Comparison of Individually-Adjusted Heparin Versus Ultra Low-Dose Aspirin for Prevention of Thromboemboli in Immune-Mediated Hemolytic Anemia in Dogs
And
The Safety of Ultrasound-Guided Fine Needle Aspiration of the Feline Pancreas: A Case-Control Study

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Sarah Kathleen Crain

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P. Jane Armstrong and David J. Polzin

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Introduction

The following thesis is a compilation of the clinical studies designed and performed over a 3-year period at the University of Minnesota College of Veterinary Medicine. The studies, which include a prospective, randomized clinical trial and a retrospective case-controlled study, are unrelated to each other in subject matter, but both proved to be challenging in their own way, and had a lot to teach as far as the challenges and benefits unique to each type of clinical trial.

The chapters first include a review of canine autoimmune hemolytic anemia, which provided significant insight into the common types of studies performed in veterinary medicine as well as provides background on a complex and interesting disease. The second and third chapters detail the studies performed, as well as discuss the specific challenges, shortcomings, and learning issues presented with each study. Despite careful planning and anticipation of the potential issues that would be encountered, each study was imperfect in its own way. Performing these studies was a very valuable way to learn about the challenges of study design, data collection, and data evaluation.

Chapter 1: Review of Immune-Mediated Hemolytic Anemia

Immune-mediated hemolytic anemia (IMHA) is a clinically important and potentially devastating disease in dogs, with a mortality rate that ranges from 20% to 70%[1,2]. The disease produces an acute, often severe, anemia, which frequently requires transfusion. IMHA also results in a systemic inflammatory state, which activates coagulation and may result in the development of thrombotic complications. Thrombosis is one of the major causes of death from IMHA, though financial demands from hospitalization and transfusion costs placing a significant burden on the client and may result in euthanasia. Therapy centers on glucocorticoid immunosuppression, as well as thrombophylaxis and supportive care (transfusions, enteral support).

There is a substantial body of literature focused on canine IMHA, but the quality of the data lacks much external validity, as was recently demonstrated in a systematic review performed by Swann and Skelly[3]. The majority of the studies available are retrospective, so there exists substantial variability in the treatments received by the dogs and in the determinants measured. The prospective studies available often have low numbers of subjects, along with variability in treatments and measurements, as is the case in many veterinary clinical trials. An excellent review by Piek in 2011[4] discussed the clinical presentation and diagnostic workup of dogs with IMHA, as well as treatment recommendations. She concluded that the optimal method for improving prognosis in dogs with this disease is through collaborative efforts, standardization of testing, and the development of a scoring

system. Indeed, the development of registries and collaboration between hospitals is the next necessary step in improving outcome for this subset of canine patients. This chapter is a review of the current literature and understanding of the immune pathology, coagulation abnormalities, prognostic indicators, and therapies for canine IMHA.

Pathophysiology of Immune-Mediated Hemolytic Anemia:

IMHA is the most common cause of hemolytic anemia in dogs[5]. IMHA results from reduced self-tolerance of red blood cell surface antigens[6] resulting in autoantibody formation and a type II hypersensitivity reaction. The majority of cases in the dog are considered to be idiopathic, or primary, although certain diseases such as underlying neoplasia or infectious causes such as parasitic or rickettsial diseases can elicit secondary IMHA. Certain drugs, such as penicillins, are also capable of eliciting a type II hypersensitivity response. Vaccination has been implicated as a trigger for the formation of autoantibodies, but there is limited data to support their role in IMHA[7].

IgG and IgM bind to the red blood cell surface through their variable regions (Fab)[8]. As a pentamer, IgM results in cross-linking of red blood cells and autoagglutination and can fix complement. IgG, as a monomer, is conversely a poor activator of complement, as opposed to IgM, a pentamer. Complement is often a component of IgM-mediated hemolytic anemia and can cause intravascular hemolysis.

The specific immune dysfunction that leads to IMHA is currently unknown in dogs. It is unknown if primary IMHA is triggered by abnormal T cell activation, deficits in B cell tolerance, or whether there is a role for dendritic cells or regulatory T cells. There is evidence that dogs with IMHA have autoreactive T cells, supporting the role of T cells in triggering the autoimmune response[9].

Tan et al[6] evaluated cell surface antigens that are commonly found in dogs with IMHA. In 12 dogs with IMHA, they found a high prevalence of reactivity to antigens relating to oxidative stress and complement compared to dogs without IMHA. It is unknown, however, if these proteins are triggers or products of the disease[6]. Once autoreactive antibodies form to a specific self-antigen, it is expected that epitope spreading to nearby or similar antigens will occur[10]. In humans, Band3 on the red blood cell surface is the most common target in IMHA[6] but it is not known if Band3 is the initial autoantibody target or a consequence of epitope spreading.

Immunoglobulin (Ig) constant regions (Fc) are recognized by the Fc-receptors on phagocytic cells of the mononuclear phagocytic system in the liver and spleen. The partial clearance of the red blood cell, via phagocytosis by the splenic macrophages or Kupffer cells of the liver, results in extravascular hemolysis and spherocyte formation. Spherocytes are visualized as smaller red blood cells on a blood smear with denser hemoglobin concentration and a lack of central pallor due to partial membrane removal. Although spherocytosis is considered a hallmark of IMHA, spherocytosis can occur in other conditions that cause red cell membrane damage such as neoplasia, rickettsial disease, or oxidative damage[11]. Spherocytes

are often found in low numbers in those conditions, whereas they can be overwhelmingly present in IMHA.

A systemic inflammatory response promotes progression of IMHA. Additional mononuclear cells which contribute to the phagocytosis of antibody-bound red blood cells are mobilized from the bone marrow and are recruited to sites of inflammation. This migration of immune cells has been a source of study in as an attempt to further understand IMHA pathophysiology. MCP-1 (monocyte chemoattractant protein-1) is a cytokine that originates from mononuclear and endothelial cells. A recent study demonstrated an increase in MCP-1 in dogs with IMHA[12]. Kjelgaard et al.[13] prospectively evaluated cytokines dogs with IMHA upon diagnosis of the disease. They demonstrated increased concentrations of cytokines IL-2, IL-4, IL-10, and keratinocyte chemoattractant protein in dogs with IMHA relative to control dogs. IL-4 and IL-10 are cytokines that are involved in antibody production, and IL-10 is a marker of B cell activation and proliferation. These findings likely reflect the increased production of self antibodies. IL-2 is a CD4+ Th-related cytokine, which stimulates B cells to produce antibody. This study also found an increase in the expression of macrophage-associated cytokines IL-18 and MCP-1 in nonsurvivors relative to survivors, indicating that macrophage/monocyte activation may have an important role in determining the outcome of IMHA patients.

Abnormalities in Coagulation and Mechanisms of Thromboembolism:

Thromboemboli:

The major cause of mortality from IMHA, the formation of thromboemboli, is poorly understood. Thromboemboli are classified as arterial or venous in origin. Arterial thrombi generally occur from activation of platelets in high-flow conditions in arteries and arterioles, and are traditionally targeted via anti-platelet medications, such as aspirin or clopidrogrel. Venous thrombi form in both veins and venules in low-flow conditions and they are considered to be fibrin rich due to activation of coagulation factors triggering conversion of fibrinogen to fibrin. Therefore, they are traditionally targeted with anticoagulant medications such as heparin[14].

Thromboemboli are reported to occur commonly in dogs with IMHA in multiple studies, with ranges up to 80% of dogs affected[7,15,16,17]. Pulmonary thromboemboli are the most common type of thromboemboli reported in dogs with IMHA. They have, however, been reported to occur in other locations, including cardiac tissue[18], arterial sytems (such as splenic, renal, iliac, and mesenteric artery thrombi) and venous systems (portal vein, splenic vein, and vena cava)[7,11,19,20].

Platelet Activation:

Coagulation abnormalities in dogs with IMHA have been evaluated increasingly in the veterinary literature. Endothelial abnormalities, increased hypercoagulability, and abnormal blood flow (Virchow's triad) have been explored as reasons for the high prevalence of thrombosis in dogs with IMHA. Importantly, dogs with IMHA have activated platelets relative to control dogs. Weiss and Brazzell[21] demonstrated that dogs with IMHA have increased P-selectin expression

on the surface of their platelets relative to healthy dogs. P-selectin is inside the platelet's alpha granule, and is expressed on the platelet surface upon activation. In their study, dogs with IMHA had P-selectin expression that was 8 times higher than the expression in healthy control dogs. They also found that 75% of dogs with IMHA had P-selectin expression over the reference range for their population of healthy control dogs. The study by Ridyard et al[22] several years later corroborated these findings. Ridyard's group found increases in fibrinogen-platelet binding, P-selectin, and platelet microparticles in dogs with IMHA compared to healthy dogs. Interestingly, they also found a relationship between platelet activation and severe thrombocytopenia, with higher platelet activation observed in the five severely thrombocytopenic dogs (n=14) in their study. In this group of fourteen dogs with IMHA, three did not survive to discharge and two of these dogs had suspect pulmonary thromboemboli. All three of these dogs were in the thrombocytopenic group. What is unknown at this point is whether the thrombocytopenia is a cause or effect of a prothrombotic state; is the platelet activation a result of systemic coagulation abnormalities or is it an effect of the inflammatory disease state and therefore causing a significant portion of the coagulation abnormalities?

The plasma membrane of the platelet and the endothelial cell is normally asymmetrically charged, with anionic phospholipids such as phosphatidylserine (PS) on the inner leaflet. Damaged red blood cell membranes and activated platelets and platelet microparticles can lose this asymmetry. This results in exposure of PS to the outer surface of the cell. This can trigger coagulation by facilitating assembly of prothrombinase and tenase complexes, which are both critical steps in the

coagulation cascade. Red blood cells expressing PS on their surfaces also bind to the macrophage-PS receptors and induce phagocytosis.

Thrombocytopenia:

Important aspects of coagulation that have been evaluated in dogs with IMHA include platelet number, tissue factor, and D-dimers. Thrombocytopenia is common in dogs with IMHA[7,11,23,24] and in several studies thrombocytopenia has been identified as a poor prognostic indicator in dogs with IMHA[2,7,16,22,24,25]. A study by Orcutt et al[18] demonstrated that without concurrent DIC, there appears to be no increased risk of mortality with concurrent IMHA and thrombocytopenia; in that study nine of twelve dogs with IMHA and a platelet count of less than 15,000 cells/uL survived. The classification of the dogs in the Orcutt study as not having DIC is important; in many of the previous studies, other hemostatic abnormalities were present in the dogs with IMHA. These changes, often consistent with DIC, could have accounted for or contributed to the dogs' poorer prognosis if they reflected different pathologic processes or more fulminant disease. Other criteria of DIC that have been reported in dogs with IMHA include prolongation of coagulation times and elevated fibrin degradation products (FDPs) or D-dimers. These findings in dogs with IMHA may be accounted for by a combination of increased procoagulant factors, the presence of free hemoglobin, decreased anticoagulant factors, and vasculitis[19].

Tissue Factor:

A study by Piek et al[25] evaluated the expression of intravascular tissue factor (TF) in dogs with IMHA and found it was elevated; TF initiates the intrinsic pathway of coagulation in the cell-based model of coagulation. Interestingly, the TF levels did not have an association with survival of dogs with IMHA.

D-dimers:

D-dimers are formed in the blood when thrombin cleaves fibrinogen, allowing Factor XIIIa, which is activated by thrombin, to cross-link fibrin and form a stable clot. D-dimers are therefore considered to be a measure of thrombin's action on fibrinogen and are a marker for active coagulation and fibrinolysis. Dogs with IMHA often have elevated D-dimers at the time of diagnosis[22,26,27]. D-dimers are thought to be more sensitive and equally as specific as FDPs for thrombosis in dogs[28]. No studies to date have found a significant association between the elevation of D-dimers and the increased morbidity or mortality from thromboembolism in dogs with IMHA.

