ALTERATIONS IN COAGULATION IN DOGS WITH HEART DISEASE

By

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(Under the Direction of Benjamin Brainard)

ABSTRACT

Dogs with heart disease have abnormalities in blood flow potentially predisposing them to alterations in coagulation. This project sought to identify the abnormalities in coagulation seen in dogs with various types of heart disease and compare these abnormalities to a cohort of dogs withnout heart disease. This was a propective observational study carried out at a university veterinary teaching hospital. Thromboelastography (TEG) and complete blood count indices were compared to dogs with heart disease (as diagnosed by echocardiography) with controls. This study found that dogs with heart disease had abnormalities in coagulation primarily as demonstrated by the TEG variables of an increased R value, increased MA and CI. We concluded that dogs with heart disease have abnormalities in their TEG indices compared to normal dogs.

INDEX WORDS: Thromboelastography, Dogs, heart disease, platelets

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B.S., The University of Illinois, 1997

D.V.M., The University of Georgia, 2006

A Thesis Submitted to the Graduate Faculty of the University of Georgia College in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2014

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May 2014

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Platelet Physiology:

Platelets are anucleate cells derived from the megakaryocytes in the bone marrow. Megakaryocytes are large multinucleate precursor cells that grow and mature in the bone marrow. Megakaryocytes develop an extensive microtubular system which assists in the formation of pseudopodia and eventually directs how the megakaryocytes will break into platelets. When stimulated by factors such as thrombopoetin or stem cell factor, megakaryocyte microtubules and microfilaments aid in breaking apart the megakaryocyte and release the individual platelets into the blood stream. [1]

In the blood stream, platelets have numerous roles in the body. Their primary role is to initiate, enhance and propagate hemostasis at the point of vascular injury. Platelets accomplish this process in a number of complex ways. First, in healthy animals, platelets are present in large numbers in the blood stream. When damage to the endothelium occurs, platelets move to the site of injury. Von Willebrand Factor (vWF), which is freely circulating in the blood stream (as well as contained in Weibel-Palade bodies in endothelial cells and in platelet α-granules in some species) bind to subendothelial collagen. This protein can then bind to platelets via the GP-Ib receptor causing adhesion of the platelet to the site of injury as well as activation of the platelet, further propagating local clotting.[2]

Activation of the platelet is the key response contributing to the coagulation process. During activation of the platelets, numerous processes occur simultaneously. The enzymes flipase, floppase and scramblase cause negatively charged phosphydidylsyrine (PS) membrane lipids to be translocated from the inner membrane of the cell to the outside of the cell. This change is an important factor in providing

the appropriate anionic surface for coagulation factor complexes to assemble during the propagation and amplification phases of the coagulation process. In addition to providing an anionic surface, the externalization of PS also induces the production of microparticles from the platelet surface. Secondly, upon activation of the platelet, the platelet releases the contents of alpha and dense granules. These granules contain numerous proteins, enzymes and molecules necessary to propagate coagulation. Glycoprotein (GP)1b-IX-V, GPIIb-IIIa, P-selectin, Platelet Endothelial Cell Adhesion Molecule (PECAM)-1, Platelet Factor-4, vWF, factor V, fibrinogen, thrombospondin, and glucose transporter-3 are stored in the alpha granules while GP-1b, GP IIb-IIIa, P-selectin, ADP, calcium, serotonin, GTP-binding proteins and vesicular monoamine transporter (MRP4) are contained in dense granules.[1] Thirdly, activation of platelets causes the expression of numerous proteins on the cell surface which enhance the platelet's ability to facilitate clot formation. Proteins such as P-selectin and PECAM-1 are liberated from internal organelles and expressed on the cell's surface allowing them to interact with other cells and proteins in the immediate environment. The interaction of surface proteins on the activated platelet with soluble factors released from the granules leads to activation of other nearby platelets primarily through the ADP and thrombin receptors .[3] Additionally, activated platelets can interact with leukocytes primarily through P-selectin and P-Selectin Glycoprotein Ligand 1 (PSGL-1) causing leukocyte activation. Lastly, the platelet undergoes a conformational change with activation transforming from a smooth discoid shape into a cell with many pseudopodia and filaform processes. This shape change increases platelet surface area, and may allow it to exert a more profound procoagulant effect in its immediate vicinity and facilitates its ability to bind to fibrin strands forming around the platelet.

