THE ABILITY OF A CELL SALVAGE SYSTEM AND LEUKOCYTE REDUCTION FILTER TO REMOVE HEMANGIOSARCOMA CELLS FROM DOG BLOOD

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2016

Major Subject: Biomedical Sciences

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ABSTRACT

Hemangiosarcoma is the neoplastic growth of primitive cells that resemble endothelial cells. Dogs with hemangiosarcoma often present on an emergency basis due to tumor rupture and intraabdominal bleeding. Treatment involves hemodynamic stabilization, surgery to remove the bleeding tumor and chemotherapy to address micrometastatic disease, which is presumed to be present at the time of diagnosis. Allogeneic blood transfusions are often required in the perioperative period to address blood loss. With recent advances in human medicine, the liberal use of allogeneic blood products in patients with cancer has been called into question. Intraoperative cell salvage offers an alternative method to restore blood volume in dogs with intraabdominal bleeding, but the concern for iatrogenic tumor cell dissemination exists. The first objective of this study was to determine if the administration of allogeneic blood products negatively affects time to disease progression in dogs undergoing surgery for hemangiosarcoma. The second objective of this study was to determine the ability of an intraoperative cell salvage system with a leukocyte reduction filter to remove hemangiosarcoma cells from dog blood *ex vivo*.

Retrospective data were collected from two hospitals to include dogs undergoing surgery for hemangiosarcoma. Six variables were analyzed to determine predictors of time to disease progression. The administration of allogeneic blood products was found to be independently associated with shorter time to disease progression. For the second objective, cultured hemangiosarcoma cells were added to dog blood to represent the blood that may be encountered during surgery to address a bleeding tumor. The solution

was processed through the intraoperative cell salvage machine followed by a leukocyte reduction filter. PCR, flow cytometry and cytologic examination were used to determine the presence of cultured hemangiosarcoma cells at different points in the intraoperative cell salvage system processing. The processing removed a majority hemangiosarcoma cells from dog blood.

ACKNOWLEDGEMENTS

I thank my mentor, co-investigator, and role model, Dr. Kelley Thieman Mankin for her understanding, vision and guidance. I would like to thank my committee for making this journey possible for me. Particular thanks to Tasha Miller, Gus Wright, Rodger Smith and Kate Nelson for support and valuable role in this project. Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University College of Veterinary Medicine a great experience.

A special thank you is for my husband and family for their support, allowing me to continually grow. I dedicate this work to Dr. Linda White, who inspired me to pursue my dreams.

NOMENCLATURE

ABT	Allogeneic blood transfusion
bFBF	Basic fibroblastic growth factor
CT	Computed tomography
DIC	Disseminated intravascular coagulopathy
HSA	Hemangiosarcoma
IOCS	Intraoperative cell salvage
LR	Leukoreduction
LRF	Leukocyte reduction filter
MST	Median survival time
PCV	Packed cell volume
pRBC	Packed red blood cells
RBC	Red blood cell
TRALI	Transfusion-related acute lung injury
TRIM	Transfusion-related immune modulation
VEGF	Vascular endothelial growth factor
WBC	White blood cell

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION TO HEMANGIOSARCOMA IN DOGS: PATHOPHYSIOLOGY TO TREATMENT

Introduction

Hemangiosarcoma (HSA) is the neoplastic growth of primitive cells that resemble endothelial cells. Dogs are the most common species affected with this tumor type. Dogs often present on an emergency basis due to tumor rupture and intraabdominal bleeding. Treatment involves hemodynamic stabilization, surgery to remove the bleeding tumor and chemotherapy to address micrometastatic disease, which is presumed to be present at the time of diagnosis. Because this tumor is intimately associated with the systemic vasculature, is quick to metastasize, and is associated with a short survival time despite treatment, it is considered highly aggressive with a grave long term prognosis.

Several options exist to stabilize a dog with an acute abdominal hemorrhage. Often crystalloid fluids and allogeneic blood products are used in combination. No evidence-based guidelines have been published regarding the use of allogeneic blood transfusions (ABT) in dogs with HSA. With proper blood typing and cross matching, acute transfusion reactions are rare which has led to the belief that ABT are safe for dogs with cancer. [1] However, more long-term consequences of allogeneic blood transfusions may exist. Recently in human medicine, the clinical effects of transfusion-related immunomodulation (TRIM) have been studied. TRIM refers to the changes in the immune system following the administration of ABT and may be especially important in

cancer patients. TRIM is believed to be the cause of a shorter tumor-free interval and survival time for some types of human cancers. [2-4] The following review will discuss the known pathophysiology, current treatment options and the potential role of blood transfusions in disease progression for dogs with intraabdominal HSA.

Epidemiology

HSA occurs more in dogs than in any other species and dogs are the only species that frequently develop naturally-occurring HSA. [5, 6] An estimated 50,000 dogs are diagnosed with HSA each year in the United States. [6] Middle-age to older dogs are most commonly affected but HSA has been reported in a dog as young as 2 years old. [7] Some breeds are overrepresented including: German shepherd, boxer, Golden retriever, Labrador retriever, flat coated retriever, poodles, and Bernese Mountain dogs but any breed can be affected. [7-9] Males may have a higher prevalence than females. [7,10,11]

The two major types of HSA are visceral and non-visceral. Most of the non-visceral HSAs are cutaneous. The spleen, right atrium and liver are the most common visceral locations of the primary tumor. [10,12] Between 25-67% of dogs with splenic HSA are estimated to have right atrial involvement at the time of presentation. [13,14] Splenic HSA is rapidly metastatic to the liver, lung, omentum, and mesentery. [13] Since it is a neoplasm of cells originating in the bone marrow theoretically any tissue with a blood supply can be affected, but the most vascular organs are more commonly affected. [15] In fact, HSA is the most common metastatic tumor to the brain. [16] Other tissues that are commonly affected by primary or metastatic HSA include: lung, kidney, oral cavity, muscle, brain, bone, urinary bladder, left ventricle, lymph nodes, aorta, uterus, tongue,

digit, adrenal glands, pleura, pancreas, small intestines, diaphragm, retroperitoneum and prostate. [7,16,17] The route of metastasis of HSA has not been studied and may be hematogenous, via lymphatics and/or though tumor cell seeding from tumor rupture.

HSA in dogs is analogous to angiosarcoma in humans. In 1974, Prisetler reported that dogs are affected with HSA of the liver at up to 100x the rate of humans and that the tumors are histologically similar. [18] The distinguishing features of the immunohistochemistry of vascular tumors of dogs and humans are comparable. [19] Human angiosarcoma and dog HSA cells both express the same cell surface markers and originate from primitive endothelial cells. [20, 15,21] Identical antibody markers are used in both species for early detection of their respective tumor types and similarities are present in the genetic abnormalities in human angiosarcoma and dog HSA. [6,22] The biologic behavior of human angiosarcoma and dog HSA is so similar that dogs have been used as a model species to study angiosarcoma in people. [23]

Pathogenesis and Tumorigenesis

Since its description in 1955, HSA was thought to arise from mutated mature endothelial cells. [24] Using multi-parameter flow cytometry, Lamerato-Kozicki *et al.* found that HSA cells actually originate in the bone marrow as stem cells that stop differentiating early in the maturation process. Whether the HSA cancer stem cells arise from de-differentiated somatic cells or if they are a product of mutated stem cells is unknown. [15]

A specific carcinogen has not been identified as the cause of abdominal HSA in dogs although there is an increased risk in dogs that are prenatally exposed to ionizating

radiation.[25] Four DNA-damaging agents responsible for an increased risk of hepatic HSA in humans have been identified: thorium dioxide (a radioactive intravenous contrast for angiography used in 1930-1955), inorganic arsenic from pesticides, vinyl chloride monomers from working with PVC pipes and anabolic steroids.[26] These agents have not been studied in dogs.

The tumorigenesis of HSA is thought to be centered on abnormal progression through the cell cycle and deregulated angiogenesis.[27] Angiogenesis is the formation of new blood vessels from existing vessels and is normally tightly regulated. Angiogenesis is required for metastasis and tumors can initiate this process in several ways. HSA cells express growth factors that normally regulate the formation of new blood vessels such as vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFBF).[21,27] HSA cells express the receptors for VEGF and bFBF as well as the platelet derived growth factor receptor.[28,29,30] VEGF and bFGF work directly on endothelium to stimulate proliferation while platelet derived growth factor works through inflammation to induce angiogenesis.[31] VEGF has been also found to enhance the crosstalk between tumor cells and the immune cells in their microenvironment.[32] Abnormal or dysregulated angiogenesis is one of the links between cancer development and inflammation.[33] Overexpression of VEGF can transform immortalized mice endothelial cells into HSA that eventually express VEGF receptors.[34] In the mouse model of HSA, the mouse will not develop HSA if the autocrine endothelial release of VEGF is deleted.[35] This shows that the uncontrolled secretion of VEGF is a requirement for development of HSA in the mouse model. Since HSA cells express both the ligands and receptors for

the growth factors responsible for angiogenesis, the stimulation for new blood vessel growth is under autocrine control. The inherent autonomous angiogenesis associated with HSA helps to explain the aggressive biologic behavior of this tumor.

The gene signatures found to be abnormal in dogs with HSA are those involved in inflammation (IL-8, IL-6, IL-1, COX-2), angiogenesis (VEGF-A, VEGF-R3), adhesion (FN-1), invasion, coagulation (Fibronectin 1), metabolism, cell cycle, wound healing (FN-1), signaling (ADAMS9), cell-cell interactions, hypoxia-inducible genes, metastasis (COX-2, FN-1) and the immune response. [6,11,27] These changes in gene expression are specific to HSA and are not simply a consequence of malignancy in general. The gene expression of cultured tumor cells is different from those tumor cells in whole tissue, which suggests interaction between tumor cells and the surrounding stroma and inflammatory cells. [6] Mast cells, which have pro-inflammatory angiogenic effects, are found in increased numbers in human angiosarcoma but their presence and significance has not been studied for HSA in dogs. [36] The relationship between inflammation and tumor progression is synergistic and multifactorial. [33]

Clinical Signs and Diagnosis

Dogs with HSA may present to the veterinarian with mild to moderate non-specific clinical signs or may present with signs related to a massive intraabdominal hemorrhage due to a ruptured tumor. [7] The length of the clinical latency period of HSA remains unknown and the diagnosis is usually made late in the course of the disease. [7,10,12] Owners may report vague changes leading up to presentation such as lethargy, weakness, decreased appetite, or distended abdomen. [13,37] Neurologic signs due to

metastatic disease may be the only complaint.[13] Although no clinical signs are pathognomonic of HSA, one retrospective study of 271 dogs showed that the observance of anorexia, collapse and hemoabdomen at presentation was significantly more common in dogs with splenic HSA, whereas abdominal pain, vomiting and diarrhea were more likely to be seen in dogs with splenic hematomas.[38] The authors postulate that anorexia is associated with cancer cachexia and lethargy is associated with abdominal bleeding and those two clinical signs are more likely associated with a malignant process. They further propose that slow progressive splenomegaly causing displacement of gastrointestinal organs and secondary vomiting, diarrhea and pain before splenic rupture and hemorrhage is more likely due to a benign lesion. Subsequent to their study, tumor size alone has been proven not predictive of the biologic behavior for splenic masses.[39,40]

More than 50% of dogs with HSA are presented to the veterinarian for acute collapse, which is likely secondary to hemorrhage or arrhythmia. Physical examination may reveal a palpable abdominal mass or abdominal distention. The abdominal distention may be from a hemoabdomen or from the tumor itself.[41] Some dogs may have a palpable fluid wave, supportive of hemoabdomen. In a retrospective study of dogs with splenic masses, 83% presented with hemoabdomen.[42] The dog may show signs of hypovolemia and/or anemic hypoxia if there is a recent or ongoing hemorrhage (pallor, weakness, collapse, delayed capillary refill time, tachycardia, tachypnea and low blood pressure if in decompensated hemorrhagic shock). If the collapse is due to hemorrhage, the extravascular blood in the abdomen may spontaneously undergo autotransfusion

after an unknown period of time. The severity of clinical signs will depend on the duration and rate of abdominal bleeding. [43] If there is cardiac involvement, the dog may show signs of cardiac tamponade, right heart failure or cardiogenic shock. The clinical picture for HSA can vary from a normal dog with an incidental splenic mass to a severely hypovolemic dog in decompensated shock.

Initial bloodwork can be variable in dogs with HSA. They often have blood loss anemia with abnormal red blood cell (RBC) morphology. Several causes for anemia are possible in dogs with HSA such as: blood loss, anemia of chronic inflammation, splenomegaly, microangiopathic hemolytic anemia from splenic disease, and paraneoplastic syndrome especially if the bone marrow is affected. [41] The complete blood count and serum biochemistry may have nonspecific changes but a neutrophilic leukocytosis, anemia and thrombocytopenia are more common in dogs with HSA compared to those with benign splenic disease. [37,44] In most dogs presenting with HSA, measures of coagulation parameters are abnormal. [37,45] About 50% of dogs with HSA meet the criteria for disseminated intravascular coagulopathy (DIC) on presentation. [10, 45] Thrombocytopenia is found in 75-97% of dogs with HSA but it is unclear if this is due to increased consumption of platelets from hemorrhage, decreased platelet formation if the bone marrow is affected, or a combination. [10,37,45] The abnormalities in the vasculature supplying the tumor are thought to contribute to the changes in RBCs morphology and coagulation parameters in dogs with HSA. [10] Around 55% of dogs with HSA presenting with a hemoabdomen will have elevated lactate, a marker of inadequate tissue perfusion.

[46] Hyperlactatemia can be caused by tissue hypoxia (Type A hyperlactatemia) or the neoplasm itself (Type B hyperlactatemia).

Determining the cause of the clinical presentation prior to performing surgery would be advantageous since the prognosis for survival with a splenic mass depends on the etiology. The owners are often faced with the decision to proceed with surgical treatment in an emergency situation with relatively few facts and no promise for cure or survival. For this reason, work has been done to find a way to accurately diagnose HSA prior to anesthesia and surgery. Several clinical factors, imaging characteristics and biomarkers have been analyzed to solve this problem.

Retrospective data have been used in attempt to identify dogs with malignant neoplasia based on initial clinical assessment. One retrospective study analyzed medical records of 71 dogs with splenic masses, anemia and hemoabdomen that required blood transfusion and attempted to predict whether the dog was affected by hemangioma or HSA. The investigators determined a cut off value for serum total protein (< 5.8 mg/dL) and platelet count (< 90,000 cells/dL) to predict the presence of HSA. These parameters may simply be a surrogate measure of hemorrhage and the positive and negative predictive values to diagnose HSA were unacceptably low.[47] This study excluded dogs with HSA that did not receive a blood transfusion, dogs with severely abnormal coagulation parameters and dogs that were euthanized due to other bleeding masses, so the study sample may not represent the population of dogs with HSA. One retrospective study of 70 dogs presenting with splenic masses showed that the severity of anemia on presentation in dogs with hemoabdomen can be predictive of a malignant splenic tumor.

The authors determined that as the PCV of the dog decreased by 10%, the odds of the presence of malignancy in the spleen increased 3-fold. In this study, the presence of abdominal effusion was associated with a 6-fold increase in the odds of a malignant mass.[48] Three other retrospective studies showed that a ruptured mass at the time of presentation is more likely HSA than a benign process but one prospective study failed to agree.[47,49-51] In one retrospective study of dogs presenting with non-traumatic hemoabdomen, those with anemia, nucleated RBC or splenic rupture were significantly more likely to be affected by a splenic neoplasm, HSA being the most common.[51] Despite the reported associations, no clinical presentation is accurately predictive for HSA.

The diagnosis of a hemoabdomen is based on collecting free abdominal fluid and determining that the fluid has a packed cell volume (PCV) over 10%. This fluid will not clot in a syringe because the platelets are consumed in the clot at the site of hemorrhage.[41] Non-traumatic hemoabdomen can be caused by several different conditions including coagulopathy, ruptured mass (benign or malignant), hepatobiliary disease, gastric dilatation volvulus, liver lobe torsion, splenic torsion and caval syndrome from heartworm disease.[37,52] Unfortunately, less than 25% of hemoabdomen fluid samples will contain detectable neoplastic cells using fluid cytology and false positives are possible if a reactive mesothelial cell is included in the sample, so attempting diagnosis of HSA from this fluid is not recommended.[41] Although sampling of a mass is recommended pre-operatively for many other types of tumors, fine needle aspirates and other biopsy techniques are often unrewarding and carry a significant risk of hemorrhage

in dogs with splenic HSA.[41] Additionally, seeding tumor cells into the abdomen during FNA is possible, although seeding is unlikely to change the clinical course of the disease on a large scale.[39] No preoperative test is accurate in predicting if a dog's hemoabdomen is caused by HSA or caused by a different process.

If HSA is suspected, imaging is recommended to attempt to determine the cause of bleeding, to look for metastatic disease and to determine if general anesthesia and surgery are indicated for the dog. Abdominal radiographs may be difficult to interpret with free abdominal fluid or a mass causing obscuring of serosal detail. Thoracic radiographs are obtained to look for metastatic disease and to help determine anesthetic candidacy. In dogs with HSA, radiographs are 78% sensitive and 90% specific for detecting metastatic disease and have a 92% positive predictive value and 74% negative predictive value when 3 orthogonal views are interpreted.[14] Abdominal ultrasound has superior image quality with the presence of abdominal fluid. The ultrasonographic features of splenic and liver HSA have been described but this test cannot differentiate between other lesions such as hematoma, hemangioma and other metastatic tumors.[53] Computed tomography (CT) provides more abdominal and thoracic detail compared to radiographs but requires sedation or anesthesia. Objective parameters obtained from contrast enhanced CT can be used to help determine if a splenic mass is benign or malignant. The presence of hepatic nodules found on CT is associated with a greater risk of splenic malignancy in all dogs with focal splenic masses.[40] Magnetic resonance imaging (MRI) can determine if a focal splenic lesion is benign or malignant with the

specificity of 90% and sensitivity of 100%.[54] MRI is not used widely because of the increased time and cost required to obtain this information.

