. 177–179.

387. Maguire PB, Wynne KJ, Harney DF, O'Donoghue NM, Stephens G, Fitzgerald DJ, Identification of the phosphotyrosine proteome from thrombin activated platelets. Proteomics, 2002. **2**: p. 642–648.

388. Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB, Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. Blood, 2004. **103**: p. 2096–2104.

389. Garcia A, Prabhakar S, Hughan S, Anderson TW, Brock CJ, Pearce AC, Dwek RA, Watson SP, Hebestreit HF, Zitzmann N, Differential proteome analysis of TRAP-activated platelets: Involvement of DOK-2 and phosphorylation of RGS proteins. Blood, 2004. **103**: p. 2088–2095.

390. Senis YA, Antrobus R, Severin S, Parguina AF, Rosa I, Zitzmann N, Watson SP, Garcia A, Proteomic analysis of integrin alphaIIbbeta3 outside-in signaling reveals Src-kinase-independent phosphorylation of Dok-1 and Dok-3 leading to SHIP-1 interactions. J Thromb Haemost, 2009. **7**: p. 1718–1726.

391. Tan S, Tan HT, Chung MC, Membrane proteins and membrane proteomics. Proteomics, 2008. **8**: p. 3924–3932.

392. Moebius J, Zahedi RP, Lewandrowski U, Berger C, Walter U, Sickmann A, The human platelet membrane proteome reveals several new potential membrane proteins. Mol Cell Proteomics, 2005. **4**: p. 1754–1761.

393. Lewandrowski U, Moebius J, Walter U, Sickmann A, Elucidation of N-glycosylation sites on human platelet proteins: A glycoproteomic approach. Mol Cell Proteomics, 2006. **5**: p. 226–233.

394. Lewandrowski U, Wortelkamp S, Lohrig K, Zahedi RP, Wolters DA, Walter U, Sickmann A. Platelet membrane proteomics: A novel repository for functional research. Blood, 2009. **114**: e10–e19.

CHAPTER III

ULTRA-PURE PLATELET ISOLATION FROM CANINE WHOLE BLOOD

Reprinted from BMC Veterinary Research 9, Trichler SA, Bulla SC, Thomason J, Lunsford KV, Bulla C, 144, Copyright (2013). doi: 10.1186/1746-6148-9-144

Abstract

Background

Several research applications involving platelets, such as proteomic and transcriptomic analysis, require samples with very low numbers of contaminating leukocytes, which have considerably higher RNA and protein content than platelets. We sought to develop a platelet purification protocol that would minimize contamination, involve minimal centrifugation steps, and yield highly pure platelet samples derived from low volume whole blood samples from healthy dogs.

Results

Using an optimized OptiPrep density gradient technique, platelet recovery was 51.56% with 99.99% platelet purity and leukocyte contamination of 100 leukocytes per 10^8 platelets, on average. Platelet samples were subjected to additional purification with CD45-labeled Dynabeads after density barrier centrifugation resulting in a 95-fold depletion of residual leukocytes. Platelets purified using these methods remained inactivated as assessed by Annexin V and P-selectin labeling with flow cytometry.

Conclusions

The use of OptiPrep density gradient is a quick method for obtaining highly purified platelet samples from low volumes of canine whole blood with minimal contamination. Additional depletion of residual leukocytes can be achieved using CD45-labeled beads. These platelet samples can then be used for many downstream applications that require ultra-pure platelet samples such as RNA and protein analysis.

Background

Platelets are known to play important roles in hemostasis and tissue repair. Recently, the role of platelets in angiogenesis is being evaluated more closely as a potential modulator of tumor progression and metastasis. However, the continued exploration of platelets and their roles in hemostasis, healing, or cancer progression and metastasis requires increasingly pure platelet isolates.

Newer methods being employed in the study of disease include proteomic, transcriptomic and metabolic studies that require platelet samples with very low levels of leukocyte contamination. Leukocytes have been estimated to contain 12,500-fold more mRNA and 65-fold more protein than platelets [1,2]. Therefore, even very low numbers of contaminating cells will produce significant contamination. Current commonly used methods for producing platelet rich plasma (PRP) can yield up to 72% platelet recovery but, in exchange for the high platelet yield, these samples often have high residual leukocyte counts (> 3,000 leukocytes per 10^8 platelet) [3,4]. High levels of leukocyte contamination would produce a sample in which the majority of isolated mRNA is likely to be of leukocyte, rather than platelet, origin.

Methodologies that have successfully been used to produce highly pure platelet isolates devoid of notable leukocyte contamination require relatively large volumes of whole blood (40mL or greater) to recover sufficient platelets for study as a significant percentage of the platelets are lost during the purification process [5]. High platelet purities may also be achieved by use of platelet aphaeresis during blood collection as well as use of specialized leukocyte filters. Specialized equipment and training are required for platelet apheresis collection methods, which are not widely available in the clinical veterinary practice setting [3,6]. Again, employing multiple filters for leukocyte depletion from platelet samples leads to considerable platelet loss, often requiring large volumes of whole blood or platelet concentrate [7]. The larger blood volumes required for these techniques also makes them impractical for use in preparing clinical veterinary samples for analysis. Other techniques for producing highly pure platelet samples often require multiple centrifugation steps. The physical stress of these centrifugation steps often leads to platelet activation and subsequent granule content release which alters results if looking at platelet contents via protein or RNA.

The dog has quickly gained recognition as an import- ant animal model for many human diseases including cancer. Not only are dogs of veterinary importance from a companion animal standpoint, but also develop many of the same naturally occurring cancers and diseases as humans [8-10]. Our goal was to provide a fast and reliable method for producing ultra-pure platelet samples suitable for proteomic and transcriptomic analysis from clinically relevant (3–5mL) whole blood volumes with minimal platelet activation.

Results

Decreased leukocyte contamination using OptiPrep density barrier

Following centrifugation of whole blood with a 1.063g/mL density barrier, distinct layering was observed (Figure 1). After optimization of the OptiPrep density barrier centrifugation and platelet layer extraction steps, collection of 1 mL of the cloudy second layer consistently yielded a highly concentrated platelet fraction with an average purity of 99.99 ± 0.01% (mean ± standard deviation) by manual count, and average platelet recovery of 51.56 ± 9.66%. These platelet fractions were highly pure with only 100.07 ± 48.77 leukocytes per 10^8 platelets. The original whole blood samples had cell counts, on average, of 2,457,405 ± 746,380 leukocytes per 10^8 platelets. Therefore, on average, optimized density barrier centrifugation resulted in a 24,574-fold decrease of the leukocyte to platelet ratio from whole blood.

Reduction of leukocyte contamination by bead separation

Throughout optimization of the OptiPrep density barrier purification technique, CD45-labeled Dynabeads were tested as a method to remove additional residual leukocytes. An average 95-fold decrease in leukocyte contamination was seen for samples containing high levels of contaminating cells. In samples containing an already low amount of contaminating leukocytes such as those seen with the OptiPrep density barrier centrifugation, there was no added benefit of continuing with the additional purification step using the leukocyte-specific beads due to additional platelet loss from the bead separation process.

Purity, contamination, and assessment of activation by flow cytometry

Flow cytometric analysis of the platelet samples obtained after OptiPrep density barrier centrifugation indicated, on average, 99.47 ± 0.21% CD61-positive events and 0.19 ± 0.04% CD45-positive events. After incubation with CD45-labeled Dynabeads the samples had, on average, 98.84 ± 0.03% CD61-positive events and 0.27 ± 0.03% CD45-positive events (Figures 2 and 3). Platelet sample activation was evaluated using Annexin V and P-selectin labeling by flow cytometry (Figures 4 and 5). After initial centrifugation with the 1.063g/mL density barrier, the platelet sample had 0.66% annexin positivity and 0.4% P-selectin positivity, and after incubation with CD45-labeled beads there was 3.00% Annexin positivity. Because of the platelet function inhibitory effect of the PECT anticoagulant, whole blood was collected in citrate to acquire PRP for use as positive controls by activating with thrombin and collagen. Thrombin activated platelets had 25.18% Annexin positivity and 13.44% P-selectin positivity while platelets activated by collagen had 88.28% Annexin positivity and 0.66% P-selectin positivity.

Discussion

The described technique allows the purification of canine platelets using density barrier centrifugation and yields a highly pure platelet sample with very low leukocyte contamination (99.99±0.01% platelet purity with only 100 leukocytes per 10^8 platelets). This technique leaves platelets in a resting, non-activated state as assessed with Annexin V and P-selectin labeling by flow cytometry. Minimal centrifugation steps and sample handling is involved along with no additional need for filtering or special aphaeresis equipment. Moreover, this procedure is quick and efficient, resulting in a platelet

recovery of approximately 52%, and can be performed on small, clinically practical whole blood sample volumes (3–5mL).

Cell counting should be performed following the density barrier centrifugation step, and if residual leukocytes are seen in the counting chamber, it is recommended that the sample be incubated with CD45-labeled Dynabeads for removal of residual leukocytes to increase the purity of the platelet sample. Our results show that with this procedure residual leukocytes can be further reduced 95-fold (up to 586-fold maximum observed with optimized protocol) with minimal platelet loss.

During our optimization trials, we determined that the volumes and the ratio of whole blood to OptiPrep density barrier should be carefully maintained. Adding more or less than 3mL of blood on top of the 5mL of density barrier can cause poor layer separation, resulting in lower platelet purity and recovery. It is also vitally important to handle samples with care as to avoid any disturbance or mixing of the post-centrifugation layers, which may re-contaminate the platelet layer.

Little comparable data exists in the current literature in regards to veterinary species, including the dog. In humans however, density gradient centrifugation methods for platelet purification are well established, but are aided by aphaeresis-derived platelet concentrates obtained from large whole blood volumes [2,11,12]. One example of human platelet purification is the 1990 study from Ford et al. which used a 1.063g/mL OptiPrep density barrier centrifugation of human whole blood to achieve a platelet recovery of 70% with 125 leukocytes per 10^8 platelets [12]. More recently, Birschmann et al. achieved platelet samples with 21.9 and 1.5 leukocytes per 108 platelets using pre-purified PRP and apheresis- derived platelet concentrates over layered OptiPrep density

barriers, respectively [2]. In clinical veterinary settings, the collection of large blood volumes from single clinical patients is neither safe nor ethical and aphaeresis equipment is neither easily accessible nor cost effective. Here we describe a platelet purification technique that uses low clinically relevant volumes of canine whole blood and yields ultra-pure non-activated platelets with minimal leukocyte contamination.

Conclusion

This study details an efficient technique for purification of canine blood platelets that results in ultra-pure resting platelet samples derived from small, clinically reasonable whole blood volumes. These ultra-pure resting platelet samples are thus suitable for use in multiple downstream analyses including proteomic and transcriptomic methodologies for the study of platelet physiology and functional roles in naturally occurring diseases in dogs.

Methods

Platelet purification

Healthy female intact Walker hound dogs were used in this study. The dogs were not exposed to any medications or vaccines for at least two months before initiation of the study. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements at a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Whole blood samples were collected by jugular venipuncture with a 20-gauge needle directly into a glass vacutainer tube containing 0.5mL of PECT medium (94nM prostaglandin E1, 0.63mM Na2CO3, 90mM EDTA, and 10mM theophylline) that had an

approximate draw of 5mL. Sample preparation was initiated within one hour on the day of collection and initial blood cell counts were performed by an automated cell counter (Cell-Dyn 3700, Abbott Laboratories, Abbott Park, IL, USA).

A 1.063 g/mL density barrier was created by combining 5 mL of 1.320 g/mL 60% iodixanol stock solution (OptiPrep density gradient medium, Sigma-Aldrich, Saint Louis, MO, USA) with 22mL diluent (0.85% NaCl, 20mM HEPES-NaOH, pH 7.4, 1mM EDTA). For platelet separation, 3mL of each sample were layered over 5mL of the 1.063g/mL density barrier. Layered samples were then centrifuged at 350xg for 15 minutes at 20°C in a swinging bucket rotor with the brake turned off [11,12]. One milliliter of the concentrated, opaque platelet fraction was collected as illustrated (Figure 1). Further removal of residual contaminating leukocytes was performed by coupling magnetic beads (Sheep anti-rat IgG Dynabeads, Invitrogen Dynal, Oslo, Norway) with rat anti-dog CD45 antibody (AbD Serotec, Raleigh, NC, USA), incubating the beads with the platelet sample for thirty minutes at room temperature with gentle rotation, and placing the mixture in a specialized magnet according to the manufacturer's protocol. Cells in before and after bead depletion samples (platelets, leukocytes, and erythrocytes) were counted manually using a hemocytometer (improved Neubauer, Hausser Scientific Co., Harsham, PA, USA) to assess sample recovery and purity.

Flow cytometry

Flow cytometric analysis was performed with a flow cytometer using Cell Quest Pro software (FACScalibur, BD Biosciences, San Jose, CA, USA). In order to assess sample purity, double staining with RPE-anti-CD45 (Rat anti-dog CD45: RPE, MCA1042PE, AbD Serotec, Raleigh, NC, USA) and FITC-anti-CD61 (Mouse anti-

human CD61: FITC, BD Pharmingen, San Jose, CA, USA) were performed. One hundred microliters of sample were incubated with 10µL of anti-CD45 and 20µL of anti-CD61 for 20 minutes at room temperature. The samples were washed and resuspended in phosphate buffered saline for analysis. Samples were displayed on log forward-angle versus log side-angle light scatter plots. Gates were determined to include all blood cells and results visualized on a dual color quadrant.

We also employed an automated leukocyte count technique, which relies on propidium iodide DNA staining and on test tubes containing precise numbers of fluorescent beads to quantify the residual leukocyte, according to manufacturer's protocol (LeucoCOUNT Reagent Kit, BD Biosciences). For each sample 30,000 bead events were counted.

Platelet activation was assessed using fluorescent-labeled Annexin V (FITC Annexin V, BD Biosciences) and RPE-anti-CD62P (P-selectin) (Mouse anti-human CD62P: RPE, MCA2419, AbD Serotec) binding and flow cytometric analysis. Annexin V binds exposed phosphatidylserine on the platelet membrane, a platelet membrane phospholipid that is trans located from the inner membrane leaflet to the exposed outer surface during platelet activation [12]. P-selectin is a platelet granule membrane protein that translocates to the outer membrane surface after activation and granule release [13]. For Annexin V labeling, around 5 million platelets (after OptiPrep and after Dynabeads) were spun down and resuspended in binding buffer before staining with 1:10 diluted Annexin V according to the manufacturer's protocol. For P-selectin labeling, 20uL of RPE-anti- CD62P antibody were added to platelet samples the same as described above for CD45 and CD61 labeling. For positive controls, platelets were activated with 1U/ml

of thrombin and 10ug/mL collagen. Platelet populations were displayed on log forward-angle versus log side-angle light scatter plots. Gates were adjusted to platelet populations, and 5,000-gated events were recorded for each labeling. Expression was quantified by the percent of positive events for Annexin V and P-selectin.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This research was funded by the Office of Research and Graduate Studies from the College of Veterinary Medicine, Mississippi State University.

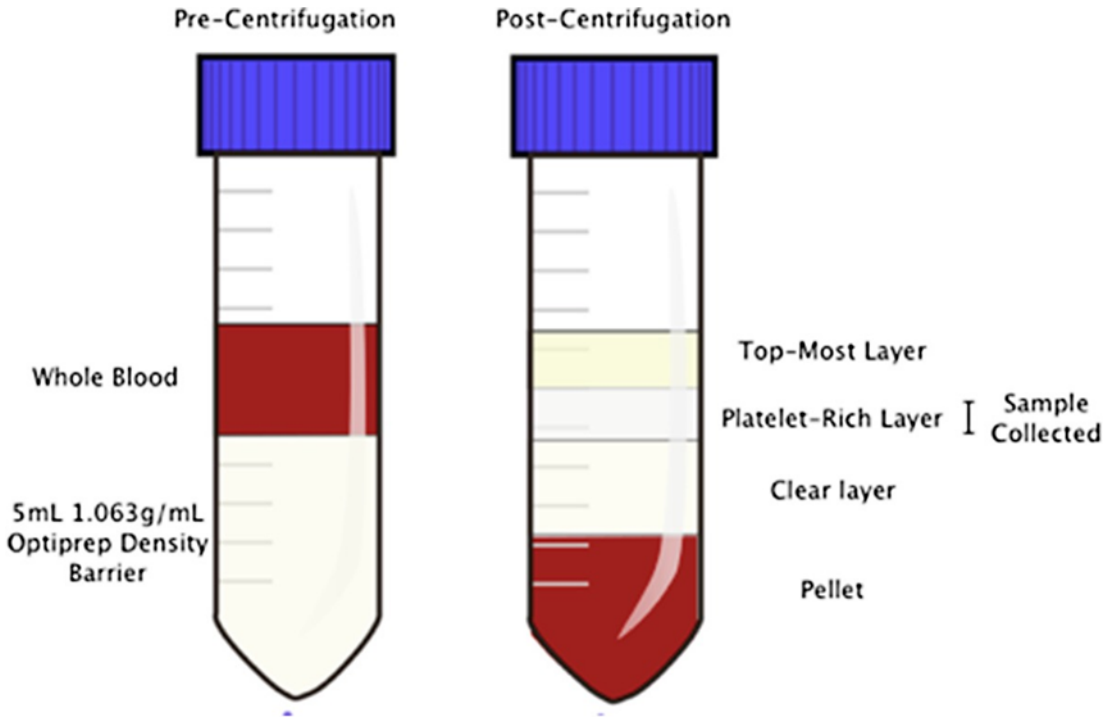

Figure 3.1 Density barrier layering before and after centrifugation with whole blood.