Coagulation Factors

Coagulation factors have traditionally been thought of in a cascade/waterfall theory. In this model one factor activates the next factor until ultimately fibrinogen is converted to fibrin. Classically this interaction is thought of as two separate paths (the intrinsic and extrinsic pathways) which converge to

form the common pathway. The coagulation cascade can proceed fully to the generation of fibrin when either arm is activated. [4]

The extrinsic pathway is composed of tissue factor and factor VII. When circulating factor VII comes into contact with tissue factor (most commonly found on exposed subendothelial collagen), factor VII binds to this tissue factor and is converted into activated factor VII. This pathway is called the extrinsic pathway because it relies on a substance (tissue factor) that resides outside of the blood stream. In the classical cascade, activated factor VII then activates factor X, which when combined with calcium and its cofactor, factor V, activates factor II. The pathway from factor X to factor II is called the common pathway. Factor II or thrombin then converts fibrinogen to fibrin generating the initial fibrin plug.

The second arm of the traditional cascade involves the intrinsic pathway. Here circulating factor XII becomes activated by interaction with factor XI. The factor XII - XI complex then activates factor IX. Factor IX along with its cofactor, factor VIII, then feed into the common pathway by activating factor X. In the intrinsic pathway, all necessary coagulation factors reside in the bloodstream.

Because this model of coagulation was discovered through in vitro experiments, it does not necessarily represent what occurs in vivo. However, common testing of coagulation relies on this model. Tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time all use this model of coagulation to discern abnormalities in coagulation. These tests are helpful to rule in or out specific clotting disorders. However a more global outlook is necessary to understand the intricacies of the coagulation system as it happens within the body. [5]

The Cell Based Model of Coagulation

The cell-based model of coagulation was described in the early 2000's. [6] In this model, cells and specifically their phospholipid membranes play a key role in the coagulation process. In the cell- based model of coagulation, coagulation progresses through three phases which overlap with each other. These phases are initiation, amplification and propagation. Coagulation typically starts with the interaction of

tissue factor with Factor VII. This interaction activates factor VII, which is then able to activate Factor X. The activated Factor X, along with its cofactor – Factor V, form the prothrombinase complex, which activates prothrombin to thrombin (also known as activated factor II). The small amount of soluble thrombin generated during the initiation phase is able to diffuse from the site of TF expression.

Thrombin is one of the most pro-coagulant substances in the body. The small amount of thrombin generated from the initiation phase can activate platelets and feed into the amplification phase of coagulation. Thrombin also activates factor IX (through activation of the TF-FVII complex) and further activates local coagulation through platelet bound factor XI. [7] The feedback causes large amounts of factor IX to be activated on a negatively charged cell surface (such as that of an activated platelet). This active factor IX along with its cofactor activated Factor VIII (Factor VIIIa) produces large amounts of activated factor X (FXa) via the tenase complex located on the negatively charged activated platelet surface. The FXa contributes to production of large amounts of thrombin. This additional thrombin continues to feedback in a self-perpetuating loop, activating local platelets and supporting fibrin production. The fibrin strands that are generated by the amplification process tether platelets via interaction with GP ligand-1b. This interaction holds platelets at the newly formed clot, allowing their degranulated products to remain in the local area, providing a source of additional Factor V and VIII, and resulting in further stimulation of local platelets and clotting factors. Lastly, the Factor XIIIa cross links the fibrin strands, increasing clot stability. [4]