Blood-based biomarkers have been investigated for their ability to predict the presence of HSA. Based on the pathogenesis of HSA, it is not surprising that plasma VEGF and urine bFGF are elevated in dogs with HSA compared to control dogs.[31] The limitations of measuring these biomarkers include the lack of normal reference ranges, low specificity, and the lack of correlation with disease stage or treatment outcome. Serum alpha-1 acid glycoprotein, a marker of inflammation that suppresses immune function, is elevated in the serum of dogs with HSA but the clinical utility of this measurement has not been further investigated.[55] Thymidine kinase, a biomarker for cellular proliferation, is significantly higher in serum of dogs with HSA compared to normal dogs; however, this marker was unable to distinguish dogs with HSA from dogs with benign splenic changes in a small study.[56] A clinical trial is being conducted to determine if this biomarker can be used for the early diagnosis of HSA in dogs.[57] The serum levels of the lipocalin region of collagen XXVII α 1 were also found to be elevated in dogs with metastatic HSA when compared to dogs with localized HSA, tumors other than HSA, and control dogs. Furthermore, serum levels of this biomarker correlate with disease remission and recurrence.[58] Clinical validation of this blood-based biomarker has not been published but may prove to be valuable in the future.

Cardiac Troponin I, a marker of myocardial damage, is elevated in the plasma of dogs with cardiac HSA compared to dogs with non-cardiac HSA, dogs with other tumors, and dogs with pericardial effusion due to other causes. This test may be used to

determine if a dog with pericardial effusion is neoplasia-induced and to determine the presence of cardiac involvement in a dog with suspected visceral HSA.[59] Multi-parameter flow cytometry of peripheral blood has been successful at identifying circulating HSA cells in affected dogs. Flow cytometry allowed for distinguishing dogs with HSA from normal dogs and from dogs with benign splenic disease. Furthermore, the number of detectable circulating HSA cells decreased when the tumor was surgically removed. Flow cytometry shows promise to detect HSA early in the disease process, detect HSA cells in abdominal effusions, and to monitor for disease recurrence.[15]

In summary, clinical factors are inaccurate at predicting the presence of HSA although some associations have been described. Advanced imaging modalities can be used to predict if a splenic mass is benign or malignant but the tumor origin cannot be determined. Biomarkers and flow cytometry are not yet validated but have promise to diagnose HSA before surgery and monitor response to treatment in the future. Histopathology of a surgically excised biopsy is the gold standard for the diagnosis of HSA. HSAs have a variable amount of hemorrhage within the tumor and can form secondary hematomas, which if examined by a pathologist may lead to the wrong diagnosis with a very different prognosis.[60] The distinction is important and can be challenging.

Clinical Staging

Despite the efforts to find a reliable method of preoperative HSA diagnosis, the diagnosis is not typically made until histopathology results are available several days after surgery. For this reason, staging the dog for metastatic disease is often

recommended as part of the pre-surgical work up in order to provide as much information to the owner as possible.

Clinical staging for HSA uses the tumor, node, metastasis (TNM) system and was first described by Brown in 1985. [7] Stage I indicates a tumor confined to the spleen, stage II indicates a ruptured splenic tumor or local lymph node metastasis and stage III indicates a tumor invading other tissues or distant metastasis. A majority of dogs present with stage III disease. [7,10,61] Several studies of dogs with HSA did not find a difference in survival time between the dogs in each of the 3 stages. [7,12,39,51] However, the dogs received a variety of treatments that have been shown to affect survival times and this variable was not taken into account when comparing survival. Others have found that the presence of metastasis at the time of diagnosis of HSA has a significant influence on survival. By examining pathologic specimens of splenic HSA, one report retrospectively determined the median survival time (MST) for dogs with and without metastatic disease to be 0 and 97 days respectively. [60] Another retrospective report of dogs with HSA treated with surgery alone showed a strong relationship between the disease stage and survival time. Groups of dogs with stage I, II and III HSA showed a MST of approximately 5 months, 2 months and less than 1 month respectively. [62] Since surgery for removal of the primary tumor in a dog with metastatic lesions would likely allow for only a short survival time, clinical staging might help to guide treatment decisions. [62,63]

Pre-Surgical Stabilization

Pre-surgical stabilization is aimed at resolving the dog's cardiac arrhythmias and hypovolemia if present. The degree of stabilization required is determined by the status

of the dog. Around 44% of dogs presenting with splenic masses and 70% of dogs presenting with a hemoabdomen develop arrhythmias.[50] Ventricular arrhythmias are best detected with continuous electrocardiography (ECG) compared to intermittent ECG readings. The indications for treatment of ventricular arrhythmias are: sustained ventricular tachycardia, superimposition of an ectopic beat on the T wave of the preceding beat (R on T), multiform beats, and if the clinical status of the dog is affected by the arrhythmias.[64] Dogs may also develop sinus tachycardia, likely as a symptom of compensation for hypovolemia in response to increasing sympathetic tone in order to preserve cardiac output. Arrhythmias may also be caused by myocardial reperfusion injury or secondary to splenic disease itself as the spleen has direct effects on left ventricular contractility.[50] The consequences of arrhythmias vary in dogs with splenic disease, from an incidental finding to life-threatening.

Transfusion Trigger

A transfusion trigger is the point when the available clinical data provokes the clinician to prescribe blood products. There are no research-based recommendations for the safe and effective use of blood products in dogs with HSA.[65] Anecdotal transfusion triggers include a PCV of 20 to 25% or lower in dogs with hemoabdomen or a PCV lower than 12%.[41, 66] A PCV lower than 12% is recommended as an absolute indication for blood component therapy in dogs since a lower PCV is associated with an increased risk of multiple organ failure in human patients.[67] The indication for administering packed red blood cells (pRBC) is to treat anemic hypoxia.[68, 65] Precise diagnosis of this condition is challenging in a clinical setting. The clinical signs caused by anemic

hypoxia are non-specific and depend on the duration and severity of anemia. The physiologic responses to anemic hypoxia are aimed toward improving oxygen delivery to specific tissues and include: increased sympathetic tone causing increased cardiac output and shunting of blood from non-essential organ systems, decreased left ventricular afterload due to decreased blood viscosity, right-shift in the oxyhemoglobin dissociation curve and recruitment of capillaries in essential organ systems. [69]Anemic hypoxia cannot be diagnosed using PCV alone. [65] Rather than relying on a PCV cut-off value, accurate methods that measure global tissue oxygenation such as arterial lactate, oxygen extraction ratio, gastric pH and cellular oxygen consumption should be used to study research-based transfusion triggers. [67]

In 1943, Adams declared a transfusion trigger for human surgical patients to be a PCV of 24-30%. [70] Since then, the use of this relatively liberal, arbitrary range has been questioned in both human and veterinary medicine. The famous CRIT Study, a prospective study with almost 5000 ICU patients, showed that administration of ABT was an independent predictor of increased morbidity and mortality during the hospital stay. [71] Since then, another large prospective study had similar findings. [72] In one retrospective study of over 10,000 severely anemic human patients undergoing non-cardiac surgery, the administration of 1 or 2 units of allogeneic blood was independently associated with a 40-90% increase in 30 day morbidity and a 29% increase in mortality. Morbidities included pulmonary, septic, wound, and thromboembolic complications. [73] Although it is possible that the complications were associated with intraoperative bleeding, the investigators included only patients with severe preoperative anemia, only

those that received 1-2 units of blood and those undergoing non-emergency surgeries in an effort to eliminate patients with severe intraoperative blood loss. Another prospective study which included general surgery patients who received 1-2 units of ABT for any reason showed that there was a dose-dependent increase in 30-day morbidity and mortality associated with ABT administration. [74] A prospective study of human patients that were treated with splenectomy for various reasons found that the administration of ABT during hospitalization was the only independent risk factor for morbidity. [75] The results from a systematic review of human critical care studies showed that in 42 out of 45 studies, the risks of ABT outweighed the benefits. [76]

The association between ABT and morbidity has been demonstrated in dogs. A retrospective study of over 200 dogs that received ABT showed that accounting for disease severity, a high pre-transfusion PCV and high dose (in mL/kg) of pRBC administered were both risk factors for dogs not surviving hospitalization. [77] This study suggests that even in the presence of anemia, the use of ABT may be detrimental to patients in whom a transfusion is not indicated.

The ideal transfusion trigger would ensure that animals with life-threatening anemic hypoxia were provided with hemoglobin support but animals with tolerable levels of anemia are not given ABT, therefore avoiding the associated risks. The tolerance to anemia in a given animal depends on many factors such as the cause of anemia and the rate of development of anemia. [65] For several subpopulations of high-risk, critically ill human patients, Spahn showed that a restrictive transfusion trigger of PCV $\leq$ 21% is associated with less morbidity and mortality in the hospital compared to a

more liberal transfusion trigger. [78] In healthy human patients, acute normovolemic reduction of PCV to 15% had no effects on the measures of tissue oxygenation. [79] These studies show that hypovolemia and anemia are relatively well tolerated in humans and must be severe to result in morbidity, mortality and poor tissue oxygenation. However, these findings may not be applicable to dogs.

One experimental dog study provides valuable information for establishing a research-based transfusion trigger. In healthy dogs, tissue oxygen delivery is normal until 50% of the blood volume is lost via hemorrhage. Tissue oxygen delivery is normal until the PCV falls below 8% in normovolemic dogs. [80] These findings demonstrate that, as in humans, anemia and hypovolemia must be severe to impair tissue oxygen delivery in healthy dogs. However, the findings in these healthy dogs may not be directly translated to dogs with HSA. Therefore, the transfusion trigger may be different in dogs with a hemoabdomen due to a bleeding splenic mass.

Because of the lack of research-based transfusion triggers, the over-use of ABT during pre-surgical stabilization for dogs with HSA cannot be accurately defined at this time. In one retrospective study of 83 dogs with hemoabdomens, all dogs were given an ABT but the median PCV on presentation was 29% with a range of 9% to 71% and the median heart rate on presentation was 146 beats per minute with a range of 80 to 225 beats per minute. [46] In another retrospective study of dogs with anemia and splenic masses, 18 dogs without hemoabdomens received 22 ABT. [47] The mean PCV of all transfused dogs in this study was 27% with a range of 13% to 47%. This wide range of transfusion triggers demonstrates the variety of dogs that receive ABT, likely due to a

lack of guidelines. Mirroring the confusion in the veterinary literature, one study of human clinicians and residents showed that ABT are prescribed by surgeons and anesthesiologist without their full understanding of the indications, risks and benefits of transfusions. [81] Since the short-term side effects are rare and ABT administration is likely to improve bloodwork values, ABT may be overprescribed in human and veterinary medicine. Until recently, the negative long-term side effects of ABT have been overlooked.

Transfusion Related Immunomodulation

Transfusion related immunomodulation, or TRIM, is the poorly understood immunologic consequence of ABT that may have long-term clinical effects. Clinical consequences attributed to TRIM in humans include: improved organ transplant survival, enhanced tumor progression, increased postoperative infection risk, improvement of symptoms of autoimmune disease, and transfusion-related acute lung injury (TRALI). [82] These effects can partially be explained by the immunomodulatory and/or pro-inflammatory changes seen after administration of ABT but the full mechanism remains undiscovered. [83]

The immunosuppressive effects of ABTs were first noticed in 1973 when Opelz reported that fewer human renal transplant recipients experienced transplant rejection after receiving ABT, in a dose-dependent manner. [84] The same finding was reported in laboratory dogs undergoing renal transplant. [85] As cyclosporine became more popular and offered the same effects as an ABT, the use of ABT to reduce transplant-rejection in humans was discontinued. [82, 86]

Ghant suggested that in cancer patients, the immunomodulatory effects of ABT may have long-term consequences in these already immunocompromised patients. He postulated that since tumor cell antigens were not different than histocompatibility antigens, ABT may cause a non-selective immunosuppression that can allow for malignant tumor cells to survive. [87] Two years later, Horimi showed that ABT enhanced implanted tumor growth in mice. [88] The tumor-promoting effect of ABT administration was seen across species and has become more apparent in humans since Gantt's findings were published. In one prospective study of over 300 human colorectal cancer patients, perioperative ABT administration and tumor stage were the only two variables to independently negatively affect disease free survival. [2] A randomized controlled trail of patients with potentially curable resectable colorectal tumors compared disease-free survival rates between patients randomized to receive ABT or autologous pre-donated blood if needed during surgery. Again, results showed ABT and tumor stage were the only two variables to independently negatively affect disease-free survival. [89] A recent meta-analysis of 36 studies showed a dose-dependent negative effect of ABT on patients with curative-intent colorectal cancer surgeries. [90] These studies highlight the importance of considering the immunomodulatory effects of ABT, especially in cancer patients.

Although a causal relationship between ABT and immunomodulation leading to tumor progression in the recipient is difficult to prove, experimental research has been performed to find the mechanisms of action. The cellular and biochemical changes detected following ABT administration in cancer patients was studied by Guo *et al*. Patients with malignant gastrointestinal tumors were grouped into patients who received

perioperative ABT and patients who received autologous blood. Several markers of immune dysfunction were identified in patients receiving ABT. The ABT group had decreased natural killer (NK) cell activity.[1] NK cells play an important role in the immune-system's tumor cell recognition and killing mechanisms. NK cells work to prevent metastasis and emboli of tumor cells.[91] In addition to lower NK cell activity, plasma IL-10 levels were increased in the ABT group, which leads to a shift of T-cell phenotype from T-helper 1 to T-helper 2 (Th2). A T-helper cell imbalance was present in people receiving ABT for at least 7 days post-surgery, with more Th-2 cells found after ABT administration.[1] The cytokines produced by Th2 cells depress cytotoxic T-cell and macrophage activation, which are important anti-tumor effects of the innate immune system.[82] Further, plasma IL-2 was significantly decreased in the ABT group. This cytokine enhances T-cell, B-cell and NK cell function. Additionally, IL-2 is involved in activating macrophages to kill tumor cells.[33] Postoperative immunoglobulin was also significantly decreased in the ABT group, indicating an impaired humoral immune response. In light of these findings, the study author suggests that autotransfusion be employed in cancer patients whenever possible.[92] A different investigator described a dose dependent increase in production of prostaglandin E 2 (PGE2) and thromboxane by macrophages following ABT.[93] PGE2 is an immunosuppressive prostanoid that downregulates antigen presentation and IL-2 production.[86] The role of PGE2 in TRIM was further supported when Perez *et al.* demonstrated that ABT-induced immunosuppression was enhanced when linolenic acid, a precursor of PGE2, is abundant in the diet of rats.[94] The role of dietary modulation of

the immune system in ABT recipients was dramatic in the rat model, but has not been further explored. The mechanism of TRIM involves inhibition of the innate and humoral immune system which has been demonstrated in people with cancer. These subclinical changes to immune system function may contribute to the negative effect of ABT administration in some patient populations.

In addition to the changes seen in the immune system, the effects of TRIM may also be due to systemic inflammation. ABT enhances the inflammatory insult of surgery and anesthesia via directly inducing the release of inflammatory mediators in human patients. [95] An acute inflammatory response to stored blood administration has been demonstrated in two veterinary studies. [96,97] Inflammation promotes tumor growth by contributing to genetic instability, recruiting factors for angiogenesis, increasing cell-cell interactions, increasing growth factor release, and increasing stromal remodeling to allow for tumor spread. [33,98,99] Systemic inflammation enhances micrometastatic lesion expansion via multiple mechanisms. [100] Tumor cells are able to use the adhesion molecules that they induce and the white blood cell (WBC)-derived cytokines to colonize distant sites. [33] Tumor-induced inflammation is one of the hallmarks for cancer. [101] The synergy between cancer and inflammation paired with the inflammatory changes secondary to ABT administration may help to explain the negative effects demonstrated with ABT administration in cancer patients.

TRALI, or transfusion-related acute lung injury, is the severe diffuse lung pathology that occurs in a small proportion of human patients within six hours of ABT administration. [102] The pathophysiology behind TRALI is poorly understood but may be

a significant link between inflammation and tumor progression for cancer patients receiving ABT. The final result of TRALI is increased capillary permeability and neutrophilic infiltration into the lungs. [65] The capillary integrity in the lungs is an important barrier to extravasation of metastatic cells in this highly perfused organ. Extravasation and colonization of metastatic cells may be achieved easier in patients with compromised capillary integrity. The incidence of TRALI in dogs receiving blood transfusions perioperatively for HSA is unknown but in a retrospective study of dogs with splenic masses, 2 of 518 (0.004%) dogs undergoing a transfusion experienced TRALI and 2 of the 41 (5%) dogs that did not survive to discharge died of TRALI. [42] Even in the absence of overt TRALI, the administration of stored autologous blood is associated with pulmonary vascular leakage in healthy dogs. Dogs given blood that had been stored for 21 days showed significant microscopic and macroscopic evidence of pulmonary edema and perivascular hemorrhage compared to dogs given fresh autologous blood. [103] Because of the propensity for HSA to metastasize to the lungs, we suspect that ABT-induced pulmonary vascular changes may be especially relevant to dogs undergoing treatment for HSA.

Soluble mediators that are released during blood storage may account for some of the inflammatory and tumor-promoting changes seen in dogs administered banked blood. In stored human blood, WBCs release inflammatory mediators such as histamine, VEGF, myeloperoxidase, and plasminogen activator inhibitor-1, which accumulate over time. [104] Stored dog blood accumulates mediators such as VEGF, IL-8 and microparticles, all which have been shown to enhance HSA growth and survival. [21,31,105-

[109] Microparticles are cell fragments that have been recently recognized for their involvement with inflammation, tumor progression, angiogenesis and metastasis. [110] The clinical consequences of these mediators are unknown but when administered to a blood recipient, can help explain the acute inflammatory response which may be especially important in dogs with HSA.