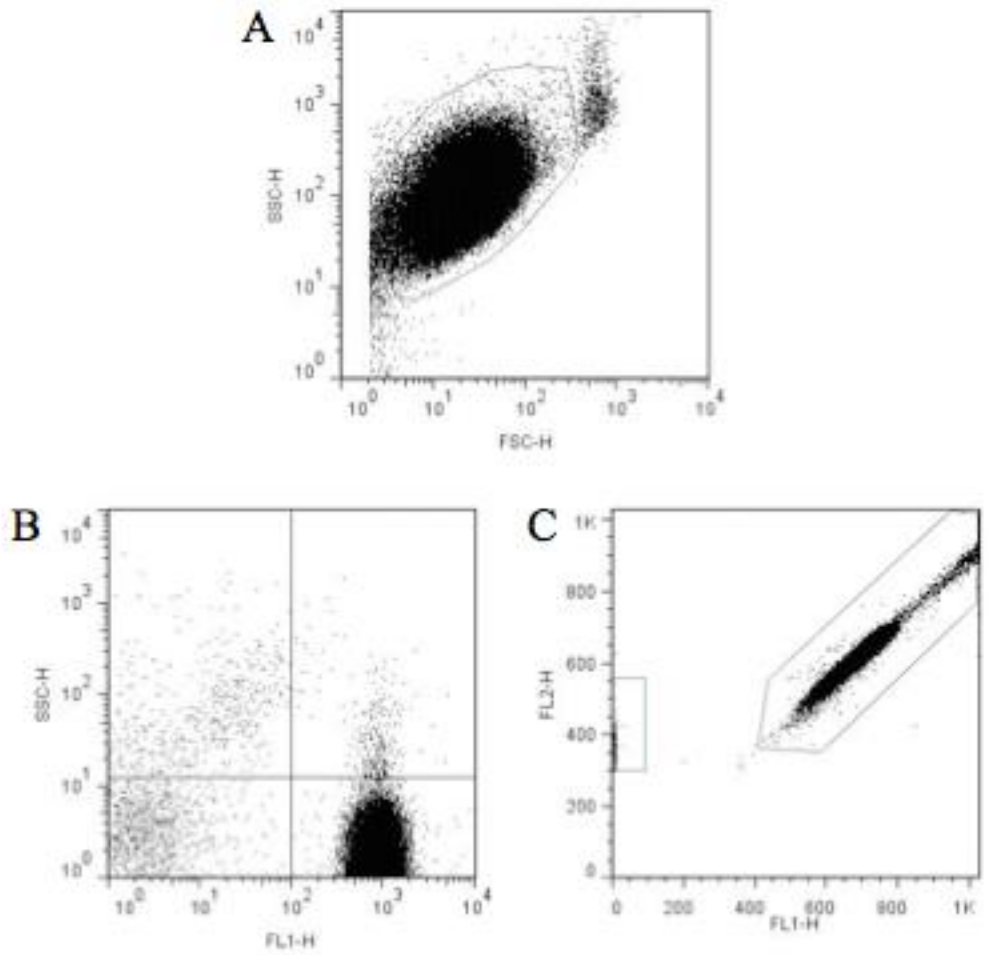

Figure 3.2 Flow cytometric analyses of optimized platelet samples after density barrier centrifugation.

(A) Log forward and side scatter chart of platelet population (gated) with minimal contaminating cells. (B) Fluorescence chart of CD45 (FL2) and CD61 (FL1) antibody-labeled samples. Average CD61+: 99.47 ± 0.21% (n = 3). Average CD45+: 0.19 ± 0.04% (n = 3). (C) LeucoCOUNT chart for sample showing residual leukocytes (left gate) and LeucoCOUNT beads (right gate).

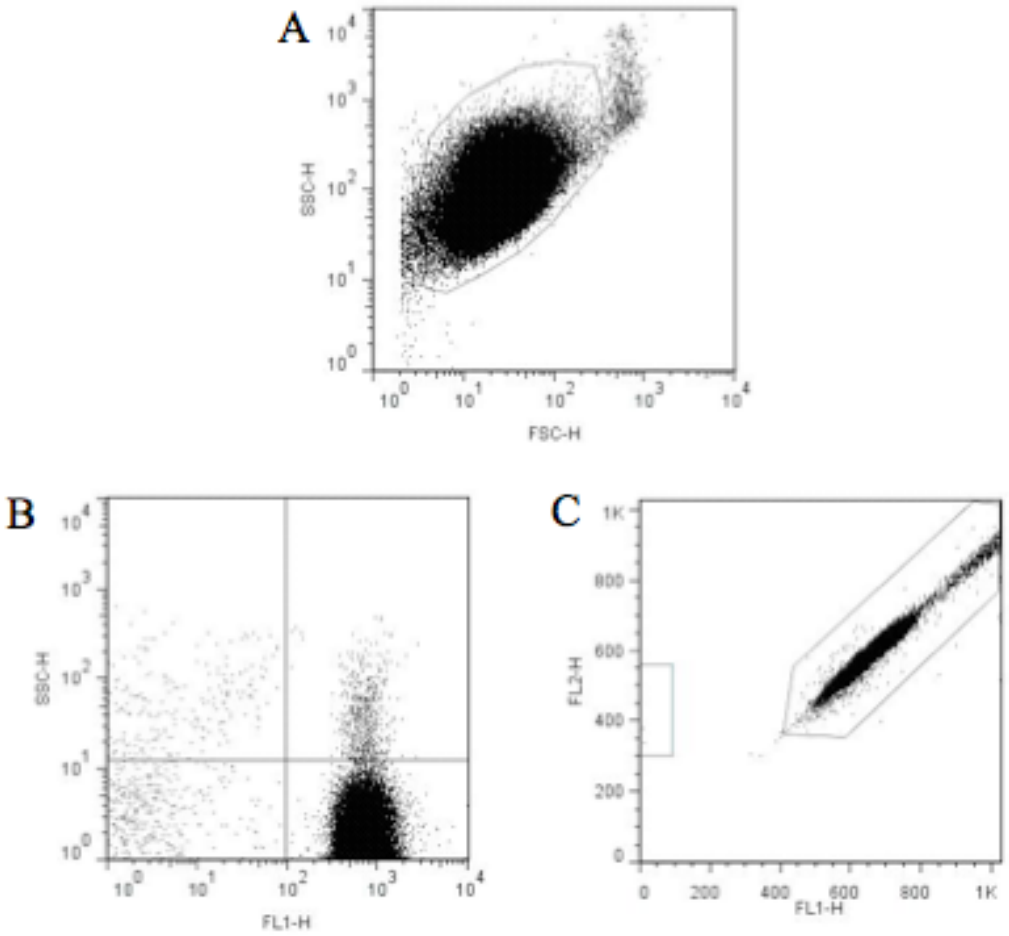

Figure 3.3 Flow cytometric analyses of optimized platelet samples after CD45-labeled Dynabead incubation.

(A) Log forward and side scatter chart of platelet population (gated) of platelet sample with minimal contaminating cells. (B) Fluorescence chart of CD45 (FL2) and CD61 (FL1) antibody-labeled sample. Average CD61+: 97.99 ± 1.38% (n = 12). Average CD45+: 0.43 ± 0.33% (n = 12). (C) LeucoCOUNT chart for sample after density barrier centrifugation showing residual leukocytes (left gate) and LeucoCOUNT beads (right gate).

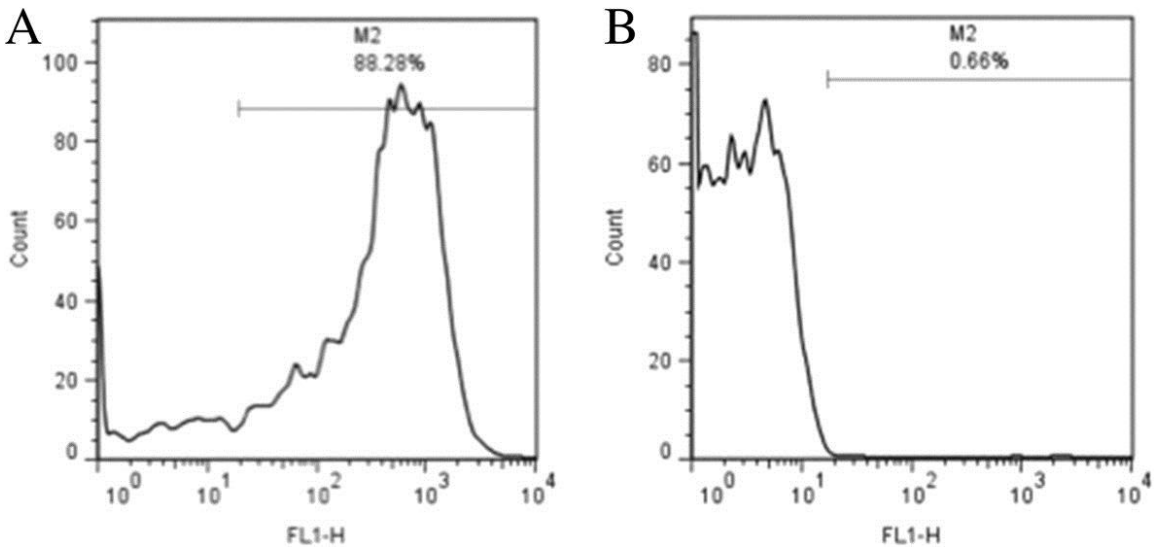

Figure 3.4 Flow cytometric analyses of platelet activation by Annexin V labeling.

(A) Collagen activated platelets. (B) Platelet sample after density barrier centrifugation.

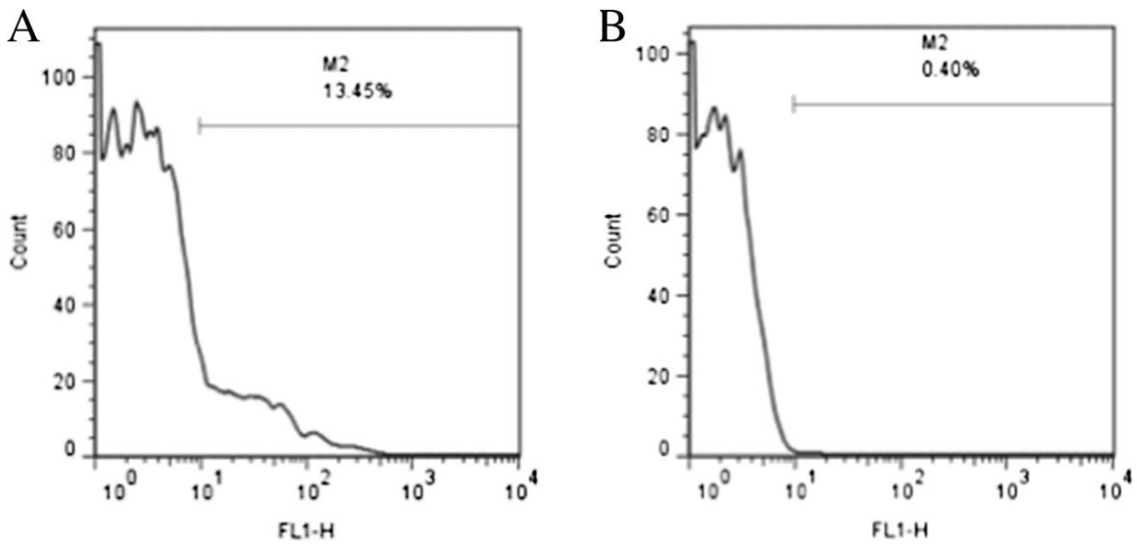

Figure 3.5 Flow cytometric analyses of platelet activation by P-selecting labelling.

(A) Thrombin activated platelets. (B) Platelet sample after density barrier centrifugation.

References

1. Fink L, Holschermann H, Kwapiszewska G, Muyal JP, Lengemann B, Bohle RM, Santoso S, Characterization of platelet-specific mRNA by real-time PCR after laser-assisted micro dissection. Thromb Haemost, 2003. **90**(4): p. 749–756.

2. Birschmann I, Mietner S, Dittrich M, Pfrang J, Dandekar T, Walter U, Use of functional highly purified human platelets for the identification of new proteins of the IPP signaling pathway. Thromb Res, 2008. **122**(1): p. 59–68.

3. Gnatenko DV, Dunn JJ, McCorkle SR, Weissmann D, Perrotta PL, Bahou WF, Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood, 2003. **101**(6): p. 2285–2293.

4. George JN, Thoi LL, McManus LM, Reimann TA, Isolation of human platelet membrane microparticles from plasma and serum. Blood, 1982. **60**(4): p. 834–840.

5. Rox JM, Bugert P, Muller J, Schorr A, Madlener K, Kluter H, Potzsch B, Hanfland Madlener K, Gene expression analysis in platelets from a single donor: evaluation of a PCR-based amplification technique. Clin Chem, 2004. **50**(12): p. 2271–2278.

6. Appleman EH, Sachais BS, Patel R, Drobatz KJ, Groman RP, Kennedy DR, O'Donnell PA, Bryan C, Callan MB, Cryopreservation of Canine Platelets. J Vet Intern Med, 2009. **23**(1): p. 138–145.

7. O'Neill EE, Brock CJ, Von Kriegsheim AF, Pearce AC, Dwek RA, Watson SP, Hebestreit HF, Towards complete analysis of the platelet proteome. Proteomics, 2002. **2**(3): p. 288–305.

8. MacEwen EG, Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Rev, 1990. **9**(2): p. 125–136.

9. Withrow SJ, Wilkins RM, Cross talk from pets to people: translational osteosarcoma treatments. ILAR J, 2010. **51**(3): p. 208–213.

10. Paoloni M, Khanna C, Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer, 2008. **8**(2): p. 147–156.

11. Ford TC, Graham J, Rickwood D, A new, rapid, one-step method for the isolation of platelets from human blood. Clin Chim Acta, 1990. **192**(2): p. 115–119.

12. Graham J, Isolation of human platelets (thrombocytes). Scientific World Journal, 2002. **2**: p. 1607–1609.

13. Tait JF, Smith C, Wood BL, Measurement of phosphatidylserine exposure in leukocytes and platelets by whole-blood flow cytometry with annexin V. Blood Cells Mol Dis, 1999. **25**(5-6): p. 271–278.

CHAPTER IV

IDENTIFICATION OF CANINE PLATELET PROTEINS SEPARATED BY DIFFERENTIAL DETERGENT FRACTIONATION FOR NONELECTROPHORETIC PROTEOMICS ANALYZED BY GENE ONTOLOGY AND PATHWAYS ANALYSIS

Reprinted from Veterinary Medicine: Research and Reports 5, Trichler SA, Bulla SC, Mahajan N, Lunsford KV, Pendarvis K, Nanduri B, McCarthy FM, Bulla C, 1-9, Copyright (2014). doi: 10.2147/VMRR.S47127

Abstract

During platelet development, proteins necessary for the many functional roles of the platelet are stored within cytoplasmic granules. Platelets have also been shown to take up and store many plasma proteins into granules. This makes the platelet a potential novel source of biomarkers for many disease states. Approaches to sample preparation for proteomic studies for biomarkers search vary. Compared with traditional two-dimensional polyacrylamide gel electrophoresis systems, nonelectrophoretic proteomics methods that employ offline protein fractionation methods such as the differential detergent fractionation method have clear advantages. Here we report a proteomic survey of the canine platelet proteome using differential detergent fractionation coupled with mass spectrometry and functional modeling of the canine platelet proteins identified. A total of 5,974 unique proteins were identified from platelets, of which only 298 (5%) had previous experimental evidence of *in vivo* expression. The use of offline prefractionation

of canine proteins by differential detergent fractionation resulted in greater proteome coverage as compared with previous reports. This initial study contributes to a broader understanding of canine platelet biology and aids functional research, identification of potential treatment targets and biomarkers, and sets a new standard for the resting platelet proteome.

Introduction

The roles that platelets play in blood hemostasis, wound healing, inflammation, and thrombus formation are well described, and more recently, platelets have also been recognized for their interactions with tumor cells [1]. During platelet development, proteins necessary for the many functional roles of the platelet are stored within cytoplasmic granules. Platelets have been shown to take up and store many plasma proteins into granules. This makes the platelet a potential novel source of biomarkers for many disease states [2]. Naturally occurring disease in the dog, a companion animal of veterinary importance, is gaining recognition as a valuable model of human disease [3,4]. Translational studies focusing on identification of novel platelet protein markers for disease using the dog first require a description of the basal canine platelet proteome. This will allow for the necessary comparison of the canine and human platelet proteome.

Approaches to sample preparation for proteomic studies vary. Compared with traditional two-dimensional polyacrylamide gel electrophoresis systems, nonelectrophoretic proteomics methods that employ offline protein fractionation methods, such as the differential detergent fractionation (DDF) method described by McCarthy et al [5], have clear advantages. This technique, for instance, increases the yield of hydrophobic membrane proteins due to protease digestion based on the

physicochemical properties of peptides. Because the DDF method assigns GO (Gene Ontology) cellular component terms to proteins based on the detergent fraction they were extracted in, as well as the number of transmembrane domains the protein contains, it allows identification of the subcellular localization of proteins expressed within the cell. Here we report a proteomic survey of the canine platelet proteome using DDF, mass spectrometry, and subsequent functional modeling. The canine platelet proteome described here provides evidence for *in vivo* expression or uptake of these proteins in the canine platelet. Additionally, since the method used for protein fractionation could predict subcellular localization of the proteins, we could also add to the "cellular component" of protein functional annotation based on subcellular localization as described below.

Materials and methods

Platelet isolation

Normal resting platelets were purified from the blood (collected in ethylenediaminetetraacetic acid) of three adult healthy female Walker hounds. Whole blood (3mL) was layered on top of 3mL of a 1.077g/mL density gradient and spun at 400*g* for 30 minutes at room temperature. The resulting top fraction was washed with HEPES-NaCl, layered over a combination of diluted 60% iodixanol 1.32g/mL density barriers (10% and 13% stock solution, from top to bottom), and spun at 300*g* for 20 minutes. The top fraction was washed, pelleted, and resuspended in HEPES-NaCl. Cell counts were performed manually with a hemocytometer and samples were found to have an d The sample was then pelleted again and stored at −80ºC. Platelet pellets from a total

of 20mL of blood from each dog were subjected to protein extraction (average of 4.7 ± 4.1 × 10^8 total platelets).

Protein extraction and identification

Proteins were isolated as previously described [5]. Briefly, four different extractions using digitonin, Triton X-100, deoxycholate plus Tween 40, and sodium dodecyl sulfate were used to sequentially isolate proteins based upon decreasing solubility. Each of the fractions was precipitated with 25% trichloroacetic acid to remove salts and detergents, and trypsin digested. Peptides were desalted and then cleaned using a reverse phase macrotrap and strong cation exchange macrotrap (Michrom Bioresources Inc., Auburn, CA, USA), respectively, according to the manufacturer's instructions. Samples were then dried and resuspended in 2% acetonitrile, 0.1% formic acid.

Peptide mass spectrometry was accomplished using an EASY-nLC (Thermo Scientific, Rockford, IL, USA) high performance liquid chromatography machine coupled with an LTQ Velos linear ion trap mass spectrometer (Thermo Scientific). The Easy-nLC was configured for reverse phase chromatography using a Hypersil Gold KAPPA C18 column (Thermo Scientific) with a flow rate of 333 nanoliters per minute. Peptides were separated for mass spectrometry analysis using an acetonitrile gradient starting at 2% acetonitrile, 0.1% formic acid and reaching 50% acetonitrile, 0.1% formic acid in 120 minutes, followed by a 15-minute wash of 95% acetonitrile, 0.1% formic acid. Column equilibration was handled automatically using the EASY-nLC. The eluate from the high performance liquid chromatography was fed directly to the LTQ Velos for nanospray ionization followed by tandem mass spectrometry analysis of detected peptides. The LTQ Velos was configured to perform one mass spectrometry scan

followed by 20 tandem mass spectrometry scans of the 20 most intense peaks repeatedly over the 172-minute duration of each high performance liquid chromatography run. Dynamic exclusion was enabled with a duration of 5 minutes, repeat count of one, and a list length of 500. The collected spectra were subsequently analyzed using the X!Tandem (Open-source software, The Global Proteome Machine Organization) search algorithm [6].