The body has various mechanisms to limit clot formation to only the area necessary to provide hemostasis. Tissue Factor Pathway Inhibitor (TFPI) is the first protein in this line of defense. TFPI is found normally circulating in the blood and inactivates factor Xa and the TF-Factor VIIa complex. [3] Its main role is to prevent further activation of coagulation in the bloodstream or anywhere away from the primary clot location. Another significant anticoagulant in the body is antithrombin. Antithrombin is a serine protease inhibitor that circulates in the body in a relatively inactive form with low affinity for its targets. It becomes at least a thousand times more potent when complexed with heparin or a heparin-like

glycosaminoglycan.[8] When this occurs antithrombin has anticoagulant activity directed primarily at the inactivation of factor Xa and factor IIa.[8] One of the most potent anticoagulants in the body is plasmin. Plasmin's role is to break down fibrin into degradation products that can be cleared by the body. Plasmin formation is stimulated by the presence of fibrin. These pathways exist in a balance (in health) between fibrin formation and fibrin degradation by plasmin. Finally, activated protein C and its cofactor protein S are potent anticoagulants. The protein C/protein S complex inactivates the tenase complex (specifically factor VIII) as well as factor V in the prothrombinase complex.[9]

Intersection of Coagulation and Inflammation

In health, the body maintains a delicate balance of coagulation to allow clot formation to occur at the point of vascular injury while preventing coagulation from continuing unimpeded or occurring at distant sites. However, pathologic conditions can influence the coagulation system, resulting in hyper or hypocaogulable states.

There is a profound bidirectional interplay between the inflammation and coagulation pathways.[10] Uncontrolled or exuberant activation of one of these pathways inevitably activates the other pathway, potentially leading to either an inflammatory response at a site of coagulation, clotting at the site of significant inflammation or pathologically, disseminated inflammation causing systemic coagulation. This is most clearly seen with tissue factor (TF) expression by monocytes. As monocytes become activated by cytokines, such as IL-6 and platelet derived growth factor, one consequence is the expression of tissue factor or tissue factor-bearing microparticles.[11] This expressed tissue factor can interact with circulating factor VIIa, initiating coagulation, generating thrombin and potentially leading to the production of microthrombi or to a consumptive coagulopathy.

While the platelet itself acts as the primary cell involved in coagulation, platelets can also be thought of as inflammatory cells. When activated platelets release the contents of their granules, many potent inflammatory mediators are released. These mediators include IL-1β, chemokines in the CC- and

CXC-subfamilies, histamine, and CD-40 ligand.[12] Additionally, P-selectin (CD 62P) is an important protein that is expressed on platelet surfaces after activation. P–selection binds to PSGL-1on the surface of as neutrophils, monocytes, monocyte-derived microparticles, and TH1 cells causing activation of these cells.[12]

Protease-activated receptors (PARs) play a crucial link between coagulation and inflammation. PARs have been found on endothelial cells, mononuclear cells, platelets, fibroblasts and smooth muscle cells.[12] PARs are G-protein coupled receptors. A total of four PARs (PAR 1-4) have been identified. While not fully elucidated, it is suspected that canines have PAR-1 and PAR-4 and may also have PAR-3.[1] Thrombin, plasmin and trypsin can activate PAR 1,3 and 4 while PAR 2 can be activated by trypsin, leukocyte proteinase-3, tissue factor-VIIa complex, factor Xa and a number of bacteria-derived proteins.[11,13] Of particular importance may be the binding of TF-factor VIIa to PAR-2 on leukocytes. This interaction results in the generation of reactive oxygen species and expression of cell adhesion molecules by monocytes and neutrophils resulting in the generation of proinflammatory cytokines such as TNF-α and IL-1β.[11] At this time it is unclear if canine PAR receptors directly lead to the generation of similar proinflammatory cytokines.

Leukocytes interact with platelets in a number of different ways. Most notably, neutrophils can interact with activated platelets tethered at the site of endothelial injury. This interaction is primarily between P-selectin on the platelet surface and PSGL-1 found at the tips of the leukocyte microvilli.[14] Association of PSGL-1 with P-selectin activates neutrophils, resulting in the release of cytokines and chemokines that promote inflammation. This can occur not only on the surface of the endothelium and subendothelial collagen but can also happen through interaction of neutrophils and monocytes with circulating activated platelets. Monocyte activation results in the expression of a number of surface proteins including tissue factor, which can then support thrombin generation at the site of injury or beyond.