Leukoreduction (LR) is the removal of WBC using a filter. The use of LR is underexplored in veterinary medicine. VEGF, WBC and platelets are removed from dog blood with pre-storage LR. [105, 111] Also, the viability of dog RBCs are normal after LR processing. [112] One study evaluated the consequences of autologous blood transfusions with or without prestorage-LR. When blood from healthy dogs was stored for 21 days and returned back to the donor, total WBC count, mature neutrophils, C-reactive protein and fibrinogen (markers of acute inflammation) were all elevated despite the absence of clinical signs of transfusion reactions. These biochemical changes did not occur in dogs given autologous blood that underwent LR before storage. [96] Therefore, the administration of stored WBC results in an acute inflammatory response in the recipient, even with autologous blood transfusions. LR may reduce some of the aspects of the transfusion-induced inflammatory response during the administration of ABT, but the absence of WBC does not completely negate the effects of TRIM. [97,113, 82]

Because negative clinical effects are demonstrated following ABT administration in cancer patients, determining if a causal relationship exists between ABT and tumor recurrence is of importance. Administration of ABT may be a marker for more severely affected patients, which would affect the results of retrospective and observational

studies. The cause of cancer recurrence is multifactorial and difficult to understand using retrospective or observational studies. [114] Several potentially confounding factors that may affect tumor recurrence exist in the perioperative setting. Prospective, double-blinded randomized control trials, taking into account all known confounders, are needed to determine if the relationship between ABT and cancer recurrence is causal. Despite the limitations of the currently available literature, the possible negative consequences of ABT administration should be considered.

Several disadvantages are associated with the administration of ABT in dogs. First, either an established blood bank or on-site blood donors are required. Second, blood typing and cross-matching are recommended in some dogs to reduce the risk of a clinically significant transfusion reaction. Third, stored blood has decreased oxygen carrying capacity and accumulated inflammatory mediators compared to fresh blood. [76, 115]Additionally, albeit uncommon and usually mild, the incidence of an immediate transfusion-related complications is 37% in dogs. [77] Furthermore, the long term consequences of ABT are poorly understood. The question remains whether autologous blood administration can ameliorate the disadvantages encountered with ABT administration. The cumulative effects of inflammatory insults (surgery, ABT) and immunosuppression (surgery, anesthesia and TRIM) may affect short and long-term outcome in dogs with HSA.

Intraoperative Cell Salvage with Leukocyte Reduction Filtration

Alternatives to ABT have been explored to address anemic hypoxia. Some alternatives include xenotransfusion with bovine hemoglobin-based oxygen carrying

products and acute normovolemic hemodilution, but neither is currently used clinically in dogs. [65,116] An alternative to ABT is intraoperative cell salvage (IOCS) for autotransfusion. IOCS was first described by Highmore in 1874 as a life-saving measure for women who lost blood during childbirth. [117] By 1968, two commercially available IOCS machines were developed for use in trauma patients. [118]

IOCS involves recovering and washing the blood shed during surgery, concentrating the RBC, filtering the WBC and platelets and returning the pRBC to the patient. [119, 120] In 1991, IOCS was first described in dogs with non-neoplastic causes of bleeding as a case series. A combination of IOCS and ABT was used to address complications from a castration, a portosystemic shunt ligation and heart worm disease. No short term complications were noted. [121,122]

The Haemonetics Cell Saver System (Fresenius Kabi AG, Ban Homburg, Germany) is one of the commercially available IOCS machines. The system allows for aspiration of blood from the surgical field using a double lumen suction tip. Dilute heparin is exposed to the salvaged blood at the distal end of the suction tip to prevent clot formation. The blood then passes through a cardiotomy reservoir with a 140μm filter to remove gross debris. Then, the blood is pumped into the disposable bowl housed in the centrifuge. In the centrifuge, the RBC settle to the bottom while the plasma, a portion of the WBC, platelets, free hemoglobin, cell debris, medications, activated clotting factors and wash fluid are removed from the top. [120,122] When the cells in the bowl reach a PCV of about 60%, they are washed with saline and pumped into a blood

bag. The pRBC are then passed through a leukocyte reduction filter (LRF) and given to the patient, during surgery or within 18 hours of blood collection. [120]

IOCS only saves healthy RBC. [123] Using the Cell Saver System, Ray *et al*. showed that RBC salvaged during spinal surgery have a normal lifespan of 30 days. [123] Compared to ABT, salvaged blood has been shown to have increased longevity, increased 2,3-disphosphoglycerate levels, increased adenosine triphosphate levels and improved conformability which is important for capillary circulation. For these reasons, tissue oxygen delivery is expected to be greater for RBC processed with IOCS when compared to ABT. [119]

There is legitimate concern for iatrogenic dissemination of tumor cells shed during oncologic surgery using IOCS. In 1984, Homann showed that carcinoma cells can survive an autotransfusion system and induce tumor formation in athymic mice. [124] The manipulation of a tumor in surgery increases the number of circulating tumor cells and the number of tumor cells shed during surgery. [125] For these reasons, it is prudent to attempt to remove tumor cells from the blood encountered during surgery before returning the blood to the patient.

LRFs have a role in IOCS for autologous transfusion. [126] In experimental settings, LRFs have been shown to remove a variety of tumor cells from different types of fluid media. The tumor cell types that have been studied include: prostatic carcinoma, hepatocellular carcinoma, lung carcinoma, breast carcinoma, colon carcinoma, pancreatic carcinoma, gastric carcinoma, lymphoma, and leukemia. LRFs are capable of removing both cultured tumor cells and primary tumor cells harvested from human

cancer patients during tumor resection. [126-135] Catling *et al.* found tumor cells in 68% of blood samples following IOCS processing but no tumor cells were found after passage through the LRF. [136] Laing *et al.* found that during liver transplantation for hepatocellular carcinoma, about 63% of patients had tumor cells in the blood shed during surgery and that the LRF removed all of these cells except for in one case, when the tumor ruptured in surgery. The researchers theorized that the LRF has a limit to how many cells it filters. [137] The role of LRF for removing tumor cells in veterinary medicine has yet to be explored.

Although the direct comparison of ABT vs. autologous blood transfusion is difficult, several studies have found improved to no change in survival time in cancer patients receiving autologous blood compared to ABT. [138-143] Long term follow up has been studied in human patients with hepatocellular carcinoma. Groups receiving either autologous blood salvaged during surgery or ABT were compared. The group that received autologous blood had a significantly longer tumor-free interval and a significantly higher 10 year survival rate. [144] This study offers the longest follow up of all IOCS studies that compared survival after surgery in cancer patients. At this point, few contraindications exist for use of IOCS in human medicine. [145] Since the LRF is capable of removing tumor cells shed during surgery and no negative effect on cancer recurrence with IOCS use has been found, IOCS with LRF has been deemed safe for use in human oncologic surgeries. [146]

The complication rate for IOCS in humans is 0.027%, significantly less than the reported complication rate of 0.14% for allogeneic blood product administration. [147]

Reported complications of ABT include: febrile nonhemolytic allergic reactions, and acute hemolytic transfusion reaction secondary to a hospital error. [147] With the use of IOCS, the concern for a coagulopathy exists since the platelets, clotting factors and fibrinogen may be removed with processing. However, in humans, up to 3 L of blood can be autotransfused using IOCS with no clinically relevant changes in coagulation. [148] The limited reports of IOCS use in dogs shows that ionized calcium and magnesium may become depleted with autotransfusion but no other electrolyte or acid/base abnormalities were found. [149]

If IOCS were to be studied in dogs with HSA, the need to filter autologous blood before returning it to the dog is in question. First, it has not been proven that tumor cells are shed and therefore collected during surgery in dogs. Although not proven in dogs, tumor cells have been found in the operating field for several tumor types in humans. [125,150,151, 137] The presence of tumor cells shed during surgery is important to determine because circulating tumor cells have distinct characteristics and behavior compared to tumor cells shed from the primary tumor in surgery. [152] HSA cells have been found in the free abdominal fluid in dogs but the concentration and viability of these cells is unknown. [15] Second, it is unknown if all tumor cell types are able to survive IOCS processing, although cultured and primary carcinoma cells prove to be tumorigenic after processing. [152, 153] Third, if tumor cells survive IOCS processing and are returned to the patient, it is unknown if those cells are capable of forming new tumors or have any other clinical consequences. Metastasis is a very inefficient and complicated process; an estimated 0.1% of disseminated tumor cells are capable of forming a metastatic lesion.

[154] Wang *et al.* estimates that in dogs with HSA, at least 1% of circulating nucleated cells are HSA cells, but their ability to form metastatic lesions is unknown. [155] Fourth, even if the cells survive IOCS and exhibit tumorigenicity, it is unknown if this affects quality of life and survival time in dogs with a life-limiting disease such as HSA.

No evidence-based guidelines are available to determine when ABT is beneficial to dogs with hemoabdomen. The potential consequences of anemic hypoxia must be fully understood by the clinician and weighed against the benefit of ABT. A restrictive transfusion trigger for certain human patients has proven beneficial. TRIM should be considered as an important drawback to ABT, especially in recipients with cancer. TRIM may be avoided by using IOCS with LRF, but further studies are needed in veterinary medicine to deem this alternative method safe. In the meantime, current recommendations are to use surrogate measures of tissue oxygenation, such as serum lactate, base deficit and mixed/central venous oxygen saturation and clinical signs to help guide decision making for ABT use in dogs. [65]

Treatment and Prognosis

The initial treatment for visceral HSA is surgical removal of the primary tumor. The prognosis for surviving splenectomy for splenic masses is good. One retrospective study of 539 dogs showed that a majority (92.4%) of dogs survived surgery. However, dogs found to have HSA were more likely to die in the hospital compared to dogs with any other types of splenic masses, both malignant and benign (OR, 2.13; 95% CI, 1.04 to 4.39). The presence of hemoabdomen was not related to survival from the hospital in dogs undergoing splenectomy. [42] In a study of 83 dogs with hemoabdomen, factors

associated with death before leaving the hospital included: tachycardia, bicavitary effusion, severe respiratory disease, and the requirement of massive blood product transfusions (volume of blood products given in excess of the dog's blood volume).[46] Reported short-term complications for splenectomy include uncontrollable hemorrhage from metastatic lesions, DIC, portal system thrombosis, pulmonary thromboemboli, and cardiac tamponade due to right atrial masses.[42]

Performing surgery alone without subsequent chemotherapy administration has been questioned as a treatment option due to the biological behavior of this tumor. The MST of dogs with splenic HSA treated with surgery alone is 1.6 months.[62] Still, surgery seems to improve quality of life of the dog in the short term.[39,51,156] Out of 43 surgeries performed for dogs with HSA, only 41% were successful at removing all macroscopic disease.[157] Knowingly leaving tumor cells behind in surgery defines palliative intent, rather than curative intent. In other words, the surgery addresses the life-threatening hemorrhage but metastatic disease remains.

Doxorubicin is the most commonly studied chemotherapy drug and if used after surgery has the potential to extend survival time to 4-8 months.[60] The administration of doxorubicin is typically initiated 10-14 days after surgery, once the histopathologic diagnosis has been made.[158] Intraperitoneal nonpegylated liposome encapsulated chemotherapy has been evaluated as a treatment option for intraperitoneal metastasis. Fewer peritoneal metastatic lesions were detected in dogs treated with intraperitoneal chemotherapy compared to those dogs treated with doxorubicin intravenously (33-81% respectively). Unfortunately, the study was unable to determine if intraperitoneal

chemotherapy affected survival time. [61] Chemotherapy without surgical treatment has been evaluated and has a low response rate (47%) and high rate of serious toxicities (21%). [159] Inhaled chemotherapy allowed for shrinkage of pulmonary metastatic lesions in one of three dogs studied with HSA. No toxic side-effects were noted in this study and this mode of chemotherapy delivery needs more investigation. [160]

Low-dose metronomic chemotherapy is an alternative to traditional chemotherapy with a low profile of side-effects. The low, continuous systemic drug levels are thought to suppress angiogenesis and therefore slow tumor growth. In a small study, a treatment group of nine dogs with stage II HSA were treated with a daily dosing chemotherapy protocol which included piroxicam, a non-steroidal anti-inflammatory drug, and two other conventional anti-tumor medications. When compared to historical controls, dogs treated with the metronomic chemotherapy protocol showed improved MST to six months. However, no difference was found in the time to disease recurrence between groups. This metronomic chemotherapy protocol was well-tolerated as no toxicity was reported. [161] More studies need to be conducted to determine its efficacy. The relative success of anti-inflammatory drugs in HSA management supports the theory that inflammation is important in the pathogenesis. [6] The prognosis following surgery and chemotherapy is nonetheless grave, with a reported 7-11% 1 year survival rate. [62]

Since angiogenesis is central in the pathogenesis of HSA, anti-angiogenic treatments have been investigated as an adjunct treatment. Tumors can make angiogenic factors themselves, recruit normal cells to make them, induce angiogenic factor activation from their surrounding stroma and suppress antiangiogenic processes both

locally and for the metastatic cells. [162] Interleukin-12 (IL-12) is a cytokine with antiangiogenic properties that also has the potential to stimulate anti-tumor natural killer cells. Mice with experimentally induced dog HSA treated with IL-12 injections showed inhibition of angiogenesis and tumor growth. [163] While this treatment modality shows promise, care must be taken when interpreting xenograft studies.

Other medications have been studied for their anti-tumor effects such as minocycline and *Yunnan Baiyao*. Matrix metalloproteinases are catabolic proteins recruited by tumors from the surrounding stroma to allow for tissue invasion, a required step for metastasis. Human and dog clinical trials are underway to determine if inhibiting these enzymes allows for a longer time to tumor metastasis. [158] Minocycline has antiangiogenic and matrix metalloproteinase inhibitor activity but when looked at in a small group of 18 dogs with HSA, the addition of minocycline to the chemotherapy protocol did not significantly improve survival time despite allowing for cure in mice with experimentally induced fibrosarcoma and lung tumors. [12,164] As with all antiangiogenic drugs, minocycline may be more beneficial in dogs with early disease, as a protective mechanism against metastasis. *Yunnan Baiyao* is a Chinese herbal medication that has been used for its poorly understood anti-inflammatory, procoagulant and wound healing properties. *In vitro*, the medication initiates apoptosis in three separate dog HSA cell lines in a time and dose dependent manner. [165] Clinically, no side effects are reported and this medication is anecdotally associated with longer survival times in dogs treated with surgery with or without chemotherapy. Further studies are needed to determine its efficacy.

Immunotherapy has been gaining popularity in cancer treatment because of the ability to target tumor cells while sparing normal cells, resulting in minimal side-effects compared to conventional chemotherapy. Liposome-encapuslated muramyl tripepdife phosphatidylethanolamine (L-MTP-PE) is a mixed killed bacterial vaccine. This injection is thought to activate macrophages to an antitumor state and to support production of anti-tumor cytokines such as IL-6 and TNFα. A prospective, randomized study was conducted with 32 dogs with HSA after splenectomy in two treatment groups. The control group received only chemotherapy and had a MST of 143 days while the group receiving chemotherapy plus L-MTP-PE had a significantly longer MST of 227 days. This improved survival time was more apparent in dogs with Stage I disease compared to Stage II disease. [166] Excluding doxorubicin, this is the only treatment shown to significantly increase survival time in dogs with HSA. L-MTP-PE has not been further studied and is unavailable in the United States. An anti-HSA vaccine was designed to trigger an antibody response from the host directed against cultured HSA cells. In 13 dogs with HSA, when this vaccine was administered with chemotherapy, the MST was 182 days. Compared to historic controls, the treatment group had an improved survival time but no difference was found in the time to tumor recurrence. This study enrolled a small number of dogs and since the rate of adverse reactions was low, more studies are needed to determine the clinical benefit of anti-HSA vaccine as an adjunct treatment. [167] As we learn more about what is required for the growth and metastasis of this tumor, more targeted therapies may be investigated.

In addition to medications and immunotherapy, dietary therapies have been developed to slow the progression of cancer in dogs. A commercial dog food designed for dogs with cancer contains low carbohydrates, high polyunsaturated omega-3 fatty acids and specific amino acids. This diet has been shown to cause less systemic inflammation compared to standard commercial dog food diets. When dogs with lymphoma were placed on this diet, the chemical indices of inflammation decreased and their survival time increased but the food has yet to be studied in dogs with HSA. [168] Since inflammation is likely important in the pathogenesis of HSA, reducing subclinical systemic inflammation by changing the diet may prove beneficial.

The estimated survival time for dogs with HSA varies slightly with the treatment used. Surgery and chemotherapy remain the mainstay of treatment for HSA. Some chemotherapeutics may prove to be more effective earlier in the disease process as they work by inhibiting migration of tumor cells and formation of new blood vessels, which are both required for metastasis. Since the surgical part of treatment is considered palliative care, the focus on quality of life in the postoperative months cannot be emphasized enough. If any perioperative variables exist that can improve quality or quantity of life for dogs with HSA, further investigation is warranted.

CHAPTER II
ALLOGENEIC BLOOD TRANSFUSIONS IN DOGS WITH HEMANGIOSARCOMA: A RETROSPECTIVE STUDY OF 104 CASES

Introduction

Hemangiosarcoma (HSA) arises from primitive endothelial cells, has continuous contact with circulating blood, and has a high metastatic rate. [169] Dogs diagnosed with HSA have a short median survival time (MST) despite surgery and chemotherapy, even when gross metastases are not detected at the time of surgery. [7,15,37,49,61,63,156] The MST following treatment of HSA has not improved over time despite advances in chemotherapeutics. [7,42] Approximately 50% of dogs that are treated for splenic HSA with surgery and a variety of chemotherapy protocols are alive at 6 months and 11% are alive at 1 year. [42,63] The frequency of disease recurrence despite surgery to remove macroscopic disease suggests that micrometastatic disease is present at the time of surgery in dogs presenting with HSA. [38]

Some dogs with HSA present on emergency with a hemoabdomen due to a ruptured tumor and are often given allogeneic blood products perioperatively to address anemic hypoxia and to improve tissue oxygenation. [37,77] Recent retrospective data shows that out of 211 dogs receiving allogeneic blood transfusions (ABT) for various reasons, transfusion-associated complications occurred in 37% of dogs ranging from a febrile reaction to acute kidney and lung injury. [77] Apart from the risk of short-term side effects, the use of ABT may carry other long-term risks that are not apparent at the time of administration.