Thromboelastography:

Several groups have evaluated global coagulation in dogs with IMHA through thromboelastography (TEG), which gives an ex-vivo assessment of overall coagulation status. One retrospective study[29] evaluated TEG tracings from dogs with IMHA. A few of these dogs were on some form of thrombophylactic medication. The authors found that 33/39 dogs had hypercoagulable TEG tracings. Interestingly, none of the 6 dogs with a normal coagulation index survived compared to 56% of

the dogs with hypercoagulable tracings. Unfortunately, we do not know the status of glucocorticoid therapy in this group of dogs.

To account for the effects of glucocorticoid-induced alterations on the TEG tracings, one study evaluated TEG upon admission in dogs with IMHA who had not received glucocorticoid therapy. The dogs with IMHA had multiple markers of hypercoagulability on their TEG tracings, including a high α-angle, high MA, and high G[27]. The inherent confounder, however, when assessing dogs with IMHA through TEG, is that TEG tracings appear hypercoagulable in anemic dogs[30], which makes it difficult to differentiate hypercoagulable tracings due to anemia from true hypercoagulability.

Three studies have evaluated TEG in healthy dogs with glucocorticoid therapy, as glucocorticoids, the mainstay of treatment for dogs with IMHA, have been suspected to promote a hypercoagulable state themselves[31,32,33]. These studies found that administration of prednisone at immunosuppressive doses resulted in hypercoagulable TEG tracings. The addition of ultra low-dose aspirin in two of the studies[31,33], to mimic the effects of standard IMHA therapy, had no reduction in the hypercoagulable readings. Aspirin therapy alone did not affect TEG tracings to reduce the MA[33].

Prognostic Factors:

Many studies have evaluated dogs with IMHA to identify factors associated with survival. The majority of these studies are retrospective. Several findings have

been inconsistent between studies, but there are some that have repeatedly been demonstrated to be abnormal in dogs that do not survive treatment.

One study[25] found an association between increased mortality within the first two weeks of diagnosis and with the presence of icterus, increased BUN, leukocytosis, a left shift, and abnormalities in coagulation parameters including thrombocytopenia, increased coagulation times, and decreased fibrinogen.

Bilirubin:

Hyperbilirubinemia has been documented in several studies to be associated with an increase in mortality [2,3,7,8]and thromboembolism[1,7,20,35]. Bilirubin >1.5 ng/dL was associated with increased mortality in one study[15] and in another, bilirubin over 5 ng/dL was associated with an increased risk of thromboembolism[7]. The increased mortality associated with high bilirubin may be due to increased hemolysis, concurrent hepatic disease, more severe IgM and complement-associated disease, or development of hepatic thromboses. Free hemoglobin may also bind nitric oxide, which normally inhibits platelet aggregation. Experimental data in humans suggests that hemolysis leads to the exposure of tissue factor (a potent procoagulant) on monocytes and endothelial cells and there is subsequent activation of coagulation[23].

Alkaline Phosphatase:

Elevated alkaline phosphatase (ALP), an enzyme associated with the plasma membrane of the hepatocytes and biliary epithelium in dogs, has also been

associated with increased mortality[15], and thromboembolism[7] in dogs with IMHA. This likely has similarities to the pathophysiology that results in hyperbilirubiemia's association with increased mortality, as it is a sensitive marker for cholestasis. It also increases with prednisone therapy, which dogs with IMHA are almost invariably administered, but ALP values in dogs with IMHA rise acutely often prior to glucocorticoid administration. However, a synergistic effect on the canalicular hepatocytes or biliary epithelium from cholestasis and glucocorticoids resulting in hepatic dysfunction or microcirculatory disturbances cannot be completely excluded.

Blood Urea Nitrogen:

Elevated blood urea nitrogen (BUN) has been found to be associated with increased mortality in a number of studies[3,25,36]. In a study by Swann et al[3], the median BUN of dogs that survived was 6.05 mmol/L and the median BUN of dogs that did not survive was 10 mmol/L. It is unknown if this elevation in BUN is due to pre-renal or renal azotemia, renal thrombosis, gastrointestinal compromise or ulceration, or a combination.

Lactate:

Holahan explored the utility of lactate[36] as a point-of-care test and a relatively easy-to-measure prognostic marker. In 84% of dogs with IMHA, the lactate levels were above the reference interval and lactate was higher in nonsurvivors compared to survivors. The authors reported that the dogs that had an

elevated lactate that normalized within 6 hours survived. Lactate levels in this study were positively correlated with BUN and ALP, and inversely correlated with PCV. This is understandable, as decreased oxygen delivery due to anemia will increase anaerobic metabolism and lactate levels. In this study, a lactate of 4.4mg/dL had a sensitivity of 60% and a specificity of 77% for mortality. As a result, its utility as a marker for mortality is not ideal, but it may be useful in monitoring endpoints of therapy or for being an indicator for the need of red blood cell transfusion.

Red Blood Cell Changes:

Hematologic changes have been evaluated for associations with thrombosis and survival. Autoagglutination was associated with decreased short-term survival in one retrospective study[15], but the severity of autoagglutination was not assessed. Interestingly, spherocytosis was found to be negatively associated with death in one study[25].

Platelets:

Thrombocytopenia is a common finding in dogs with IMHA, with 65-70% of dogs with IMHA also having some degree of thrombocytopenia, and 20% of dogs having platelet counts <50,000/ul[7,11]. Thrombocytopenia has been correlated with an increase in mortality[7,15,22]. As mentioned, a study evaluating dogs with severe thrombocytopenia (<15,000 cells/uL) with concurrent IMHA and no evidence of disseminated intravascular coagulopathy (DIC) failed to find a significant increase in mortality in this patient population[18]. This suggests that other aspects of abnormal

coagulation, potentially independent of platelets, may account for the increased mortality in thrombocytopenic dogs with IMHA. Other studies have evaluated platelet activation itself in dogs with IMHA. Ridyard et al[22] noted a strong correlation between increased markers of platelet activation and severe thrombocytopenia. As mentioned, dogs with IMHA had increased levels of P-selectin, platelet-derived microparticles (PMPs), and platelet-fibrinogen binding relative to unaffected dogs. Weiss and Brazzell[21] also found increased levels of P-selectin in dogs with IMHA, which is a marker of platelet and endothelial activation.

The cause for thrombocytopenia is likely multifactorial in dogs with IMHA. The breakdown in self-tolerance leads to red cell-antibody complex formation, which results in red cell destruction and consumptive thrombocytopenia[37]. The general prothrombotic state in these dogs also may likely lead to additional platelet consumption. It is also possible that concurrent immune-mediated thrombocytopenia is underdiagnosed.

White Blood Cell Changes:

Leukocytosis, particularly a moderate to marked neutrophilia with a left shift and toxic change in neutrophils are other hematologic changes associated with a poor outome in dogs with IMHA[2,13,25]. In one study[19], a moderate to marked leukocytosis was correlated with more severe postmortem lesions in dogs, the majority of these being ischemic lesions secondary to hypoxic tissue damage from anemia or thromboembolism. Leukocytosis is considered to be a reflection of

moderate to marked tissue damage. Bands have been implicated in several studies [2,19], and may represent systemic inflammation or marrow hypoxic damage.

Other markers of coagulation have been evaluated in dogs with IMHA, including coagulation time testing, fibrinogen levels, and antithrombin III levels. Increased activated partial thromboplastin time (aPTT) has been associated with increased mortality in several studies[15,25,38]. Increased prothrombin time (PT) has also been demonstrated to be associated with a poor outcome in one study[2]. These findings are often concurrent with other markers of inflammation. Kuzi et al[38] demonstrated that elevated aPTT was found concurrently with elevated bilirubin, low antithrombin levels, hypoalbuminemia, and leukocytosis[38].

Antithrombin III:

Antithrombin III (ATIII) has been of clinical interest in hypercoagulable diseases. ATIII is a liver-generated large serine protease inhibitor, which is critical for regulating coagulation by inactivating Factors X, II, VIIa, and plasmin. It accounts for about 80% of the total inhibitory effect of plasma on coagulation[38] . ATIII is also important for the appropriate function of heparin. Heparin is used as antithrombotic therapy for dogs with IMHA (below). Antithrombin III significantly increases in activity when interacting with endogenous heparan sulfate proteoglycans[39,40]. ATIII can be decreased in several disease states due to loss, production deficiencies, or increased degradation, which can lead to a hypercoagulable state. Kuzi et al[38] retrospectively evaluated the incidence of ATIII abnormalities in 20 dogs with IMHA and found decreased ATIII levels in 10 of them.

This study found an increased odds ratio for mortality, in all dogs with hypoantithrombinemia, when ATIII levels were less than 60% (OR 9.9) and 30% (OR 14.7). However, within the subset of dogs with IMHA, low ATIII levels did not result in an increase in the odds of mortality. A study by Scott-Moncreiff et al[11] demonstrated that 76% of the 20 dogs evaluated with IMHA had low ATIII levels. This study evaluated the risk of low ATIII levels for thromboembolic complications and it could not identify a relationship between the two variables.

Fibrinogen:

Fibrinogen levels have been evaluated in IMHA patients in several studies. Fibrinogen is a soluble plasma protein that is converted to fibrin by thrombin. Thrombin converts fibrinogen to an insoluble form, and it is then cross-linked by factor XIII. Fibrinogen is used diagnostically as a marker of inflammation, as it is an acute-phase protein and marker of prothrombotic state. Fibrinogen is often found to be elevated in dogs with IMHA[19,24,25,27]. The elevation in this acute phase protein also has implications for anticoagulant therapy.

Anticoagulant Therapy:

As thromboembolic complications can be devastating in patients with IMHA, and dogs with IMHA are hypercoagulable, it is critical to employ aggressive, effective antithrombotic therapy in these patients. Currently, different methods for anticoagulant therapy are used. The most common are anti-platelet therapy with aspirin or clopidogrel or prolongation of the coagulation cascade with

unfractionated or low molecular-weight heparin. Preferences for the use of one or a combination of these therapies in treatment of IMHA vary among veterinarians, and are based on personal experience, ease of administration, cost, and monitoring requirements. As there are both venous and arterial thromboses in these dogs, it is difficult to say that one method of anticoagulant therapy (targeting platelets or coagulation factors) is superior to another.

Aspirin:

Ultra low-dose aspirin (ULDA), given at 0.5 mg/kg orally daily, is a convenient method of attempting coagulation control in dogs with IMHA. Aspirin irreversibly inhibits cyclooxygenase, which decreases thromboxaneA_2 and prevents platelet activation. It requires no additional monitoring. The popularity of the use of ULDA in dogs with IMHA came from the results of a large retrospective study out of Cornell[15], which found that dogs that survived were more likely to receive ULDA than unfractionated heparin or no therapy. However, the efficacy of this dose on platelets in dogs with IMHA has been questioned. In vitro studies assessing the effects of ULDA on platelet aggregation have had conflicting results[41,42]. A recent study by Hoh et al[43] demonstrated that even doses of 1 mg/kg/day may not sufficiently inhibit platelets in healthy dogs, as measured by their metabolites. As the platelets in dogs with IMHA are known to be excessively activated, this dose may need to be even higher to have a sufficient antiplatelet effect.

Clopidogrel:

Clopidogrel is an irreversible inhibitor of the ADP-receptor P2Y12 on the platelet membrane, which is an important receptor for triggering platelet aggregation. It is may be used in conjunction with aspirin in some conditions in humans. There has been one controlled, open-label study in dogs[44] evaluating clopidogrel and aspirin alone or together in dogs with IMHA. This study failed to note any differences between survival or thrombotic complications among the three groups, leading the authors to conclude that clopidogrel is not superior to aspirin. It may, however, be a safe and useful alternative, particularly in dogs with gastrointestinal dysfunction.