Raw spectral data from the LTQ Velos were converted to mzML format using the msConvert tool from the ProteoWizard (Open-source software) software project [7] because X!Tandem cannot read the Thermo raw format directly. The FASTA database used for peptide spectrum matching (target database) was the *Canis familiaris* RefSeq protein database from the National Center for Biotechnology Information. X!Tandem was configured to use tryptic cleavage sites with up to two missed cleavages. Precursor and fragment mass tolerance were set to 1000 ppm and 500 ppm, respectively. Four amino acid modifications were included in the database search, ie, single and double oxidation of methionine and both carboxymethylation and carboxamidomethylation of cysteine. A decoy search was also performed using a randomized version of the target database with the same search parameters as before. The search results were filtered using the methods described previously [8,9]. A decoy score distribution of X!Tandem hyperscores and e-values was created and each match from the target database was evaluated as a possible outlier and assigned a probability of being such. Peptides from the target database were accepted if the probability of being an outlier was 95% or higher. A list of proteins and identified peptides was generated for each experimental sample.

Functional analysis of platelet proteome

Platelet proteins identified using mass spectrometry were analyzed using AgBase tools (Open-source software, Agbase, Mississippi State University) [10]. GORetriever (Open-source software, Agbase, Mississippi State University) was used to obtain GO annotation and these results summarized using GOSlimViewer with the Generic Slim set [11]. We compared the function of the platelet proteome against the GO for the entire GO proteome by downloading the dog gene association file from AgBase (December 14, 2012) and comparing the relative proportions for each slimmed GO term.

Platelet proteins identified by mass spectrometry were also analyzed using DDF2GO [12], a tool that groups proteins into GO cellular component categories based on the fraction they were extracted in as well as their number of transmembrane domains, predicted using PSORTII [13] (Open-source software, Human Genome Center, Tokyo, Japan) and TMpred [14] (Open-source software, TMbase) online prediction softwares. DDF2GO uses a GO CC mapping table, previously established by studies from McCarthy et al using proteins' DDF fractions and transmembrane domains, which dictates GO CC assignment conditions based on DDF fraction percentages and average transmembrane domains [5]. Since platelets are anucleate, the annotations to the GO term "nucleus" were not included in the results.

Pathways analysis

We used Ingenuity Pathways Analysis (Ingenuity Systems Inc., Redwood City, CA, USA) to identify significant molecular functions and canonical pathways represented by platelet proteins as described earlier [15]. A right-tailed Fisher's Exact test was used to calculate a *P*-value determining the probability that each biological function assigned

to that data set is due to chance alone. All molecular functions and metabolic and signaling pathways identified at a *P*-value of <0.05 were considered to be statistically significant.

Results

Protein identification

We identified 5,974 unique proteins from platelets collected from three healthy dogs (http://www.dovepress.com/cr_data/ supplementary_file_47127.pdf). Only 298 (5%) of these identified proteins had previous experimental evidence of *in vivo* expression. The remaining 5,676 (95%) of the identified proteins are computationally predicted based on homology to known proteins in related species.

Functional analysis

Our GO analysis of the proteins identified by mass spectrometry indicated that 86% of the 5,974 proteins we identified had GO annotation. Using the generic GO Slim to summarize the GO function for our proteins, we identified the general functions represented in our data set. The most under-represented and over-represented GO functions in the canine platelet proteome dataset compared with the canine genome are shown in Figure 1, and the top ten GO terms for each ontology are shown in Figure 2.

Compared with the canine genome, our canine platelet proteome was highly enriched in the GO biological process terms “cytoskeleton organization”, “cell recognition”, “cellular component organization”, “cell communication”, “cell-to-cell signaling”, and “response to endogenous/ exogenous stimulus”, which can be related to important functions in platelets. The GO terms “translation” and “secondary metabolic

process" were found to be under-represented in the canine platelet compared with the genome as would be expected considering the platelets' lack of nuclei. The most represented GO biological process terms were "unknown" (36.20%), "anatomical structure development" (7.33%), and "signal transduction" (5.15%). The last two terms reflect the importance of intracellular signaling and structural changes in platelet function. In hemostasis, platelet surface receptors will bind to exposed subendothelial proteins upon vascular injury, which leads to platelet activation and morphologic change from a discoid to a spherical shape.

In comparison with the dog genome, our platelet proteome was highly enriched in the GO cellular component terms "cytoplasmic membrane-bound vesicle", "endosome", "proteinaceous extracellular matrix", "organelle", "plasma membrane", and "cytoskeleton". All of these GO terms highly enriched in the platelet are important to platelet function and morphology in normal hemostasis. Some under-represented GO terms in the platelet versus the genome included "nuclear", "ribosome", and "lipid particle", which is not surprising considering platelets are anucleate and lack normal nucleated cell translational levels and membrane assembly. The most represented GO terms were "unknown" (22.03%), "cell" (14.49%), "intracellular" (13.46%), "cytoplasm" (8.73%), and "organelle" (8.28%). These localization categories are rather general and only indicate that within the proteins with known localization, the majority are annotated as being anywhere in the cell, including the membrane (cell) or not (intracellular).

Highly enriched protein GO molecular function terms present in the platelet compared with the dog genome included "protein binding", "carbohydrate binding", "enzyme regulator activity", "cytoskeletal protein binding", "actin binding", and

"receptor binding", all of which are related to important platelet functions. Some of the most under-represented GO terms in this category are "structural constituent of ribosome" and "mRNA binding" which, again, make sense considering the anucleate nature of platelets and their limited protein translation functions. The most represented GO molecular function terms were "unknown" (49.16%), "ion binding" (12.63%), "kinase activity" (4.59%), "DNA binding" (3.92%), and "transmembrane transporter activity" (3.13%), which emphasize the importance of signaling in platelets.

In order to evaluate pathways and processes further, we used Ingenuity Pathway Analysis (Ingenuity Systems Inc.) software to obtain the significantly represented functions and pathways in the canine platelet proteome, as shown in Figures 3 and 4. In the analysis of the canine platelet proteome, "hematological system development and function", "inflammatory response", "cancer", and "cellular movement" were among the top represented functional categories found in the canine platelet. These categories indicate the multifunctionality of platelets, which play important roles in inflammation and cancer development in addition to their hemostasis function [1]. The three most represented functions include "cellular assembly and organization", "cellular function and maintenance", and "cell morphology", which illustrates functions that are important during platelet activation and hemostatic function.

The most significant canonical pathways are shown in Figure 4. "Thrombin signaling", "HGF signaling", "EGE signaling", "PDGF signaling", "integrin signaling", and "thrombopoietin signaling" were among the top represented canonical pathways listed for the canine platelet. The proteins in these canonical pathways have significant roles in hemostasis, clot formation, wound healing, and angiogenesis. The presence of the

"actin cytoskeleton" pathway as being the fifth most significant corroborates with the over-representation of cytoskeleton proteins indicated by the GO analysis results.

Subcellular localization of proteins

Platelet proteins were extracted using DDF, a multistep protein extraction method that uses four detergents (digitonin, Triton X-100, deoxycholate plus Tween 40, and sodium dodecyl sulfate) to separate proteins into four protein fractions based on decreasing solubility. Subsequent analysis of proteins extracted using DDF was performed using DDF2GO [12] software which assigns cellular component-specific GO terms to each protein based on the detergent fraction the protein was extracted in and the number of transmembrane domains in the protein, if any.

Three healthy dog platelet samples were used for this proteomic study with an average of 2,668 proteins identified from each dog. We identified 1,803 proteins common to more than one dog which we then further analyzed to identify where these proteins were located in the platelet based on GO terms from DDF2GO.

The DDF method we used isolates proteins from throughout the entire platelet, as graphically illustrated in Figure 5 using cellular component terms from GOSlimViewer (Open- source software, Agbase, Mississippi State University) for proteins found in more than one of the three dogs. About 42% of the protein annotations were related to membranes, approximately 17% of which were to the plasma membrane in particular. Nearly 36% were related to organelles, with 10%, 3%, and 2% belonging to the mitochondrion, vesicle, and endoplasmic reticulum categories, respectively. About 22% of annotations related to the cytoplasm. These annotations indicate that the proteins found in more than one dog are located in the same subcellular regions.

In our platelet protein analysis we observed up to a 4.9- fold increase in information about subcellular localization using DDF2GO for protein functional analysis compared with AgBase's GORetriever. DDF2GO provides protein annotation outputs that are different from traditionally used software like GORetriever. Tools such as GORetriever assign protein annotations in light of what is already known about the proteins. In addition to what is already known about the proteins, DDF2GO allows us to look at proteins based on the detergent fraction(s) that the protein was extracted in as well as the number of transmembrane domains the protein has. Proportionally, we observed a 4.0-fold increase in protein annotations related to "plasma membrane" and a 2.3-fold increase related to "cytoplasm". The "mitochondrion" and "endoplasmic reticulum" GO cellular component categories had 4.9-fold and 1.6-fold increases in annotations for proteins in their categories, respectively. Use of DDF2GO software for protein annotation adds experimentally derived functional information, improving our functional analysis of the canine platelet proteome.

Discussion

In this report, we describe the canine platelet proteome and provide the first *in vivo* expression evidence of these proteins in the dog. We identified 2.4-fold more proteins than the most comprehensive platelet proteome previously reported [16].M The number of proteins we found is also higher than previous human platelet predictions of 2,000–3,000 proteins [17] and the most recent estimate of 5,000 proteins [18]. This is likely due to the fact that previous studies used two-dimensional electrophoresis for protein separation. This technique is commonly used for proteomics but has the disadvantage of low yields of membrane, basic, high molecular weight and some

signaling proteins [19,20]. Our non-gel-based mass spectrometry coupled with DDF ensured a comprehensive proteome.

The fact that the majority of the proteins are categorized as "unknown" in all three ontologies reflects that there are still a significant number of proteins for which we have little or no functional information. This is a recognized problem across a large range of species [21]; however, tissue expression data frequently provides the first clues to protein function and we report that these proteins are expressed in platelets.

The DDF technique offers unique advantages to protein extraction in that it does not disrupt cellular architecture. The first detergent used in DDF, ie, digitonin, efficiently isolates soluble cytosolic proteins due to how it interacts with cholesterol to form pores in the cell membrane. Triton X-100, the second sequential detergent used, solubilizes membrane and organelle proteins, while the third detergent used, ie, a combination of deoxycholate and Tween 40, extracts the soluble nuclear fraction. Sodium dodecyl sulfate is the final detergent used in DDF which primarily isolates the more insoluble proteins as well as nuclear matrix proteins [5]. Because of this, proteins isolated in different detergent fractions prior to mass spectrometry analysis can allow identification of protein subcellular localization.

In our platelet protein analysis, we observed up to a 4.9-fold increase in information about subcellular localization using DDF2GO for protein functional analysis compared with AgBase's GORetriever. Traditional protein functional analyses employ software such as GORetriever, which annotates proteins based on existing annotations for a given list of proteins. Not only does DDF2GO software factor in a protein's existing annotation, but also considers the detergent fraction(s) that the protein was extracted in as

well as the number of transmembrane domains if the given protein is a membrane protein. These additional considerations involved in annotation assignment by DDF2GO allow addition of knowledge as to where the protein came from and the proportion found in that subcellular location. This addition of experimentally derived functional information not only improves our functional analysis of the canine platelet proteome, but also confirms the suspicion that the knowledge of protein function and subcellular localization is incomplete. Use of DDF2GO allowed us to have improved coverage of subcellular localization data throughout the platelet.

Interestingly, there was a relatively high amount (7.15%) of proteins categorized as "nuclear", which at first seems counterintuitive, since platelets are anucleate, although this finding is in accordance with reports from other studies [22-24]. The presence of nuclear protein in platelets is not well understood and their possible role in platelet function is yet to be determined [22,25]. One of the explanations for this finding takes into account the nature of the GO terms and how functional modeling relies on biological knowledge; while proteins are annotated to the nucleus, they also have other subcellular localizations and the GO annotation is not context-dependent. In our data, 71% of the proteins annotated as "nuclear" were also found to have other annotations in the GO cellular component category. Moreover, nuclear proteins may represent, in part, contamination by residual leukocytes. However, we believe that was not an issue in our analysis, since our samples were of high purity (99.98% by manual count), in accordance with some of the few studies that actually address sample contamination [16, 23, 24]. Lastly, it has been stated that dog platelets can sometimes present fragments of nuclear

material [26]. Thus, considering this affirmation, the presence of nuclear components in platelet proteome analysis would be normal and expected.

Using DDF2GO software for platelet protein annotation analysis, the annotations we add are specific for proteins expressed in platelets. The nuclear category of GO terms and annotations are excluded from the output production. For the currently described rat and human platelet proteomes [16-18, 24], the technique for platelet protein analysis described here has not been used.

Conclusion

This initial study contributes to a broader understanding of canine platelet biology and aids functional research, identification of potential treatment targets and biomarkers, and sets a new standard for the resting platelet proteome.

Acknowledgments

The authors would like to acknowledge Tony Arick and Lakshmi Pillai, who developed the pipeline and GO CC mapping script, and Allen Shack for assistance with the DDF technique.

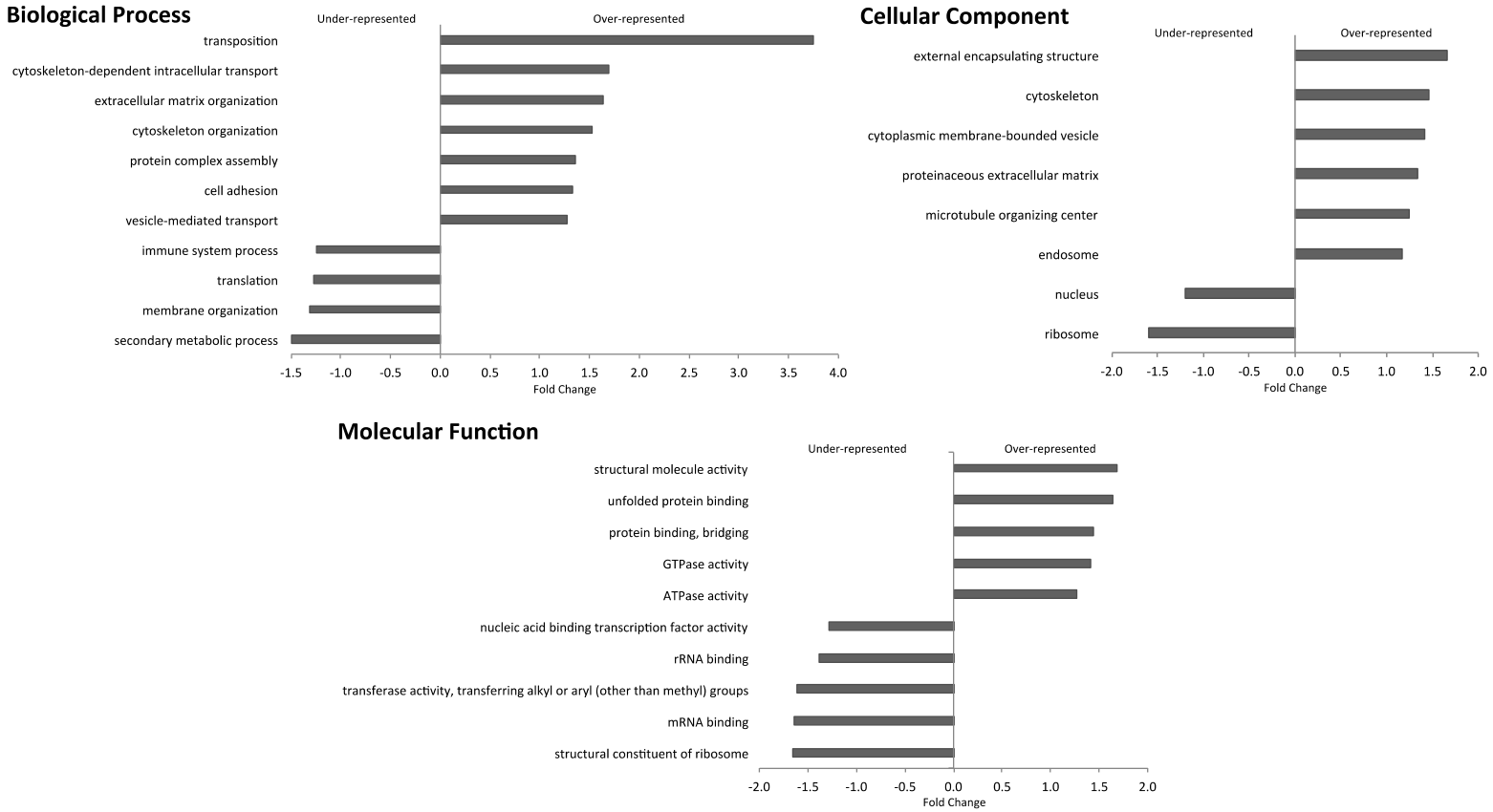

Figure 4.1 Canine platelet proteome GO categories compared with the canine genome.

The graphs show important under-represented and over-represented GO functions in canine platelets. The y-axis shows the categories within functional groups and the x-axis displays the relative difference between the percentages of the categories in the platelet proteome and the percentages in all canine GO annotations.