Possible long-term risks are related to the storage of blood products and the immunomodulatory effects seen in ABT recipients. Stored blood products accumulate mediators that may be especially detrimental to recipients with preexisting tumor burden.[114] Allogeneic blood transfusion-related immune modulation (TRIM) is an unintended consequence of ABT that has been well described in humans.[83,114] TRIM results in a nonspecific inflammatory response and immune dysfunction, which may contribute to tumor progression.[82] In light of the cellular and biochemical changes that occur with ABT, several observational studies have been performed in humans with various tumor types to determine if ABT administration affects tumor recurrence and patient survival. In a prospective study of 339 patients with colorectal cancer, Tartter *et al.* showed that tumor stage and ABT administration were the only factors to independently affect survival time after accounting for the other confounding variables such as blood loss, tumor differentiation and duration of surgery.[2] Hirano *et al.* compared the 10-year survival rate of colorectal cancer patients who received ABT to those who received autologous blood during surgery. The group who received ABT at the time of surgery had a significantly shorter tumor-free time interval and lower 10-year survival rate, implying long-term consequences of ABT in cancer patients.[144] Meta-analyses looking at the relationship between ABT and cancer recurrence show that ABT is an independent predictor of cancer recurrence.[90,170]

ABT contributes to perioperative immune dysfunction, inflammation and inferior postoperative outcome in human patients resulting in the recommendation to perform autologous blood transfusions rather than ABT.[92,95] The effect of ABT

administration on outcome in dogs has not been previously investigated. We hypothesize that the administration of ABT to dogs undergoing splenectomy for HSA is associated with a shorter time to clinical decline.

Materials and Methods

An electronic medical record search was conducted to identify dogs undergoing splenectomy for a splenic mass between January 2006 to May 2014 at the University of Florida and January 2005 to May 2014 at Texas A&M University. Dogs that underwent splenectomy, were diagnosed with HSA based on histopathology, and were discharged from the hospital were included. A total of 104 dogs were included, 38 from the University of Florida and 66 from Texas A&M University. Dogs were excluded from the study if they did not survive to discharge from the hospital.

Information obtained from the medical records included: age, sex, breed, presence or absence of a hemoabdomen, whether or not ABT was administered, presence and location of metastatic disease diagnosed at the time of surgery, if the dog received chemotherapy or *Yunnan Baiyao*, the time to clinical decline, if time to clinical decline was related to HSA progression, and the days of survival following surgery. The time to clinical decline was determined from the medical record based on client communications or recheck appointments at the participating institution or through communication with a referring veterinarian. Clinical decline was determined to be related to HSA progression based on clinical signs and subsequent diagnostics or necropsy findings confirming the presence of concurrent diseases.

The presence of metastatic disease at the time of surgery was determined by results of preoperative thoracic radiographs and biopsies obtained at surgery. Dogs were retrospectively staged into categories first described by Brown in 1985 with stage I defined as HSA confined to the spleen, stage II defined as HSA that has ruptured, and stage III defined as HSA with multiple gross lesions in more than one organ.[7]

Statistical Analysis

Disease-free interval following surgery was the primary variable of interest. For each dog, this was defined as the interval between the date of surgery and the date of clinical decline. Dogs that had not experienced clinical decline by study's end were right-censored.

The Kaplan Meier method was used to obtain univariate descriptive statistics for survival data, including the median survival time, and compare the survival experience for the enrolled cases by chemotherapy (yes/no), hemoabdomen (yes/no), blood transfusion (yes/no), gross metastasis (yes/no), and *Yunnan Baiyao* administration (yes/no). The log-rank test for equality across strata was used to assess whether a categorical predictor variable should be included in the final model. A Cox proportional hazard model with a single continuous predictor was used to assess cases by age. The Wald chi-square test was used to evaluate the association of this continuous variable [i.e., age] with time to clinical decline.

Multivariable Cox regression models were used to analyze the association or effect of the potential predictor variables with a p-value of 0.25 or less on univariate analysis. Possible interactions were assessed between all predictor variables. The

assumption of proportionality was confirmed by including time-dependent covariates in the model. Model fit was evaluated using Cox-Snell residuals. Analyses were performed using STATA software, Intercooled version 12.0.

Results

One hundred and four dogs met the inclusion criteria. Of the 35 represented breeds, mixed breed (n=20), Labrador (n=17), Golden retriever (n=13), German Shepherd (n=7) and boxers (n=7) were the most common. The mean age at surgery was 10.4 years (range, 4.1 to 15.4 years). The sex distribution included intact females (n=5), intact males (n=10), spayed females (n=48) and castrated males (n=41). Dogs with splenic HSA were classified as clinical stage I in 18/104 (17.3%), stage II in 39/104 (37.5%) and stage III 47/104 (45.2%) **(Table 1)**. Of the dogs with distant metastases, 29 were to the liver, 11 were to the lung, 11 were to the omentum, two were to abdominal lymph nodes, two were to the falciform fat, one was to the ilial wing, and one was to the subcutaneous tissue. A total of 75/104 (72.1%) dogs presented with a hemoabdomen, 36/75 (48.0%) of those had gross metastatic disease at the time of surgery. Eleven dogs had metastasis to distant organs but did not have a hemoabdomen and were categorized to stage III. Except for the four dogs that were alive at the end of the study, all died or were euthanized as a result of HSA progression.

Six months after surgery, 25/104 (24%) dogs had not clinically declined and 30/104 (29%) were alive. One year after surgery, 9/104 (8.7%) dogs had not clinically declined and 13/104 (12.5%) were alive. The mean interval between time to clinical decline and time to death was 36 days (range 0 – 1461 days). The time to clinical decline

was the same as date of death in 34/104 (33 %) of dogs. Four dogs were alive at the completion of the study, two of which were administered ABT, none had gross metastatic disease and one received chemotherapy and *Yunnan Baiyao*. At the time of manuscript preparation, the mean survival time following surgery for the four dogs that are still alive is 560 days (range 166 – 1143 days).

	Stage I	Stage II	Stage III
Total dogs	N=18 (17%)	N=39 (38%)	N=47 (45%)
ABT administration (%)	4 (22%)	29 (74%)	30 (64%)
Chemotherapy [+ABT administration]	14 [3]	17 [13]	16 [15]
Yunnan Baiyao [+ABT administration)	4 [3]	1 [1]	8 [7]
Alive at end of study [+ABT administration)	2 [1]	2 [1]	0 [0]
Median time to decline (range) [+ABT administration] (range)	175 (48-481) [238] (161-274)	95 (5-439) [89] (5-439)	60 (1-916) [43] (1-916)
Median survival time [+ABT administration]	193 (48-693) [256] (173-274)	105 (5-1586) [100] (5-1586)	63 (1-916) [56] (1-916)

Table 1. Dogs with HSA who survived hospitalization after surgical treatment were categorized based on clinical stage. The number of dogs in each stage that were administered ABT, chemotherapy and *Yunnan Baiyao* are reported here. The number of dogs in each stage that were administered ABT as well as the other two treatments is reported here in brackets. The median time to decline and MST are reported in days.

The dogs were divided into groups based on whether an ABT was administered while in hospital. **Table 2** summarizes the characteristics of the two groups. The groups were similar in age and sex distribution. As expected, ABT administration was more common in dogs that presented with a hemoabdomen. In the present study population, 75/104 (72%) of dogs with HSA presented with hemoabdomen and 59 of those 75 dogs (78%) were administered ABT. The groups had a similar prevalence of gross metastatic disease on presentation. A similar proportion of dogs received chemotherapy in each group. For dogs not given ABT (n=37), the median time to decline was 120 days (range 38 - 916 days) and MST was 136 days (range 41 - 916 days). For dogs who received ABT (n=67), the median time to decline was 76 days (range 1 – 836 days) and the MST was 97 days (range 1-1586 days).

	No ABT N=37	ABT N=67
Mean age in years (range)	11.1 (7.4 – 15.4)	10.1 (4.1-14.7)
Hemoabdomen	15 (41%)	60 (90%)
Gross metastatic lesion	13 (35%)	34 (51%)
Chemotherapy	16 (43%)	31 (46%)
Yunnan Baiyao	2 (5%)	11 (16%)
Median time to decline (range)	120 (38 - 916)	76 (1 - 836)
Median survival time (range)	136 (41 - 916)	97 (1 - 1586)

Table 2. Dogs treated for HSA were divided into groups based on if an allogenic blood transfusion was administered at the time of surgery. The number of dogs in each category is reported. The median time to decline and MST are reported for each group in days.

The Kaplan Meier method showed that ABT administration (p=0.0145), chemotherapy administration (p=0.0612), the presence of a hemoabdomen (p=0.0009), the presence of gross metastatic lesions (p=0.0009) and the administration of *Yunnan Baiyao* (p=0.162) were all associated with time to disease progression using univariate analysis. Age of the dog at the time of surgery (p=0.588) was not associated with time to disease progression. The administration of *Yunnan Baiyao* decreased the time to decline by 64% (p=0.008, CI 23-83%).

Multivariable Cox regression model was used to determine the effect of these predictor variables on time to tumor progression. No interactions were found between predictor variables. While holding all other variables constant, ABT administration increased the time to decline by 69% (p=0.042, CI 2-179%). The presence of gross metastasis increased the time to decline by 146% (p=0.00, CI 60-279%). The presence of metastatic lesions indicated a shorter time to disease progression when compared to the presence of a hemoabdomen in this study (OR 2.4 versus 1.7). The administration of chemotherapy decreased the time to decline by 33% (p=0.063, CI -2%, 67%).

The chemotherapy protocol consisted of doxorubicin for most dogs but other protocols were used for a variety of reasons. Reasons for a non-doxorubicin protocol that were recorded in the medical record included preexisting cardiac disease and drug intolerance. *Yunnan Baiyao* was prescribed to 13 dogs, five of which had presented with a hemoabdomen. Because of the small number of dogs that received *Yunnan Baiyao* and the uncertainty of how often this was offered as a treatment option, this variable was not included in the multivariate analysis.

Discussion

This study sought to determine if ABT administration has clinically relevant consequences for dogs diagnosed with HSA. In this study population, the use of ABT was associated with a shorter time to clinical decline in dogs with splenic HSA. The shortened disease-free interval found in dogs receiving ABT is possibly due to the effects of TRIM in dogs with cancer, the negative effects associated with blood storage, the advanced disease in dogs requiring ABT, or, likely, a combination of factors.

Our study demonstrates a shorter time to clinical decline in dogs with HSA that are administered an ABT. If this relationship is causal, it may be explained in part by the effects of TRIM. TRIM refers to the immunomodulatory and pro-inflammatory changes in a recipient following ABT administration. In human patients, TRIM has been implicated in decreased post-surgical disease-free interval and survival time for a variety of tumor types. [2,89,144,170] The effects of TRIM may be especially relevant in recipients with metastatic disease at the time of surgery. [171] Studies have shown that dogs with HSA often have either gross or micro-metastases at the time of diagnosis and 45% of the dogs in the present study were diagnosed with gross metastasis at the time of surgery. Therefore, if TRIM affects dogs like people, we expect this group of dogs to be especially susceptible to the immunomodulatory effects of ABT.

Since the clinical effects of TRIM have been defined in human medicine, the cellular and biochemical changes associated with ABT in humans and lab animals have been studied in order to elucidate the mechanism by which the immunomodulatory effects occur. The mechanisms of TRIM are currently divided into two overlapping

categories: the pro-inflammatory effects and immunomodulatory effects, both of which may support metastasis.[33,83] The administration of ABT in human gastrointestinal cancer patients causes depressed natural killer cell activity, a shift of T-cell phenotype from T-helper 1 to T-helper 2, elevated interleukin-10 (IL-10) levels, decreased interleukin-2 (IL-2) levels and decreased immunoglobulin levels in the recipient. These changes collectively reflect a dampened innate and humoral immune function.[92] Natural killer cells and the cytokines produced by T-helper 1 cells have anti-tumor activity. T-helper 2 cytokine production can be initiated directly by tumor cells and promotes tumor invasiveness and metastasis.[32] IL-10 levels are elevated following ABT, which mediates the shift from a T-helper 1 to T-helper 2 phenotype. Following ABT, plasma IL-2 levels are significantly decreased.[1] IL-2 normally enhances T-cell, B-cell and natural killer cell function as well as activates macrophages to kill tumor cells.[33] Prostaglandin E2, an immunosuppressive prostanoid, is elevated in ABT recipients.[93] These immunologic changes seen in human cancer patients with ABT use warrants further study into the clinical consequences of immunomodulation in dogs with HSA.

The shortened disease-free interval found in dogs receiving ABT is possibly due to the negative effects associated with blood storage. In addition to diminished function of stored RBC, administration of stored blood products is associated with dramatic vascular changes in experimental dogs. Dogs given 21-day-old autologous blood showed significant microscopic and macroscopic evidence of pulmonary edema and perivascular hemorrhage compared to those given fresh autologous blood.[103] Importantly, the changes in the lung were subclinical and would likely be missed in a hospitalized dog.

Because of the propensity for HSA to metastasize to the lungs, we suspect that ABT-induced pulmonary vascular changes may be especially relevant to dogs undergoing treatment for HSA. Inflammation and inflammatory mediators play a role in cancer metastasis. Specifically, vascular endothelial growth factor, interleukin-8 and microparticles may be especially relevant to dogs with HSA receiving stored ABT. Each of these substances increases in stored blood. Further, each of these substances has been linked to cancer development or metastasis and inflammation, suggesting that "storage lesion" may be a clinically significant downside to giving stored blood products to dogs with HSA. However, the relationship between storage lesion and tumor progression has not been explored in dogs.

The presence of a ruptured tumor and gross metastasis in the abdomen were each independently associated with shorter time to clinical decline in this study population. Clinical staging for dogs with HSA was first described by Brown in 1985.[7] Stage I indicates a tumor confined to the spleen, Stage II indicates a ruptured tumor or local lymph node metastasis and Stage III indicates a tumor invading other tissues or macroscopic distant metastasis. Although stages are described, controversy exists over whether stage predicts outcome. Several retrospective studies with heterogeneous patient populations have shown that survival time for dogs with HSA is not consistently influenced by the stage of disease at presentation.[38,51,61,156] However, one prospective and one retrospective study of dogs with splenic HSA showed that the presence of a hemoabdomen at the time of diagnosis is associated with shorter survival time.[38,42] The present study demonstrates that dogs with metastatic lesions and hemoabdomen have a

shorter time to clinical decline. Furthermore, the presence of metastatic lesions indicated a shorter time to disease progression when compared to the presence of a hemoabdomen in our study (OR 2.4 versus 1.7). This result suggests that in dogs with HSA, staging influences time to disease progression and prognosis. We expect that dogs with stage I and II HSA had a longer time to disease progression compared to stage III because they presented with a heavier tumor burden.

While the dogs in the current study demonstrated a decreased time to disease progression when ABT was administered, the administration of chemotherapy was associated with significantly increased survival time. Despite the heterogeneity of the chemotherapy protocols, our findings agree with the current literature that chemotherapy administration significantly increases MST in dogs with hemangiosarcoma.[12,60,157] No specific chemotherapy protocol has been determined superior in treating dogs with hemangiosarcoma.[42,158] For this reason, the type of chemotherapy administered was not included in the analysis.

The effect of *Yunnan Baiyao* was evaluated to determine if administration is associated with improved outcome in dogs with HSA. *Yunnan Baiyao* is a Chinese medication and is purported to mitigate bleeding and inflammation. The mechanism of action of the medication is unknown, and any explanation of this finding is speculative. However, anti-tumor effects of *Yunnan Baiyao* have been studied and the medication has been shown to slow the growth of cultured HSA cells *in vitro*.[165] Our data shows slower time to disease progression associated with the administration of *Yunnan Baiyao* to dogs presenting with HSA. The beneficial effect may be due to the anti-inflammatory,

hemostatic, pain relieving, or wound healing properties of the medication or due to a Type I error because of the small number of study dogs that received this treatment.

Study Limitations

Several limitations exist in this study. The first is that the mechanism behind a shorter time to disease progression in dogs administered ABT is not identified in this study. While possibilities proposed in human medicine are presented, disease severity or other undefined confounding variables may necessitate ABT in dogs and may represent an unidentified underlying cause of shorter time to disease progression. Evidence-based transfusion guidelines ("triggers") for dogs with non-traumatic hemoabdomen do not exist. Individual clinicians were responsible for determining whether or not ABT were administered in this study. While ABT use may be a surrogate marker for the presence more severe disease, our statistical analysis showed that ABT was an independent predictor of decreased time to disease progression and that no interaction was found between predictor variables. Interestingly, 7 (7/29 = 24%) of the dogs without a hemoabdomen were given ABT. The reason for ABT administration in dogs without hemoabdomen is unclear from the medical record and demonstrates the variety of "transfusion triggers" among clinicians. Previous studies have suggested that liberal ABT use can result in negative consequences including non-survival.[77] Human studies show that the use of ABT in critical patients is an independent predictor of morbidity and mortality and that liberal transfusion triggers may negatively affect patient outcome.[71,72] A causal relationship between ABT use and decreased time to disease progression is impossible to prove in this study.

An additional limitation is that a variety of different allogeneic blood products were administered to the dogs in our study including: packed red blood cells, fresh whole blood and fresh frozen plasma. The contribution of specific blood components to TRIM and storage lesions are not well-defined in dogs. The effects of TRIM may be mediated by WBC-derived cytokines or soluble peptides found in plasma.[83] Additionally, allogeneic fresh frozen plasma has been shown to increase tumor-promoting factors such as TNFα, IL-10, contain growth factors such as insulin-like growth factor and promote tumor growth in nude mice.[172, 173,174] Because of the existing evidence that any component of blood may contribute to the inflammatory and immunomodulatory effects of ABT, dogs given any type of allogeneic blood product were grouped together. Further, the duration of pre-transfusion blood product storage varied in the study population and this may have affected our results since blood storage is associated with clinically relevant consequences.