Systemic inflammation can also cause wide-spread activation of the coagulation system. Cytokine (most importantly IL-1, IL-6, I-12 and TNF-α) induction of tissue factor expression can activate the coagulation system. Inflammation upregulates TF production in endothelial cells, monocytes and dendritic cells. It also increases the production of microparticles from endothelial cells, monocytes and neutrophils.[15] Microparticles (MPs) are small membrane blebs that are released from cells exposed to inflammatory stimuli. MPs carry the phenotypic fingerprint of the cell of origin, which can include a procoagulant phenotype if the cell of origin is expressing TF. Systemic inflammation downregulates endogenous anticoagulant systems – principally the protein C system-- shifting the balance in the body toward a procoagulant state. Protein C is activated principally by the thrombin/thrombomodulin complex. Neutrophil elastase, liberated from activated neutrophils, cleaves thrombomodulin, preventing the activation of Protein C and tipping the balance towards a more hypercoagulable state. In addition, protein S can be proteolytically inactivated by neutrophil proteases.[16] Fibrinolysis is also inhibited when systemic inflammation is present. Plasminigen activator inhibitor (PAI-1), which is the major inhibitor of plasminogen activators (e.g., tPA, urokinase), is upregulated in the face of inflammation, which results in the inhibition of fibrin breakdown and a subsequent hyperfibrinolytic state.[10]

The same systems that control the coagulation response also modulate the inflammatory effects of coagulation. Antithrombin directly inhibits factors IIa and Xa. Antithrombin levels are significantly reduced in severe inflammation because of impaired synthesis, degradation by neutrophil proteases and consumption.[11] Antithrombin is decreased in both people and dogs with significant systemic inflammation such as that caused by sepsis.[17,18] In one study of dogs with naturally occurring septic peritonitis, a hypercoagulable state as measured by thromboelastography has been documented, coinciding with decreased antithrombin activity.[19]

Protein C is an acute phase protein made by the liver in response to systemic inflammation. Activated protein C (aPC) activity is enhanced by binding to the thrombin/thrombomodulin complex on endothelial cells.[11] aPC is also a potent modulator of systemic inflammation and has been shown to

decrease the production of IL-1, IL-6, IL-8 and TNF-α as well as decrease leukocyte activation and cytokine release.[9] The activity of aPC activity is decreased in both animals and people with systemic inflammation.[20,21] This may be due to a number of factors including a consumption, impaired synthesis, and the inability of platelets to be activated secondary to lack of thrombomodulin on the endothelial cell surface (down-regulated by proinflammatory cytokines). [16]

Assessment of Platelet Activity

Attempting to assess the specific state of the coagulation and inflammation systems in the body at a specific point in time is challenging. This was traditionally assessed using global markers, such as total platelet count, white blood cell counts and morphology, and coagulation testing.

Platelet counts have been performed using both cytologic counting via light microscopy as well as through the use of automated cell counters. Automated cells counters are standard practice in most veterinary and human hospitals across the country. They use either electrical impedance or laser optics to obtain their cell counts. In electrical impedance devices, cells pass through a small aperture and the degree of resistance to an electrical current is detected which corresponds to specific cell types. In the optical method a laser is directed at a column of single blood cells moving past it. The degree and type of scatter from the laser is detected and converted to an electric signal. This signal corresponds to both the size and complexity of the cells, allowing identification as specific cell types.[22] Both of these methods are accurate for estimating the number of circulating platelets in dogs and people. In addition, the Advia 120 cytometer reports mean platelet component (MPC), which has been correlated with the activation status of platelets. This value is calculated from the mean platelet volume and the refractive index of the platelets. A low MPC (indicating platelet degranulation and thus a decrease in complexity) is correlated with platelet CD62P expression in people.[23]

There are numerous types of monitoring tools available today to attempt to both identify platelet abnormalities as well as to quantify the global coagulation status of an animal. These include oral or