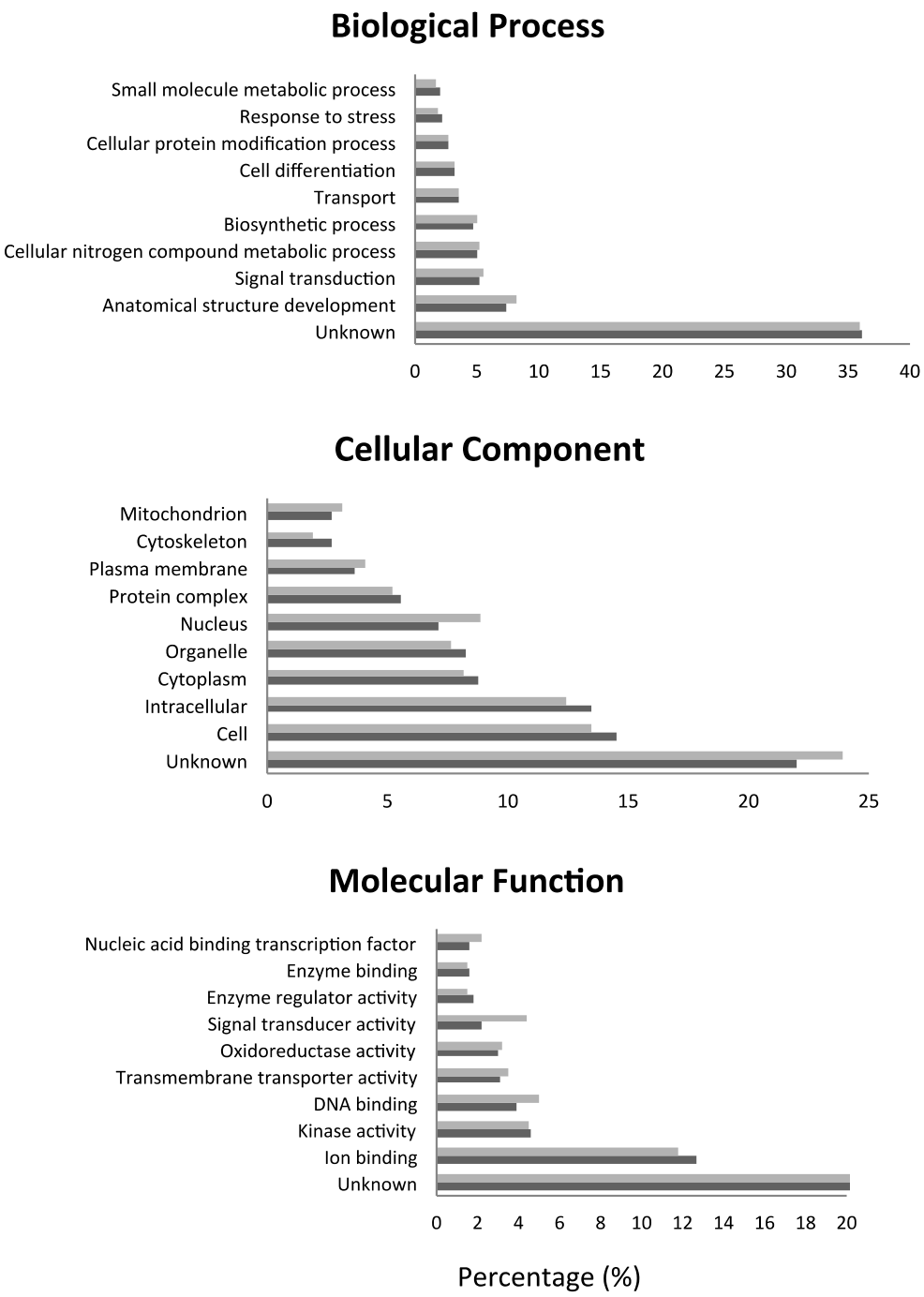

Figure 4.2 Top ten GO categories in resting canine platelets compared with all canine annotations.

Top ten GO categories in resting canine platelets (dark gray bars) compared with all canine annotations (light gray bars) for the three gene ontologies: biological process, cellular component, and molecular function. Categories are represented as percentages of the total.

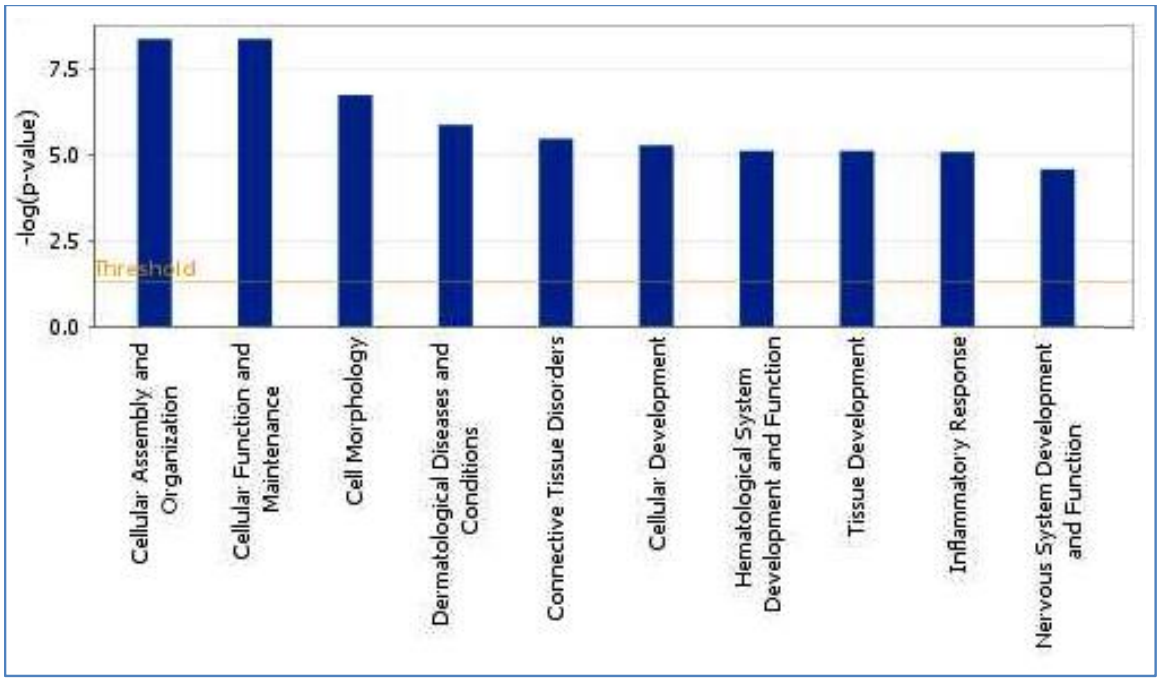

Figure 4.3 Ten most significant biological functions represented in resting canine platelets proteome.

The chart displays biological functions along the x-axis and significance in y-axis. The threshold line demonstrates the limit for significance ($P = 0.05$).

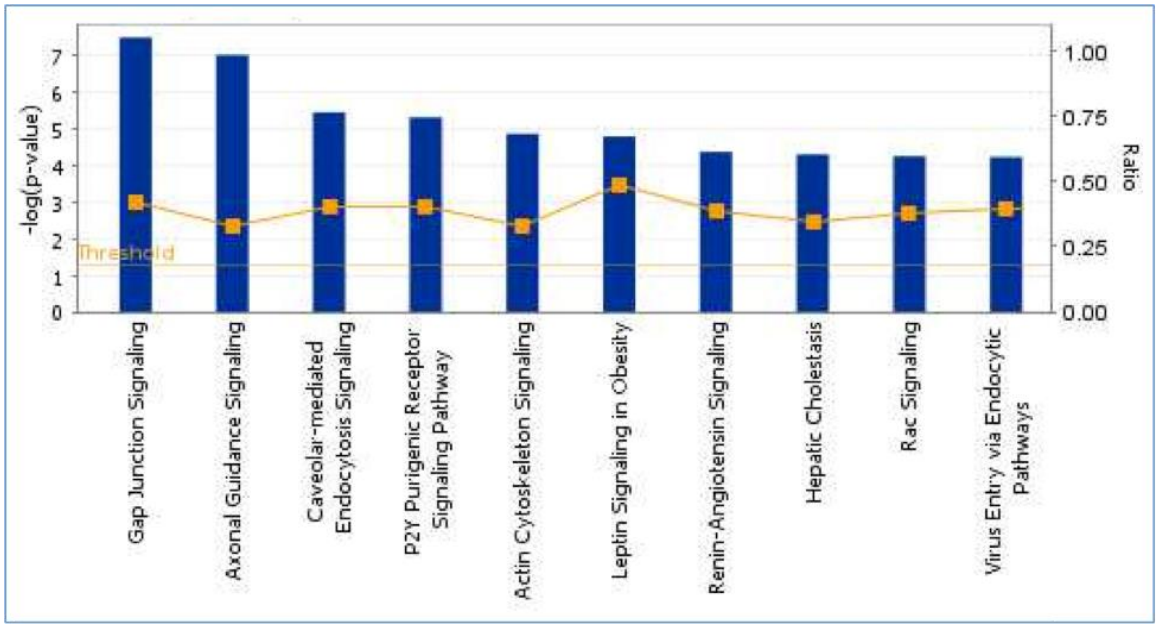

Figure 4.4 Ten most significant canonical pathways represented in resting canine platelet proteome.

Pathways are shown along the x-axis and the significance in the left y-axis. The threshold indicates the limit for significance ($P = 0.05$). The ratio is the number of proteins that meet the cutoff criteria divided by the total number of proteins in the pathway.

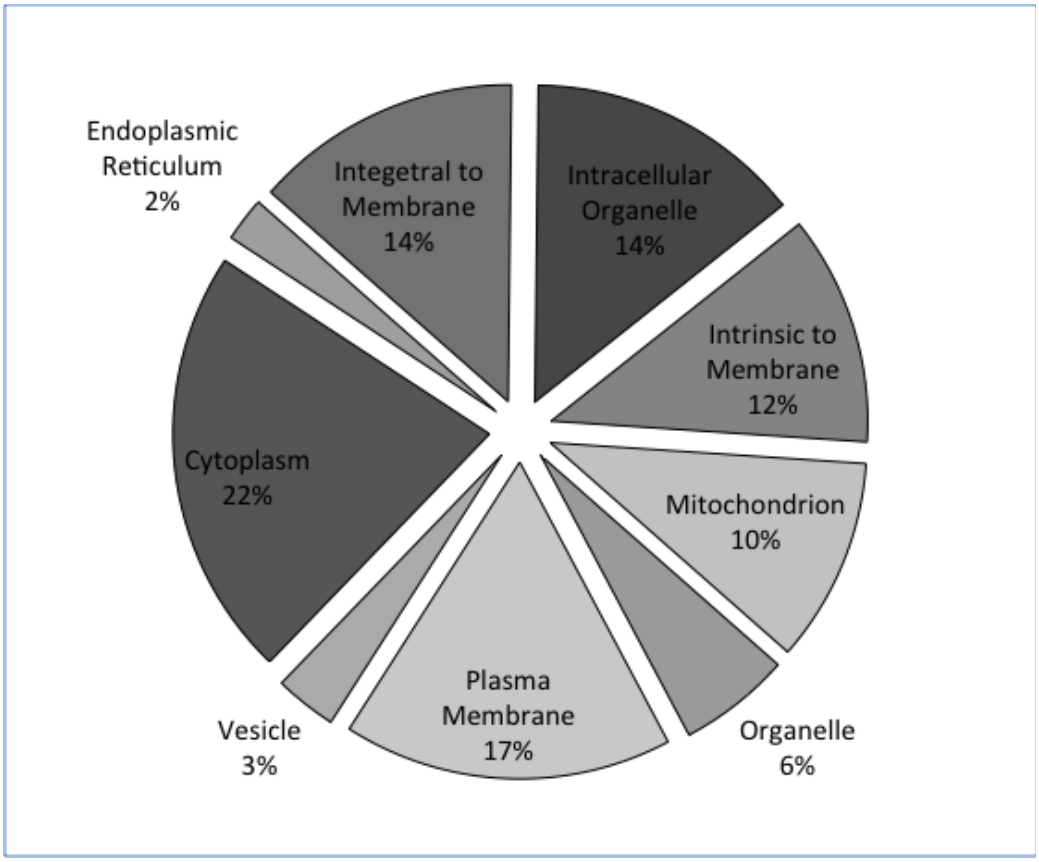

Figure 4.5 Subcellular localization of the platelet proteins found in more than one dog by DDF2GO.

The complete list of 1,803 platelet proteins from this study was used in determining the subcellular localization of the identified proteins using DDF2GO software. This figure shows the results for each of the identified categories as a pie chart. Each category and the relative percentage of total proteins present in that category are shown in the graph.

References

1. Nurden AT, Platelets, inflammation and tissue regeneration. Thromb Haemost, 2011. **105**(Suppl 1): p. 13–33.

2. Cervi D, Yip TT, Bhattacharya N, et al, Platelet-associated PF-4 as a biomarker of early tumor growth. Blood, 2008. **111**(3): p. 1201–1207.

3. MacEwen EG, Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Rev, 1990. **9**(2): p. 125–136.

4. Withrow SJ, Wilkins RM, Cross talk from pets to people: translational osteosarcoma treatments. ILAR J, 2010. **51**(3): p. 208–213.

5. McCarthy FM, Burgess SC, van der Berg BH, Koter MD, Pharr GT, Differential detergent fractionation for non-electrophoretic eukaryote cell proteomics. J Proteome Res, 2005. **4**(2): p. 316–324.

6. Craig R, Beavis RC, TANDEM: matching proteins with tandem mass spectra. Bioinformatics, 2004. **20**(9): p. 1466–1467.

7. Kessner D, Chambers M, Burke R, Agus D, Mallick P, ProteoWizard: open source software for rapid proteomics tools development. Bioinformatics, 2008. **24**(21): p. 2534–2536.

8. Rousseeuw PJ, Van Driessen K, A fast algorithm for the minimum covariance determinant estimator. Technometrics, 1999. **41**(3): p. 212–223.

9. Filzmoser P, Garrett RG, Reimann C, Multivariate outlier detection in exploration geochemistry. Comput Geosci, 2005. **31**(5): p. 579–587.

10. McCarthy FM, Gresham CR, Buza TJ, et al, AgBase: supporting func- tional modeling in agricultural organisms. Nucleic Acids Res, 2011. **39**(database issue): p. D497–D506.

11. McCarthy FM, Wang N, Magee GB, et al, Agbase: a functional genomics resource for agriculture. BMC Genomics, 2006. **7**: p. 229.

12. Pillai LR, Arick T, Burgess SC, McCarthy FM, A high-throughput experimentally based method for identifying subcellular localization. (Manuscript in preparation.)

13. Nakai K, Horton P, PSORT: a program for detecting sorting signals in proteins and detecting their subcellular localization. Trends Biochem Sci, 1999. **24**(1): p. 34–36.

14. Cserzo M, Wallin E, Simon I, von Heijne G, Elofsson A, Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng, 1997. **10**(6): p. 673–676.

15. Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC, Memili E, Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. BMC Syst Biol, 2008. **2**: p. 19.

16. O'Neill EE, Brock CJ, von Kriegsheim AF, et al, Towards complete analysis of the platelet proteome. Proteomics, 2002. **2**(3): p. 288–305.

17. Dittrich M, Birschmann I, Mietner S, Sickmann A, Walter U, Dandekar T, Platelet protein interactions: map, signaling components, and phosphorylation groundstate. Arterioscler Thromb Vasc Biol, 2008. **28**(7): p. 1326–1331.

18. Burkhart JM, Vaudel M, Gambaryan S, et al, The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. Blood, 2012. **120**(15): p. 73–82.

19. Maguire PB, Fitzgerald DJ, Platelet proteomics. J Thromb Haemost, 2003. **1**(7): p. 1593–1601.

20. Garcia A, Watson SP, Dwek RA, Zitzmann N, Applying proteomics technology to platelet research. Mass Spectrom Rev, 2005. **24**(6): p. 918–930.

21. Hanson AD, Pribat A, Waller JC, de Crecy-Lagard V. 'Unknown' proteins and 'orphan' enzymes: the missing half of the engineering parts list – and how to find it. Biochem J, 2009. **425**(1): p. 1–11.

22. Martens L, Van Damme P, Van Damme J, et al, The human platelet proteome mapped by peptide-centric proteomics: a functional protein profile. Proteomics, 2005. **5**(12): p. 3193–3204.

23. Qureshi AH, Chaoji V, Maiguel D, et al, Proteomic and phospho-proteomic profile of human platelets in basal, resting state: insights into integrin signaling. PLoS One, 2009. **4**(10): p. e7627.

24. Yu Y, Leng T, Yun D, et al, Global analysis of the rat and human platelet proteome – the molecular blueprint for illustrating multi-functional platelets and cross-species function evolution. Proteomics, 2010. **10**(13): p. 2444–2457.

25. Gnatenko DV, Perrotta PL, Bahou WF, Proteomic approaches to dissect platelet function: half the story. Blood, 2006. **108**(13): p. 3983–3991.

26. Stockham SL, Scott MA, Platelets. In: Fundamentals of Veterinary Clinical Pathology. 2nd ed. Ames, IA: Blackwell Publishing; 2008.

CHAPTER V

EFFECTS OF PLATELET LYSATE ON THE PROTEOME OF CANINE OSTEOSARCOMA AND MAMMARY TUMOR CELLS IN VITRO

Abstract

Background

Osteosarcoma and mammary tumors affect both humans and canines with high metastatic rates and poor prognosis in many cases. Most canine patients (and many human patients) have pulmonary metastases already in place at the time of diagnosis, which illustrates the importance of early detection of these neoplasms for earlier treatment initiation to improve outcome and prognosis overall. Platelets have been shown to have an active and important role in tumor cell growth and metastasis, long since expanding their function beyond just hemostasis. Limited evidence is present, though, concerning the specific roles and effects of canine platelets in cancer growth and progression. In this study, platelet lysate was incubated with three tumor cell lines (OSA8, OSA40, and CMT28) *in vitro*. Proteomic analysis was performed on the samples after protein isolation and mass spectrometry was performed.

Results

Multiple proteins were found to be differentially expressed for each of the cell lines including proteins that have been proven to have roles in tumor cell survival, growth, migration, and metastasis, as well as proteins involved in tumor cell apoptosis

and suppression. Some proteins were found identified to be up or down regulated for more than one tumor cell line.

Conclusions

Overall, the many of the proteins found to be differentially expressed after incubation of platelet lysate with canine osteosarcoma and mammary carcinoma cell lines suggest that platelets promote tumor cell growth and migration when compared to controls. In contrast, some proteins identified as having differential expression following platelet lysate incubation have anti-tumor and anti-metastatic functions. These contrasting findings further represent the dynamic nature of cancer itself in the battle between tumor survival and progression and the host response to combat the invading erroneous cells.

Introduction

Cancer is a disease that research has been devoted to tirelessly over the past century in an attempt to understand and counteract its wide range of effects that ultimately lead to high morbidity and mortality worldwide in both humans and animals. A novel theory has recently been accepted that cancer is not merely normal cells of the body that have gone rogue, but rather a disease caused by the body's inability to manage these erroneous cells – a disease of imbalance [1]. Regardless of species, the majority of deaths caused by cancer are due to metastasis (more than 90%). In the body, some tumor cells undergo a cascade of events starting with epithelial to mesenchymal transition (EMT) [2]. From this transition, these cells detach from the primary tumor site, enter into the vasculature, survive circulation despite shearing forces and immune system surveillance, and ultimately start a new tumor at a distant site within the body [3, 4].

Tumor cell invasion and metastasis are the main foundation of tumor biology that ultimately lead to death.