Other limitations are inherent to the retrospective nature of the study. The data collection is reliant on the accuracy and completeness of medical records. Omission of information from owner-reported history or medical records may have affected the results. For example, the number of dogs who had a ruptured tumor and spontaneous resolution of a hemoabdomen at home is unknown. Evidence of prior hemorrhage was not recorded from surgery reports. In these instances, the dog may have been inaccurately categorized as having stage I disease. Many unknown perioperative clinical factors may contribute to HSA recurrence such as: degree of resectability of the tumor, the presence of postoperative complications and concurrent disease. The genetic,

biochemical and microenvironmental factors of each individual tumor may also affect outcome and were not accounted for in this study. Additionally, including data from two institutions allowed for a heterogeneous sample, the variability between clinical variables at the two locations is not defined. The individual owner's decision to pursue treatment may be influenced by an unknown number of factors existing at the time of presentation and this may affect the population of dogs in our study. Time to disease progression was used as an outcome measure rather than mean survival time to reduce the variability associated with owner's decision to euthanize.

The biochemical and clinical consequences of ABT in dogs are incompletely understood at this time. These data argue for the study of an evidence-based transfusion trigger point and the consideration of exploring alternative practices in appropriate clinical patients. In order to determine if the administration of ABT directly affects disease progression in dogs with HSA, a prospective randomized control study is required. Since withholding blood products from a patient in need is unethical and not advised, an alternative to ABT is required. Autotransfusion may be used for dogs needing to improve tissue oxygen delivery in the control group. Bower has shown that intraoperative cell salvage is a practical option for human cancer patients; however neoplasia remains listed as a contraindication for autotransfusion in veterinary literature.[121,146, 175] The safety of this blood conservation technique must be established for dogs with HSA before being used in a clinical setting.

In conclusion, the dogs included in this study that were administered an ABT had accelerated disease progression compared to dogs that were not administered ABT.

Because the use of ABT in our study was associated with the presence of advanced disease, we cannot exclude the possibility that ABT is a surrogate marker of a hemoabdomen, less stable patients and possibly more biologically aggressive disease. Confounding our retrospective observations is the inability to control for disease severity. If the apparent relationship is causal, ABT may contribute to progression of HSA because of the consequences of TRIM, storage lesion or a combination of factors. The tumor-promoting effects of ABT have been demonstrated in experimental animal models but the clinically relevant consequences for dogs with HSA have yet to be proven.[171] Future prospective studies should be aimed towards determining whether ABT affects disease progression for HSA patients when compared to other methods used to improve tissue oxygenation.

CHAPTER III

THE ABILITY OF A CELL SALVAGE SYSTEM AND LEUKOCYTE REDUCTION FILTER TO REMOVE HEMANGIOSARCOMA CELLS FROM DOG BLOOD

Introduction

Hemangiosarcoma (HSA) is the most common splenic tumor in dogs and is the most common cause of non-traumatic hemoabdomen in dogs. [169, 37] Dogs with HSA frequently present on an emergency basis due to clinical signs related to a ruptured tumor. A hemoabdomen can result in hemorrhagic shock and some dogs require blood transfusions to address the blood lost into the abdomen and surgery to remove the bleeding tumor. Allogeneic blood transfusion (ABT), specifically packed red blood cells (pRBC), are the most commonly used blood products in veterinary medicine. [176] The goal of administering pRBC is to improve oxygen delivery to tissues by increasing hemoglobin levels. [65,68]

Although ABT administration can be lifesaving when indicated, recent advances in human medicine have questioned the liberal use of ABT. Two research groups have prospectively demonstrated that as the quantity of ABT increases, patient morbidity and mortality each increase independently of other variables. [71,72] The long-term consequences of ABT administration are becoming more apparent in patients with cancer. [1] In one prospective cohort study, human patients that received autologous blood transfusions during hepatocellular carcinoma resection had a significantly longer time to

tumor recurrence and a higher 10-year survival rate compared to the patients that received ABT.[144]

The known disadvantages of ABT administration in dogs include: the requirement for a blood bank or on-site donors, the time associated with blood typing and cross matching, and a 37% incidence of transfusion-related complications.[77] The oxygen carrying capacity of stored red blood cells (RBC) diminishes over time.[76] Furthermore, immunomodulation is found in human cancer patients following ABT administration.[114] In an attempt to avoid the side effects of ABT, alternative methods to restore tissue oxygenation in the face of blood loss have been explored.

Intraoperative cell salvage (IOCS) for autotransfusion was first described by Highmore in 1874 as a lifesaving measure for obstetrics.[117] Since then, the technique has evolved and is now used for a variety of human surgeries, including oncologic surgeries.[119,146] IOCS has been used successfully as a blood conservation technique to address non-neoplastic hemorrhage in dogs but has yet to be studied for oncologic surgery.[120,122] Albeit a controversial topic, recent human literature advocates for use of IOCS oncologic surgery.[135,177,178] Although IOCS use may even provide superior outcomes for human oncologic surgery, neoplasia is listed as a contraindication for the use of IOCS in the veterinary literature.[122,179]

The concern for returning tumor cells to the systemic circulation and iatrogenic tumor metastasis exists with IOCS use in dogs presenting with acute, non-traumatic hemoabdomens since about 80% of this population are diagnosed with cancer.[37] However, the use of a leukocyte reduction filter (LRF) before returning the salvaged

blood to the patient may alleviate this theoretical concern. The LRF has been shown to remove a variety of tumor cell types from saline, donated blood and intraoperatively salvaged human blood. [126,128-132,135,136,177,178]

The goal of this study is to determine the ability of IOCS with LRF to remove HSA cells from dog blood *ex vivo*. This technique has the potential to serve as an alternative to ABT. We hypothesize that LRF in combination with the IOCS system will remove detectable HSA cells from both culture media and dog blood samples. To test this hypothesis, we used flow cytometry, RT-PCR and cytological examination to determine the presence of cultured HSA cells at various points in IOCS and LRF processing.

Materials and Methods

An established HSA cell line, DEN-hemangiosarcoma, was used for this experiment. [180] HSA cells were maintained at 37°C and protected from light before use. The cells were cultured in monolayer and were detached using 0.25% trypsin (Lonza, Houston, TX). For the first portion of the study, the cells were stained using PKH26 Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling, (PKH26GL, Sigma-Aldrich, St. Louis, MO) according to manufacturer instructions. RBC lysis buffer (eBioscience, San Diego, CA) was added to a sample of the cells then the cells were examined visually for morphologic evidence of damage. The cells were counted using a cellometer before dilution.

Prior to assessing the ability of LRF and IOCS to remove the HSA cells, we tested the ability of cytology, flow cytometry and RT-PCR to detect small numbers of

HSA cells. Briefly, HSA cells that were stained with PKH26 were used to create a 50 cell / mL solution in culture media. This solution was tested with RT-PCR, flow cytometry and cytological examination by standard technique detailed below.

Part 1

The ability of a LRF to remove HSA cells from solution was evaluated. Briefly, 35 mL of a 1,000,000 cell / mL solution of PKH26-stained HSA cells was created in culture media. The solution was added to a sterile transfer bag (Terumo Corporation, Tokyo, Japan) then processed through the Leukocyte Reduction Filter for Intraoperatively Salvaged Blood (RS1 Haemonetics Manufacuturing Inc., Braintree, MA) using a pressure bag under 300 mmHg. The post-LRF filtrate was collected in a second sterile transfer bag. Samples were obtained pre and post-LRF from the two transfer bags. Samples of 5 mL were collected in EDTA-tubes for cytological examination and samples of 2 mL were collected in Eppendorf tubes (VWR International, Houston, TX) for RT-PCR and flow cytometry**.** Time to perform filtration was recorded. The steps of Part 1 were repeated 3 times obtaining the same samples each time.

Part 2

Fresh, whole dog blood anticoagulated with heparin was obtained from a commercial blood bank within 24 hours of collection (Whole Canine Blood, Animal Blood Bank Resources , Stockbridge, MI). In a sterile bowl, 300 mL of whole blood was mixed with a solution of culture media containing 300 million HSA cells for a final concentration of 500,000 HSA cells / mL to represent abdominal hemorrhage

encountered during surgery for ruptured HSA.[15] The blood/HSA cell mixture was processed through the Fresenius C.A.T.S Continuous Autotransfusion System (#9CAA0574 Fresenius Kabi AG, Ban Homburg, Germany), using previously described techniques.[120,128] The blood/HSA cell mixture was suctioned from a sterile bowl using a sterile double lumen suction tip with heparinized saline, as would be done clinically.[149] The mixture was collected in a cardiotomy reservoir with a 140 μm filter and centrifuged to separate RBC from other blood components. In the centrifuge, the healthy RBC settle to the bottom while the plasma, a portion of the white blood cells (WBC), platelets, free hemoglobin, cell debris, medications, activated clotting factors and wash fluid are removed with the supernatant.[120,122] When the cells in the centrifuge reached a packed cell volume (PCV) of about 60%, they were washed with saline and pumped into a collection bag from the IOCS system. The washed pRBC were processed through the LRF which was primed with 35 mL of sterile saline. Four separate samples were collected at different times during the IOCS process. Sample 1 was collected from the sterile bowl prior to IOCS system processing, sample 2 was collected after IOCS system processing, sample 3 was collected after LRF processing and sample 4 was collected from the discarded saline used to wash the RBC **(Figure 1)**. Samples of 5 mL were collected in EDTA-tubes for cytological examination. Samples of 2 mL for RT-PCR and flow cytometry were collected and immediately RBC lysis buffer was added. This process was repeated for three trials on different days with sterile supplies and a different dog's blood. The duration of processing through the IOCS system and duration of processing through the LRF were recorded.

On the second and third trials of the IOCS and LRF system, the four samples collected in EDTA-tubes were pelleted and frozen at -80 degrees Celsius until testing using RT-PCR and flow cytometry. The samples were thawed at 37 degrees Celsius in a water bath immediately before RT-PCR and flow cytometry.

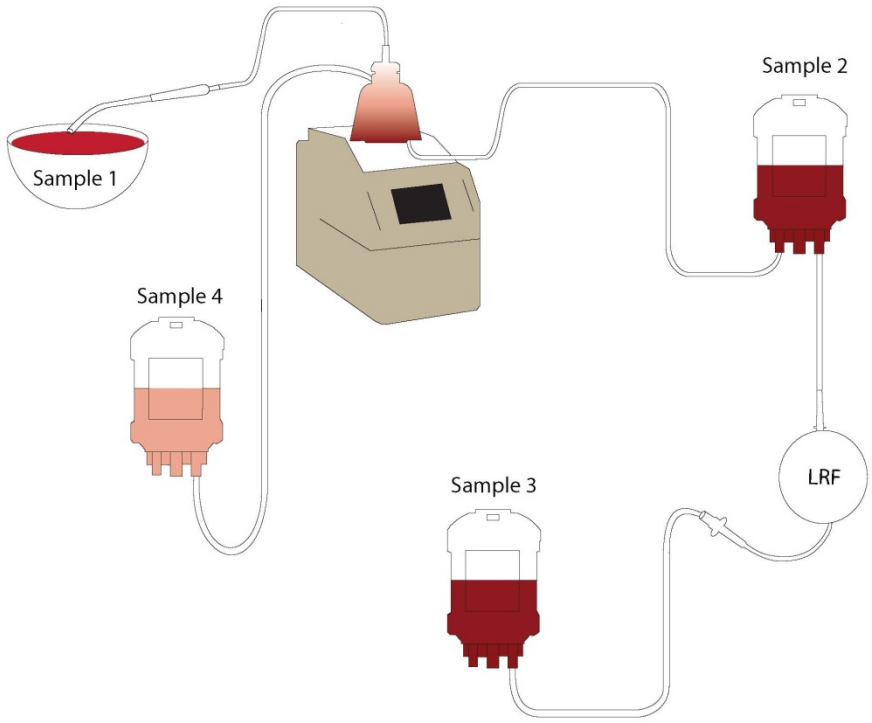

Figure 1. IOCS with LRF and sampling locations

Cell Detection Methods

RT-PCR Part 1: A total RNA sample was obtained from PKH26-stained HSA cells. The cells were lysed and RNA was extracted using the RNAqueous®-Micro Total RNA isolation kit (Ambion, Grande Island, NY). The RNA preparation was treated with TURBO DNA-free™ Kit (Ambion, Grande Island, NY) to remove any remaining traces

of DNA. RNA quantity was assessed using a NanoDrop spectrophotometer in preparation for cDNA synthesis. cDNA was synthesized using 35 ng of RNA and the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The thermocycler used for cDNA synthesis was an Eppendorf Mastercycler epgradient S (Eppendorf, Hamburg, Germany). The expression of von Willebrand Factor was confirmed through RT-PCR by mixing one-tenth of the synthesized cDNA, SsoFast™ qPCR Mastermix (Bio Rad, Hercules, CA), and 1 µM of both forward (5'-TGACAAGGTGTGTGTCCACC-3') and reverse (5'-ACAGGCAGTTGTCCTCACAG-3') primers. RPS19 was amplified as an internal control. All reactions were performed in triplicate. The thermocycler used for RT- PCR was a Bioradicycler IQ (Biorad, Hercules, CA).

RT-PCR Part 2: The same process as above was performed with two exceptions: HSA cells were not PKH26-stained and RBC lysis was performed prior to RNA extraction.

Flow Cytometry Part 1: PKH26-stained HSA cells were mixed with 100 µL of concentrated unstained HSA cells to increase the detection yield of flow cytometry. Acquisition of the flow cytometric data was performed using a MoFlo Astrios and the Summit software package (Beckman Coulter, Brea, CA). Analysis of the data was performed using FloJo X software package (Tree Star Inc., Ashland, OR). The samples were tested for 2 replicates. HSA cell fragments and cell aggregates were included in the data collection. This method was repeated for the pre- and post-LRF samples in Part 1.

Flow Cytometry Part 2: The samples 1-4 from Part 2 were washed with PBS to remove residual serum, incubated in 1 mM EDTA for 5 min, and detached by gentle scraping with a sterile rubber policeman. Staining buffer consisted of PBS with 0.5% bovine serum and 0.1% sodium azide. One million cells were incubated with anti-CD51/CD61 antibody (2 mug/ml, EMD Millipore, Billerica, MA), anti-CD18 antibody (0.5 mg/mL, AbdSerotec, Raleigh, NC), or anti-CD117 antibody (2 mug/ml, eBiosciences,San Diego, CA) for 30 min at 4°C. Cells were then washed four times in staining buffer and incubated with a goat-anti-mouse IgG/IgM antibody conjugated to Alexa 488, PE, and Alexa 647 for 20 min. Cells were again washed four times in staining buffer and then fixed in 2.5% neutral buffered formalin. Samples were kept at 4°C protected from light until analysis using a Beckman Coulter MoFlo Astiros FACS instrument (Beckman Coulter, Berea, CA, USA) running the Summit software package. [15] The flow data analysis was conducted using FlowJo 10 (Tree Star Inc., Ashland, OR).[15]

Cytology Part 1 and Part 2: A board-certified clinical pathologist (G.L.) prepared and examined three slides of each sample using a method modified from Catling *et al*. [136] The clinical pathologist was blinded to whether the sample was pre- or post-LRF. The aliquot of each sample was concentrated by centrifugation at 3000 rpm for 10 minutes. The supernatant was removed and the remaining cells and solution were re-suspended in 5 mL of phosphate buffered saline. Five drops of a RBC lysing agent (ZAP OGLOBIN II Lytic Reagent, Beckman Coulter, Ireland) were added to the re-suspended liquid, which was re-centrifuged then the supernatant was decanted to leave 1

mL of liquid. The tube was vortexed for 30 seconds to re-suspend the cells. Three cytocentrifuge funnel/slide combinations were loaded with 200 µL of the solution. Cytocentrifuge preparations were centrifuged at 1500 rpm for 10 min. Preparations were stained using a modified Wright's stain (Wescor, Logan, UT) and evaluated for the presence of HSA cells. The number of whole cells and number of cell fragments present were recorded. Slide preparations were made immediately after sample collection.

Leukocyte Reduction Filter Evaluation

Filter material was removed from the plastic housing and placed in 10% neutral buffered formalin for more than 24 hours. Following fixation, the filter material was dehydrated in a series of graded ethanol. The layered filter material was separated to allow representative evaluation. A low-vacuum scanning electron microscope[2] at 5 to 15 kV was used to obtain images of HSA cells captured within the filter material. Images were captured at magnifications of 300x to 3000x.

Statistical Analysis

Descriptive statistics were generated for time for processing using JMP Pro 11 (SAS Institute Inc, Cary,NC).

Results

RT-PCR, flow cytometry and cytological examination were all able to detect the presence of PKH26-stained HSA cells in the 50 cell/ml solution. Therefore, all three tests were performed to evaluate samples in both part 1 and part 2 of the study.

Part 1

Analysis by RT-PCR, flow cytometry and cytological evaluation all detected the presence of PKH26-stained HSA cells in the pre-LRF samples. Following LRF processing, RT-PCR, flow cytometry and cytological evaluation did not detect any PKH26-stained HSA cells in the post-LRF samples.

The mean duration required for 55 mL of the 50 cells/mL of culture media solution to pass through the LRF under 300 mmHg of pressure was 7.6 s or 7.2 mL/s (range, 7.4-7.9 s).

Part 2

When the HSA cells were suspended in dog blood, all three methods of detection revealed the presence of HSA cells in sample 1 (control). Following IOCS (sample 2), RT-PCR, flow cytometry and cytological evaluation detected HSA cells. Additionally, flow cytometry detected signals consistent with cell fragments and cell fragment aggregates in samples following IOCS. Following LRF (sample 3), HSA cells were not detected by RT-PCR or cytological analysis. Flow cytometry did not detect HSA cells in the post-LRF sample for the first trial. Flow cytometry detected the presence of approximately 16 HSA cells and 56 HSA cells in post-LRF samples in the second and third trials, respectively. When the discarded wash solution (sample 4) was evaluated, RT-PCR and cytological analysis did not detect any HSA cells. Flow cytometry did not detect HSA cells in sample 4 for the first trial. Flow cytometry determined the presence of approximately 14 HSA cells and 94 HSA cells in the wash solutions of the second and third trials, respectively **(Table 3)**

Cell Detection Method	Sample 1 Pre-IOCS	Sample 2 Post-IOCS	Sample 3 Post-LRF	Sample 4 IOCS Wash
Flow cytometry	+ + +	+ + +	- + +	- + +
RT-PCR	+ + +	+ + +	- - -	- - -
Cytologic examination	+ + +	+ + +	- - -	- - -

Table 3. Results from three cell detection methods. Each plus sign (+) denotes HSA cells were detected from one trial. Each negative sign (-) denotes no HSA cells were detected from one trial.