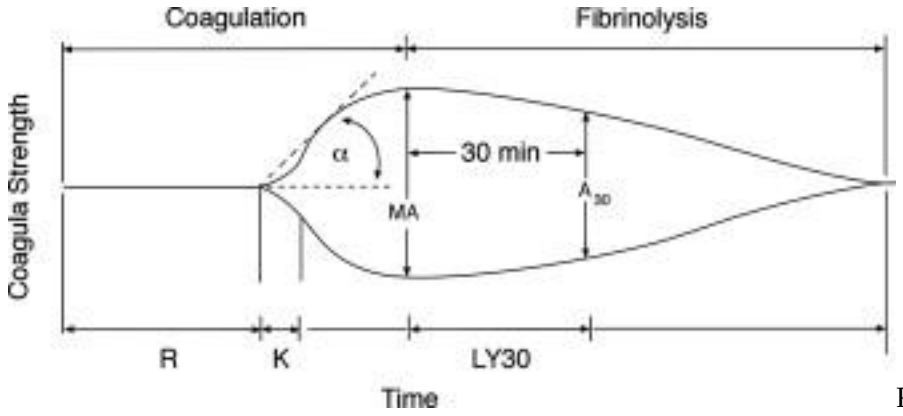
buccal muscosal bleeding time, thromboelastography, platelet aggrogometry, platelet function analyser (PFA)-100, Sonoclot and ROTEM. Buccal mucosal bleeding time involves a small incision on the buccal surface of an animal's gums and the time to clot formation is measured. This test identifies defects of primary hemostasis but is relatively non-specific, does not differentiate between platelet defects and vWD, and has high interoperator variability.[24] The PFA-100 is a very sensitive but non-specific test for disorders of primary hemostasis. The PFA-100 forces citrated anticoagulated blood and the associated platelets under high shear through a narrow opening and measures the time necessary for a platelet plug to form at the aperture thereby stopping the flow.[25] The results are reported as closure times and increased closure times are seen with platelet function disorders, vWD and anti-platelet medications, in addition to low platelet counts.[26] Platelet aggregometry uses agonists such as epinephrine, collagen and ADP to activate platelets and document their ability to form platelet aggregates, an essential part of primary hemostasis. Either light transmittance (in the case of PRP) or impedance (in the case of whole blood) aggregometry is used to detect platelet aggregation. Abnormal test results can be seen with inherited platelet disorders as well as due to the effects of anti-platelet medications.[26]

Thromboelastography

Thromboelastography was originally described in Germany in 1948 and has been used to characterize the entire progression of coagulation from initiation of clot formation to fibrinolysis.[27] TEG uses a plastic cup that is filled with citrated whole blood and is maintained at 37^{o}C. A set amount of calcium is infused into the sample with or without an additional activating agent. A pin is then inserted into the sample and the cup is oscillated around the pin. As fibrin forms between the pin and the cup, torque is generated on the pin. This torque is translated into an electrical signal, which is converted by the TEG into a tracing that graphs the torque diversion from midline in millimeters. The tracing that is produced can be divided into three zones – precoagulation, coagulation and fibrinolysis.[28]

There are four standard values in the TEG tracing that are consistently reported. These are R, K, angle (α) and MA.[5] See figure 1 below.

Figure 1 – Standard TEG tracing with indices

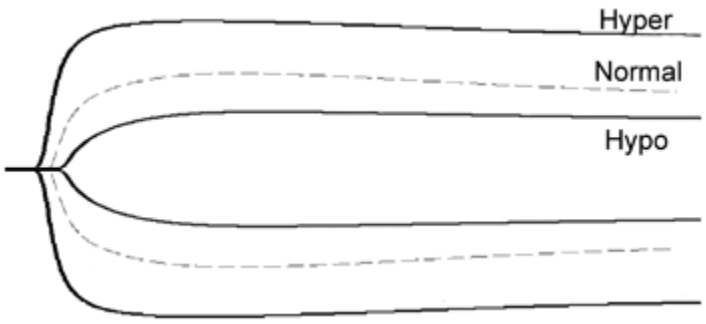


Reikvam 2009[29]