Osteosarcoma (OSA) is the most common bone tumor of both humans and canines with a notorious reputation for being highly aggressive in both species [5, 6]. In humans, with children and adolescents primarily being affected, distant metastasis occurs in 45% of cases regardless of treatment protocols utilized [7]. In canines, although the standard treatments including limb amputation/sparing, chemotherapy and radiotherapy have significantly increased survival rates, almost 90% of dogs will eventually develop predominantly pulmonary metastases [6]. The majority of these tumors occur in the appendicular skeleton of middle-aged large and giant breeds. Roughly 10,000 new cases of OSA are identified in dogs annually [6]. Prognosis remains poor, with average disease free intervals of 10 months and median survival times ranging from 3 months to 1 year. Less than 20% of dogs survive for more than 2 years following diagnosis [8, 9]. Since most cases already have pulmonary metastasis by the time of diagnosis (which may or not be visual on imaging), early detection is key in being able to treat and improve prognosis in these patients.

Some of the most common gynecological neoplasias in both humans and canines are mammary tumors [10, 11]. Malignant canine mammary tumors have been reported to have an incidence of up to 50% [12, 13]. Distant and local metastasis may occur in both human and canine malignant mammary tumors, and relapse often occurs following surgical excision [14]. Canine mammary tumor cells have successfully been used for years as *in vitro* models for human breast cancer research based on clinical similarities,

spontaneous occurrence, intraepithelial lesions, hormonal influences, and age of onset [15, 16, 17, 18].

Platelets have many well-known roles involving blood hemostasis, wound healing, inflammation, thrombus formation, and more recently recognized interactions with tumor cells and metastasis [19]. Thrombocytosis has long been associated and documented with neoplasias, particularly metastatic malignant neoplasms [20, 21]. These anucleate, disc-shaped cells contain three different types of granules, alpha granules, dense granules, and lysosomes [22], which have more recently been believed to have large roles in contributing to metastatic disease, beyond their more well-known roles in coagulation and hemostasis. Platelets have the ability to take up and store angiogenesis-related proteins into specific granules, making them potential sources of biomarkers for tumor dormancy and growth [23]. Alpha granules contain many proteins primarily associated with platelet adhesion and aggregation. Dense granules contain very small substances that aid in platelet recruitment when secreted. Lysosomes mainly have the task of releasing hydrolase enzymes that are utilized in the breakdown of platelet aggregates [21]. In addition to angiogenesis-modulating molecules, activated platelets have been shown to release factors that can modulate hemostasis, inflammation, and cell proliferation - all of which are necessary for tumors to make a transition from tumor dormancy to progression. In one study, the injection of tumor cells previously incubated for 48 hours with washed platelets resulted in increased pulmonary metastasis in mice, indicating that platelets seem to prime the tumor cells for metastasis [23]. Similarly, exposure of tumor cells to platelet-derived microparticles (vesicles that are shed from platelets after activation that contain several adhesive proteins expressed in activation)

also increased the metastatic potential of the tumor cells [24]. Many studies have shown that platelets promote metastasis of cancer by providing protection to tumor cells within the vasculature and cloak the tumor cells from immune surveillance through still undetermined mechanisms [26, 27, 28].

Limited proteomic information exists regarding canine platelet-tumor interactions and their effects. Our previous study mapped and described the normal canine platelet proteome [29]. Here we describe our proteomic findings on the effects of *in vitro* incubation of platelet lysate with three different tumor cell lines (OSA8, OSA40, CMT28).

Methods

Cell Lines and Culture

This experiment utilized two canine osteosarcoma cell lines (OSA8 and OSA40) established from dogs with spontaneously occurring osteosarcoma [30]. A canine mammary carcinoma cell line (CMT28) was established from dogs with spontaneously occurring mammary carcinoma [31]. Cell lines were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, New York, USA) containing 10% FBS (Gibco, ThermoFisher Scientific, USA), 0.2% Primocin (InvivoGen, San Diego, California, USA), and 1% HEPES buffer solution (Sigma-Aldrich) at 37ºF and maintained at 5% CO_2.

Platelet Purification

Whole blood was collected from three healthy female Walker hounds via jugular venipuncture with a 20-gauge needle using 3.2% sodium citrate anticoagulant. Special

care was taken on blood draw technique to prevent platelet activation. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements at a facility accredited by the American Association for Accreditation of Laboratory Animal Care. To maintain a ratio of 100 platelets per tumor cell, 20mL of blood was collected from each dog. Sample preparation was initiated within one hour of collection. Platelet-rich plasma (PRP) was obtained by spinning the blood at 1200g for 2 min with the brake setting at level 2 in 5mL Eppendorf tubes in a spinning bucket rotor. The top ¾ of PRP was collected and the protocol for PRP repeated. PRP was then layered over an Optiprep density barrier using the technique reported in Trichler et al 2013 [29]. Briefly, 3mL of PRP was placed over 5mL of a 1.063g/dL Optiprep density barrier in a 15mL Falcon tube. The tubes were then centrifuged at 350g for 15min in a spinning bucket rotor with the brake turned off and the temperature set to 20ºC. After spinning, 1mL of the 'platelet rich layer' was collected for further processing and lysis. The 'platelet rich layer' sits below the plasma layer and has a very opaque to white appearance around the 4-5mL mark on the 15mL conical centrifuge tube.

All samples were analyzed for platelet, leukocyte, and erythrocyte numbers using a hemocytometer (improved Neubauer, Hausser Scientific Co., Harsham, PA, USA). Platelets and erythrocytes were counted by diluting the final sample obtained after the Optiprep step 1:20 with phosphate buffered saline (PBS). To monitor for any residual leukocytes in the samples, a 1:2 dilution with Turk's solution was used to stain any leukocytes present. For all samples collected and purified, no leukocytes or erythrocytes were observed after staining. Cell counts were used to calculate the volume of each

sample needed to produce an overall ratio of 100 platelets per cancer cell for each experimental sample.

Platelet Lysate preparation

Platelet lysate was prepared using a sonicator (Q Sonica LLC, Newtown, CT, model Q125, probe model CL-18) on each Optiprep sample for 10 bursts of 5 seconds with 1 minute intervals between each burst. After lysis, samples were checked for residual intact platelets using a hemocytometer (improved Neubauer, Hausser Scientific Co., Harsham, PA, USA). Once residual platelets were no longer observed, samples were centrifuged at 10,000g for 10 minutes at 4ºC. Supernatants were pooled for all of the samples from the three different dogs. The pooled samples were used with the experimental samples *in vitro*.

Cell culture with platelet lysate

Cancer cells from each cell line (OSA8, OSA40, and CMT28) were harvested from T75 flasks (Thermo Scientific, Waltham, Massachusetts, USA) that were at 90-100% confluency and counted using Trypan Blue staining. One million cells were seeded into nine T25 flasks (Thermo Scientific, Waltham, Massachusetts, USA) and allowed to attach overnight in 10% FBS containing DMEM medium at 37ºC with 5% CO_2. The following day, existing medium was removed while leaving cells attached to the flask. Platelet lysate was combined with cell culture medium using the previously established volume to represent a ratio of 100 platelets per cancer cell (100,000,000 platelets). To prevent clot formation in the flasks due to lysate addition, 4U/mL Heparin (Sigma-Aldrich) was prepared with 10% FBS containing DMEM medium. For control samples,

Optiprep density barrier was added in place of platelet lysate at the same volume. Experimental samples for each cell line included 8 hour incubations of lysate and control samples as well as a zero hour control. All samples were run in triplicate.

After incubation, samples were trypsinized using 0.25% Trypsin-EDTA (Gibco, ThermoFisher Scientific, USA) and centrifuged at 450g for 5 minutes at room temperature. All samples were stained with Trypan Blue and cell recovery was estimated by determining cell counts with a Neubauer hemocytometer to evaluate cell recovery. Cell pellets were then washed three times using 10mL sterile PBS (Sigma-Aldrich) and centrifuged at 450g for 10 minutes at room temperature. The final pellet was resususpended in 1mL sterile PBS, pelleted at 450g for 10 minutes, and immediately frozen in liquid nitrogen after removal of the supernatant.

Sample Digestion

Protein pellets were solubilized in 100µL of 6M urea in 50mM ammonium bicarbonate (AMBIC). 200mM of dithiothreitol (DTT) was added to a final concentration of 5mM and samples were incubated for 30min at 37°C. Next, 20mM iodoacetamide (IAA) was added to a final concentration of 15mM and incubated for 30min at room temperature, followed by the addition of 20 µL DTT to quench the IAA reaction. Lys-C/trypsin (Promega) was next added in a 1:25 ratio (enzyme:protein) and incubated at 37°C for four hours. Samples were then diluted to <1M urea by the addition of 50mM AMBIC and digested overnight at 37°C. The following day, samples were desalted using C18 Macro Spin columns (Nest Group) and dried down by vacuum centrifugation.

LC-MS/MS Analysis

LC separation was done on a Waters Nano Acquity UHPLC (Waters Corporation) with a Proxeon nanospray source. The digested peptides were reconstituted in 2% acetonitrile /0.1% trifluoroacetic acid and roughly 3µg of each sample was loaded onto a 100 micron x 25 mm Magic C18 100Å 5U reverse phase trap where they were desalted online before being separated on a 75 micron x 150 mm Magic C18 200Å 3U reverse phase column. Peptides were eluted using a gradient of 0.1% formic acid (A) and 100% acetonitrile (B) with a flow rate of 300nL/min. A 120-minute gradient was run with 5% to 35% B over 100 minutes, 35% to 80% B over 8 minutes, 80% B for 1 minute, 80% to 5% B over 1 minute, and finally held at 5% B for 10 minutes. Each of the gradients was followed by a 1h column wash.

Mass spectra was collected on an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) in a data-dependent mode with one MS precursor scan followed by 15 MS/MS scans. A dynamic exclusion of 15 seconds was used. MS spectra were acquired with a resolution of 70,000 and a target of 1×10^6 ions or a maximum injection time of 30ms. MS/MS spectra were acquired with a resolution of 17,500 and a target of 5×10^4 ions or a maximum injection time of 50ms. Peptide fragmentation was performed using higher-energy collision dissociation (HCD) with a normalized collision energy (NCE) value of 27. Unassigned charge states as well as +1 and ions >+5 were excluded from MS/MS fragmentation.

Data Analysis

Scaffold (version Scaffold_4.5.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications

were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Scaffold then probabilistically validates these peptide identifications using the Peptide Prophet algorithm [31] and subsequently verifies peptide identifications assigned by SEQUEST, Mascot, and other search engines using the X!Tandem database searching program [32, 33]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [34]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The FASTA database used for peptide spectrum matching (target database) was the *Canis familiaris* RefSeq protein database from National Center for Biotechnology Information (NCBI). Proteins were also annotated with GO terms from NCBI (downloaded Feb 29, 2016) [35]. Cell proteins identified using mass spectrometry were analyzed using Scaffold 4.0 (Proteome Software, Portland, OR, USA) to generate peptide and protein lists using different filtering methods and statistical tests.

Conservative filter parameters were set for quantitative protein analysis – 99% protein threshold, minimum number of peptides at 2, and 95% peptide threshold. Statistical tests including ANOVA and Fishers Exact Tests run on each sample set to determine proteins/spectra of statistical significance. A Fisher's Exact test was used to calculate a *P*-value determining the probability that each biological function assigned to that data set is due to chance alone. All molecular functions and metabolic and signaling

pathways identified at a *P*-value of <0.05 were considered to be statistically significant via Scaffold Viewer version 4 software statistical analyses.

Results

Protein Identification

A total of 1632 proteins (376956 spectra) were identified from the OSA8 cell line samples, 1718 proteins (373864 spectra) for the OSA40 cell line, and 1856 proteins (372908 spectra) for the CMT28 cell line using conservative filtering parameters in Scaffold software for quantitative protein analysis as detailed in the Materials and Methods section.

Identification of differentially expressed proteins

Triplicate samples for each of the three tumor cell lines were analyzed for samples containing tumor cell lines only, tumor cells briefly exposed to platelet lysate before being washed, and tumor cells incubated with platelet lysate for eight hours, for a total of twenty-eight samples to be analyzed by mass spectrometry. The tumor cells were harvested and subjected to detergent fractionation and digestion before being run through LC-MS/MS. Entire protein lists are available in supplemental file 2. Using Scaffold software, protein spectra identified as having a significant difference ($P < 0.05$) between samples incubated with platelet lysate for 8 hours and those tumor cells incubated for 8 hours without any exposure to platelet lysate were identified for all three cell lines. Proteins that also were identified to be significantly different when comparing the platelet lysate exposed (but not incubated, 0h group) to the tumor cell control samples (no platelet lysate exposure) were separated out and are explained elsewhere in this manuscript.

Proteins identified as either significantly increased or decreased after incubation with platelet lysate are shown in Table 5.1 and illustrated in Figure 5.1 for each cell line studied.

For all cell lines studied (OSA 8, OSA 40, CMT 28), collagen alpha-1 (Accession number F1Q3I5_CANLF) was found to be present at significantly lower amounts in the samples incubated with platelet lysate for 8 hours. No other significant protein changes were found to be shared between the two OSA cell lines studied. Two proteins were found to have statistically significant differences in expression between the OSA8 and CMT28 cell lines, though. Collagen alpha-2 had decreased expression after incubation with platelet lysate and an 'uncharacterized protein' (Accession number F1PBI6_CANLF) was overexpressed in the platelet lysate incubated samples. This protein was later identified as being thrombospondin 1 via a Uniprot database search.

OSA8 cell line

After 8 hours of incubation with platelet lysate, seven proteins were identified as having a significant difference in total spectral count (discounting those that also had a significant difference at 0h exposure). Increases in protein expression of DNA topoisomerase 2 (2.9-fold increase), polypeptide N-acetylgalactosaminyltransferase (3.2-fold), thrombospondin 1 (8.2-fold), and small nuclear ribonucleoprotein polypeptide A (14-fold) were identified. Alternatively, decreases in collagen alpha-1 (12-fold decrease), 'uncategorized protein' (accession # E2RLJ3_CANLF; 5.3-fold), and collagen alpha-2 (62-fold) were identified.

OSA40 cell line

Ten proteins were found to be statistically significant in over or under expression for the OSA40 cell line samples incubated with platelet lysate for 8 hours. Expression of collagen alpha-1 (4-fold decrease), Nuclear Transport Factor 2 (1.3-fold), and NADH Dehydrogenase 1 subunit C2 (3-fold) were lower after 8 hours of OSA40 cell incubation with platelet lysate for 8 hours. In addition, levels of Plasminogen Activator Inhibitor 1 Precursor (16-fold increase), Fibronectin (12.7-fold), Proteasome subunit beta type (4.3-fold), Protein phosphatase (14-fold), Zinc finger CCCH-type (6.3-fold), Solute carrier family 3 (7-fold), and replication protein A2 (5.3-fold) were found to be significantly increased in samples after 8 hours of incubation with platelet lysate.

CMT28 cell line

Proteins that were increased in total spectra counted for the platelet lysate incubated samples included Thrombosponsin 1 (4.7-fold increase), Ubiquitin-conjugating Enzyme E2C (3.2-fold), Small Ubiquitin-related Modifier 2 (3.4-fold), and Nuclear Casein Kinase and Cyclin-dependent Kinase Substrate 1 (4.8-fold). Collagen alpha-1 (7.6-fold decrease), Collagen alpha-1 Type III (27-fold), Collagen alpha-1 Type V (3.9-fold), Collagen alpha-2 (15-fold), NIN1/RPN12 Binding Protein 1 homolog (6.5-fold), DEAH box polypeptide 15 (3.3-fold), and 'uncategorized protein' (access ion number F1PKU9_CANLF; 11-fold) all had decreased expression after incubation with platelet lysate. The identification for 'uncategorized protein' (F1PKU9_CANLF) in this sample was not present in different database searches, but GO analysis revealed GO terms associated including cytochrome-c oxidase activity, being an integral part to the cell membrane, and also involvement in hydrogen ion transmembrane transport.

Discussion

Collagen alpha-1 Type I was found to have significantly decreased expression in all three tumor cell lines studied after incubation with platelet lysate. Collagen alpha-1 is a fibrillar forming collagen classified as type 1 collagen important in development and differentiation of different aspects of the skeletal system including endochondral ossification, osteoblast differentiation, and intramembranous ossification as identified by GO analysis. Significant decreases in collagen alpha-2 Type I were found for the OSA8 and CMT28 cell line samples in platelet lysate incubated samples. Type 1 collagen is the archetypal collagen with its primary role being in tissue [36]. The extracellular matrix (ECM) is an important component of the tumor microenvironment, with collagen being the most abundant constituent. Traditionally the ECM and its components were thought of as a scaffold system that formed a generalized barrier against tumor spread. More recently, though, studies have shown that ECM is able to elicit signaling with effects on cell migration, cell adhesion, tissue repair and morphogenesis, and angiogenesis [37]. Studies have also shown changes in the ECM in response to cancer – notably a down regulation in ECM proteolysis compared to the tight regulation seen under normal circumstances [38, 39]. Interestingly, studies have shown that either an increased or decreased deposition of collagen can have an association with increased malignancy [40, 41]. During cancer invasion, changes are made within the tumor stroma including collagen degradation, re-deposition, cross-linking, and stiffening, which ultimately encourages tumor progression by destabilizing cell polarity and cell-to-cell adhesions [1]. In our samples, the decrease seen across all three cell lines after platelet incubation could suggest decreased collagen production or increased degradation taking place in response

to tumor cell exposure. The exposure of platelet proteins and components present in the lysate over an extended period of time (8 hours) provided the tumor cells with the ability to overall lead to a decrease in the amount of collagen alpha-1 fragments detectable – the mechanisms include an increase in degradation or a decreased production of collagen.

Lysyl oxidase (LOX) is a critical enzyme involved in remodeling of the ECM, and is synthesized either by stromal cells in early carcinogenesis or hypoxic tumor cells during late stage progression [42, 43]. LOX contributes to collagen cross-linking, increasing matrix deposition, and increasing tissue stiffness, which in turn enhances tumor cell adhesion and migration via activated integrins. LOX has been identified as essential for driving tumor cells to escape from primary tissue sites extravasate, and grow at secondary sites [44, 45, 46]. Other studies have shown that LOX introduces EMT (endothelial to mesenchymal transition) and promotes metastatic dissemination in aggressive and poorly differentiated tumors [47]. In our study, all three cell line samples contained LOX (Accession number J9NZK5_CANLF). In the OSA8 samples, an ANOVA test revealed that there were significantly greater LOX proteins present in the 0h platelet 'exposed' samples when compared to the 8h and control samples. Although not statistically significant for the other cell lines, similar trends were observed for the LOX protein in the 0h samples. This could suggest a decreased production of LOX after exposed to the platelet proteins/lysate. LOX has been shown to work synergistically with MMPs (matrix metalloproteinases) to aid tumor progression via ECM remodeling [1], but MMP proteins were not able to be identified in our samples after searching for various accession numbers associated with different MMPs.