The mean time for IOCS processing was 15 min (range 13-17 min) or 0.64 mL/s and the mean time for LRF processing using gravity was 6 min (range 3-10 min) or 0.43 mL/s.

The filter imaging revealed cells adhered to the fibers of the filter **(Figure 2).** The cells appeared intact.

Discussion

The findings of this study indicate that IOCS in combination with LRF is able to remove a majority of the 300 million HSA cells added to the dog blood. The IOCS process filters, washes, and centrifuges the blood, however, is inadequate for HSA cell removal as HSA cells were detected after the IOCS processing. Evaluation of the sample of pRBC following IOCS (sample 2) in Part 2 of the experiment revealed the presence of HSA cells. A small number of HSA cells were detected in the wash solution in two of

the three experimental trials, which shows that the IOCS system alone is relatively ineffective at removing HSA cells during the saline wash. We suspect that the LRF is responsible for the majority of HSA cell removal.

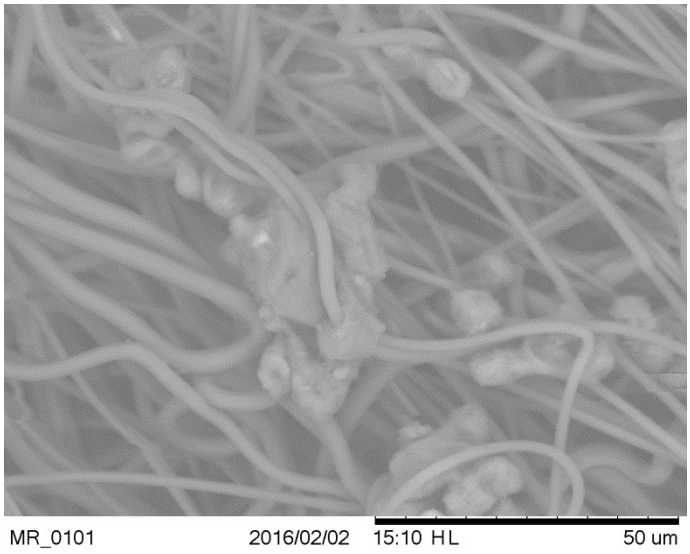

Figure 2. Scanning electron micrograph of a used LRF

Flow cytometry detected HSA cells in the post-LRF filtrate (sample 3) in 2 of the 3 trials. HSA cells may have passed through the LRF if the filter material has a limit to the number of cells that it can trap and that number was exceeded in the second and third trial. Previous studies have shown that with high concentrations of cultured tumor cells, the LRF fails to remove all detectable tumor cells.[127,129] Fruhauf *et al.* found that the depletion capacity, or percent of cells removed by the LRF, of a LRF using cultured colorectal carcinoma cells and primary tumor cells in human blood was 99.6% to 100%.[130] Because flow cytometry cannot be used to accurately quantitate cells, we cannot calculate the precise depletion capacity of the LRF in this experiment. However, visual

comparison the scatterplots from the pre- and post-LRF samples shows a drastic reduction of cell number in the area gated for HSA cells **(Figure 3).** In a different study, hepatocellular carcinoma cells were detected post-LRF when a tumor ruptured in surgery. The authors theorized that the filter was overwhelmed allowing overflow of tumor cells.[127] In the present study, the number of tumor cells may have overwhelmed the filters, allowing overflow. It is difficult to explain why HSA cells were detected in only two of the three trials.

The LRF removes WBC and tumor cells. Therefore, increasing either WBC or tumor cell numbers would theoretically contribute to overwhelming the filter. A WBC count was not performed on the blood used in this study, so we are unable to determine if a slightly higher WBC count was present in blood from trials 2 and 3, resulting in "spill over" of HSA cells into the post-LRF sample. However, a study using human blood showed that the patient's WBC count did not correlate with the ability of the LRF to remove tumor cells.[181]

Alternatively, other differences in the blood could have contributed to the detection of HSA cells in two of the three trials. Brownlee *et al.* found that the efficiency of the LRF to remove dog WBC increased when the dog blood was cooled.[112] Increasing blood viscosity with lower temperatures may slow the flow and increase the contact time between the cells and the filter material. If this speculation is true, other factors that increase blood viscosity, such as PCV, would be expected to change the efficiency of the LRF as well. The temperature of the blood/HSA cell mixture was not measured in our study design but all trials were performed at ambient temperatures. The

variation in temperature, viscosity and PCV of the blood/HSA cell mixture in each of the three trials may have contributed to the detection of HSA cells by flow cytometry in two of the three trials.

A discrepancy between the three methods of cell detection was found in the post-LRF and wash fluid samples for the second and third IOCS and LRF trials. Flow cytometry detected the presence of HSA cells in both of the samples but RT-PCR and cytological examination did not detect HSA cells. RT-PCR is used to detect small amounts of DNA and is considered a sensitive method of cell detection. With low numbers of HSA cells, false negative results are possible with RT-PCR if the DNA was damaged during sample handling. False positive results are possible with flow cytometry. False positive results could have occurred if HSA cells from a previous run were stuck to the plastic tubing inside the analyzer despite efforts to flush the tubing of all debris between samples. Further, flow cytometry detects surface markers in cell membrane. It is possible that clumps of fragmented cell membrane were interpreted as cells in the second and third trials. However, because of the number of cells found, we consider this possibility unlikely.

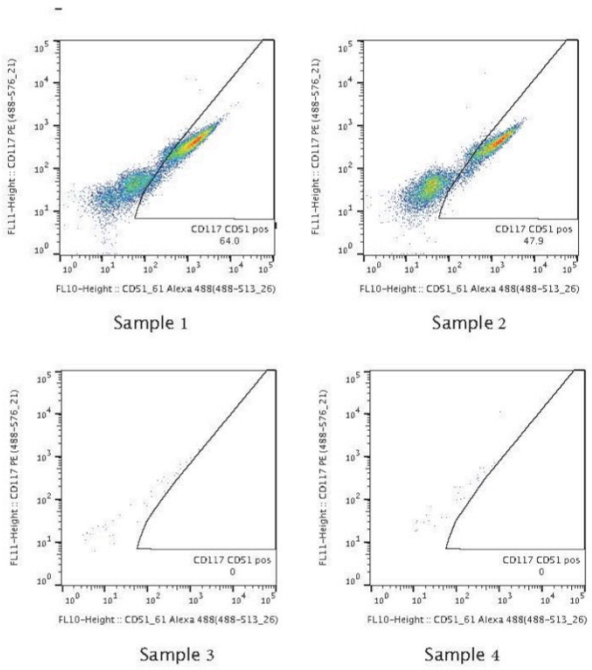

Figure 3. Flow cytometry results from Part 2 second trial. A marked reduction in HSA cell numbers is found in the post-LRF sample (sample 3). A small number of HSA cells are found in the wash fluid obtained from the IOCS processing.

The number of tumor cells expected to be encountered during surgical treatment for HSA is unknown. The number of HSA cells to be processed through the IOCS and LRF in this experiment is based on the findings of Lamerato, Kozicki *et al.* An estimated 0.8% of all circulating nucleated cells in dogs with HSA are tumor-like cells. [15] In a dog with a high-normal WBC count of 136 WBC/μL of blood, about 136,000 HSA cells/mL of blood are circulating in dogs with HSA. This experimental design tested the ability of the LRF to remove over 2000 times the estimated amount of circulating HSA cells in attempt to recreate the clinical scenario of a dog undergoing surgery to address a

ruptured tumor. Specifically, in Part 1, 35 million cells were added to culture media and processed through the LRF. No HSA cells were detected following LRF processing in Part 1. In Part 2, a total of 300 million HSA cells were added to dog blood and processed through the IOCS and LRF. The administration of ABT has a negative effect on the success of cancer treatment in some human patient populations.[3,90,2] When used in place of ABT, autologous blood transfusions may ameliorate some of the negative effects on tumor progression seen with ABT administration. A randomized controlled trial of patients with colorectal tumors compared disease-free survival rates between patients randomized to receive ABT or autologous pre-donated blood during curative-intent surgery. Results showed ABT administration and tumor stage were the only two variables to independently negatively affect disease-free survival.[89] When compared to ABT administration, IOCS use was shown to be associated with an equal survival time in human patients with late stage hepatocellular carcinoma but an improved survival time when used in patients with early stage hepatocellular cacinoma.[139,144] While a clear benefit has not been proven in all patient populations, IOCS use has been deemed safe for use in oncologic surgery for humans.[146,19]

The mechanism by which ABT affects disease progression in cancer patients is poorly understood and multifactorial but is believed to involve transfusion-related immunomodulation (TRIM) and the negative effects seen with storage of blood products.[114] TRIM refers to the subclinical changes in the immune system following the administration of ABT, which may be especially important in cancer patients. Patients with malignant gastrointestinal tumors who received ABT showed impaired tumor cell

recognition and killing compared to those patients receiving autologous blood.[1] These tumor-promoting effects of ABT are seen across species as ABT has been shown to enhance implanted tumor growth in mice but the clinical consequences of TRIM in dogs have not been described.[88]

The storage of blood products may also contribute to the negative clinical effects seen with ABT use, and may be especially relevant in HSA patients.[76,96,104] Stored dog blood accumulates mediators which have been shown to enhance HSA growth and survival, such as VEGF, IL-8 and microparticles.[21,31,105-107] Microparticles are cell fragments that have been recently recognized for their involvement with inflammation, tumor progression, angiogenesis and metastasis.[108] The clinical consequences of the presence of these mediators are unknown. While the benefit of ABT administration in dogs with life-threatening hemorrhage and anemic hypoxia cannot be denied, these potentially tumor-promoting mediators are inadvertently administered to dogs undergoing surgery for HSA. Currently, alternative methods to address hemorrhage and anemic hypoxia in dogs with HSA are limited.

IOCS for dogs with cancer has yet to be explored as an alternative to ABT administration. The previously described veterinary use of the IOCS did not include a LRF.[122,149,182] The viability of canine RBCs is normal immediately after LRF processing.[112] Although not yet studied in dogs, human RBC salvaged during spinal surgery have a normal lifespan of 30 days after IOCS processing.[123] Compared to allogeneic blood products, intraoperatively salvaged blood has been shown to have increased longevity, increased 2,3-disphosphoglycerate levels, increased adenosine

triphosphate levels and improved conformability, which is important for capillary circulation. For these reasons, tissue oxygen delivery should be greater when IOCS is used compared to ABT administration.[120]

Because of the potential consequence of returning tumor cells to a dog with HSA through autotransfusion, we suspect it would be prudent to include a LRF when using IOCS in oncologic surgery as the LRF greatly reduced the number of HSA cells. The LRF failed to eliminate all tumor cells from blood that was processed through the IOCS system, however the need to remove all HSA cells from blood before autotransfusion is questioned for several reasons. First, it has not been proven that tumor cells are shed and therefore collected during surgery in dogs. Tumor cells have been found in the operating field for several tumor types in humans.[125,137,150] The presence of tumor cells shed during surgery is important to determine because circulating tumor cells have distinct characteristics and behavior compared to tumor cells shed from the primary tumor in surgery.[152] HSA cells have been found in the free abdominal fluid in dogs but the concentration and viability of these cells shed during surgery is unknown.[15] Second, it is unknown if all tumor cell types are able to survive IOCS processing. Results from this experiment show that whole tumor cells are present after IOCS processing but their viability was not tested. Third, if tumor cells survive IOCS processing and are returned to the patient, it is unknown if those cells are capable of forming new tumors or contribute to the progression of disease. Human carcinoma cells salvaged at surgery have been shown to be tumorigenic in immunodeficient mice, however these results may not apply to patients with a normal immune system or other tumor cell types.[152]

Metastasis is an inefficient and complicated process; an estimated 0.1% of disseminated tumor cells are capable of forming a metastatic lesion.[154] Wang *et al.* estimates that in dogs with HSA, 1% of circulating nucleated cells are HSA cells, but their ability to form metastatic lesions is unknown.[155] Fourth, even if the cells survive IOCS and exhibit tumorigenicity, it is unknown if this affects quality of life and survival time in dogs with a life-limiting disease such as HSA.

Study Limitations

This study has limitations, especially in regards to cell detection. The number of HSA cells encountered during surgery is unknown and may not have been accurately recreated in this study. The acceptable number of remaining tumor cells before returning autologous blood to a patient has not been established. While a majority of HSA cells can be removed with IOCS and LRF processing, the possible presence of other soluble mediators that permit tumor progression were not evaluated in this study. We cannot comment on the safety for use in clinical patients.

All three cell detection methods used in this experimental approach have limitations. In Part 1, we were able to determine the ability of flow cytometry, RT-PCR and cytological examination to detect 50 HSA cells/mL of culture media. Although no HSA cells were found in the post-LRF samples in the first trial for Part 2, we cannot determine if fewer than 50 HSA cells/mL of blood were present. A highly sensitive and specific cell detection method has been shown to detect small concentrations of cultured HSA cells, but this technology requires more validation and was not available to the investigators.[155]

Because of the potential consequences of ABT listed above, IOCS is a viable consideration for animals needing blood in the perioperative period. The veterinary literature states that IOCS should be avoided in oncologic surgery to avoid iatrogenic tumor cell dissemination. We have shown that the LRF is capable of removing a majority of 300 million cultured HSA cells from dog blood. With further studies, IOCS with LRF has the potential to negate some of the disadvantages of ABT when used for dogs with HSA.

CHAPTER IV

SUMMARY

Hemangiosarcoma (HSA) is a biologically aggressive tumor with a poorly understood pathogenesis and natural history. Despite advances in veterinary oncology, the survival time for this tumor type has not improved in the past thirty years. The retrospective data from medical records of dogs undergoing surgical treatment for HSA showed that allogeneic blood transfusion (ABT) use was an independent predictor for shorter time to clinical decline. We cannot determine if ABT use is a surrogate variable for increasing disease severity in dogs with HSA. If the relationship between ABT and shorter time to clinical decline is causal, transfusion-related immunomodulation may be involved.

Intraoperative cell salvage offers a practical advantage to ABT use in dogs with HSA that need a blood transfusion. The addition of a leukocyte reduction filter to the intraoperative cell salvage system may allow for this technique to be used to restore intravascular hemoglobin and volume support to dogs undergoing surgery to address bleeding intraabdominal tumors. We showed that the intraoperative cell salvage system and leukocyte reduction filter are able to remove a majority of HSA cells from dog blood. Our results allow for future experiments to determine if this blood-conservation technique can be used safely in clinical settings and if intraoperative cell salvage could ameliorate the negative consequences of ABT use in dogs with HSA.

REFERENCES

1. Guo JR, Xu F, Jin XJ, Shen HC, Liu Y, Zhang YW, Shao Y. Impact of allogenic and autologous transfusion on immune function in patients with tumors. Asian Pac J Cancer Prev. 2014;15(1):467-74.

2. Tartter P. The association of perioperative blood transfusion with colorectal cancer recurrence. Ann Surg. 1992;216(6):633-638.

3. Hyung WJ, Noh SH, Shin DW, Huh JHJ, Huh BJ, Choi SH, Min JS. Adverse effects of perioperative transfusion on patients with stage III and IV gastric cancer. Ann Surg Oncol. 2002;9(1):5-12.

4. Rosenberg SA, Seipp CA, White DE, Wesley R. Perioperative blood transfusions are associated with increased rates of recurrence and decreased survival in patients with high-grade soft-tissue sarcomas of the extremities. J Clin Oncol. 1985 05;3(5):698-709.

5. Spangler WL, Kass PH. Pathologic factors affecting postsplenectomy survival in dogs. Journal of Veterinary Internal Medicine. 1997;11(3):166-71.

6. Tamburini BA, Phang TL, Fosmire SP, Scott MC, Trapp SC, Duckett MM, Robinson SR, Slansky JE, Sharkey LC, Cutter GR, Wojcieszyn JW, Bellgrau D, Gemmill RM, Hunter LE, Modiano JF. Gene expression profiling identifies inflammation and angiogenesis as distinguishing features of canine hemangiosarcoma. BMC Cancer. 2010;10(619)

7. Brown NO, Patnaik AK, MacEwen EG. Canine hemangiosarcoma: Retrospective analysis of 104 cases. J Am Vet Med Assoc. 1985 Jan 1;186(1):56-58.

8. Spangler WL. Pathologic factors affecting postsplenectomy survival in dogs. Journal of veterinary internal medicine. 1997;11(3):166-71.

9. Moe L, Gamlem H, Dahl K, Glattre E. Canine neoplasia - population-based incidence of vascular tumours. APMIS.Acta pathologica, microbiologica et immunologica Scandinavica. 2008;116(125):63-8.

10. Hammer AS, Couto CG, Swardson C, Getzy D. Hemostatic abnormalities in dogs with hemangiosarcoma. Journal of Veterinary Internal Medicine. 1991;5(1):11-4.

11. Sabattini S, Bettini G. Immunohistochemical analysis of canine haemangioma and haemangiosarcoma. J Comp Pathol. 2009;140(2-3):158-168.

12. Sorenmo K, Duda L, Barber L, Cronin K, Sammarco C, Usborne A, Goldschmidt M, Shofer F. Canine hemangiosarcoma treated with standard chemotherapy and minocycline. Journal of Veterinary Internal Medicine. 2000;14(4):395-8.

13. Waters D. Metastatic pattern in dogs with spleenic hemangiosarcoma - clinical implications. J Small Anim Pract. 1988;29(12):805-14.

14. Holt D. Correlation between thoracic radiographs and postmortem findings in dogs with hemangiosarcoma: 77 cases (1984-1989). J Am Vet Med Assoc. 1992

15. Lamerato Kozicki A, Lamerato Kozicki K, Helm C, Jubala G, Cutter J. Canine hemangiosarcoma originates from hematopoietic precursors with potential for endothelial differentiation. Exp Hematol. 2006;34(7):870-8.