Heparin:

Heparin has been widely used in dogs with IMHA. Unfractionated heparin facilitates antithrombin-mediated inactivation of thrombin and factor Xa, inactivates factors IXa, XIa, VIIa, and XIIa, and increases the release of tissue factor plasminogen inhibitor from endothelial cells. Heparin can also inhibit the binding of phosphatidylserine to thrombospondin on endothelial cells, which reduces the prothombinase and tenase complex formation.

Unfractionated heparin, however, requires appropriate monitoring. Historically, this has been through monitoring of aPTT; however, there is considerable variability in the reagents used for aPTT monitoring which leads to erratic results for heparin targets[41]. Additionally, the aPTT assay is affected by a variety of factors, including hyperfibrinogenemia, which is present in many dogs with IMHA (above). Increased circulating factor VIII levels can shorten aPTT times.

Currently, the more ideal test for monitoring and adjusting unfractionated heparin doses are levels of anti-factor Xa, since this is a direct target of unfractionated heparin. Breuhl et al[24] evaluated the effects of unfractionated heparin on anti-Xa levels in dogs with IMHA, using standard doses of heparin (300U/kg q 6 hours). In this study, less than 50% of dogs achieved target ranges of anti-Xa within the first 40 hours of therapy. This study demonstrated that standard doses of heparin were not optimal for dogs with IMHA. Since many dogs have elevated fibrinogen, and fibrinogen and other acute phase proteins bind to heparin preferentially, it is entirely possible that dogs with IMHA require higher doses. Studies in humans have demonstrated that subtherapeutic heparin can, in fact, increase the risk of thromboembolism in certain diseases [41,46,47]. Helmond et al[48] compared dogs with standard dose heparin therapy to dogs who received heparin adjusted to achieve anti-Xa levels within the therapeutic target (0.35-0.7 U/mL). In this study, 7/8 dogs with individually adjusted heparin survived (for how long?), with one dog in the treatment group succumbing to thromboembolic complications. In the standard dose group, 1/7 survived, and 5 of the 6 dogs that did not survive had documented thrombotic complications. Interestingly, this study also demonstrated that the trough anti-Xa levels were subtherapeutic, which may indicate that these dogs require more frequent heparin dosing. Additionally, heparin may not be as effective when antithrombin levels are low (below 60%)[38], which is a common laboratory finding in dogs with IMHA (above).

Low molecular weight heparin differs from unfractionated heparin in that it is comprised of smaller heparin molecules, as opposed to a mixture of small and larger molecules. Low molecular weight heparin cannot be monitored by aPTT levels, since the smaller heparin molecules are not large enough to inhibit thrombin when complexed to

antithrombin. One study examined dalteparin in dogs, and the study found that it failed to increase anti-Xa levels from baseline[49]. Low molecular weight heparin may, however, be a safe alternative to unfractionated heparin, particularly if unfractionated heparin has a short half-life in dogs with IMHA.

Immune-Modulatory and Adjunctive Therapies:

The mainstay of therapy for IMHA is immunosuppression with prednisone. However, because prednisone has considerable side effects, it may contribute to thromboembolic tendencies (above), and may not be sufficient alone to suppress IMHA. Second-line immunosupressives and other therapies have been explored in IMHA patients.

Azathioprine and Cyclosporine:

Azathioprine and cyclosporine have been evaluated retrospectively in several studies[3,25]. Azathioprine is a purine synthesis inhibitor, which inhibits T cell responses[50]. Side effects are common with azathioprine and include hepatotoxicity and marrow suppression due to the mercaptopurine metabolite. Cyclosporine is a calcineurin inhibitor, which also suppresses T-cell function[50]. Cyclosporine can be cost-prohibitive in large dogs and it can result in gastrointestinal upset and gingival hyperplasia. In one study[3], there was an effect of treatment on survival. Dogs who received azathioprine and prednisone or prednisone alone had improved survival relative to dogs who received cyclosporine and prednisone. However, it was also noted in this study that the dogs receiving prednisone alone had lower bilirubin

levels and received fewer blood transfusions than dogs in the other groups, and the dog numbers in each group were too small to draw significant conclusions. Another study[25] retrospectively evaluated a large number of dogs with IMHA who either received prednisone or prednisone and azathioprine. There was no difference in the survival between these two groups of dogs.

Mycophenolate mofetil:

One paper[51] has examined mycophenolate mofetil in dogs with IMHA. Mycophenolate is appealing as an adjunct immunosuppressive, since its mechanism of action as a purine metabolite inhibitor is similar to azathioprine, but the side effects are considered to be more tolerable and tend to be restricted to large-bowel diarrhea. This paper evaluated five dogs with IMHA who received mycophenolate at 10-15 mg/kg three times daily, which is higher than the doses currently recommended[50]. In this study, one dog died at day 20 due to progressive IMHA, and one dog died due to mycophenolate-induced gastrointestinal toxicity. Mycophenolate at a lower dose warrants further evaluation in dogs with IMHA.

Human Intravenous Immunoglobulin:

Human intravenous immunoglobulin (hIVIg) has also been evaluated in several, mostly retrospective, studies as a therapy for dogs with IMHA[11,26,52,53]. Human IVIg modulates the expression and function of Fc receptors on phagocytic cells, possibly via sialylation of the receptors[54] and interferes with B and T cell function, as well as complement. Many of the studies are inconclusive,

demonstrating no significant adverse effects but no clear benefit in terms of survival or complications. A prospective study[26] evaluated hIVIg as an initial therapy in 28 dogs with IMHA. This study did not seem to demonstrate any significant improvement in initial response to therapy with steroids or shortening of the treatment time.

Splenectomy:

Finally, splenectomy has been examined in dogs with IMHA[55]. This study evaluated ten cases of dogs with IMHA who had undergone splenectomy, on the basis that humans with IMHA are often splenectomized with good results. Humans who have warm immune-mediated hemolytic anemia, which is the closest disease that parallels the canine condition, do respond initially to splenectomy but may relapse; overall it is a promising treatment for them[56]. Horgan[55] found that nine of ten dogs who received prednisone as well as splenectomy within 4 days of initiating treatment survived, with one dog succumbing perioperatively. They did not report the thromboembolic complications in these dogs. However, despite the good outcomes in these dogs, splenectomy is not a popular therapeutic intervention, likely due to the morbidity, risk for thrombosis, and cost associated with surgery.

Conclusions and Future Directions:

Although the veterinary community has come far in its understanding of canine IMHA, there remains a great deal to evaluate and consider. There has been considerable effort to determine both the specific pathophysiology of the disease as

well as the optimal treatment. Most studies in the veterinary literature are retrospective, however, clinical trials are costly and time-consuming to perform.

The goals for treatment include faster time to remission (with medications that are affordable to our clients) and effective anticoagulation that requires minimal monitoring. Some therapies warrant additional evaluation, including mycophenolate mofetil, low-molecular weight heparin, or individually adjusted unfractionated heparin, possibly in conjunction with clopidogrel, for more aggressive anticoagulation. Future studies should be prospective, randomized, and blinded.

Ideally, an IMHA patient registry would be established across the veterinary schools and large referral hospitals to provide the community with a bank of patients to evaluate. This would allow us to perform collaborative studies more easily, increase the number of patients we can enroll, and improve transparency in the patient database.

Chapter 2: Comparison of Individually-Adjusted Heparin Versus Ultra Low-Dose Aspirin for Prevention of Thromboemboli in Immune-Mediated Hemolytic Anemia in Dogs

Abstract:

Immune-mediated hemolytic anemia is a common cause of anemia in dogs, and results in considerable mortality and significant thromboembolic risk. Ultra-low dose aspirin (ULDA) is most commonly used to try and prevent thrombosis, but individually-adjusted heparin (IAD), based on anti-factor Xa levels, may be more effective. Dogs were randomized to receive ULDA and a placebo injection, or IAD injections and a placebo capsule. Time to thrombosis or death up to 180 days was recorded. As the sample size was small at the time of interim analysis (n=22), additional dogs were included from a previous trial (IAD) or from review of the medical records (ULDA), resulting in 40 subjects. There were no differences in the baseline laboratory values between the two groups, although the total bilirubin was higher in the ULDA group than the IAD group (p=0.1). There was no difference in survival to 180 days (p=0.18), but the difference in thromboembolic complications approached significance (p=0.07), with dogs in the IAD group less likely to have a thrombotic complication. Increased total bilirubin (p=0.06), aPTT (p=0.04), and BUN (p=0.04) at admission were associated with an increased risk of thrombosis. IAD in dogs warrants further investigation to prevent thromboembolic complications in dogs with IMHA over ULDA.

Introduction:

Immune-mediated hemolytic anemia (IMHA) is an autoimmune disease characterized by autoreactive self antibodies targeting the surface of the red blood cells and marking them for destruction. This disease is an important and common cause of morbidity and mortality in dogs. IMHA can result as a secondary process from the presence of cancer, infection, or exposure to certain medications, although the majority of cases of IMHA in dogs are considered to be primary.

Currently, prednisone is the only medication that has been shown to improve survival in dogs with IMHA. Other immunosuppressants have been evaluated in addition to prednisone, but none have shown clear benefit, and most have been evaluated in retrospective cohort studies. Prednisone, although effective, can result in considerable morbidity in dogs, including iatrogenic hypercortisolism, increased risk for infection due to chronic immunosuppression, and increased tendency for thromboembolism.

Thromboembolism is an important and common complication in dogs with IMHA, and these dogs are noted to be hypercoagulable prior to prednisone therapy[7, 11, 15, 19, 20, 27, 57]. Thromboembolism is thought to be multifactorial in origin in this population of dogs; systemic inflammation, activated platelets[21], agglutination, and hemolysis are all thought to be components of the hypercoagulable state IMHA produces. Dogs with IMHA tend to have several markers of coagulation abnormalities; many have increased d-dimers, prolonged coagulation times, and platelet abnormalities[23, 26]. Survival of this disease in the canine population ranges

from 30% to 80%, with thromboembolic complications a leading cause of mortality, especially within the first several weeks of treatment[1, 2, 7].

Currently, no reliable method of anticoagulation exists for dogs with IMHA. Commonly accepted as standard of care is ultra low-dose aspirin (ULDA); this became standard therapy after retrospective data assessing outcome in a population of dogs with IMHA was evaluated, and noted improved survival in dogs receiving ULDA therapy over standard-dose heparin or no antithrombotic therapy[15]. Since that study, however, new data has been generated demonstrating that ULDA therapy may be unreliable in dogs, and may not inhibit platelet activity to the extent necessary to prevent thromboembolism[43]. A subsequent study looking at dogs with IMHA demonstrated that ULDA therapy did not reliably inhibit platelet activity in that population[58]. Clopidogrel, an irreversible inhibitor of the ADP-receptor P2Y12 on the platelet, was shown in one study to be no better than ULDA therapy[44].

Heparin therapy is also considered to be ineffective in dogs with IMHA[24]. Targeting heparin therapy to achieve a certain level of inhibition of Factor Xa is considered to be the most reliable way to prevent thromboembolism, and an anti-Xa assay has been validated for use in dogs. One study demonstrated that 300 U/kg heparin in dogs with IMHA was not sufficient to achieve appropriate anti-Xa levels[24] and a second study compared standard-dose heparin therapy to individually-adjusted therapy (IAD) that targeted anti-Xa levels within the therapeutic range [48]. This study demonstrated significant improvement in survival in the dogs who received individually-adjusted therapy as opposed to standard dose heparin therapy. The data from the dogs with individually-adjusted heparin was promising,

and therefore needed to be compared to current standard therapy of ULDA. The purpose of this study was to determine the effect of individually-adjusted heparin (IAD) therapy in dogs compared to ULDA in preventing thrombosis in dogs with IMHA.