Fibronectin (Accession number F1P6H7_CANLF) was identified in OSA40 and CMT28 samples. An ANOVA test revealed a significant increase in the expression of fibronectin for samples incubated with platelet lysate for 8 hours when compared to the 0h and Control samples. Although not significant in the CMT28 cell line, a similar trend was present. Fibronectin has been discovered to have a role in early stages of cancer metastasis, as it has roles in mediation of collagen reorganization [48, 49].

Two proteins were found to be statistically different in both the OSA8 and CMT28 cell lines. The first protein, collagen alpha-2 (Accession Number: F1PHY1_CANLF), was significantly underexpressed in the OSA8 and CMT28 samples after platelet lysate incubation. Like collagen alpha-1, collagen alpha-2 is a type 1 collagen that has a fibrillar formation function. Functions including skeletal system development, blood vessel development, involvement in the TGF-b signaling pathway, blood pressure regulation, organization of collagen fibrils, protein binding, and PDGF binding were identified on GO analysis of the protein. The other protein was an "Uncharacterized protein" (Accession number F1PBI6_CANLF) upon initial identification, but was able to be identified as Thrombospondin 1 (TSP1) after a database search in UniProt. Thrombospondin 1 had increased expression in the OSA8 and CMT28 cells after 8 hours of platelet lysate exposure. TSP1 is a protein that can be found within platelet alpha granules and is released upon platelet activation. More recent studies have illustrated the anti-angiogenic function and effect of TSP1 on different types of tumor cells - overall representing a host response to attempt to prevent the angiogenic switch by tumor cells [50]. Interestingly, though, the 0h control samples did not see a significant change in TSP1 levels after being exposed to, but not incubated with, the same platelet

lysate, therefore eliminating the possibility of activated platelets having been used for the samples. The TSP1 thus appears to have had a tumor cell line source.

OSA8 up-regulated proteins

In OSA8 cell line samples, increased expression of topoisomerase II, polypeptide N-acetylgalactosaminyltransferase, small nuclear ribonucleoprotein polypeptide A (as well as the previously mentioned thrombospondin-1) were identified after incubation with platelet lysate. Topoisomerase II (TOPII) has an important function in the unwinding of DNA during replication and transcription, via its ability to cut a single strand of DNA's phosphate backbone. This allows unwinding to take place, therefore preventing overwinding ahead of the replication fork which would ultimately halt the actions of the DNA or RNA polymerases. In recent years, it has been discovered that the actions of chemotherapeutic agents such as doxorubicin and etoposide [51] specifically target DNA Topoisomerase II to either promote formation of DNA strand breaks within the tumor cells (TOPII poisons) or to prevent the catalytic activity of TOPII (catalytic inhibitors) [52]. The reason as to why this protein would have increased expression after incubation with platelet lysate is unclear. In previous publications, increased levels of polypeptide N-acetylgalactosaminyltransferase was found to be significantly correlated with shorter progression-free survival intervals in patients with ovarian cancer as opposed to experiments in which the gene for polypeptide N-acetylgalactosaminyltransferase was suppressed [53]. Small nuclear ribonucleoprotein polypeptide A is associated with transcription and protein biosynthesis and has previously been reported to be associated with tumors including hepatocellular carcinomas [54]. Increases of this protein in samples of OSA cells after incubation with platelet lysate could indicate the progression

of tumor cells towards replication and growth. In addition to the decreased collagen alpha-1 seen for all three cell lines, decreased protein expression of an 'uncategorized protein' (Accession number E2RLJ3_CANLF) and collagen alpha-2 were identified for the OSA8 cell line samples after incubation with platelet lysate. No additional information could be determined about the protein identified with accession number E2RLJ3_CANLF upon multiple database searches. Details about the collagens identified, including this one, were detailed previously in the discussion section.

OSA8 down-regulated proteins

Three proteins (collagen alpha 1 type 1, uncategorized protein (accession number E2RLJ3_CANLF), and collagen alpha 2 type 1) were identified as being significantly decreased in samples after platelet lysate incubation took place as compared to the control samples. Both of these collagens were discussed in greater detail above. Information is limited in the literature regarding the uncategorized protein with accession number E2RLJ3_CANLF (gene name LSM12), but there are reports of this protein playing an active role in translation machinery, and the protein has been localized to stress granules (cytoplasmic mRNP granules) [55, 56]. Suggested roles of LSM12 may include mRNA degradation or tRNA splicing [57]. Current literature does not describe a role of LSM12 with regards to any type of neoplasia or tumor cell line. In our study, a decrease in the amount of LSM12 present after incubation with platelet lysate could suggest a decreased cellular stress response.

OSA40 up-regulated proteins

Many proteins (Plasminogen activator inhibitor 1 precursor, Fibronectin,

Proteasome subunit beta type 4, Mg/Mn dependent protein phosphatase 1F, Zinc-finger CCCH-type containing 4, Solute carrier family 3, and Replication protein A2) were identified as having significantly increased expression in the OSA40 cell line after platelet lysate incubation. Plasminogen activator inhibitor 1 (PAI-1) is a well-studied protein due to its numerous physiologic effects including inhibition of fibrinolysis, cell migration, cell signaling, and, more recently, effects on tumor development [58]. PAI-1 can be produced by both normal, nonmalignant cells as well as tumor cells. PAI-1 has been shown to have a dose-dependent proangiogenic activity associated with a negative prognosis when elevated in human cancer patients [59, 60, 61, 62]. Various studies have illustrated that PAI-1 can also protect and prevent various cancer cells from apoptosis as well [63, 64, 65]. In mouse studies, suppression of PAI-1 led to poor tumor uptake by the mice and slower tumor growth [63, 66, 67, 68]. Increased expression of PAI-1 by OSA40 cells after incubation with platelet lysate suggests that platelet proteins had a pro-tumorigenic effect on the cells *in vitro* as compared to cells not exposed to any platelet proteins or those simply washed with the proteins but not incubated for a time period.

Fibronectin is an ECM protein that has multiple physiologic roles in wound healing, neovascularization, and angiogenesis, as well as pathologic roles including fibrosis and cancer [69]. Studies have shown increased concentrations of fibronectin in the presence of tumors, especially within the tumor stroma [70, 71, 72]. Platelets have been shown to enhance the assembly of fibronectin via their lysophosphatidic acid which mediates Rho-activated stress fiber formation and enhances fibronectin matrix assembly [73, 74]. Fibronectin undergoes conformational changes and alternative splicing which ultimately leads to promotion of tumorigenesis and neovascularization for metastases

[69]. Fibronectin had a statistically significant increase in protein expression for OSA40 cells following platelet lysate incubation, which is in agreement with previous research. The presence of platelet lysate had a directly positive impact on the production of fibronectin in these samples.

Proteasome subunit beta type 4 (PSMB4) has been established to be responsible for assembly of the proteasome. Proteasomes function to degrade various proteins via proteolysis which is a feature required in numerous proliferative and anti-apoptotic pathways by tumors [75, 76, 77, 78]. Proteosomes have therefore been targeted in therapeutic research with current therapies against the PSMB5, PSMB6, and PSMB7. Targeting PSMB4, though, would potentially prevent catalytic activity of all three of these subunits, making it a novel potential therapeutic target [79, 80, 81]. Zheng et al recently demonstrated the overexpression of PSMB4 in human multiple melanoma plasma and cell lines as well as positive correlation between upregulation of PSMB4 on cell proliferation and colony formation [82]. In our canine OSA40 cell samples, incubation with platelet lysate resulted in an increased expression of PSMB4, suggesting a pro-tumor effect on the OSA40 cells based on this finding and previous research published on the effects of PSMB4 in tumor progression.

Mg/Mn dependent protein phosphatase 1F (PPM1F) is a serine/threonine phosphatase that has been found to be overexpressed in invasive forms of human breast cancer cells [83]. PPM1F promotes cell migration and metastasis as described by Susila et al [84]. Silencing of the PPM1F gene resulted in reduced cell migration and invasiveness [85]. In our samples, OSA40 cells incubated with platelet lysate had an increased expression of PPM1F as compared to other control samples. This finding fits

with platelet proteins having a pro-tumor effect overall on the OSA40 cells via promoting up-regulation of this protein associated with cell migration and metastasis.

The protein "Zinc Finger CCCH-type Containing 4" was found to have increased expression in OSA40 cells after incubation with platelet lysate. Specific details regarding the gene name (ZC3H4) in association with tumor cells was not evident in the literature, but a similar protein that has similar translational properties in the cell, Zinc finger CCCH-type containing 15 (ZC3H15), increases hepatocellular carcinoma (HCC) growth and overall suggests a poor prognosis when elevated following surgical resection [86]. Increased expression levels of ZC3H4, a similar protein within the same family as ZC3H15, shows a potential role of this protein in having similar effects as well as being up-regulated by the presence of platelet proteins, promoting tumor growth overall.

RPA2 (Replication Protein A2) upregulation in breast cancer cell lines has been shown to promote cell proliferation, adhesion, migration, invasion, as well as induction of EMT [87]. This protein has increased expression in OSA40 cells after incubation with platelet lysate *in vitro*, suggesting platelet proteins promote these pro-neoplastic functions for this particular cell line.

OSA40 down-regulated proteins

Decreased protein expression of NADH Dehydrogenase (ubiquinone) 1 subunit C2 and an ‘uncategorized protein’ (Accession number J9P558_CANLF) were identified in samples from the OSA40 cell line. The ‘uncategorized protein’ was identified as nuclear transport factor 2 (NTF2) after searching via UniProt database. NTF2 is a protein that promotes efficient nuclear import by a cell through its ability to bind and recycle RanGDP. NTF2 translocates RanGDP back into the nucleus for conversion to RanGTP to

be used to release imported molecules bound to importin proteins [88, 89, 90]. Recent reports in the literature have shown a correlation between decreased NTF2 expression and tumor progression associated with increased nuclear size. In addition, increases in NTF2 levels found in melanoma cells resulted in decreased nuclear size [91]. The decreased expression of NTF2 by OSA40 cells after being incubated with platelet lysate suggests that the platelet proteins had an inhibitory effect on the production of NTF2 by the tumor cells, therefore creating a 'protumor' environment for the tumor cells by reduced inhibition of nuclear growth. NADH dehydrogenase (to be abbreviated using gene name: NDUFC2) has a critical role in cell physiology within the mitochondria. NDUFC2 transports electrons via NADH oxidation and then reduces ubiquinone [92, 93]. In human research, NDUFC2 has been associated with many neonatal diseases as well as malignancies including breast, colon, and thyroid cancers [94, 95, 96, 97]. Igci et al reported down regulation of the NDUFC2 gene in thyroid carcinomas [98], while amplification in the NDUFC2 gene region correlated with worse prognosis in estrogen receptor negative breast cancer [99]. In our OSA40 samples, the platelet lysate caused an interesting down regulation in the expression of NDUFC2. This effect was not repeated (not statistically significant) in the other two cell lines studied, suggesting a specific effect with the OSA40 cells alone. Research is still needed on the details and trends associated with NDUFC2 and different neoplasms since current literature shows various results for this protein and gene expression.

CMT28 up-regulated proteins

The CMT28 cells incubated with platelet lysate had significantly increased amounts of four proteins (thrombospondin-1, ubiquitin-conjugating enzyme E2C, small

ubiquitin-related modifier 2, nuclear casein kinase and cyclin-dependent kinase substrate 1). The function of ubiquitin-conjugating enzyme E2C (UBE2C) in the M phase of the cell cycle contributes to cell cycle progression as well as genetic stability [100]. Normally present in low levels in healthy tissues, increased expression and abundance has been found for several different tumor types including breast carcinomas with a high suggestive role in cancer progression [101, 102]. Nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) is a protein that has been identified as having an important role in cell cycle progression and over-expression of NUCKS1 has been identified in several different cancers [103, 104, 105, 106, 107, 108]. In our study, platelet proteins appear to play a role in increasing expression of both UBE2C and NUCKS1 in CMT28 cells, which may promote tumor cell proliferation and progression. Small ubiquitin-related modifier 2 (SUMO3) is a protein involved in a process known as sumolyation – an important post-translational modification – that has been associated with the development of HCC [109]. Some studies have shown that over-expression of SUMO proteins caused a decreased proliferation of tumor cells with little effect on migration (same). In contrast, a study by Yang et al illustrated that silencing SUMO protein expression in glioblastoma cells led to blocked DNA synthesis, cell growth, and clonogenic cell survival [110]. In our samples of CMT28 cells incubated with platelet lysate for 8 hours, significant increases in SUMO3 protein were detected. Based on the majority of other increased protein expression and their associations with tumor cell survival, this finding seems to fit with the pro-tumor function of SUMO3 and other SUMO proteins identified in some studies in the literature and further suggests that platelet proteins have a positive effect on tumor cells to allow increased expression to

take place.

CMT28 down-regulated proteins

Incubation with platelet lysate resulted in a significant decrease in expression of seven proteins (collagen alpha 1 type I, collagen alpha 2 type I, collagen alpha 1 type III, collagen alpha 1 type V, uncategorized protein (accession number F1PKU9_CANLF), NIN1/RPN12 binding protein 1 homolog, DEAH (Asp-Glu-Ala-His) box polypeptide 15) in samples using the CMT28 cell line. The associations with collagen in the tumor microenvironment and growth were previously discussed. Interestingly, silencing and down regulation of NOB1 (NIN1/RPN12 binding protein 1 homolog) has been shown to inhibit growth and proliferation of various tumor cells [111, 112]. NOB1 is closely related to regulation of the cell cycle and transcription with involvement in many different tumors. Recently, specific targeting of NOB1 resulted in inhibition of cell growth in gastric cancer [113]. Decreased expression of NOB1 in our CMT28 samples after platelet lysate incubation suggests platelet proteins having an inhibitory role in this case by causing a down regulation of this protein that has been found to have tumor-promoting properties. DEAH (Asp-Glu-Ala-His) box polypeptide 15 (DHX15) is a member of the DEXD/H box helicase family that plays important roles in the immune system response to viral infection. Increased expression led to inhibition of proliferation and metastasis in gastric cancer in a study by Xiao et al [114]. In our CMT28 cell samples incubated with platelet lysate, decreased expression of DHX15 illustrates effects of platelet proteins that promote tumor cell proliferation and metastasis by decreasing amounts of this inhibitory protein found.

Conclusions

Overall, most of the changes seen in the proteins found to have increased or decreased expression after incubation with platelet lysate have previously been established in the promotion of tumor cell growth, cell migration, and metastasis. Many of the proteins in our study that were present at statistically higher levels following incubation with platelet lysate for 8 hours have been studied or noted for their roles in aiding tumor cell survival and the progression of cancer. In addition, many of the proteins found to be down regulated or have decreased expression after incubation with platelet lysate were often proteins previously shown to have inhibitory or negative effects on tumor cell survival. Some proteins (collagen alpha 1, collagen alpha 2, thrombospondin 1) shared the same significant up- or down-regulation for more than one tumor cell line. This finding could suggest that similar changes are seen in protein expression across different types of tumors including sarcomas and carcinomas.

In contrast, there were two notable proteins, thrombospondin 1 and NIN1/RPN12 binding protein 1 homolog, whose changes following platelet lysate incubated fit more with a negative response to tumor cell growth, survival, and metastasis based on previous findings in the literature in their roles and trends with various cancers. These negative tumor cell responses were both seen in the CMT28 cell line samples while an increase in thrombospondin 1 (an anti-tumor response) was also seen in the OSA8 cell line. An increased occurrence of this response in the CMT28 cell line could suggest that mammary tumors are more easily subjected to the protective and host response factors present in platelets themselves along with the other proteins and factors that can aid tumor cells in their development and progression – overall leading to a mixed response of

the tumor cells to the platelet contents. In addition, this finding could also suggest that perhaps osteosarcoma cells are more resistant to these host response protective measures and more easily favor the pro-tumor effects from the platelet contents provided. This could also fit clinically as osteosarcomas are such aggressive tumors with rapid metastasis and poor prognosis. In addition to these theories, the nature of the conflicting responses in protein expression and tumor survival effect could be due to the exposure of the tumor cells to the entire platelet content rather than particular components selectively released if the platelets were intact and in an *in vivo* setting.

Extended time (8 hours in this case) exposed to platelet proteins leads to various changes and responses of tumor cells in the proteins that they produce. The majority of the protein changes identified as significant were those that had an overall positive effect on tumor survival and metastasis based on current literature findings for each of the significant proteins discussed. Most proteins found to be increased following platelet lysate incubation promote tumor cell survival and migration. Most of the proteins that were found to have decreased production or were suppressed following incubation with platelet lysate inhibit or prevent tumor cell survival and progression. Merely exposing the tumor cells to, but not incubating them, with the platelet lysate did not produce the significant changes in protein expression. This indicates that the changes produced require time for the platelet contents to be in contact with the tumor cells and their environment.

This information is in agreement with other research illustrating the important and complex roles that platelets play in the tumor microenvironment, tumor growth, and metastasis by promotion of cell migration and EMT. Interpretation of results, however,

must take into consideration the appropriate use of statistical tests in the large data sets seen with proteomics, and the P-values used. In our case, a P-value of 0.05 was used to determine which findings were significant or not. While a P-value of 0.05 provides a 95% confidence that observed changes in proteins are, in fact, correctly identified and significant, there is a 1 in 20 chance that each of these results are in fact random occurrences. In order to confirm these results and to identify those proteins that are truly significantly altered, further studies would be required. One of the simplest confirmatory tests would be to test the findings' repeatability via repeating the experiments, to see if the same proteins were identified as significantly increased or decreased in levels of expression following incubation with platelet lysate. Alternatively, simply changing the way in which the data is presented could offer increased confidence in the significance of the findings. An example of this would be to consider the P-value for each individual data point rather than looking at all of the proteins that fall within the P-value <0.05 category. Observing data for their individual P-values would mean that a P-value of 0.0001 would be more likely to be represent a real change that a P-value 0.001, which is still more likely to indicate a real effect than a P-value of 0.05. The proteins could be ranked based on the 'weight' of their significance associated with their specific P-value. In addition, further detailed studies could be conducted looking at specific protein levels and expression in smaller studies, using different methodologies such as immunofluorescent assays (IFA) or other similar methods.

Interestingly, our study found that not all protein changes fit with an overall pro-tumor response. This suggests and further illustrates the dynamic nature of cancer itself, and how there are multiple mechanisms occurring in response to the proteins and

contents found within and on platelets, therefore potentially producing ambiguous responses following platelet exposure. This information supports various findings within the literature illustrating the various different functions of proteins found within the different platelet granules, as well as differential release of platelet granular content based on the stimulus at hand. In this case, the entire platelet was lysed, exposing the tumor cells to all the contents present within and making up the platelet itself, including those proteins that may be pro- or anti-tumor in nature that would not be in contact with the tumor cells in all *in vivo* situations.