16. Waters D. Metastatic patterns in dogs with hemangiosarcoma and clinical implications. J Small Anim Pract. 1988;29(12):805-14.

17. Oksanen A. Haemangiosarcoma in dogs. J Comp Pathol. 1978;88(4):585-595.

18. Priester WA. Hepatic angiosarcomas in dogs: An excessive frequency as compared with man. Journal of the National Cancer Institute. 1976 August 01;57(2):451-4.

19. Gamlem H. Canine vascular neoplasia--histologic classification and inmunohistochemical analysis of 221 tumours and tumour-like lesions. APMIS.Acta pathologica, microbiologica et immunologica Scandinavica.Supplementum. 2008(125):19-40.

20. Liu L, Kakiuchi-Kiyota S, Arnold LL, Johansson SL, Wert D, Cohen SM. Pathogenesis of human hemangiosarcomas and hemangiomas. Hum Pathol. 2013 10;44(10):2302-11.

21. Foamier SP, Dickerson EB, Scott AM, Bianco SR, Pettengill MJ, Meylemans H, Padilla M, Frazer-Abel AA, Akhtar N, Getzy DM, Wojcieszyn J, Breen M, Helmand SC, Modiano JF. Canine malignant hemangiosarcoma as a model of primitive angiogenic endothelium. Lab Invest. 2004;84(5):562-7.

22. Modiano JF, Helfand SC, inventors; The Regents of the University of Colorado, assignee. Early detection of hemangiosarcoma and angiosarcoma. Boulder, CO, USA patent US 7,910,315 B2. March 22, 2011.

23. Vail DM. Spontaneously occurring tumors of companion animals as models for human cancer. Cancer Invest. 2000;18(8):781-92.

24. Lieberman L.L. Malignant hemangioendothelioma of the canine heart. J Am Vet Med Assoc. 1955;126(937):296.

25. Benjamin SA, Lee AC, Angleton GM, Saunders WJ, Keefe TJ, Mallinckrodt CH. Mortality in beagles irradiated during prenatal and postnatal development. II. contribution of benign and malignant neoplasia. Radiat Res. 1998 Sep;150(3):330-48.

26. Falk H, Herbert J, Crowley S, Ishak KG, Thomas LB, Popper H, Caldwell GG. Epidemiology of hepatic angiosarcoma in the united states: 1964-1974. Environ Health Perspect. 1981 Oct;41:107-13.

27. Yonemaru K, Sakai H, Murakami M, Kodama A, Mori T, Yanai T, Maruo K, Masegi T. The significance of p53 and retinoblastoma pathways in canine hemangiosarcoma. J Vet Med Sci. 2007 Mar;69(3):271-8.

28. Yamamoto T, Umeda T, Yokozeki H, Nishioka K. Expression of basic fibroblast growth factor and its receptor in angiosarcoma. J Am Acad Dermatol. 1999;41(1):127-9.

29. Dickerson EB, Marley K, Edris W, Tyner JW, Schalk V, MacDonald V, Loriaux M, Druker BJ, Helfand SC. Imatinib and dasatinib inhibit hemangiosarcoma and implicate PDGFR-ß and src in tumor growth. Translational Oncology. 2013;6(2):158-68.

30. Hashimoto M, Ohsawa M, Ohnishi A, Naka N, Hirota S, Kitamura Y, Aozasa K. Expression of vascular endothelial growth factor and its receptor mRNA in angiosarcoma. Lab Invest. 1995;73(6):859-63.

31. Clifford CA. Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. Journal of veterinary internal medicine. 2001;15(2):131-5.

32. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: Role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007 01;7(1):41-51.

33. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860-7.

34. Arbiser JL, Larsson H, Claesson-Welsh L, Bai X, LaMontagne K, Weiss SW, Soker S, Flynn E, Brown LF. Overexpression of VEGF 121 in immortalized endothelial cells causes conversion to slowly growing angiosarcoma and high level expression of the VEGF receptors VEGFR-1 and VEGFR-2 in vivo. The American Journal of Pathology. 2000 4;156(4):1469-76.

35. Farhang Ghahremani M, Radaelli E, Haigh K, Bartunkova S, Haenebalcke L, Marine JC, Goossens S, Haigh JJ. Loss of autocrine endothelial-derived VEGF significantly reduces hemangiosarcoma development in conditional p53-deficient mice. Cell Cycle. 2014;13(9):1501-7.

36. Yamamoto T, Umeda T, Nishioka K. Immunohistological distribution of stem cell factor and kit receptor in angiosarcoma. Acta Derm Venereol. 2000;80(6):443-5.

37. Pintar J. Acute nontraumatic hemoabdomen in the dog: A retrospective analysis of 39 cases (1987-2001). J Am Anim Hosp Assoc. 2003;39(6):518-22.

38. Prymak C. Epidemiologic, clinical, pathologic, and prognostic characteristics of splenic hemangiosarcoma and splenic hematoma in dogs: 217 cases (1985). J Am Vet Med Assoc. 1988;193(6):706-12.

39. Johnson K, Powers B, Withrow S, Sheetz M, Curtis C, Wrigley R. Splenomegaly in dogs . J Vet Internal Med. 1989;3(3):160-166.

40. Fife WD, Samii VF, Drost WT, Mattoon JS, Hoshaw-Woodard S. Comparison between malignant and nonmalignant splenic masses in dogs using contrast-enhanced computed tomography. Vet Radiol Ultrasound. 2004 07/20;45(4):289-97.

41. Nelson RW, Couto G. Selected Neoplasms in Dogs and Cats In: Nelson RW, Couto G, editors. Small Animal Internal Medicine. 3rd ed. ; 2003; p. 1142-1143.

42. Wendelburg KM. Risk factors for perioperative death in dogs undergoing splenectomy for splenic masses: 539 cases (2001-2012). J Am Vet Med Assoc. 2014;245(12):1382-90.

43. Wood CA, Moore AS, Gliatto JM, Ablin LA, Berg RJ, Rand WM. Prognosis for dogs with stage I or II splenic hemangiosarcoma treated by splenectomy alone: 32 cases (1991-1993). J Am Anim Hosp Assoc. 1998 09/01; 2015/06;34(5):417-21.

44. Wong R, Gonsalves M, Huber M, Rich L, Strom A. Erythrocyte and biochemical abnormalities as diagnostic markers in dogs with hemangiosarcoma related hemoabdomen. Vet Surg. 2015;44(7):852-7.

45. Maruyama H, Miura T, Sakai M, Koie H, Yamaya Y, Shibuya H, Sato T, Watari T, Takeuchi A, Tokuriki M, Hasegawa A. The incidence of disseminated intravascular coagulation in dogs with malignant tumor. J Vet Med Sci. 2004;66(5):573-5.

46. Lux C. Perioperative outcome in dogs with hemoperitoneum: 83 cases (2005-2010). J Am Vet Med Assoc. 2013;242(10):1385-91.

47. Hammond TN, Pesillo-Crosby SA. Prevalence of hemangiosarcoma in anemic dogs with a splenic mass and hemoperitoneum requiring a transfusion: 71 cases (2003-2005). J Am Vet Med Assoc. 2008;232(4):553-8.

48. Wierenga J, Rosenstein D, Hauptman J. Prognostic indicators of splenic masses. In: Society of Veterinary Soft Tissue Surgeons Annual Conference Proceedings ; 2002 2002; Keystone, CO. 2002

49. Johnson KA. Splenomegaly in dogs: Predictors of neoplasia and survival after splenectomy. J Vet Internal Med. 1989;3(16):160-8.

50. Marino DJ. Ventricular arrhythmias in dogs undergoing splenectomy: A prospective study. Veterinary surgery. 1994;23(2):101-6.

51. Johnson KA, Powers BE, Withrow SJ, Sheetz MJ, Curtis CR, Wrigley RH. Splenomegaly in dogs. Journal of Veterinary Internal Medicine. 1989;3(3):160-6.

52. Brockman D. A practical approach to hemoperitoneum in the dog and cat. The Veterinary clinics of North America.Small animal practice. 2000;30(3):657-70.

53. Wrigley RH. Ultrasonographic features of splenic hemangiosarcoma in dogs: 18 cases (1980-1986). J Am Vet Med Assoc. 1988;192(8):1113-7.

54. Clifford CA. Magnetic resonance imaging of focal splenic and hepatic lesions in the dog. J Vet Internal Med. 2004;18(3):330-8.

55. Yuki M, Machida N, Sawano T, Itoh H. Investigation of serum concentrations and immunohistochemical localization of a1-acid glycoprotein in tumor dogs. Vet Res Commun. 2011 01;35(1):1-11.

56. Thamm DH, Kamstock DA, Sharp CR, Johnson SI, Mazzaferro E, Herold LV, Barnes SM, Winkler K, Selting KA. Elevated serum thymidine kinase activity in canine splenic hemangiosarcoma. Vet Comp Oncol. 2012 12;10(4):292-302.

57. Sharp C. Diagnosis of Hemangiosarcoma in Dogs with Hemoabdomen [Internet]. cited 7/16/15]. Available from: http://sites.tufts.edu/vetclinicaltrials/?qa_faqs=diagnosis-of-hemangiosarcoma-in-dogs-with-hemoabdomen

58. Kirby GM, Mackay A, Grant A, Woods P, McEwen B, Khanna C, Macri J, Hayes MA, Stalker M. Concentration of lipocalin region of collagen XXVII alpha 1 in the serum of dogs with hemangiosarcoma. J Vet Intern Med. 2011 05/20;25(3):497-503.

59. Chun R.1., Kellihan H.B., Henik R.A., Stephen R.L. Comparison of plasma cardiac troponin I concentrations among dogs with cardiac hemangiosarcoma, noncardiac hemangiosarcoma, other neoplasms, and pericardial effusion of nonhemangiosarcoma origin. J Am Vet Med Assoc. 2010;237(7):806-11.

60. Göritz M. Neoplastic disease: Canine splenic haemangiosarcoma: Influence of metastases, chemotherapy and growth pattern on post-splenectomy survival and expression of angiogenic factors. J Comp Pathol. 2013;149(1):30-9.

61. Sorenmo K, Samluk M, Clifford C, Baez J, Barrett JS, Poppenga R, Overley B, Skorupski K, Oberthaler K, Winkle TV, Seller G, Shofer F. Clinical and pharmacokinetic characteristics of

intracavitary administration of pegylated liposomal encapsulated doxorubicin in dogs with splenic hemangiosarcoma. Journal of Veterinary Internal Medicine. 2007;21(6):1347-54.

62. Wendelburg K, Price L, Burgess K, Lyons J, Lew F, Berg J. Survival time of dogs with splenic hemangiosarcoma treated by splenectomy with or without adjuvant chemotherapy: 208 cases (2001–2012). J Am Vet Med Assoc. 2015;247(4):393-403.

63. Göritz M, Müller K, Krastel D, Staudacher G, Schmidt P, Kühn M, Nickel R, Schoon H-. Canine splenic haemangiosarcoma: Influence of metastases, chemotherapy and growth pattern on post-splenectomy survival and expression of angiogenic factors. J Comp Pathol. 2013;149(1):30-9.

64. Pariaut R. Chapter 47 - Ventricular Tachyarrhythmias In: Hopper DCS, editor. Small Animal Critical Care Medicine. Saint Louis: W.B. Saunders; 2009; p. 200-3.

65. Prittie J. Controversies related to red blood cell transfusion in critically ill patients. J Vet Emerg Crite Car. 2010;20(2):167-176.

66. Ludwig, L VMD, MS, Dipl. ACVS,. Surgery STAT: Emergency management of hemoabdomen [Internet]. http://veterinarynews.dvm360.com: Oct 01, 2010 cited 7/13/2015]. Available from: http://veterinarynews.dvm360.com/surgery-stat-emergency-management-hemoabdomen

67. Prittie JE. Triggers for use, optimal dosing, and problems associated with red cell transfusions. The Veterinary clinics of North America.Small animal practice. 2003;33(6):1261-75.

68. Kerl ME, Höhenhaus AE. Packed red blood cell transfusions in dogs: 131 cases (1989). J Am Vet Med Assoc. 1993;202(9):1495-1499.

69. Giger U. Chapter 120 - Anemia In: Hopper DCS, editor. Small Animal Critical Care Medicine. Saint Louis: W.B. Saunders; 2009; p. 518-23.

70. Adams R , Lundy J . Anesthesia in cases of poor surgical RiskSome suggestions for decreasing the risk. Anesthesiology. 1942 September 1;3(5):603-7.

71. Corwin HLM, Gettinger AM, Pearl RGM. The CRIT study: Anemia and blood transfusion in the critically ill - current clinical practice in the united states. Critical Care Medicine. 2004;3(1):39-52.

72. Vincent J, Baron J, Reinhart K, et al:. Anemia and blood transfusion in critically ill patients. J Am Med Assoc. 2002;288(12):1499-1507.

73. Glance LG, Dick AW, Mukamel DB. Association between intraoperative blood transfusion and mortality and morbidity in patients UndergoingNoncardiac surgery. Anesthesiology. 2011;114(2):283-292.

74. Bernard A. Intraoperative transfusion of 1 U to 2 U packed red blood cells is associated with increased 30-day mortality, surgical-site infection, pneumonia, and sepsis in general surgery patients. J Am Coll Surg. 2009;208(5):931-7.

75. Gianchandani Moorjani R, Marchena-Gomez J, Casimiro-Perez J, Roque-Castellano C, Ramirez-Felipe J. Morbidity- and mortality-related prognostic factors of nontraumatic splenectomies. Asian Journal of Surgery. 2014;37(2):73-9.

76. Napolitano LM, Corwin HL. Efficacy of red blood cell transfusion in the critically ill. Crit Care Clin. 2004;20(2):255-68.

77. Holowaychuk MK, Leader JL, Monteith, G. Risk factors for transfusion-associated complications and nonsurvival in dogs receiving packed red blood cell transfusions: 211 cases (2008–2011). J Am Vet Med A. 2014;244(4):431-437.

78. Spahn D, Spahn G, Stein P. Evidence base for restrictive transfusion triggers in high-risk patients. Transfus Med Hemother. 2015;42(2):110-4.

79. Weiskopf RB, Viele MK, Feiner J, Kelley S, Lieberman J, Noorani M, Leung JM, Fisher DM, Murray WR, Toy P, Moore MA. Human cardiovascular and metabolic response to acute, severe isovolemic anemia. JAMA. 1998 01/21;279(3):217-21.

80. Schwartz S. Sequential hemodynamic and oxygen transport responses in hypovolemia, anemia, and hypoxia. Am J Physiol. 1981;241(6):H864-871.

81. Salem-Schatz SR. Influence of clinical knowledge, organizational context, and practice style on transfusion decision making. implications for practice change strategies. JAMA: the Journal of the American Medical Association. 1990;264(4):476-83.

82. Kirkley S. Proposed mechanisms of transfusion-induced immunomodulation. Clin Diagn Lab Immunol. 1999;6(5):652-7.

83. Vamvakas EC, Blajchman MA. Transfusion-related immunomodulation (TRIM): An update. Blood Rev. 2007 Nov;21(6):327-48.

84. Opelz G, Sengar DP, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplants. Transplant Proc. 1973;5(1):253-9.

85. Marquet RL, Heineman E, Tank B, Obertop H, Niessen GJ, Bijnen AB, Westbroek DL, Jeekel J. Abrogation of the beneficial blood transfusion effect in dogs by splenectomy. World J Surg. 1984;8(3):408-13.

86. Brunson ME, Alexander JW. Mechanisms of transfusion-induced immunosuppression. Transfusion. 1990;30(7):651-8.

87. Gantt C. Red blood cells for cancer patients. The Lancet. 1981 8/15;318(8242):363.

88. Horimi T, Kagawa S, Ninomiya M, Yoshida E, Hiramatsu S, Orita K. Possible induction by blood transfusion of immunological tolerance against growth of transplanted tumors in mice. Acta Med Okayama. 1983;37(3):259-63.

89. Heiss MM, Tempel W., Delanoff C., Jauch KW, Gabka C, Mempel M, Dieterich HJ, Eissäer HJ, Schildberg FW. Blood transfusion-modulated tumor recurrence: First results of a randomized study of autologous versus allogeneic blood transfusion in colorectal cancer surgery. Journal of Clinical Oncology. 1994;12(9):1859-67.

90. Amato A, Pescatori M. Perioperative blood transfusions for the recurrence of colorectal cancer. Cochrane Database Syst Rev. 2006 Jan 25;1(1):CD005033.

91. Journal Of Biomedicine & Biotechnology [Internet]. United States: Hindawi Pub. Corp Vol. 2011, 2011 - Available from: doi:10.1155/2011/676198

92. Guo JR, Xu F, Jin XJ, Shen HC, Liu Y, Zhang YW, Shao Y. Impact of allogenic and autologous transfusion on immune function in patients with tumors. Asian Pac J Cancer Prev. 2014;15(1):467-74.

93. Lenhard V, Maassen G, Opelz G. Transfusion-induced enhancement of prostaglandin and thromboxane release in prospective kidney graft recipients. Proc Eur Dial Transplant Assoc Eur Ren Assoc. 1985;21:923-7.

94. Perez RV, Munda R, Alexander JW. Dietary immunoregulation of transfusion-induced immunosuppression. Transplantation. 1988;45(3):614-7.

95. Fransen E, Maessen J, Dentener M, Senden N, Buurman W. Impact of blood transfusions on inflammatory mediator release in patients undergoing cardiac surgery. Chest. 1999 Nov;116(5):1233-9.

96. McMichael MA, Smith SA, Galligan KS, et al:. Effect of leukoreduction on transfusion-induced inflammation in dogs. J Vet Internal Med. 2010;24(5):1131-7.

97. Callan MB, Patel RT, Rux AH, Bandyopadhyay S, Sireci AN, O'Donnell,P.A., Ruane T, Sikora T, Marryott K, Sachais BS, Hod EA. Transfusion of 28-day-old leucoreduced or non-leucoreduced stored red blood cells induces an inflammatory response in healthy dogs. Vox Sang. 2013 11;105(4):319-27.