Materials and Methods:

Study Design:

This study was a prospective, randomized, double-blinded placebo controlled clinical trial. The individually adjusted dose (IAD) group of dogs received unfractionated heparin (UH) to a target plasma concentration of 0.35-0.7 U/mL. The low-dose aspirin group (LDA) received aspirin at 0.5 mg/kg/day PO; dogs who fell between capsule sizes were rounded up to the nearest dose.

Dog Selection:

Eligible subjects were client-owned dogs who were admitted to the University of Minnesota Veterinary Medical Center (UMN VMC) between November 2010 and May 2013 with suspected IMHA. Informed consent was obtained from all owners before enrollment. The University Institutional Animal Care and Use Committee approved the study. All eligible subjects were evaluated and enrolled within 24 hours. A physical examination, complete blood count (CBC), serum biochemical profile, urinalysis, prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, fibrinogen, antithrombin level (AT), 4DX SNAP, arterial blood gas (ABG), and thoracic radiographs were performed on all patients within 24 hours of

admission. A Coomb's test was performed if there was no evidence of saline agglutination on the CBC.

Inclusion Criteria:

Dogs were considered candidates for enrollment if there was a confirmed diagnosis of IMHA, characterized by regenerative anemia (hematocrit <30% with reticulocytes >60,000/uL) with evidence of hemolysis (hyperbilirubinemia, bilirubinuria, and/or hemoglobinuria) and one or more of the following: positive agglutination, spherocytosis, or a positive Coomb's test. Slide agglutination was verified by the technical staff at the University of Minnesota Diagnostic lab by saline dispersion testing and microscopic review of the blood film. Coomb's testing was performed using Standard Operating Procedures at the University of Minnesota[a].

Exclusion Criteria:

Dogs with IMHA were excluded if there was any evidence that their hemolytic anemia was secondary to an identifiable cause based on thoracic radiographs, positive 4DX[b] test (heartworm antigen, antibodies to *Ehrlichia canis, Anaplasma phagocytophilum,* or *Borrelia burgdorferi*), or examination of blood film for erythroparasites or neoplastic cells, or history of exposure to drugs or compounds known to cause hemolysis.

Dogs with body weight <5.0 kg were excluded due to the need for repeat sampling for anti-Xa levels. Dogs were also excluded if they had a platelet count

<40,000/ul due to the need for jugular venipuncture, or PT or aPTT greater than twice the normal upper reference limit.

Dogs were excluded if they received any treatment with any immunosuppressive medication for their anemia for greater than 3 days prior to enrollment or any immunosuppressive drug within the last six months, had any therapy with aspirin prior to enrollment, or were not enrolled and randomized within 36 hours after admission to the hospital.

Randomization:

Dogs were randomized to either the IAD group or the LDA group the day of enrollment; dogs were randomized in groups of 6 using a table of random numbers and group assignments were placed in sequentially numbered sealed envelopes to be opened at the time of enrollment. Randomization and preparation of the envelopes was performed by an individual not directly involved in the study patient management. The primary clinician(s) involved in patient management were unaware of the study group designation for each dog.

Antithrombotic Therapy:

Dogs in the IAD group were started on 300IU/kg heparin SC every six hours for the first seven days, then every eight hours until day 30, at which point the dose was reduced by 15% each day for five days and discontinued. The heparin doses were adjusted based on the anti-Factor Xa chromogenic assay checked on days 3, 4, 5, 7, 14, 21, and 28 to maintain the UH levels within the therapeutic range (0.35-

0.7IU/ml). The adjustments were made by an unmasked investigator and relayed to the attending clinician. Dogs in this group also received an oral placebo capsule (calcium carbonate powder and yellow food coloring) that would be the equivalent of 0.5 mg/kg/day of 5 mg aspirin capsules.

Dogs in the LDA group were started on aspirin at a dose of 0.5 mg/kg PO every 24 hours. 5 mg capsules were compounded for use (aspirin USP, calcium carbonate powder, and yellow food coloring). In cases where 0.5 mg/kg was between capsule doses, the number of capsules administered was rounded up to the next whole number. Aspirin was continued for 30 days, then discontinued. Dogs in the LDA group received injections of 0.9% saline subcutaneously (SC) every six hours for seven days, then every 8 hours until day 35, with a 15% dose taper daily for the last 5 days. This was started assuming a concentration of 10,000IU/ml heparin at a dose of 300 U/kg. The dose was adjusted randomly by an unmasked investigator and relayed to the attending clinician to maintain masking.

Treatment and Monitoring Plan:

Within the fist 24 hours after examination at the UMN VMC, dogs were managed with a standard treatment protocol in addition to their antithrombotic therapy. Therapy included immunosuppression, transfusions, crystalloid fluids, antibiotics, gastroprotectants, oxygen supplementation, and nutritional support according to this protocol.

Immunosuppression was administered as dexamethasone SP at 0.1 mg/kg IV q12 h if the dog was not tolerating oral medications. If the dog was tolerating oral medications, prednisone at 1 mg/kg PO q 12 hours, or 30mg/m2/day (if the patient was >30kg) was administered. Prednisone was tapered when the hematocrit was within normal range (>36%) with no further evidence of hemolysis (lack of spherocytosis, reticulocytes <60,000/uL). Additional immunosuppressive medication was given at the clinician's discretion.

Packed red blood cell transfusions were administered in cases with a PCV <12%, acute decreases in PCV to <15%, tachycardia, or other clinical signs that were attributable to severe anemia. IV crystalloids were administered if clinically indicated to maintain hydration. Oxygen therapy, in the forms of nasal oxygen or oxygen cage, was provided if there were clinical signs of tachypnea, increased respiratory effort, or tachycardia were present, or at the discretion of the attending clinician. Antiemetics (ondansetron[c], maropitant[d], or metoclopramide[e] were administered in patients who were vomiting or inappetent, or at the discretion of the attending clinician. Gastric protection was administered at the discretion of the attending clinician. All patients were fed enterally if possible. A nasoesophageal or nasogastric tube was placed in cases of patients who were anorexic for >48 hours. Antibiotic therapy was used if there was evidence of localizable infection.

All samples aside from anti-Factor Xa levels were drawn from a peripheral vein. PCV and vital signs (temperature, heart rate and pulse quality, respiratory rate and effort, mucous membrane color) were monitored every 6-12 hours at the discretion of the attending clinician. Weight was monitored every 6-12 hours. Urine

production was estimated daily. Red blood cell morphology and the presence of autoagglutination was monitored daily during hospitalization.

Packed cell volume (PCV) was monitored in all dogs and trough and peak (2 hours post dose) heparin levels were monitored in IAD dogs on days 3, 4, and 5. A CBC was monitored in all dogs and trough and peak heparin levels were monitored in IAD dogs on days 7, 14, 21, and 28. Heparin levels were assessed using an anti-Factor Xa chromogenic assay[59]. Dogs in the LDA group had blood drawn to maintain blinding, but no anti-Factor Xa test was run. Follow up examinations and a CBC were performed on days 60, 90, and 180.

Anti-Factor Xa Chromogenic Assay:

Quantitative determination of unfractionated heparin was determined using a commercially available chromogenic assay kit for anti-Factor Xa that has been validated for use in dogs[59, g]. The assays were performed using a FLOUstay OPTIMA microplate reader[h].

The blood samples were obtained via jugular venipuncture; 2.7 ml blood was collected into a 3mL tube containing 3.8% sodium citrate to result in a 1:9 ratio of citrate:whole blood. The sample was then centrifuged at 2500 x *g* at room temperature for ten minutes, and the plasma was harvested. 1.2 ml of plasma was diluted with 0.4 mL of Owren-Kohler buffer[59]. The sample was then transported to the UMN Fairview Acute Care Laboratory and evaluated for anti-Xa activity.

Additional Subjects:

Due to the low enrollment in the prospective clinical trial, the treatment groups were augmented.

Additional LDA dogs were identified by searching the electronic medical record for dogs with a diagnosis of primary IMHA that were seen at the UMN VMC between November 2010 and April 2013. These dogs had to have a diagnosis of IMHA as for the dogs in the prospective clinical trial as well as meet inclusion criteria regarding diagnostic testing. These dogs also had to receive ultra low-dose aspirin as part of their therapy for IMHA from the time of diagnosis. However, these dogs could be <5 kg or could have been administered aspirin prior to admission.

Additional IAD dogs were identified from a previous study performed at the UMN VMC[48] evaluating individually-adjusted heparin therapy (versus standard dose therapy) in dogs with IMHA that had identical inclusion and exclusion criteria as well as the heparin dosing and monitoring protocol.

Study Endpoints:

The primary endpoint of the study was survival time from enrollment of the study until death or to the end of the study period (180 days). For dogs that were not enrolled in the prospective study, death or survival to 180 days was considered the primary endpoint.

Secondary endpoints included the presence of thromboembolic complications based on clinical observation, (hypoxemia, ascites, neurologic signs, limb swelling) imaging studies, and/or necropsy findings. Hemorrhagic complications were detected by clinical examination findings.

Statistical Analysis:

The IAD and the LDA dogs were evaluated for differences on enrollment or initial evaluation using a Wilcoxon signed-rank test. The effect of group on survival and thromboembolic complications by day 180 was evaluated using a Fischer's Exact Test. The association between laboratory abnormalities and survival or thromboembolism was evaluated using a Wilcoxon signed-rank test. The associations between Antithrombin III levels and fibrin degradation products with thrombosis were evaluated with a Fisher's Exact Test. Significance was set at $P<0.05$. Statistical analysis was performed using a standard statistical software package[g].

Results:

Ten dogs were randomized to the IAD group. This group included 7 male neutered dogs and 3 female spayed dogs. The mean age of this group was 6.4 years (range, 3-10 years). Breeds represented included 1 each of the following: Welsh Terrier, Shih Tzu, American Staffordshire Terrier, English Springer Spaniel, Cocker Spaniel, English Setter, Lhasa Apso, Viszla, Labrador Retriever, and Dachshund.

Twelve dogs were randomized to the LDA group. Included were 6 male neutered dogs and 6 female spayed, with a mean age of 6.5 years (range, 1-9 years). Breeds included American Staffordshire Terrier (3), English Springer Spaniel (2), and one each of the following: Pembroke Welsh Corgi, Toy Poodle, Boston Terrier, German Shepherd Dog, Labradoodle, and Australian Cattle Dog.

Eight dogs who received individually-adjusted heparin therapy were extracted from a previous study[17]. This group included 7 neutered males and 1 spayed female. The mean age of this group was 7.75 years (range, 1-13 years).

The following breeds were represented in this group: Shih Tzu (2) and 1 each of the following: Standard Poodle, Fox Terrier, Cavalier King Charles Spaniel, Collie, Dachshund, and Airedale.

Ten additional dogs with IMHA who received aspirin therapy were identified. This group included 2 neutered males and 8 spayed females. Mean age of this group was 6.6 years (range, 1-13 years).

Breeds included in this group were Shih Tzu (2), Miniature Schnauzer (2), and one each of Brittany Spaniel, Shetland Sheepdog, Collie, Boston Terrier, Pekingese, and German Shepherd Dog.

With the treatment groups together, there were 18 dogs who received IAD therapy. Overall, this group included 14 male neutered dogs and 4 spayed female dogs, with a mean age of 6.4 years. There were 22 dogs who received ultra low-dose aspirin. This group included 8 neutered males and 14 spayed females; the mean age of this group was 5.7 years.

There was no significant difference in the age of the dogs between the groups. The male dogs were overrepresented in the IAD group, and the female dogs in the LDA group. There was no difference between the groups as far as BUN, total serum bilirubin, PT and aPTT, or band neutrophil counts at the time of diagnosis (table 1). The average total bilirubin values for the groups were 6.1mg/dl for LDA and 1.4 mg/dl for aspirin, but this difference was not statistically significant (p=0.1).