Further analyses or experiments are needed to confirm the value of the findings described here. Overall, though, it does appear that canine platelets stimulate changes in protein expression of various canine tumor cell lines.

Declarations

Acknowledgements

Protein digestion and mass spectrometry performed by the lab of Dr. Phinney at UC Davis.

Table 5.1 Significantly different proteins following incubation with platelet lysate.

Protein	Accession Number	Gene Name	OSA8	OSA40	CMT28
Collagen α1	CO1A1_CANLF	COL1A1	↓	↓	↓
Uncharacterized Protein	E2RLJ3_CANLF	LSM12	↓		
DNA Topoisomerase IIα	F1Q2M4_CANLF	TOP2A	↑		
Polypeptide N-acetylgalactosaminyltransferase	F1P835_CANLF	GALNT2	↑		
Thrombospondin 1	F1PBI6_CANLF	THBS1	↑		↑
Small Nuclear Ribonucleoprotein Polypeptide A	F1PFC6_CANLF	SNRPA	↑		
Collagen α2	F1PHY1_CANLF	COL1A2	↓		↓
Plasminogen Activator Inhibitor 1 Precursor	F1PUI4_CANLF	SERPINE1		↑	
Fibronectin	F1P6H7_CANLF	FN1		↑	
Proteasome subunit β-type	E2RBR6_CANLF	PSMB4		↑	
Nuclear Transport Factor 2	J9P558_CANLF	NUTF2		↓	
Protein Phosphatase, Mg2+/Mn2+ dependent	E2R912_CANLF	PPM1F		↑	
Zinc finger CCCH-type	E2RSL2_CANLF	ZC3H4		↑	
Solute Carrier Family 3	F1PRC5_CANLF	SLC3A2		↑	

Table 5.1 (Continued)

Replication Protein A2	E2RQP7_CANLF	RPA2	⬆	
NADH Dehydrogenase 1 subunit C2	E2RLB6_CANLF	NDUFC2	⬇	
Collagen α1 Type III	F1PG69_CANLF	COL3A1		⬇
Collagen α1 Type V	F1PHX8_CANLF	COL5A1		⬇
Ubiquitin-conjugating enzyme E2C	E2RN95_CANLF	UBE2C		⬆
Small Ubiquitin-related Modifier 2	E2RGN5_CANLF	SUMO3		⬆
Uncategorized Protein	F1PKU9_CANLF	N/A		⬇
NIN1/RPN12 Binding Protein 1 Homolog	E2RBA4_CANLF	NOB1		⬇
Nuclear Casein Kinase and Cyclin-dependent Kinase Substrate 1	F1P9H9_CANLF	NUCKS1		⬆
DEAH (Asp-Glu-Ala-His) Box Polypeptide 15	E2R2U3_CANLF	DHX15		⬇

This table lists all proteins found to be significantly increased or decreased in expression following incubation with platelet lysate for 8 hours as compared to control samples. The protein name, accession number, associated gene name, and an arrow representing increased (⬆) or decreased (⬇) expression is shown.

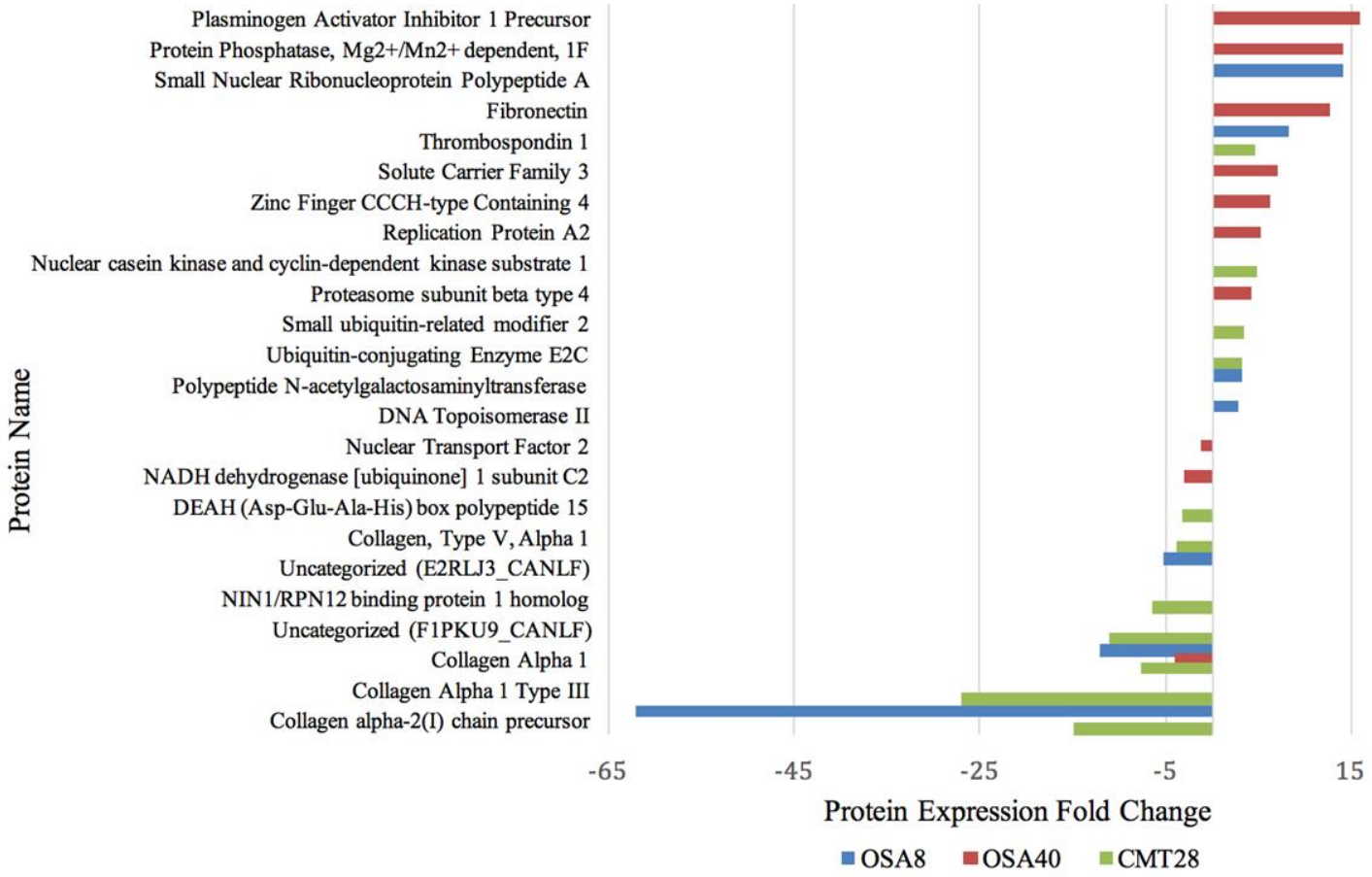

Figure 5.1 Significant increased or decreased protein fold changes following platelet lysate incubation.

This graph shows the proteins found to be significantly increased or decreased following incubation with platelet lysate for 8 hours in vitro for each of the three tumor cell lines used in the study. The y-axis shows the name of the protein and the x-axis displays the fold change difference for protein expression in the samples incubated with platelet lysate for 8 hours as compared to the control samples.

References

1. Fang M, Yuan J, Peng C, Li Y, Collagen as a double-edged sword in tumor progression. Tumour Biol, 2014. **35**(4): p. 2871-2882.

2. Lou XL, Sun J, Gong SQ, Yu XF, Gong R, Deng H, Interaction between circulating cancer cells and platelets: clinical implication. Chin J Cancer Res, 2015. **27**(5): p. 450-460.

3. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, et al, Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med, 2004. **351**(8): p. 781-791.

4. Yang J, Weinberg RA, Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell, 2008. **14**(6): p. 818-829.

5. Ritter J, Bielack SS, Osteosarcoma. Ann Oncol, 2010. **21**(Suppl 7): vii320–5.

6. O'Donoghue LE, Ptitsyn A, Kamstock DA, Siebert J, Thomas RS, Duval DL, Expression profiling in canine osteosarcoma: identification of biomarkers and pathways associated with outcome. BMC Cancer, 2010. **10**: p. 506.

7. Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM, Genome-wide analyses on high-grade osteosarcoma: making sense of a genomically most unstable tumor. Int J Cancer, 2013. **133**: p. 2512–21.

8. Selvarajah GT, Kirpensteijn J, Prognostic and predictive biomarkers of canine osteosarcoma. Vet J, 2010. **185**(1): p. 28-35.

9. Bacon NJ, Ehrhart NP, Dernell WS, Lafferty M, Withrow SJ, Use of alternating administration of carboplatin and doxorubicin in dogs with microscopic metastases after amputation for appendicular osteosarcoma: 50 cases (1999-2006). J Am Vet Med Assoc, 2008. **232**(10): p. 1504-1510.

10. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D, Global cancer statistics. CA Cancer J Clin, 2011. **61**(2): p. 69–90.

11. Misdorp W, Tumours of the mammary gland. In: Meuten DJ (eds). Tumors in Domestic Animals. Iowa: Iowa State Press; 2002. p. 575–606.

12. Brodey RS, Goldschmidt MH, Roszel JR, Canine mammary gland neoplasms. J Am Anim Hospital Assoc, 1983. **19**: p. 61-90.

13. Hamilton JM, Comparative aspects of mammary tumors. Adv Cancer Res, 1974. **19**: p. 1-45.

14. Owen LN, A comparative study of canine and human breast cancer. Invest Cell Pathol, 1979. **2**(4): p. 257–275.

15. Chrisp CE, Spangler WL, The malignant canine tumour as a model for the study of human breast cancer. In: Shifrine M, Wilson FD (eds). The Canine as a biomedical research model: Immunological, hematological and oncological aspects. Washington DC: National Technical Informaion Center, Department of Energy; 1980. p. 331-349.

16. Owen LN, Morgan DR, Bostock DE, Flemans RJ, Tissue culture and transplantation studies on canine mammary carcinoma. Eur J Cancer, 1977. **13**(12): p. 1445-1449.

17. MacEwen EG, Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Rev, 1990. **9**(2): p. 125-136.

18. Strandberg JD, Goodman DG, Animal model of human disease: Canine mammary neoplasia. Am J Pathol, 1974. **75**(1): p. 225-228.

19. Nurden AT, Platelets, inflammation and tissue regeneration. Thromb Haemost, 2011. **105**(Suppl1): S13-33.

20. Francis JL, Biggerstaff J, Amirkhosravi A, Hemostasis and malignancy. Semin Thromb Hemost, 1998. **24**(2): p. 93-109.

21. Sierko E, Wojtukiewicz MZ, Platelets and angiogenesis in malignancy. Semin Thromb Hemost, 2004. **30**(1): p. 95-108.

22. Rendu F, Brohard-Bohn B, The platelet release reaction: granules' constituents, secretion and functions. Platelets, 2001. **12**(5): p. 261-273.

23. Cervi D, Yip TT, Bhattacharya N et al, Platelet-associated PF-4 as a biomarker of early tumor growth. Blood, 2008. **111**(3): p. 1201-1207.

24. Labelle M, Begum S, Hynes RO, Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell, 2011. **20**(5): p. 576-590.

25. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, et al, Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer, 2005. **113**(5): p. 752-760.

26. Gay LJ, Felding-Habermann B, Contribution of platelets to tumour metastasis. Nat Rev Cancer, 2011. **11**(2): p. 123-134.

27. Li J, Zhang L, Sun Y, Molecular basis of the initial platelet adhesion in arterial thrombosis: molecular dynamics simulations. J Mol Graph Model, 2012. **37**: p. 49-58.

28. Stegner D, Dutting S, Nieswandt B, Mechanistic explanation for platelet contribution to cancer metastasis. Thromb Res, 2014. **133**(Suppl2): p. S149-157.

29. Trichler SA, Bulla SC, Thomason J, Lunsford KV, Bulla C, Ultra-pure platelet isolation from canine whole blood. BMC Vet Res, 2013. **9**: p. 144.

30. Scott MC, Sarver AL, Gavin KJ, Thayanithy V, Getzy DM et al, Molecular subtypes of osteosarcoma identified by reducing tumor heterogeneity through an interspecies comparative approach. Bone, 2011. **49**: 356e367.

31. Keller A, Nesvizhskii AI, Kolker E, Aebersold R, Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem, 2002. **74**(20): p. 5383–5392.

32. Craig R, Beavis RC, A method for reducing the time required to match protein sequences with tandem mass spectra. Rapid Communications in Mass Spectrometry, 2003. **17**(20): p. 2310-2316.

33. Searle BC, Turner M, Nesvizhskii AI, Improving sensitivity by probabilistically combining results from multiple MS/MS search methodologies. J Proteome Res, 2008. **7**(1): p. 245-253.

34. Nesvizhskii AI, Keller A, Kolker E, Aebersold R, A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem, 2003. **75**(17): p. 4646–4658.

35. Ashburner M, Ball CA, Blake JA et al, Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet, 2000. **25**(1): p. 25-29.

36. Boot-Handford RP, Tuckwell DS, Fibrillar collagen: the key to vertebrate evolution? A tale of molecular incest. BioEssays, 2003. **25**: p. 142–151.

37. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al, Tensional homeostasis and the malignant phenotype. Cancer Cell, 2005. **8**: p. 241–254.

38. Butcher DT, Alliston T, Weaver VM, A tense situation: forcing tumour progression. Nat Rev Cancer, 2009. **9**: p. 108–122.

39. Page-McCaw A, Ewald AJ, Werb Z, Matrix metalloproteinases and the regulation of tissue remodeling. Nat Rev Mol Cell Biol, 2007. **8**: p. 221–233.

40. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al, Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell, 2009. **139**: p. 891–906.

41. Arnold SA, Rivera LB, Miller AF, Carbon JG, Dineen SP, Xie Y, et al, Lack of host SPARC enhances vascular function and tumor spread in an orthotopic murine model of pancreatic carcinoma. Dis Model Mech, 2010. **3**: p. 57–72.

42. Xiao Q, Ge G, Lysyl Oxidase, Extracellular matrix remodeling and cancer metastasis. Cancer Microenviron, 2012. **5**: p. 261–273.

43. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al, Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell, 2009. **15**: p. 35–44.

44. Galbraith CG, Yamada KM, Sheetz MP, The relationship between force and focal complex development. J Cell Biol, 2002. **159**: p. 695–705.

45. Riveline D, Zamir E, Balaban NQ, Schwarz US, Ishizaki T, Narumiya S, et al, Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol, 2001. **153**: p. 1175–1186.

46. Erler JT, Giaccia AJ, Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res, 2006. **66**: p. 10238–10241.

47. Polyak K, Weinberg RA, Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer, 2009. **9**: p. 265–273.

48. Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S, Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. J Biol Chem, 2002. **277**: p. 37377–37381.

49. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al, VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature, 2005. **438**: p. 820–827.

50. Zaslavsky A, Baek KH, Lynch RC, Short S, Grillo J, Folkman J, Italiano JE Jr, Ryeom S, Platelet-derived thrombospondin-1 is a critical negative regulator and potential biomarker of angiogenesis. Blood, **115**(22): p. 4605-4613.

51. Liu LF, DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem, 1989. **58**: 351-375.

52. Nitiss JL, Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer, 2009. **9**(5): p. 338-350.

53. Wang ZQ, Bachvarova M, Morin C, Plante M, Gregoire J, Renaud MC, Sebastianelli A, Bachvarov D, Role of the polypeptide N-acetylgalactosaminyltransferase 3 in ovarian cancer progression: possible implications in abnormal mucin O-glycosylation. Oncotarget, 2014. **5**(2): p. 544-560.

54. Liu Y, Zhu X, Zhu J, Liao S, Tang Q, Liu K, Guan X, Zhang J, Feng Z, Identification of differential expression of genes in hepatocellular carcinoma by suppression subtractive hybridization combined cDNA microarray. Oncol Rep, 2007. **18**(4): p. 943-951.

55. Swisher KD, Parker R, Localization to, and Effects of Pbp1, Pbp4, Lsm12, Dhh1, and Pab1 on Stress Granules in Saccharomyces cerevisiae. PLoS One, 2010. **5**(4): e10006.

56. Fittschen M, Lastres-Becker I, Halbach MV et al, Genetic ablation of ataxin-2 increases several global translation factors in their transcript abundance but decreases translation rate. Neurogenetics, 2015. **16**(3): p. 181-192.

57. Ralser M, Albrecht M, Nonhoff U, Lengauer T, Lehrach H et al, An integrative approach to gain insights into the cellular func- tion of human ataxin-2. J Mol Biol, 2005. **346**: p. 203–214.

58. Placencio VR, DeClerck YA, Plasminogen activator inhibitor-1 in cancer: rationale and insight for future therapeutic testing. Cancer Res, 2015. **75**(15): p. 2969-2974.

59. McMahon B, Kwaan HC, The plasminogen activator system and cancer. Pathophysiol Haemost Thromb, 2008. **36**: p. 184–194.

60. Foekens JA, Peters HA, Look MP, Portengen H, Schmitt M, Kramer MD, et al, The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res, 2000. **60**: p. 636–643.

61. Bajou K, Masson V, Gerard RD, Schmitt PM, Albert V, Praus M, et al, The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. J Cell Biol, 2001. **152**: p. 777–784.

62. Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, et al, Plasminogen activator inhibitor-1 promotes angiogenesis by stimulating endothelial cell migration toward fibronectin. Cancer Res, 2001. **61**: p. 5587–5594.

63. Fang H, Placencio VR, DeClerck YA, Protumorigenic activity of plasminogen activator inhibitor-1 through an antiapoptotic function. J Natl Cancer Inst, 2012. **104**: p. 1470–1484.

64. Valiente M, Obenauf AC, Jin X, Chen Q, Zhang XH, Lee DJ, et al, Serpins promote cancer cell survival and vascular co-option in brain metastasis. Cell, 2014. **156**: p. 1002–1016.

65. Balsara RD, Castellino FJ, Ploplis VA, A novel function of plasminogen activator inhibitor-1 in modulation of the AKT pathway in wild type and plasminogen activator inhibitor-1-deficient endothelial cells. J Biol Chem, 2006. **281**: p. 22527–36.

66. Bajou K, Peng H, Laug WE, Maillard C, Noel A, Foidart JM, et al, Plasminogen activator inhibitor-1 protects endothelial cells from FasL-mediated apoptosis. Cancer cell, 2008. **14**: p. 324–334.

67. McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, et al, Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. J Biol Chem, 2001. **276**: p. 33964–8.

68. Maillard C, Jost M, Romer MU, Brunner N, Houard X, Lejeune A, et al, Host plasminogen activator inhibitor-1 promotes human skin carcinoma progression in a stage-dependent manner. Neoplasia, 2005. **7**: p. 57–66.