98. Luo JL. Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer cell. 2004;6(3):297-305.

99. Morrison WB. Inflammation and cancer: A comparative view. Journal of Veterinary Internal Medicine. 2012;26(1):18-31.

100. Giancotti F. Mechanisms governing metastatic dormancy and reactivation. Cell. 2013;155(4):750-64.

101. Raposo TP, Beirão BCB, Pang LY, Queiroga FL, Argyle DJ. Inflammation and cancer: Till death tears them apart. The Veterinary Journal. 2015 8;205(2):161-74.

102. Sachs UJH. Pathophysiology of TRALI: Current concepts. Intensive Care Med. 2007;33(1):S3-S11.

103. Bennett SH. Pulmonary effects of autotransfused blood. A comparison of fresh autologous and stored blood with blood retrieved from the pleural cavity in an in situ lung perfusion model. The American journal of surgery. 1973;125(6):696-702.

104. Nielsen HJ. Time-dependent, spontaneous release of white cell- and platelet-derived bioactive substances from stored human blood. Transfusion. 1996;36(11-12):960-5.

105. Graf C. Effect of leukoreduction treatment on vascular endothelial growth factor concentration in stored canine blood transfusion products. Am J Vet Res. 2012;73(12):2001-6.

106. Corsi R, McMichael M, Smith S, O'Brien M, Herring J, Ngwenyama T, Galligan A, Beloshapka A, Deng P, Swanson K. Cytokine concentration in stored canine erythrocyte concentrates. Journal of veterinary emergency and critical care. 2014;24(3):259-63.

107. Herring J, Smith S, McMichael M, O'Brien M, Ngwenyama T, Corsi R, Galligan A, Beloshapka A, Deng P, Swanson K. Microparticles in stored canine RBC concentrates. Vet Clin Pathol. 2013;42(2):163-9.

108. Herring JM, McMichael MA, Smith SA. Microparticles in health and disease. J Vet Intern Med. 2013 09/20;27(5):1020-33.

109. Kim JH, Frantz AM, Anderson KL, Graef AJ, Scott MC, Robinson S, Sharkey LC, O'Brien TD, Dickerson EB, Modiano JF. Interleukin-8 promotes canine hemangiosarcoma growth by regulating the tumor microenvironment. Exp Cell Res. 2014 Apr 15;323(1):155-64.

110. Greening DW, Gopal SK, Mathias RA, Liu L, Sheng J, Zhu HJ, Simpson RJ. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. Semin Cell Dev Biol. 2015;40:60-71.

111. Kisielewicz C, Self I. Canine and feline blood transfusions: Controversies and recent advances in administration practices. Vet Anaesth Analg. 2014;41(3):233-42.

112. Brownlee L. Use of a prestorage leukoreduction filter effectively removes leukocytes from canine whole blood while preserving red blood cell viability. Journal of veterinary internal medicine. 2000;14(4):412-7.

113. Blajchman MA. Clinical and molecular basis of transfusion-induced immunomodulation: Summary of the proceedings of a state-of-the-art conference. Transfus Med Rev. 2001;15(2):108-35.

114. Cata JP, Wang H, Gottumukkala V, et al:. Inflammatory response, immunosuppression, and cancer recurrence after perioperative blood transfusions. Br J Anaesth. 2013;110(5):690-701.

115. Price GS, Armstrong PJ, McLeod DA, Babineau CA, Metcalf MR, Sellett LC. Evaluation of citrate-phosphate-dextrose-adenine as a storage medium for packed canine erythrocytes. J Vet Intern Med. 1988;2(3):126-32.

116. Driessen B, Jahr JS, Lurie F, Gunther RA. Inadequacy of low-volume resuscitation with hemoglobin-based oxygen carrier hemoglobin glutamer-200 (bovine) in canine hypovolemia. J Vet Pharmacol Ther. 2001;24(1):61-71.

117. Highmore W. Overlooked source of blood supply for transfusion in post-partum haemorrhage. The Lancet. 1874 Jan 17:89.

118. Brzica Jr SM. Autologous blood transfusion. Mayo Clin Proc. 1976;51(11):723-37.

119. Ashworth A, Klein AA. Cell salvage as part of a blood conservation strategy in anaesthesia . Br J Anaesth. 2010;105(4):401-16.

120. Kellett-Gregory LM, Seth M, Adamantos S, et al:. Autologous canine red blood cell transfusion using cell salvage devices. J Vet Emerg Crit Car. 2013;23(1):82-86.

121. Niebauer GW. Autotransfusion for intraoperative blood salvage: A new technique. Compendium for the Practicing Veterinarian. 1991;13(7):1105-8.

122. Hirst C, Adamantos S. Autologous blood transfusion following red blood cell salvage for the management of blood loss in 3 dogs with hemoperitoneum. Journal of Veterinary Emergency and Critical Care. 2012;22(3):355-60.

123. Ray JM. Erythrocyte survival following intraoperative autotransfusion in spinal surgery: An in vivo comparative study and 5-year update. Spine (Philadelphia, Pa.1976). 1986;11(9):879-82.

124. Homann B, Zenner HP, Schauber J, Ackermann R. Tumor cells carried through autotransfusion. are these cells still malignant? Acta Anaesthesiol Belg. 1984;35 Suppl:51-9.

125. Yamaguchi K, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. Ann Surg. 2000;232(1):58-65.

126. Martin RCG, Wellhausen SR, Moehle DA, et al:. Evaluation of intraoperative autotransfusion filtration for hepatectomy and pancreatectomy. Ann Surg Oncol. 2005;12(12):1017-1024.

127. Gwak MS, Lee KW, Kim SY, et al:. Can a leukocyte depletion filter (LDF) reduce the risk of reintroduction of hepatocellular carcinoma cells? Liver Transplantation. 2005;11(3):331-5.

128. Futamura N, Nakanishi H, Hirose H, et al:. The effects of storage on the survival of cancer cells in blood and efficient elimination of contaminating cancer cells by a leukocyte depletion filter. Am Surgeon. 2005;71(7):585-590.

129. Miller GV, Ramsden CW, Primrose JN. Autologous transfusion: An alternative to transfusion with banked blood during surgery for cancer. Br J Surg. 1991;78(6):713-5.

130. Fruhauf N. Filtration of malignant cells: Tumour cell depletion in an ex vivo model using a leukocyte adhesion filter. Perfusion. 2001;16:51-5.

131. Kongsgaard UE, Wang MY, Kvalheim G. Leukocyte depletion filter removes cancer cells in human blood. Acta Anaesth Scand. 1996;40(1):118-120.

132. Stoffel JT, Topjian L, Libertino JA. Analysis of peripheral blood for prostate cells after autologous transfusion given during radical prostatectomy. BJU Int. 2005 08;96(3):313-5.

133. Perseghin P, Viganò M, Rocco G, Della Pona C, Buscemi A, Rizzi A. Effectiveness of leukocyte filters in reducing tumor cell contamination after intraoperative blood salvage in lung cancer patients. Vox Sang. 1997;72(4):221-4.

134. Torre GC, Ferrari M, Favre A. A new technique for intraoperative blood recovery in the cancer patient. Eur J Surg Oncol. 1994;20(5):565-570.

135. Kumar N, Chen Y, Zaw AS, et al:. Use of intraoperative cell-salvage for autologous blood transfusions in metastatic spine tumour surgery: A systematic review. Lancet Oncol. 2014;15(1):33-41.

136. Catling S, Williams S, Freites O, Rees M, Davies C, Hopkins L. Use of a leucocyte filter to remove tumour cells from intra-operative cell salvage blood. Anaesthesia. 2008;63(12):1332-8.

137. Liang TB, Li DL, Liang L, et al:. Intraoperative blood salvage during liver transplantation in patients with hepatocellular carcinoma: Efficiency of leukocyte depletion filters in the removal of tumor cells. Transplantation. 2008;85(6):863-869.

138. Connor JP, Morris PC, Alagoz T, Anderson B, Bottles K, Buller RE. Intraoperative autologous blood collection and autotransfusion in the surgical management of early cancers of the uterine cervix. Obstet Gynecol. 1995;86(3):373-8.

139. Fujimoto J, Okamoto E, Yamanaka N, Oriyama T, Furukawa K, Kawamura E, Tanaka T, Tomoda F. Efficacy of autotransfusion in hepatectomy for hepatocellular carcinoma. Arch Surg. 1993;128(9):1065-9.

140. Zulim RA, Rocco M, Goodnight JE, Smith GJ, Krag DN, Schneider PD. Intraoperative autotransfusion in hepatic resection for malignancy. is it safe? Arch Surg. 1993;128(2):206-11.

141. Davis M, Sofer M, Gomez Marin O, Bruck D, Soloway MS. The use of cell salvage during radical retropubic prostatectomy: Does it influence cancer recurrence? BJU Int. 2003;91(6):474-6.

142. Akbulut S, Kayaalp C, Yilmaz M, Ince V, Ozgor D, Karabulut K, Eris C, Toprak H, Aydin C, Yilmaz S. Effect of autotransfusion system on tumor recurrence and survival in hepatocellular carcinoma patients. World J Gastroenterol. 2013;19(10):1625-31.

143. Klimberg I, Sirois R, Wajsman Z, Baker J. Intraoperative autotransfusion in urologic oncology. Arch Surg. 1986;121(11):1326-9.

144. Hirano T, Yamanaka J, Iimuro Y, et al:. Long-term safety of autotransfusion during hepatectomy for hepatocellular carcinoma. Surg Today. 2005;35(12):1042-1046.

145. Esper S, Waters J. Intra-operative cell salvage: A fresh look at the indications and contraindications. Blood Transfus. 2011;9(2):139-47.

146. Bower MR, Ellis SF, Scoggins CR, McMasters KM, Martin RC. Phase II comparison study of intraoperative autotransfusion for major oncologic procedures. Ann Surg Oncol. 2011 Jan;18(1):166-73.

147. Domen RE. Adverse reactions associated with autologous blood transfusion: Evaluation and incidence at a large academic hospital. Transfusion. 1998;38(3):296-300.

148. Li MH, Yan LN, Wang SR. Autologous transfusion with modified total hepatic vascular exclusion for extracapsular resection of giant hepatic cavernous hemangioma. Hepatobiliary Pancreat Dis Int. 2007 Feb;6(1):43-8.

149. Lamb J, Thieman Mankin K, Levine G, Thompson J. Electrolyte and acid/base changes in dogs undergoing autologous blood transfusion via a cell salvage device. Can Vet J. 2015;56(9):947-52.

150. Oefelein MG, Kaul K, Herz B, Blum MD, Holland JM, Keeler TC, Cook WA, Ignatoff JM. Molecular detection of prostate epithelial cells from the surgical field and peripheral circulation during radical prostatectomy. J Urol. 1996;155(1):238-42.

151. Kudo H, Fujita H, Hanada Y, Hayami H, Kondoh T, Kohmura E. Cytological and bacteriological studies of intraoperative autologous blood in neurosurgery. Surg Neurol. 2004;62(3):195,9; discussion 199.

152. Hansen E, Wolff N, Knuechel R, et al:. Tumor cells in blood shed from the surgical field. Arch Surg. 1995;130(4):387-393.

153. Edelman MJ, Potter P, Mahaffey KG, Frink R, Leidich RB. The potential for reintroduction of tumor cells during intraoperative blood salvage: Reduction of risk with use of the RC-400 leukocyte depletion filter. Urology. 1996 2;47(2):179-81.

154. Weiss L. Chapter 6: Metastatic inefficiency. Adv Cancer Res. 1990;54(159211.7):159-211.

155. Weina Wang, Kisker DW, Thamm DH, Hua Shao, Lear KL. Optofluidic intracavity spectroscopy of canine hemangiosarcoma. Biomedical Engineering, IEEE Transactions on. 2011;58(4):853-60.

156. Wood CA, Moore AS, Gliatto J.M., Ablin L.A., Berg R.J., Rand W.M. Prognosis for dogs with stage I or II splenic hemangiosarcoma treated by splenectomy alone: 32 cases (1991-1993). J Am Anim Hosp Assoc. 1998;34(5):417-21.

157. Ogilvie GK. Surgery and doxorubicin in dogs with hemangiosarcoma. Journal of veterinary internal medicine. 1996;10(6):379-84.

158. Clifford CA, Mackin AJ, Henry CJ. Treatment of canine hemangiosarcoma: 2000 and beyond. Journal of Veterinary Internal Medicine. 2000;14(5):479-85.

159. Dervisis NG, Dominguez PA, Newman RG, Cadile CD, Kitchell BE. Treatment with DAV for advanced-stage hemangiosarcoma in dogs. J Am Anim Hosp Assoc. 2011 05/20;47(3):170-8.

160. Hershey AE, Kurzman ID, Forrest LJ, Bohling CA, Stonerook M, Placke ME, Imondi AR, Vail DM. Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: Proof of principle using dogs with spontaneously occurring tumors as a model. Clin Cancer Res. 1999;5(9):2653-9.

161. Lana S, U'ren L, Plaza S, Elmslie R, Gustafson D, Morley P, Dow S. Continuous low-dose oral chemotherapy for adjuvant therapy of splenic hemangiosarcoma in dogs. J Vet Intern Med. 2007 Jul-Aug;21(4):764-9.

162. Folkman J. Clinical applications of research on angiogenesis. N Engl J Med. 1995 12/28; 2015/06;333(26):1757-63.

163. Akhtar N. Interleukin-12 inhibits tumor growth in a novel angiogenesis canine hemangiosarcoma xenograft model. Neoplasia. 2004;6(2):106-16.

164. Sotomayor EA. Minocycline in combination with chemotherapy or radiation therapy in vitro and in vivo. Cancer Chemother Pharmacol. 1992;30(5):377-84.

165. Wirth KA, Kow K, Salute ME, Bacon NJ, Milner RJ. In vitro effects of *yunnan baiyao* on canine hemangiosarcoma cell lines. Vet Comp Oncol. 2014 Jun 29;doi: 10.1111(vco.12100):1-14.

166. Vail DM, MacEwen EG, Kurzman ID, Helfand SC, Kisseberth WC, London CA, Dubielzig RR, Obradovich JE, Madewell BR, Rodriguez Jr. CO, Fidel J, Susaneck S(7), Rosenberg M(8). Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine adjuvant immunotherapy for splenic hemangiosarcoma in the dog: A randomized multi-institutional clinical trial. Clinical Cancer Research. 1995 / 10 / 01 /;1(10):1165-70.

167. U'Ren LW, Biller B, Elmslie R, Thamm D, Dow S. Evaluation of a novel tumor vaccine in dogs with hemangiosarcoma. J Vet Intern Med. 2007;21(1):113-20.

168. Ogilvie GK. Interventional nutrition for the cancer patient. Clin Tech Small Anim Pract. 1998 11;13(4):224-31.

169. Thamm DH. Hemangiosarcoma In: Stephen J. Withrow, David M. Vail, Rodney Page, editor. Withrow and MacEwen's Small Animal Clinical Oncology. 4th ed. Elsevier Health Sciences; 2013; p. 679-687.

170. Brand A, Houbiers J. Clinical studies of blood transfusion and cancer In: Vamvakas E, Blajchman E, editors. Immunomodulatory effects of allogeneic blood transfusion. Bethesda, MD: AABB Press; 1999; p. 145-90.

171. Bordin JO, Bardossy L, Blajchman MA. Growth enhancement of established tumors by allogeneic blood transfusion in experimental animals and its amelioration by leukodepletion: The importance of the timing of the leukodepletion. Blood. 1994;84(1):344-8.

172. Schneider SO, Rensing H, Graber S, Kreuer S, Kleinschmidt S, Kreimeier S, Muller P, Mathes AM, Biedler AE. Impact of platelets and fresh frozen plasma in contrast to red cell concentrate on unstimulated and stimulated cytokine release in an in vitro model of transfusion. Scand J Immunol. 2009 Aug;70(2):101-5.

173. Hansen-Pupp I, Engstrom E, Niklasson A, Berg AC, Fellman V, Lofqvist C, Hellstrom A, Ley D. Fresh-frozen plasma as a source of exogenous insulin-like growth factor-I in the extremely preterm infant. J Clin Endocrinol Metab. 2009 Feb;94(2):477-82.

174. Barnett CC,Jr, Beck AW, Holloway SE, Kehler M, Schluterman MK, Brekken RA, Fleming JB, Silliman CC. Intravenous delivery of the plasma fraction of stored packed

erythrocytes promotes pancreatic cancer growth in immunocompetent mice. Cancer. 2010 Aug 15;116(16):3862-74.

175. Silverstein D, Campbell J. Fluid Therapy In: Tobias K.M., Johnston S.A., editors. Veterinary Surgery: Small Animal. 1st ed. St. Louis, MO: Elsivier; 2011; p. 43-83.

176. Callan MB, Oakley DA, Shofer FS, Giger U. Canine red blood cell transfusion practice. J Am Anim Hosp Assoc. 1996;32(4):303-311.

177. Waters J. Indications and contraindications of cell salvage. Transfusuon. 2004;44(12 Suppl):40S-44S.

178. Trudeau JD, Waters T, Chipperfield K. Should intraoperative cell-salvaged blood be used in patients with suspected or known malignancy? Can J Anesth. 2012;59(11):1058-70.

179. Day TK. Shock: Pathophysiology, Diagnosis and Treatment In: Slatter DH, editor. Textbook of small animal surgery. 3rd ed. Philadelphia, PA: Philadelphia, PA : Saunders; 2003; p. 1-17.

180. Thamm DH, Dickerson EB, Achtar N, Lewis R, Auerbach R, Helfend SC, MacEwen EG. Biological and molecular characterization of a canine hemangiosarcoma-derived cell line. Res Vet Sci. 2006;81(1):76-86.

181. Alexiou C, Sheppard S, Smith D, Gibbs R, Haw M. Effect of blood temperature on the efficacy of systemic leucodepletion during cardiopulmonary bypass: A prospective randomized clinical study. ASAIO journal. 2005;51(6):802-7.

182. Higgs VA, Rudloff E, Kirby R, Linklater AKJ. Autologous blood transfusion in dogs with thoracic or abdominal hemorrhage: 25 cases (2007-2012). J Vet Emerg Crit Car. 2015 Nov;25(6):731-8.