Four of the 18 dogs who received LDA had a total serum bilirubin over 5 mg/dl. Two dogs were euthanized the day after diagnosis due to severe ongoing hemolysis; one had a bilirubin of 53.3 mg/dl and one had a value of 6.9 mg/dl. Two dogs survived past 180 days; one dog had a bilirubin of 20.6 mg/dl and one had a value of 12.9 mg/dl.

Survival:

Thirteen of the 22 dogs in the LDA group survived to the endpoint of 180 days. Of the 9 dogs that did not survive, 7 died or were euthanized within 1 week of diagnosis; one dog was euthanized at day 10, and one at day 27.

Thirteen of the 18 dogs in the IAD group survived to 180 days. Of the 5 dogs who did not survive, 4 died within one week of diagnosis. One dog died at 9 days post-diagnosis.

There was no difference in the proportion of dogs between the groups that did not survive ($p=0.18$).

There was no difference in the age of the dogs between groups who survived. There was no difference in the BUN ($p=0.2$), ALP ($p=0.6$), PT (0.44), aPTT (0.4), or band neutrophil count (0.25). The difference in total bilirubin between the dogs who survived and those who did not was approaching significance, but was not significant at a p of 0.09. There was no difference in antithrombin level ($p=0.19$), FDPs ($p=0.86$), or fibrinogen ($p=0.13$) between the dogs who survived and those who did not.

Thrombosis and Causes of Death:

Eight of the 22 dogs in the LDA group had possible or confirmed thromboembolic complications. Two dogs had suspected pulmonary thromboemboli (PTE), based on acute dyspnea and hypoxemia. One of these dogs survived to study completion and one was euthanized due to the thrombus as well as refractory hemolysis. One dog had arterial thrombosis to 3 feet, and one had venous thrombosis to a foreleg. Both of these dogs were euthanized due to complications from these thromboses. Two dogs had PTE concurrent with other organ thrombi (spleen and liver in one, adrenal in the second), which were found on post-mortem examination. These dogs were euthanized due to dyspnea and deteriorating clinical condition. One dog was euthanized due to poor clinical condition, and had hepatic venous and sinusoidal thrombosis found on post-mortem examination. One dog developed a thrombus in the caudal vena cava; this thrombus was identified on ultrasound after ascites was noted on physical examination. This dog had eventual resolution of her thrombus and survived to completion. Overall, two dogs who experienced thromboembolic complications survived to 180 days.

Other causes of death in the LDA group included one case of pyelonephritis leading to renal failure, and two dogs who had continued hemolysis that was not responsive to immunosuppressive therapy.

Two of the 18 dogs in the IAD group had possible or confirmed thromboembolic complications. One dog developed acute dyspnea and hypoxemia and had a suspected PTE; the second developed a suspected PTE and had a portal

thrombus noted on post-mortem examination. Both of these dogs were euthanized within 24 hours of onset of clinical signs.

Three additional dogs in the IAD group did not survive; one dog experienced hematemesis and deteriorating clinical condition, one was refractory to therapy and had a decline in clinical condition, and one dog developed non-regenerative anemia.

There was no statistical difference in the number of dogs with thromboembolic complications between the two groups of dogs, but it did approach significance (p=0.07) (Table 2).

Hemorrhagic Complications:

Two dogs in the AID group had non-fatal hemorrhagic complications. No dogs in the LDA group had hemorrhagic complications. There was no significant difference in the number of dogs who had hemorrhagic complications between the groups (p=0.2).

Hospitalization and Therapy:

The median length of hospitalization for the dogs in the IAD group was 3 days (range, 0-9 days) and 4 days (range, 0-9 days) for the dogs in the LDA group. In the IAD group, 4 dogs received no packed red blood cell transfusion, 8 dogs received one, 5 dogs received 2, and one dog received 3. In the LDA group, 4 dogs received no packed red blood cell transfusions, 4 dogs received one, 9 dogs received two, 3 dogs received 3, and 1 dog received 4.

No dog had been treated with any immunosuppressive or anticoagulant therapy prior to admission. All dogs received prednisone.

In the IAD group, concurrent immunosuppressive therapies included azathioprine in 14 dogs, cyclosporine in 3 dogs, and mycophenolate in 1 dog. In the LDA group, 9 dogs received azathioprine and 5 received mycophenolate in addition to prednisone.

Relationship of Coagulation Parameters and Thromboembolic Complications:

Elevated BUN and aPTT were significantly associated with the development of thromboembolic complications in all dogs (p=0.01 and p=0.04, respectively). The association of elevated bilirubin with thrombosis was not significant, but approached significance with a p-value of 0.07.

There was no relationship between the development of thromboembolic complications and age of the dog (p=0.7), ALP (0.6), PT (p=0.6), fibrinogen (p=0.3), antithrombin III level (p=0.8), or band neutrophils (p=0.4).

The relationship between the degree of spherocytosis and development of thromboemboli was evaluated. There was no association with the degree of spherocytosis and thromboemboli (p=1).

Twenty-nine dogs had FDP levels measured on admission. There was no association between the level of FDPs and the development of thrombosis (p=0.6). Eighteen dogs had d-dimers measured at the time of admission. The results for all dogs was <0.5 (Table 2).

Discussion:

The results of this study are encouraging for the use of individually-adjusted heparin therapy for thrombophylaxis in dogs with IMHA. Critical to note in this study is that it was underpowered; power analysis prior to enrollment demonstrated that to achieve 80% power, 30 dogs in each group would need to be enrolled. 22 dogs were enrolled in the blinded, placebo-controlled study, and 18 other dogs were identified as cohorts to add statistical power, although appropriate power (80%) was still not met by a deficit of 20 dogs. The slow enrollment was the largest challenge encountered in this study. Given that the study was underpowered and therefore at risk for Type II error, the near significant difference in thrombotic complications between the two groups of dogs is very compelling, implying that a true difference may be present, and strongly warrants further investigation into the possible benefits of IAD therapy for prevention of thrombosis. The results obtained lend confidence to continuation of study and possibility that individually adjusted heparin could improve outcome.

This study corroborated previous studies evaluating risk factors for thrombosis in dogs with IMHA. It has been documented that elevations in BUN, total bilirubin, and increased aPTT are associated with an increased risk for thromboembolic complications[2, 19]. As has also been reported, the majority of the thromboses occurred within the first few weeks of therapy[11, 20]. Similar to previous findings[21], there was no association between AT III and increased risk of thrombosis although many of the dogs in our study had antithrombin III levels that measured below normal. Previous studies[2, 15] have also found an increased risk of thrombosis with elevated band neutrophil counts, which was not found in the current study.

Interestingly, there was no difference in the mortality between the two groups of dogs, nor was there an association between any of the clinicopathologic findings and mortality, although again, Type II error may account for that. Many of the thromboses that were encountered by the dogs were non-fatal and were survived. Another reason for the lack of difference in survival between groups, while a difference in thrombosis is suggested, is that some dogs did not die from their thromboses. Those dogs tended to succumb to their disease due to persistent hemolysis, often resulting in overwhelming transfusion demands. This places a significant financial demand on the client, and is often a cause for electing euthanasia. It would be interesting to evaluate whether survival would have been different if finances had not been a factor. Returning to the data and censoring the dogs who did not die of thrombosis, and repeating the analysis on the relationship between treatment group and survival is the next step to take.

Thromboses were found to be both venous and arterial in our study. Other causes of death were due to the refractory nature of some of the cases; repeated transfusions are often financially limiting for many clients, and in some cases the IMHA caused sufficient morbidity to impact quality of life significantly.

It is possible that there were more thrombotic complications, possibly in both groups of dogs, than were accounted for. This may be a source of misclassification that could also contribute to the lack of statistical significance in the present study. Not all dogs had post-mortem examinations, and clot lysis is reported to occur quickly post-mortem, so it is possible that there were thromboses that were missed in dogs who did have a post-mortem examination.

With the small number of dogs in this study, we were unable to correlate additional therapies, such as the use of secondary immunosuppressives, antibiotics, or anti-nausea medications with mortality. The study size was the largest challenge encountered in this trial. The exclusion criteria were the largest reason for this, as many dogs were admitted that had received aspirin therapy already. Additionally, more active recruitment from the surrounding primary care hospitals could have been pursued.

In addition to pursuing more cases to continue this study, there are other questions that would be interesting to pursue. The anti-Xa target range is specifically for humans, although the test has been validated in dogs. Because dogs with IMHA often have low ATIII levels, it would be interesting to evaluate whether dogs with IMHA have a different anti-Xa target range. The relationship between anti-Xa and low ATIII has not been evaluated in dogs, and given that ATIII is necessary for unfractionated heparin to work, it may be important to determine whether the range for these dogs differs from the normal population if it is to be used for heparin adjustment.

Another interesting issue that arose when performing this study was inadvertent unmasking of the attending clinicians. There were communication errors that included the dog group assignment, or poor placement of the assignment list by staff. As the outcome measure was fairly objective, however, it is unlikely that occasional unmasking affected the endpoint assessment.

One issue that also came up when performing this study was the owner stress regarding the enrollment of their dog when they have just been diagnosed

with a new, potentially fatal, and likely expensive, disease. Some owners were excited to participate, and perhaps felt a sense of control, whereas others felt overwhelmed. It was impossible to predict the reactions owners would have, so both had to be anticipated and treated with equal respect.

In conclusion, this study provides support for the continued evaluation of individually adjusted heparin therapy for the use of thrombophylaxis in dogs with IMHA. There is a strong trend towards a significant decrease in thromboembolic complications in dogs who received individually adjusted heparin relative to those on ultra low-dose aspirin.

Chapter 3: Safety of ultrasound-guided fine-needle aspiration of the feline pancreas: A case-control study

Abstract:

The safety of fine-needle aspiration (FNA) of the feline pancreas has not been reported. The incidence of complications following ultrasound-guided pancreatic FNA in 73 cats (PA cats) with clinical and sonographic evidence of pancreatic disease was compared to complications in two groups of matched control cats also diagnosed with pancreatic disease who either had abdominal organs aspirated other than the pancreas (Control-FNA, n=63) or no aspirates performed (Control-No FNA, n=61). The complication rate within 48 hours of the aspirate procedure did not differ among the PA cats (11%), Control-FNA (14%), or Control-No FNA (8%) cats. There was no difference in rate of survival to discharge (82%, 84%, and 83%, respectively) or length of hospital stay among groups. The cytologic recovery rate for the pancreatic samples was 67%. Correlation with histopathology, available in 7 cases, was 86%. Pancreatic FNA in cats is a safe procedure that appears to be diagnostically useful.

Introduction:

The clinical importance of feline pancreatic disease is increasingly being recognized. A pathologic classification has been proposed, including various histopathological types of pancreatitis, nodular hyperplasia, neoplasia, pancreatic pseudocyst, abscess, amyloid deposition, and pancreatic atrophy[60]. In addition to the broad spectrum of diseases, the clinical picture is further complicated by the potential for pancreatic disease to present concurrently with inflammatory bowel disease and/or cholangitis[61]. Given the significance of pancreatic diseases in cats, there is a pressing need for minimally-invasive diagnostic tests to distinguish among inflammatory, hyperplastic, and neoplastic lesions.

Ultrasound is often used in conjunction with clinical and laboratory findings to identify pancreatic disease. Abdominal ultrasonography is safe and has good specificity for the presence of pancreatitis, but has suboptimal sensitivity, reportedly 30-80% in cats[62, 63, 64]. Additionally, ultrasonography cannot reliably differentiate among different pathologic processes in the pancreas [64, 65, 66]. Nodular pancreatic change raises concern for neoplasia, but is not specific[65], and may also occur with inflammatory disease[67].