69. Wang K, Seo BR, Fischbach C, Gourdon D, Fibronectin mechanobiology regulates tumorigenesis. Cell Mol Bioeng, 2016. **9**: p. 1-11.

70. Zardi L, Cecconi C, Barbieri O, Carnemolla B, Picca M, Santi L, Concentration of fibronectin in plasma of tumor-bearing mice and synthesis by Ehrlich ascites tumor cells. Cancer Res, 1979. **39**: p. 3774–3779.

71. Asch BB, Kamat BR, Burstein NA, Interactions of normal, dysplastic, and malignant mammary epithelial cells with fibronectin in vivo and in vitro. Cancer Res, 1981. **41**: p. 2115–2125.

72. Stenman S, Vaheri A, Fibronectin in human solid tumors. Int J Cancer, 1981. **27**: p. 427–435.

73. Zhang Q, Checovich WJ, Peters DM, Albrecht RM, Mosher DF, Modulation of cell surface fibronectin assembly sites by lysophosphatidic acid. J Cell Biol, 1994. **127**: p. 1447–1459.

74. Zhang Q, Magnusson MK, Mosher DF, Lysophosphatidic acid and microtubule destabilizing agents stimulate fibronectin matrix assembly through Rho-dependent actin stress fiber formation and cell contraction. Mol Biol Cell, 1997. **8**: p. 1415–1425.

75. Peters JM, Franke WW, Kleinschmidt JA, Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. J Biol Chem, 1994. **269**: 7709e7718.

76. Shen M, Schmitt S, Buac D, Dou QP, Targeting the ubiquitin-proteasome system for cancer therapy. Expert Opin Ther Targets, 2013. **17**: 1091e1108.

77. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T, The ubiquitinproteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell, 1994. **78**: 773e785.

78. Orlowski RZ, Small GW, Shi YY, Evidence that inhibition of p44/42 mitogen-activated protein kinase signaling is a factor in proteasome inhibitor mediated apoptosis. J Biol Chem, 2002. **277**: 27864e27871.

79. Pellom ST Jr, Shanker A, Development of proteasome inhibitors as therapeutic drugs J Clin Cell Immunol, 2012. **S5**: p. 5.

80. Murata S, Yashiroda H, Tanaka K, Molecular mechanisms of proteasome assembly. Nat Rev Mol Cell Biol, 2009. **10**: 104e115.

81. Hirano Y, Kaneko T, Okamoto K, Bai M, Yashiroda H, Furuyama K, Kato K, Tanaka K, Murata S, Dissecting beta-ring assembly pathway of the mammalian 20S proteasome. EMBO J, 2008. **27**: 2204e2213.

82. Zheng P, Guo H, Li G, Han S, Luo F, Liu Y, PSMB4 promotes multiple myeloma cell growth by activating NF-kB-miR-21 signaling. Biochem Biophys Res Commun, 2015. **458**(2): p. 328-333.

83. Zhang S, Guo T, Chan H, Sze SK, Koh CG, Integrative transcriptome and proteome study to identify the signaling network regulated by POPX2 phosphatase. J Proteome Res, 2013. **12**: p. 2525-2536.

84. Susila A, Chan H, Loh AX, Phang HQ, Wong ET, Tergaonkar V, Koh CG, The POPX2 phosphatase regulates cancer cell motility and invasiveness. Cell Cycle, 2010. **9**: p. 179-187.

85. Singh P, Gan CS, Guo T, Phang HQ, Sze SK, Koh CG, Investigation of POPX2 phosphatase functions by comparative phosphoproteomic analysis. Proteomics, 2011. **11**: p. 2891-2900.

86. Jiang BG, Wan ZH, Huang J et al, Elevated ZC3H15 increases HCC growth and predicts poor survival after surgical resection. Oncotarget, 2016. **7**(24): p. 37238-37249.

87. Chen CC, Juan CW, Chen KY, Chang YC, Lee JC, Chang MC, Upregulation of RPA2 promotes NF-κB activation in breast cancer by relieving the antagonistic function of menin on NF-κB-regulated transcription. Carcinogenesis, 2016. [Epub ahead of print].

88. Ribbeck K, Lipowsky G, Kent HM, Stewart M, Görlich D, NTF2 mediates nuclear import of Ran. EMBO J, 1998. **17**: p. 6587-6598.

89. Madrid AS, Weis K, Nuclear transport is becoming crystal clear. Chromosoma, 2006. **115**: p. 98-109.

90. Stewart, M, Molecular mechanism of the nuclear protein import cycle. Nat Rev Mol Cell Biol, 2007. **8**: p. 195-208.

91. Vukovic LD, Jevtic P, Zhang Z, Stohr BA, Levy DL, Nuclear size is sensitive to NTF2 protein levels in a manner dependent on Ran binding. J Cell Sci, 2016. **129**(6): p. 1115-1127.

92. Loeffen J, van den Heuvel L, Smeets R, et al, cDNA sequence and chromosomal localization of the remaining three human nuclear encoded iron sulphur protein (IP) subunits of complex I: the human IP fraction is completed. Biochem Biophys Res Commun, 1998. **247**: p. 751–758.

93. Chomova M, Racay P, Mitochondrial complex I in the network of known and unknown facts. Gen Physiol Biophys, 2010. **29**: p. 3–11.

94. Woerner SM, Kloor M, Mueller A, et al, Microsatellite instability of selective target genes in HNPCC-associated colon adenomas. Oncogene, 2005. **24**: p. 2525–2535.

95. Chin SF, Teschendorff AE, Marioni JC, et al, High-resolution aCGH and expression profiling identifies a novel genomic subtype of ER negative breast cancer. Genome Biol, 2007. **8**: p. R215.

96. Igci YZ, Arslan A, Akarsu E, et al, Differential expression of a set of genes in follicular and classic variants of papillary thyroid carcinoma. Endocr Pathol, 2011. **22**: p. 86–96.

97. Santidrian AF, Matsuno-Yagi A, Ritland M, et al, Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression. J Clin Invest, 2013. **123**: p. 1068–81.

98. Igci YZ, Arslan A, Akarsu E, et al, Differential expression of a set of genes in follicular and classic variants of papillary thyroid carcinoma. Endocr Pathol, 2011. **22**: p. 86–96.

99. Chin SF, Teschendorff AE, Marioni JC, et al, High-resolution aCGH and expression profiling identifies a novel genomic subtype of ER negative breast cancer. Genome Biol, 2007. **8**: p. R215.

100. van Ree JH, Jeganathan KB, Malureanu L, van Deursen JM, Overexpression of the E2 ubiquitin–conjugating enzyme UbcH10 causes chromosome missegregation and tumor formation. J Cell Bio, 2010. **188**: p. 83-100.

101. Berlingieri MT, Pallante P, Sboner A, Barbareschi M, Bianco M, Ferraro A, Fusco A, UbcH10 is overexpressed in malignant breast carcinomas. European Journal of Cancer, 2007. **43**: p. 2729-2735.

102. Palumbo A Jr, Da Costa NM, De Martino M, Sepe R, Pellecchia S, de Sousa VP, Neto PN, Kruel CD, Bergman A, Nasciutti LE, Fusco A, Pinto LF, UBE2C is overexpressed in ESCC tissues and its abrogation attenuates the malignant phenotype of ESCC cell lines. Oncotarget, 2016. **7**(40): p. 65876-65887.

103. Whitfield ML, Sherlock G, Saldanha AJ, et al, Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell, 2002. **13**: p. 1977–2000.

104. Drosos Y, Kouloukoussa M, Østvold AC, et al, NUCKS overexpression in breast cancer. Cancer Cell Int, 2009. **9**: p. 19.

105. Sargent LM, Ensell MX, Ostvold AC, et al, Chromosomal changes in high- and low-invasive mouse lung adenocarcinoma cell strains derived from early passage mouse lung adenocarcinoma cell strains. Toxicol Appl Pharmacol, 2008. **233**: p. 81–91.

106. Thompson HG, Harris JW, Wold BJ, et al, Identification and confirmation of a module of coexpressed genes. Genome Res, 2002. **12**: p. 1517–1522.

107. Schaner ME, Ross DT, Ciaravino G, et al, Gene expression patterns in ovarian carcinomas. Mol Biol Cell, 2003. **14**: p. 4376–4386.

108. Naylor TL, Greshock J, Wang Y, et al, High resolution genomic analysis of sporadic breast cancer using array-based comparative genomic hybridization. Breast Cancer Res, 2005. **7**: R1186–R1198.

109. Liu J, Sha M, Wang Q et al, Small ubiquitin-related modifier 2/3 interacts with p65 and stabilizes it in the cytoplasm in HBV-associated hepatocellular carcinoma. BMC Cancer, 2015. **15**: p. 675.

110. Yang W, Wang L, Roehn G et al, Small ubiquitin-like modifier 1-3 conjugation [corrected] is activated in human astrocytic brain tumors and is required for glioblastoma cell survival. Cancer Sci, 2013. **104**(1): p. 70-77.

111. Yin J, Wang J, Jiang Y, Wang L, Wu H, Liu H, Downregulation of NOB1 inhibits proliferation and promotes apoptosis in human oral squamous cell carcinoma. Oncol Rep, 2015. **34**(6): p. 3077-87.

112. Gao X, Wang J, Bai W, Ji W, Wang L, NOB1 silencing inhibits the growth and metastasis of laryngeal cancer cells through the regulation of JNK signaling pathway. Oncol Rep, 2016. **35**(6): p. 3313-20.

113. Ji S, Zhang B, Kong Y, Ma F, Hua Y, MiR-326 inhibits gastric cancer cell growth through down regulating NOB1. Oncol Res, 2016. [Epub ahead of print].

114. Xiao YF, Li JM, Wang SM, Yong X, Tang B, Jie MM, Dong H, Yang XC, Yang SM, Cerium oxide nanoparticles inhibit the migration and proliferation of gastric cancer by increasing DHX15 expression. Int J Nanomedicine, 2016. **11**: p. 3023-34.

CHAPTER VI

FINAL CONCLUSIONS

While animal models of human disease, like the mouse, have proven to be a valuable contributor in the history of medical research due to their abilities to elucidate details behind chemical signaling and biomechanisms, their translation to clinical applications in humans can be deficient. Limitations to the mouse model include the requirement to induce various disease states and tumors via immunosuppression, inoculation, and implantation. Genetic variants and mutants have been developed to allow the study of various cellular mechanisms and the results of gene suppression or deletions. The dog, on the other hand, is a species that shares many of the same naturally occurring diseases as humans, including neoplasias like lymphoma, osteosarcoma, mammary tumors, prostate cancer, and mast cell tumors [1, 2, 3] – therefore avoiding the need to artificially induce different disease states, suppress the immune system, or create genetic alterations. Dogs share the same environment as humans which, therefore, exposes them to the same potential environmental triggers and carcinogens for cancer development (e.g. cigarette smoke, pesticides, UV exposure). In addition, dogs share many of the same clinical presentations, biological behavior, hormonal influences, histopathologic appearances, response to treatments, and prognosis in many of the tumors found to naturally affect humans.

Platelets have been shown to play various roles in different cancers affecting different species. While limited research is currently available regarding the roles of platelets in canine tumor development, growth, and metastasis, similarities shared between dog and human tumors such as lymphoma, osteosarcoma, and mammary tumors offer an important opportunity for the research and discovery of new information that will benefit both veterinary medicine and human medicine. While limited canine cancer proteomic studies are available currently, promise has been shown in the identification of potential biomarkers for various cancers that highlight the value of proteomics in the future of cancer research as well as the value of the dog as an excellent model of human cancers. The application of proteomics research in platelet-tumor cell interactions holds promise for uncovering the mechanisms and protein components by which platelets are involved in cancer.

At the beginning of this study, limited information existed regarding methodologies for obtaining highly purified samples of canine platelets from relatively small (clinically relevant) blood samples. Since platelets are anucleate cells with much less protein and mRNA than other blood cells, particularly leukocytes, future research into platelet-tumor cell interactions required that a pure platelet sample be obtainable. Clinical relevance was important for this study, as the ability to collect small samples of whole blood from clinical patients for potential use in serial monitoring of platelet samples was desirable.

With one of the main goals being to perform proteomic studies looking at platelet-tumor cell interactions, the lack of any known research into the canine platelet proteome created the motivation for the second part of this study. The first normal canine platelet

proteome was described using function and pathways analyses. Differential detergent fractionation, a novel and less widely used methodology for protein digestion in proteomic analysis, was utilized to ensure comprehensive yields of membrane, basic, high molecular weight, and signaling proteins compared to 2D electrophoretic methods that may limit these protein findings [4, 5].

In the last chapter of this study, evidence of the direct impact that platelet contents have on tumor cell expression of proteins was described for two tumor types (osteosarcoma and mammary tumors) that have a valuable role as canine models of human cancer. Most of the protein expression alterations that were found have been described as having positive effects on tumor cell survival, growth, and metastasis elsewhere in the literature for studies conducted in other species. Interestingly, some significant changes in protein expression have been linked to negative or anti-tumor effects elsewhere in the literature. Lack of direct information regarding the roles and mechanisms that these various proteins play in canine platelets and canine cancers, however, poses a gap in the research as comparisons often had to be made based on findings from human and mouse studies.

Since platelet lysate was utilized in the last study, additional experiments would be value utilizing either whole, intact platelets or platelet releaseate. Performing additional experiments using the products of activated platelets rather than platelet lysate would allow comparisons to be made looking at the effects of platelet activation on tumor cell protein expression, as many studies in the literature have shown that platelets are activated directly or indirectly by tumor cells [6, 7]. To do this, platelets could be activated after collection and purification using known platelet activators like collagen,

thrombin, or ADP, centrifuged to remove the platelets, and the releaseate added to tumor cell samples in vitro. In addition, whole platelets could be added to the tumor cell samples in vitro to see the effects of direct intact platelet contact with tumor cell protein expression, although preliminary studies have proven difficult in this regard as clot formation is a critical obstacle to overcome experimentally. Excessive wash steps are undesirable in platelet studies as activation could occur during attempts to separate the platelets from the tumor cells for proteomic analysis, altering the protein composition of the samples collected before analysis and not truly representing the effects of the platelets on the tumor cells.

Many of the findings presented by the work done in these studies leave questions unanswered, as this niche in the world of canine platelet proteome research with regards to tumor cell interactions and models of human cancer has to date attracted very little research. These initial findings have proven that canine platelet contents do stimulate tumor cell changes in protein expression, the effects of which may be contrasting in nature with regards to tumor cell survival or death. Many of the proteins that were identified as having significant alterations in expression could be monitored and studied individually for the specific roles that they play in the physiology of different canine cancers. In addition, changes that occur within the platelet itself following tumor cell exposure could be studied at the level of the proteome. This could potentially identify alterations in the platelet as a result of tumor cell direct contact or signaling proteins, and could aid in identifying increases and decreases in protein expression in response to the tumor cell presence. Since most current studies show evidence that platelets promote

tumor metastasis [8-11], it would be interesting to see the feedback and stimulation occurring from the tumor cell to the platelet itself.

Ideally, in the future this research will lead to clinical case applications. Serial monitoring of canine platelet proteomes from cancer patients at the time of diagnosis, during treatment, at and following remission, and until relapse would provide critical information about changes in platelet protein expression throughout the course of cancer development and treatment. Since many of the naturally occurring cancers that dogs share with humans tend to recur following treatment (lymphoma, osteosarcoma, mammary tumors), this could lead to identification of biomarkers for early return of cancer from remission. Identifying relapses before they are clinically apparent would aid in quicker treatment and potentially increased survival times and prognosis overall.

In conclusion, the technique described allows the collection of highly pure platelet samples from small volumes of canine whole blood, which is important for highly sensitive methods like proteomics. The results from the proteomic studies described here have revealed that canine platelet contents stimulate various changes in protein expression by select canine osteosarcoma and mammary tumor cell lines *in vitro*. Our findings describe initial mapping of the canine platelet proteome as well as suggest that platelets most likely have a positive role in tumor cell growth in dogs.

References

1. Paoloni M, Khanna C, Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer, 2008. **8**(2): p. 147–156.

2. Porrello A, Cardelli P, Spugnini EP, Oncology of companion animals as a model for humans, an overview of tumor histotypes. J Exp Clin Cancer Res, 2006. **25**(1): p. 97-105.

3. Morris JS, Genomic and proteomic profiling for cancer diagnosis in dogs. The Veterinary Journal, 2016. **215**: p. 101-109.

4. Maguire PB, Fitzgerald DJ, Platelet proteomics. J Thromb Haemost, 2003. **1**(7): p. 1593–1601.

5. Garcia A, Watson SP, Dwek RA, Zitzmann N, Applying proteomics technology to platelet research. Mass Spectrom Rev, 2005. **24**(6): p. 918–930.

6. Bambace NM, Holmes CE, The platelet contribution to cancer progression. J Thromb Haemost, 2011. **9**: p. 237-249.

7. Zucchella M, Dezza L, Pacchiarini L, Meloni F, Tacconi F, Bonomi E et al, Human tumor cells cultured “in vitro” activate platelet function by producing ADP or thrombin. Haematologica, 1989. **74**: p. 541-545.

8. Cervi D, Yip TT, Bhattacharya N et al, Platelet-associated PF-4 as a biomarker of early tumor growth. Blood, 2008. **111**(3): p. 1201-1207.

9. Sierko E, Wojtukiewicz MZ, Platelets and angiogenesis in malignancy. Semin Thromb Hemost, 2004. **30**(1): p. 95-108.

10. Gay LJ, Felding-Habermann B, Contribution of platelets to tumour metastasis. Nat Rev Cancer, 2011. **11**(2): p. 123-134.

11. Stegner D, Dutting S, Nieswandt B, Mechanistic explanation for platelet contribution to cancer metastasis. Thromb Res, 2014. **133**(Suppl2): p. S149-157.

APPENDIX A

SUPPLEMENTAL FILE INFORMATION

The following supplemental files were created using Microsoft Office Excel for Mac 2016 (Version 15.20) and saved as PDF files. The PDF files were compressed into a .rar file using WinRAR. They can be accessed on the Mississippi State University Electronic Thesis and Dissertation Database, available at http://sun.library.msstate.edu/ETD-db/. Once downloaded, the files can be extracted using an unzipping program such as WinRAR or WinZip. Files can be viewed using Adobe Reader software or other software for viewing PDF files.

Supplemental File 1: Protein List from Normal Canine Platelet

A list of all unique proteins identified from platelets collected from three healthy dogs. Included in the file is a list of accession numbers and the protein description if available.

Supplemental File 2: Tumor Cell Proteins

A list of all the proteins identified from the three different tumor cells lines used in the study in Chapter V. Included in the file is a list of accession numbers and protein descriptions when available for the OSA8, OSA40, and CMT28 cell samples.