Obtaining samples of abdominal organs by ultrasound-guided fine-needle aspiration (FNA) for cytologic evaluation is a reasonably safe and accurate diagnostic modality that often augments imaging studies. In human medicine, pancreatic FNA has a complication rate of 1.5%[68] -3%[68, 69, 70]. Diagnostic performance is good, with reported sensitivity of 92.5%[71] and specificity of 68%[72]- 100%[68] for neoplasia. Few studies have addressed pancreatic FNA in cats[64. 65. 72]. Although no complications have been reported in the 17 cats evaluated in these studies, speculation about potential risks following

pancreatic sampling may contribute to reluctance to perform pancreatic FNA. Currently, there seems to be a tendency among some clinicians to shy away from performing feline pancreatic FNA. It is a commonly-held belief that aspirating an inflamed pancreas will contribute to the inflammatory state and result in more severe pancreatitis. Although this is reported as a rare side effect in humans, it has not been described in the veterinary literature, and no safety data for this procedure exists.

The primary objective of this study was to evaluate the safety of FNA of the feline pancreas with clinical and ultrasonographic evidence of pancreatic disease. Secondary objectives were to evaluate the diagnostic yield of samples obtained by ultrasound-guided pancreatic FNA and the type of diagnostic information obtained. We hypothesized that FNA of the feline pancreas would be safe, providing diagnostically useful information in the majority of cases.

Materials and methods:

Case selection and matching with controls:

The electronic medical record system at the University of Minnesota Veterinary Medical Center was searched for cats having pancreatic cytology performed from September 2004 through September 2011. Inclusion criteria for pancreatic aspirate (PA) cats consisted of 1) abdominal ultrasound performed by a board certified veterinary radiologist in cats with clinicopathologic changes compatible with pancreatic disease, 2) ultrasonographically-identified pancreatic lesions, 3) FNA of the pancreas performed with ultrasound guidance, 4) successful pancreatic aspirate confirmed during imaging or by cytologic evaluation and 5) a

complete medical record with hospitalization for a minimum of 48-hours post-ultrasound +/- aspirate procedure. Cases were *excluded* if the required information was not available.

The population from which control cats were matched to PA cats was identified by searching the ultrasound log from September 2004 to September 2011 for consecutive feline cases having ultrasonographic abnormalities of the pancreas or peripancreatic tissue, with no pancreatic FNA performed. Inclusion criteria 1, 2 and 5 were otherwise identical to PA cats. Two controls groups were formed: control FNA cats had ultrasound-guided FNA or biopsies of abdominal organs other than the pancreas and control-No FNA cats did not have any sampling of intra-abdominal organs or tissues performed during the ultrasound procedure. We attempted to match PA cats to one control from each group. PA cats and controls were first matched based on age group (0-8 years, 9-15, and >16 years of age) and date of ultrasound (within 6 months of the PA case). To attempt to control for illness severity, controls were then matched with PA cases based on location of hospitalization (Intensive Care Unit or general wards) and, finally, on type and severity of pancreatic and peripancreatic tissue changes on ultrasound. Cases and controls were matched first on pancreatic size, echotexture, and margin irregularity, then on presence of masses, nodules, and peritoneal effusion. Ultrasound reports for cats in all groups were evaluated and the following information about the pancreas was recorded: pancreatic size, margin irregularity, echotexture, and the presence or absence of masses or nodular lesions (size, shape, number). Peripancreatic changes, including

mesenteric echogenicity changes, peritoneal effusion, and hepatic echotexture abnormalities were also recorded.

Data collection:

Signalment, historical illnesses, serum liver enzyme activities, serum bilirubin, and creatinine concentrations prior to imaging were recorded for cats in all groups. Length of hospital stay, discharge status (alive, died, or euthanized/discharged to be euthanized within 24 hours), clinical diagnosis, and concurrent diagnoses based on record review were noted. Records were evaluated to determine if any of the following, designated as "complications", occurred within 48 hours after the abdominal ultrasound: hemoabdomen, hypotension, need for corrective clinical action (transfusion, diagnostic abdominocentesis or peritoneal lavage), or respiratory distress. It was also recorded if suspicion of any of the above complications lead to recheck abdominal ultrasound within 48 hours of the original procedure.

Pancreatic FNA procedure and cytologic classification:

Aspirates were obtained with a 20 or 22 gauge hypodermic or spinal needle of an adequate length to reach the pancreas or other organ or tissue using either aspiration or fenestration techniques, or a combination of both[67]. This is the procedure typically used for intra-abdominal aspirates of solid tissues. Multiple FNA attempts, usually 2-3, are standardly made for tissue sampling in our hospital; one attempt is typically performed for fluid. Pancreatic cytology reports generated by a

board-certified veterinary clinical pathologist were reviewed and the cellularity of the cytologic samples and cytologic diagnosis(es) for the pancreas noted. Cytologic recovery rate was defined as the percent of samples from the total submitted that were diagnostic. Cytologic diagnoses were categorized as normal exocrine tissue, cyst, necrosis/inflammation, hyperplasia, neoplasia, or nondiagnostic. For a diagnosis of neoplasia, the cell of origin or specific tumor type was recorded, if determined. Histopathologic diagnosis from surgical or necropsy samples were recorded when available, and the interval between cytologic and histopathologic evaluation recorded.

Statistical analysis:

The chi-squared test of association was used to assess differences among groups in sex, frequency of complications, location of hospitalization, and discharge status. Cats that died or were discharged to be euthanized were combined for analysis. Factors used for matching cases to controls were not evaluated for differences, except for ultrasonographic features of the pancreas, as matching for these criteria was incomplete. The data were tested for normality, and ANOVA was used to evaluate differences among the groups in pre-ultrasound serum liver enzyme activities, bilirubin, creatinine, and frequency of concurrent diseases. Differences in length of hospital stay were evaluated using a linear mixed model with group (PA, control-FNA, or control-No FNA), location of hospitalization, age group, and discharge status as fixed effects and the matched cats as a random effect.

The most common distributions of clinical pathologic category of pancreatic disease for PA cats were described. Values of $p<0.05$ were considered significant.

Results:

Study population:

Ninety-five possible PA cats were identified; 22 were excluded due to inability to confirm pancreatic aspiration. Of 73 remaining PA cats, 51 were matched to two controls (21 with both controls from the same control group and nine with one from each control group). Twenty-two PA cats had a single control. Of the 162 cats identified as potential controls, 38 were excluded for inability to be satisfactorily matched to a PA cat.

Overall, 197 cats were included in this study, of which 77 were spayed females and 120 were neutered males. The mean age was 12.2 years (range 3-19). The PA group (n=73) consisted of 28 spayed females and 45 neutered males with mean age of 13.4 years (range 3-18). Of 63 Control-FNA cats, 20 were spayed females and 43 were neutered males. The mean age was 12.0 years (range 6-18). Of 61 Control-No FNA cats, 29 were spayed females and 32 were neutered males. The mean age was 11.1 years (range 3-19). Sex distribution did not differ among groups ($p=0.24$) (Table 3).

Hospitalization:

The means for lengths of hospital stay were: PA 2.77 days (range 1-7), Control-FNA 2.49 days (range 1-11) and Control-No FNA 3.0 days (range 1-9), with

no significant differences among the three groups (p=0.7). Control-No FNA cats were 3 times as likely to be hospitalized in the general wards than the ICU (p<0.001), while the other groups were more evenly distributed between locations (Table 3).

Biochemical analyses:

There were no significant differences in serum activities of ALT (p=0.17), ALP (p=0.68), GGT (p=0.69), or serum creatinine concentration (p=0.15) (Table 3). The Control-FNA group had a higher mean bilirubin than the PA and Control-No FNA cats (p<0.001).

Concurrent diseases:

In the PA group 23 (32%) had no additional diagnoses, 26 (36%) had one additional diagnosis, 14 (19%) had two, 7 (10%) had three, and three (4%) had four additional diagnoses. Of Control-FNA cats, 29 (46%) had no additional diagnoses, 27 (43%) had one, six (10%) had two, and one had four. Of Control-No FNA cats, 27 (44%) had none, 25 (41%) had one, six (10%) had two, two (3%) had three, and one (2%) had four additional diagnoses. The most common concurrent diseases were diabetes mellitus, present in 10/73 (14%) PA cats, 3/63 (5%) Control-FNA cats, and 8/61 (18%) Control No-FNA cats; hyperthyroidism in 7/73 (10%), 4/63 (6%), and 3/61 (6%); and chronic kidney disease in 14/73 (19%), 13/63 (21%), and 10/61 (16%), respectively. There was no significant difference in the frequency of concurrent diagnoses among the groups (p=0.6).

Ultrasound and pathology results:

The most common ultrasonographic abnormalities of the pancreas observed are reported in Table 4. All cats had multiple ultrasonographic abnormalities. Despite attempts to match on all major ultrasonographic findings, the PA cats were significantly more likely to have nodular or mass-like lesions ($p<0.01$) than cats in either control group. Of PA cats, 12 had pancreatic aspirates only, while 61 had multiple organs sampled, including the liver (40), spleen (9), abdominal lymph nodes (14), mesentery (10), kidney (5), cystocentesis (2), and abdominal effusion (15). One PA cat had an ultrasound-guided liver biopsy in addition to pancreatic FNA. Of Control-FNA cats, 39 had one organ aspirated, 22 had two, and one cat each had aspirates of three and four organs. Organs sampled included the liver (40), spleen (9), intestine (3), abdominal lymph nodes (6) mesentery (7), kidney (2), cystocentesis (4), and abdominal effusion (15). Four cats also had liver biopsies performed.

Twenty-four of 73 cytologic samples of the pancreas were nondiagnostic, for a cytologic recovery rate of 67%. The cytologic distribution of pathologic processes is reported in Table 5. Nine cats also had pancreatic histopathology performed two days to one year post-aspiration (one surgical, eight at necropsy). Histopathologic and cytologic diagnoses are indicated in Table 6. The correlation between pancreatic cytology and histopathology was 86%.

Complications:

There was no difference among groups in complication incidence (p=0.9) or in the number of cats with complications that survived to discharge. (Table 7). Eight PA cats (11%), 9 control-FNA cats (14%) and 5 control-no FNA cats (8%) developed complications within 48-hours of their pancreatic aspirate procedure. Complications occurred in 7/62 PA cats that had the pancreas and other tissues sampled (three liver, three peritoneal effusion, and one both**),** and in 1/11 that had only pancreatic aspiration. Two PA cats, one Control-FNA cat and 2 Control-no FNA cats had recheck ultrasounds while hospitalized to evaluate for complications. Overall, 3/8 (37%) of the PA cats and 5/9 (56%) of the Control-FNA cats experiencing complications survived to discharge compared with 57/65 (88%) and 49/54 (91%), respectively, without clinical complications. There was a 40% survival rate (2/5) in the Control-No FNA cats developing complications after their ultrasounds compared with 48/56 (86%) without complications.

Discussion:

This study failed to demonstrate any increase in complication or mortality rates in cats undergoing pancreatic aspiration compared to those who did not. The incidence of complications in our study for cats undergoing FNA of any intra-abdominal organ is higher than that in one previous report of about 5%[67]. We evaluated changes in clinical status up to 48 hours post-ultrasound to ensure that we accounted for all potential complications. Many of the complications we report are unlikely to be due to the aspiration procedure. This is supported by the lack of difference in complication rate experienced by cats that did or did not have

aspiration procedure(s) performed. The majority of complications in the PA cats were noted when a second organ, typically the liver, was aspirated. The major complications encountered in PA and Control-FNA cats were hemorrhage and hypotension, which together occurred in 3% (PA cats) and 6% (Control-FNA) of the cats.

The Control-No FNA cats were more likely to be hospitalized in the general wards than the ICU, which suggests that they may not have been as critically ill as the cats in the other groups. We attempted to match for hospitalization between groups, but due to the numerous matching criteria this was difficult. However, their complication rate was not different from the rate of the other groups. This supports that the complication risk is not greater for more critically ill cats.

The Control-FNA group cats had higher serum bilirubin concentrations relative to the other groups of cats. This may reflect a propensity for this group of cats to have primary hepatic or biliary pathology, likely resulting in a decision to aspirate the liver rather than the pancreas, despite ultrasonographic pancreatic abnormalities. PA cats had a higher proportion of pancreatic nodules which may have led to clinician bias towards aspirating the pancreas due to suspicion of neoplasia. One previous study demonstrated overlap in ultrasonographic abnormalities between cats with pancreatic neoplasia and nodular hyperplasia[67]. Due to the high variability in ultrasonographic findings and the small number of cats in each cytologic category, we did not attempt to correlate ultrasound findings with cytologic appearance.

The pancreatic cytologic recovery rate of 67% is similar to the 86% recovery that is reported for aspiration of abdominal masses in dogs and cats.[73] In the current study, samples were diagnostically useful in cases of various forms of pancreatitis (n=21), neoplasia (n=14), and septic abscessation (n=1). There was good correlation with histopathology in the small number of cases available for comparison, consistent with reports that specificity for cytology is generally very good.[15,16]. Negative results may not be accurate, as the distribution of lesions within the pancreas is known to be multifocal[74]. It is interesting that although the cyologic yield was not typically fair-excellent, it still had a fairly good recovery rate.

There are several limitations to this study. As a retrospective study, we were unable to control for variations in clinician bias for or against pancreatic aspiration, clinical pathologist and radiologist interpretation, radiologist aspiration technique, and client permission for FNA to be performed. Ideally, each PA cat would have been matched to one cat from each control group; this was not possible based on the multiple matching criteria established. There was also some variation in matching by ultrasound lesions, since each cat had multiple lesions and it was not possible to match on all of them.

The largest challenge in this study that was encountered was the matching. The matching, as it involved multiple criteria as far the cats were concerned (time of admission, location of hospitalization, age group, etc), as well as ultrasonographic findings, made it difficult to match the cats perfectly. The interpretations of the ultrasound findings varied based on the radiologist, and there were many ultrasound findings to match the cases and controls by. This resulted in preferential

matching by certain criteria (pancreas size, echotexture, margin) before other findings (masses, peritoneal effusion). These issues likely introduced variability into the groups.

Another issue that was encountered was the grouping of the cats. Initially, there were two groups in the study design: a Case group and a Control group. However, after matching was performed, it was realized that the controls needed to be split into two groups: those who had had aspirates (Control-FNA) and those who had not (Control-No FNA). This made matching of the cats imperfect, as there were some Case cats who were matched with two controls from one specific group.

Our data support previous smaller studies demonstrating that ultrasound-guided aspiration of the feline pancreas is a safe diagnostic procedure. The combination of acceptable risk and good diagnostic yield, with initial indications of good correlation with histopathology, suggests that pancreatic cytology can be a safe and valuable tool in the diagnosis of feline pancreatic disease.

Tables

	LDA Group	IAD Group	P-value
Age (years)	5.7	6.4	0.55
BUN (9-31 mg/dl)	33.2	24.7	0.16
Total Bilirubin (0-0.3 mg/dl)	6.1	1.4	0.1
ALP (8-139 u/l)	333.8	246.3	0.88
PT (6.2-7.7 sec)	7.8	7.4	0.15
aPTT (9.8-14.6 sec)	13.6	13.5	0.93
Fibrinogen	0.56	0.48	0.26
ATIII%	69%	70%	0.8
Bands (x1,000/μl)	0.79	1.04	0.43

Table 1: Differences in baseline parameters between two treatment groups. Reference range for each parameter provided.

		No Thrombosis	Thrombosis	P-value
Age (years)		5.9	6.5	0.7
BUN (9-31 mg/dl)		14.9	25.4	0.04
Total Bilirubin (0-0.3 mg/dl)		2.3	8.4	0.06
ALP (8-139 u/l)		302.2	270.5	0.6
PT (6.2-7.7 sec)		7.5	7.7	0.6
aPTT (9.8-14.6 sec)		13	14.9	0.04
Fibrinogen		0.48	0.6	0.3
ATIII%		68%	72%	0.8
Bands (x1,000/μl)		0.87	1.0	0.4
Treatment Group	LDA	14	8	0.07
	IAD	16	2	

Table 2: Differences in the baseline variables, and number of dogs in each treatment group, between the dogs with confirmed/suspected or no evidence of thromboembolism.

		PA (n=73)	Control-FNA (n=63)	Control-No FNA (n=61)
Hospitalization	General Wards	47 (64%)	28 (44%)	46 (75%)*
	ICU	26 (36%)	35(56%)	15 (25%)
Age Group	0-8 years	10 (14%)	14 (22%)	21 (35%)
	9-15 years	39 (53%)	42 (67%)	30 (49%)
	>16 years	24 (33%)	7 (11%)	10 (16%)
Sex	Neutered Male	45 (62%)	43 (68%)	32 (52%)
	Spayed Female	28 (38%)	20 (32%)	29 (48%)
Discharge Status	Alive	60 (82%)	53 (84%)	51 (83%)
	Euthanized	10 (17%)	8 (13%)	7 (12%)
	Died	1 (1%)	2 (3%)	3 (5%)
Biochemistry Results	ALP (U/L)	30 (61-275)	31 (63-230)	34 (44-110)
	ALT (U/L)	94 (157-423)	92 (114-312)	75 (77-214)
	GGT (U/L)	3 (2.8-7.8)	3 (2.8-5.2)	3 (3.1-8.8)
	Bilirubin(mg/dL)	0.3 (1.4-4.6)	3 (2.8-5.2)**	0.4 (1.4-5.3)
	Creatinine(mg/dL)	1.8 (1.6-2.9)	1.5 (1.5-2.9)	1.75 (2.2-4.3)

Table 3: Distribution of cats in each group in terms of location of hospitalization (ICU vs general wards), discharge status, and pre-ultrasound chemistry values expressed as median (95% Confidence Interval for the mean) in each group.
*Control-No FNA cats were more likely to be hospitalized in the wards than in the

ICU (p<0.001). There was no significant difference in the discharge status among cats. **Control-FNA cats had a higher total bilirubin compared to the other two groups (P<0.001).

Ultrasonographic Finding	PA Cats (n=73)	Control-FNA (n=63)	Control-No FNA (n=61)
Enlarged Pancreas	27 (40%)	26 (41%)	22 (36%)
Irregular Pancreas	28 (38%)	21 (33%)	25 (40%)
Hypoechoic Pancreas	34 (46.5%)	34 (54%)	37 (61%)
Peritoneal Effusion	33 (45%)	22 (35%)	17 (28%)
Pancreatic Masses/Nodules	33 (45%)*	16 (25%)	11 (18%)

Table 4: Prevalence of the most common ultrasonographic abnormalities among cats within each study group. *PA cats were significantly more likely to have mass lesions or nodular change to their pancreas (p<0.01).

Category of Pathology		Number of Cats
Inflammation	Suppurative	14 (1 septic)
	Lymphocytic	7
Cyst		3
Necrosis		11
Hyperplasia		6
Neoplasia	Carcinoma	11
	Round Cell	2
	Unknown	1
No Abnormalities		11
Inconclusive		24

Table 5: Distribution of pathologic diagnoses from PA cats.

Cytology Results	Histopathology Results
Mild increase in plasma cells and lymphocytes	No significant microscopic lesions
Carcinoma (n=2)	Carcinoma (n=2)
Suggestive for carcinoma	Carcinoma
Lymphocytic inflammation	Lymphocytic and plasmacytic inflammation
Lymphocytic inflammation (n=2)	Lymphocytic and plasmacytic inflammation with fibrosis (n=2)
Nondiagnostic (n=2)	Amyloidosis (n=2)

Table 6: Correlation between FNA pancreatic samples and pancreatic histopathology.

Complication	PA Cats (n=73)	Control-FNA (n=63)	Control-No FNA (n=61)
Hypotension	1	3	1
Respiratory Distress	3	0	0
Hemoabdomen	1	1	0
Whole Blood	1	0	0
Packed Red Blood Cells	2	3	2
Fresh Frozen Plasma	2	0	2
Oxyglobin[i]	0	1	0
Pleural Effusion	0	1	0
Total Complications	10*	9	5
Survival in cats with complications (%)	3 (37%)	5 (56%)	2 (40%)

*10 complications occurred in 8 cats. One cat in this group experienced hypotension and received whole blood, and a second cat developed hemoabdomen and received a packed red blood cell transfusion.

Table 7: Complications encountered within 48 hours of abdominal ultrasound, listed as number of cats and percent of cats in each group.

Bibliography:

a. University of Minnesota Coombs' test, St. Paul, MN

b. IDEXX Laboartories, Westbook, ME

c. Emcure Pharma, Bosari, Pune, India

d. Cerenia©, Zoetis, Florham Park, NJ

e. Hospira, Lake Forest, IL

f. Chromatic Heparin; Chromogenix-Instrumentation Laboratory SpA; Milano, Italy

g. R, Vienna, Austria

h. BMG Labtech, Ortenberg, Germany

i. Oxyglobin, OPK Biotech, Cambridge, MA, USA

1. Reimer ME, Troy GC, Warnick LD. Immune-mediated hemolytic anemia: 70 cases (1988-1996). JAAHA 1999;35:384-391

2. Piek CJ et al. Idiopathic Immune-Mediated Hemolytic Anemia: Treatment Outcome and Prognostic Factors in 149 Dogs. JVIM 2008;22:366-373

3. Swann JW and Skelly BJ. Evaluation of immunosuppressive regimens for immune-mediated haemolytic anemia: a retrospective study of 42 dogs. JSAP 2011:52;353-358

4. Piek CJ. Canine immune-mediated hemolytic anemia: a review with recommendations for future research. Vet Q 2011:31(3);129-141

5. Giger U. Regenerative anemia caused by blood loss or hemolysis. In: Ettinger JE, Feldman EC, eds. Textbook of Veterinary Internal Medicine. 6th Ed, 2005. St Louis MO: Elsevier Saunders. 1886-1907

6. Tan E, Bienzle D, et al. Potentially antigenic RBC membrane proteins in dogs with primary immune-mediated hemolytic anemia. Vet Clin Pathol 2012;41(1):45-55

7. Carr AP, Panciera DL, Kidd L. Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. J Vet Intern Med. 2002;16(5):504-9

8. Harkin KR, Hicks JA, Wilkerson MJ. Erythrocyte-bound immunoglobulin isotypes in dogs with immune-mediated hemolytic anemia: 54 cases (2001-2010). JAVMA 2012;241:227-232

9. Corato A Shen CR et al. Proliferative responses of peripheral blood mononuclear cells from normal dogs and dogs with autoimmune hemolytic anemia to red blood cell antigens. Vet Immunol Immunopathol 1997;59(3-4):191-204

10. Salinas GF, Braza F, et al. The role of B lymphocytes in the progression from autoimmunity to autoimmune disease. Clin Immunol 2013;146(1):34-45

11. Scott-Moncreiff JC, Treadwell NG, et al. Hemostatic abnormalities in dogs with primary immune-mediated hemolytic anemia. J Am Anim Hosp Assoc 2001;37(3):220-227

12. Duffy AL, Olea-Popelka FJ, et al. Serum concentrations of monocyte chemoattractant protein-1 in healthy and critically ill dogs. Vet Clin Pathol 2010;39:302-305

13. Kjelgaard-Hansen M, Goggs R, Wiinberg B, Chan DL. Use of Serum Concentrations of Interleukin-18 and Monocyte Chemoattractant Protein-1 as Prognostic Indicators in Primary Immune-Mediated Hemolytic Anemia in Dogs. J Vet Intern Med 2011;25(1):76-82

14. Becker RC. DVT: a new era in anticoagulant therapy. Arterioscler Thromb Vasc Biol 2010;30(3):369-371.

15. Weinkle TK, Center SA, Randolph JF et al. Evaluation of prognostic factors, survival rates, and treatment protocols for immune-mediated hemolytic anemia in dogs: 151 cases (1993-2002). JAVMA 2005;22:1869-1880

16. Mason N, Duval D, et al. Cyclophosphamide exerts no beneficial effect over prednisone alone in the initial treatment of acute immune mediated hemolytic anemia in dogs: a randomized controlled clinical trial. J Vet Intern Med 2003;17(2):206-212

17. Lunsford KV and Mackin AJ. Thromboembolic therapies in dogs and cats: an evidence-based approach. Vet Clin North Am Small Anim Pract 2007;37(3):579-609

18. Orcutt ES, Lee JA, Bianco D. Immune-mediated hemolytic anemia and severe thrombocytopenia in dogs: 12 cases (2001-2008) JVECC 2010;20(3):338-345

19. McManus PM and Craig LE. Correlation between leukocytosis and necropsy findings in dogs with immune-mediated hemolytic anemia: 34 cases (1994-1999). J Am Vet Med Assoc 2001;218(8):1308-1313

20. Klein MK, Dow SW, and Rosychuk RA. Pulmonary thromboembolism associated with immune-mediated hemolytic anemia in dogs: ten cases (1982-1987). J Am Vet Med Assoc 1989;195(2):246-250

21. Weiss, DJ and Brazzell JL. Detection of Activated Platelets in Dogs with Primary Immune-mediated hemolytic anemia. JVIM 2006;20:682-686

22. Ridyard AE, Shaw DJ, and Milne EM. Evaluation of platelet activation in canine immune-mediated hemolytic anemia. J Small Anim Pract 2010;51(6):296-304

23. Kidd L and Mackman N. Prothrombotic mechanisms and anticoagulant therapy in dogs with ummune-mediated hemolytic anemia. J Vet Emer Crit Care 2013;23(1):3-13

24. Breuhl EL, Moore G, Brooks M, Scott-Moncrieff JC. A Prospective Study of Unfractionated Heparin Therapy in Dogs with Primary Immune-Mediated Hemolytic Anemia. JAAHA 2009; 45: 125-133

25. Piek CJ, van Spil WE, Junius G, and Dekker A. Lack of evidence of a beneficial effect of azathioprine in dogs treated with prednisolone for idiopathic immune-mediated hemolytic anemia: a retrospective cohort study. BMC Veterinary Research 2011; 7:15

26. Whelan MF et al. Use of human immunoglobulin in addition to glucocorticoids for the initial treatment of dogs with immune-mediated hemolytic anemia. J Vet Emer Crit Care 2009:19(2) 158-164

27. Fenty RK, deLaforcade AM, Shaw SP, and O'Toole TE. Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J Am Vet Med Assoc. 2011;238(4):463-7

28. Stokol T et al, D-dimer concentrations in healthy dogs and dogs with disseminated intravascular coagulation. Am J Res 2000;61:393-398

29. Sinnot VB and Otto CM. Use of thromboelastography in dogs with immune-mediated hemolytic anemia: 39 cases (2000-2008). JVECC 19(5) 2009, 484-488

30. McMichael MA and Smith SA. Viscoelastic coagulation testing: technology, applications, and limitations. Vet Clin Pathol 2011;40(2):140-153

31. O'Kell AL, Grant DC, et al. Effects of oral prednisone administration with or without ultralow-dose acetylsalicylic acid on coagulation parameters in healthy dogs. Am J Vet Res 2012;73(10):1569-1576

32. Rose LJ, Dunn ME, et al. Effect of prednisone administration on coagulation variables in healthy Beagle dogs. Vet Clin Pathol 2011;40(4):426-434.

33. Flint SK, Abrams-Ogg AC, et al. Independent and combined effects of prednisone and acetylsalicylic acid on thromboelastographic variables in healthy dogs. Am J Vet Res 2011;72(10):1325-1332

34. Harkin KR, Hicks JA, Wilkerson MJ. Erythrocyte-bound immunoglobulin isotypes in dogs with immune-mediated hemolytic anemia: 54 cases (2001-2010). J Am Vet Med Assoc 2012;241:227-232

35. Klag AR, Giger U, Shofer FS. Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990). J Am Vet Med Assoc 1993;202(5):783-788

36. Holahan ML, Brown AJ, and Drobatz KJ. The association of blood lactate concentration with outcome in dogs with idiopathic IMHA: 173 cases (2003-2006). J Vet Emer Crit Care 2010;20(4): 413-420

37. Gow DJ, Gow DA, et al. Serum cardiac troponin I in dogs with proimary immune-mediated haemolytic anemia. J Sm Anim Prac 2011(52), 259-264

38. Kuzi S, Segev G, Haruvi E, Aroch I. Plasma Antithrombin Activity as a Diagnostic and Prognostic Indicator in Dogs: A Retrospective Study of 149 Dogs. J Vet Intern Med 2010;24:587-596

39. Green RA. Pathophysiology of antithrombin III deficiency. Vet Clin North Am Small Anim Pract 1988;18(1):95-104

40. Welles EG. Antithrombotic and fibrinolytic factors. A review. Vet Clin North Am Small Anim Pract 1996;26(5):1111-1127

41. Rackear D, Feldman B, Farver T, et al. The effect of three different dosages of acetylsalicylic acid on canine platelet aggregation. J Am Anim Hosp Assoc 1988;24:23–26

42. Shearer L, Kruth SA, Wood D. Effects of aspirin and clopidogrel on platelet function in healthy dogs (abstr). J Vet Intern Med 2009;23:745

43. Hoh CM, Smith SA, McMichael MA, Byron JK. Evaluation of effects of low-dose aspirin administration on urinary thromboxane metabolites in healthy dogs. Am J Vet Res 2011;72:1038-1045

44. Mellett, AM, Nakmura RK, Bianco D. A Prospective Study of Clopidogrel Therapy in Dogs with Primary Immune-Mediated Hemolytic Anemia. J Vet Intern Med 2011;25:71-75

45. Raschke R, Hirsh J, Guidry JR, Suboptimal Monitoring and Dosing of Unfractionated Heparin in Comparative Studies with Low-Molecular-Weight Heparin. Ann Intern Med 2003;138(9):720-723

46. Kelly J and Hunt BJ. Do anticoagulants improve survival in patients presenting with venous thromboembolism? J Intern Med 2003; 254(6):527-539

47. Hull RD, Pineo GF. Unfractionated and low-molecular-weight heparin. Comparisons and current recommendations. Med Clin North Am 1998;82(3):587-599

48. Helmond SE, Polzin DJ, et al. Treatment of immune-mediated hemolytic anemia with individually adjusted heparin dosing in dogs. J Vet Intern Med 2010;24(3):597-605

49. Scott KC, Hansen BD, DeFrancesco TC. Coagulation effects of low molecular weight heparin compared with heparin in dogs considered to be at risk for significant venous thrombosis. J Vet Emerg Crit Care 2009;19(1):74-80

50. Plumb, DC. Veterinary Drug Handbook, 5th Ed, 2005. Ames, IA: Wiley Blackwell.

51. West, LD and Hart JR, Treatment of idiopathic immune-mediated hemolytic anemia with mycophenolate mofetil in five dogs. J Vet Emerg Crit Care 2013

52. Kellerman DL and Bruyette DS. Intravenous human immunoglobulin for the treatment of immune-mediated hemolytic anemia in 13 dogs. J Vet Intern med 1997;11(6):327-332

53. Grundy SA and Barton C. Influence of drug treatment on survival of dogs with immune-mediated hemolytic anemia: 88 cases (1989-1999). J Am Vet Med Assoc 2001;218(4):543-546

54. Kaneko Y, Nimmerjahn F, and Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 2006;313:670-3

55. Horgan JE, Roberts BK, and Schermerhorn T. Splenectomy as an adjunctive treatment for dogs with immune-mediated hemolytic anemia: ten cases (2003-2006). J Vet Emerg Crit Care 2009;19(3): 254-261

56. Patel NY, Chilsen AM, et al. Outcomes and complications after splenectomy for hematologic disorders, Am J Surg 2012;204(6):1014-1019

57. Thompson MF, Scott-Moncreiff JC, Brooks MB. Effect of a single plasma transfusion on thromboembolism in 13 dogs with primary immune-mediated hemolytic anemia. J Am Anim Hosp Assoc 2004;40(6):446-454

58. Stiller A, Armstrong P, Polzin D, Smith S. Effect of Low-Dose Aspirin or Heparin on Platelet-derived Urinary Thromboxane Metabolite in Dogs with Immune-mediated Hemolytic Anemia. J Vet Intern Med. 2013;27(3):712 (abstract)

59. Brooks MB. Evaluation of a chromogenic assay to measure the factor Xa inhibitory activity of unfractionated heparin in canine plasma. Vet Clin Pathol 2004;33:208-214

60. Washabau RJ. Feline Pancreatic Disease In: Ettinger S and Feldman E (eds) Textbook of
Veterinary Internal Medicine. 7th ed. pp. 1704-1709

61. Armstrong PJ and Williams DA. Pancreatitis in cats. Top Companion Anim Med 2012;27:140-7

62. Saunders HM, VanWinkle TJ, Drobatz K, et al. Ultrasonographic findings in cats with
clinical, gross pathologic, and histologic evidence of acute pancreatic necrosis: 20 cases (1994-2001). J Am Vet Med Assoc 2002;221:1724-1730

63. Forman MA, Marks SL, DeCock HE, et al. Evaluation of serum feline pancreatic lipase
immunoreactivity and helical computed tomography versus conventional testing for the diagnosis of feline pancreatitis. J Vet Intern Med 2004;18:807-815

64. Bennett PF, Hahn KA, Toal R, and Legendre AM. Ultrasonographic and cytopathological
diagnosis of exocrine pancreatic carcinoma in the dog and cat. J Am Anim Hosp Assoc 2001;37:466-473

65. Seaman RL. Exocrine Pancreatic neoplasia in the cat: A case series. J Am An Hosp Assoc
2004;40:238-245

66. Hecht S and Henry G. Sonographic evaluation of the normal and abnormal pancreas. Clin
Tech Sm An Pract 2007;22:115-121

67. Bonfanti U, Bussadori C, Zatelli A, et al. Percutaneous fine-needle biopsy of deep thoracic
and abdominal masses in dogs and cats. J Small Anim Pract 2004;45:191-198

68. Zamboni GA, D'Onofrio M, Idili A, et al. Ultrasound-guided percutaneous fine-needle
aspiration of 545 focal pancreatic lesions. Am J Roentgenol 2009;193:1691-1695

69. Sharkey LC et al. Maximizing the diagnostic value of cytology in small animal practice. Vet
Clin North Am Small Anim Pract 2007;37:351-72

70. Mueller PR, Miketic LM, Simeone JF et al. Severe acute pancreatitis after percutaneous
biopsy of the pancreas. Am J Radiol 1988;151:493-494

71. Lewitowicz P, Matykiewicz J, Heciak J et al. Percutaneous fine needle biopsy in pancreatic tumors: A study of 42 cases. Gastroenterol Res Pract 2012: 908963

72. VanEnkevort BA, O'Brien RT, Young, KM. Pancreatic pseudocysts in 4 dogs and 2 cats:
Ultrasonographic and clinicopathologic findings. J Vet Intern Med 1999;13:309-313

73. Binek J, Spieler P, Hurlimann R, et al. Ultrasound-guided fine-needle aspiration of abdominal masses: Accuracy and short-term complications. Eur J Ultrasound 1995;2: 199-203

74. DeCock HEV, Forman MA, Farver TB, and Marks SL. Prevalence and histopathologic
characteristics of pancreatitis in cats. Vet Pathol 2007;44:39-49

75. Wypji JM. Getting to the point: indications for fine-needle aspiration of internal organs
and bone. Top Companion Anim Med 2011;2:77-85