The reaction time (R) is a measure of the precoagulation time and is the time until the tracing starts to diverge. This value most closely represents the intrinsic pathway of the coagulation system and therefore is influenced by the activity of factors VIII, IX, XI and XII.[29] The K value (kinetics) is the time from the reaction time (the initial diversion of the tracing) until the tracing arms diverge by 20 mm, and is related to the speed of clot formation. The α angle measures the speed of fibrin build up and thus demonstrates the rate of clot formation.[30] The angle and the K values are affected by fibrinogen concentrations, Factor XIII and to a lesser extent platelet number and function.[5] The MA is the maximum amplitude of the tracing (distance between the arms of the tracing) and is usually seen 45-90 minutes after the reaction has begun. This value is dependent on the cross linking of fibrin to the GP IIb/IIIa receptor on platelets. As such the amount of fibrinogen and ability of platelets to function properly to a large extent determine the clot strength and MA. The TEG also provides measures of fibrinolysis in samples, described as the LY 30 or LY 60. This is a measure of fibrinolysis and is determined primarily by the ability of plasmin to break down the formed clot. The Lys 30 or Lys 60 is measured either 30 or 60 minutes, respectively, after the MA is measured.[5]

TEG has been used in a number of veterinary and human applications. In dogs, hypercoagulable states (typically defined as decreased R and K values with increased α and MA values) have been identified in dogs with evidence of thrombosis, hyperadrenocorticism, extrahepatic biliary tract obstruction, protein losing nephropathy, carcinomas, portosystemic shunts, immune mediated hemolytic anemia and parvoviral enteritis, although none of these have been linked to outcome such as thrombus formation.[31-39] Hypocoagulable states, as demonstrated by increased R and K values with decreased MA and G values, have been reported in the initial phases of immune mediated thrombocytopenia and in certain phases of DIC.[40] TEG has also been used to monitor particularly heparin therapy in both human and veterinary patients with varied success.[27,30]

Figure 2 – TEG tracing for hyper-, normo- and hypocoagulable states



from McMichael 2011[41]

Determining the activation status of platelets in the systemic circulation is an area of active research. For more information on platelet activation and flow cytometry see appendix.

Heart Disease

Heart disease (of nearly any etiology) can cause a number of changes to the body's coagulation and inflammatory systems. According to Virchow, abnormal coagulation may be caused by blood stasis or turbulent flow, abnormalities in the clotting factors, or disruption of the endothelium.[42] P-selection expression on platelets is elevated in people with acute myocardial infarction, peripheral arterial disease, heart failure, diabetes mellitus and acute congestive heart failure.[43-46] Therefore it is plausible to presume

that cardiac disease (which has not yet progressed to failure) may also lead to coagulation abnormalities and/or platelet activation, and that appropriate therapy for heart disease may reduce the number of circulating activated platelets in the body, depending on the underlying cause of the cardiac disease.

Of particular interest is the increased activation of platelets in patients with heart failure. In these patients, numerous factors are believed to be responsible for this activation. An increased level of catecholamines, which occurs in heart failure, causes platelet activation primarily though α2-adrenoreceptor stimulation. Cytokines that are released in heart failure (e.g. TNF-α, macrophage chemoattractant protein-1 and macrophage chemoattractant proinflammatory protein-1α) have been shown to activate platelets as demonstrated by an increase in surface P-selectin or increased platelet oxygen production.[45] Also, the up-regulation of the renin-angiotensin-aldosterone system in patients with heart failure can increase the platelet surface expression of P-selectin and β-thromboglobulin via the angiotensin II-induced release of cytokines (KF-κB and IL-18). Simultaneously, angiotensin II causes a thrombin-induced, dose-related elevation in intraplatelet free calcium and increased platelet aggregation.[46] In one study in dogs, it appears that platelet P-selection expression may be elevated in dogs with congestive heart failure,[47] while in another study, P-selectin expression was not significantly different in dogs with subaortic stenosis or valvular disease versus those without.[48]

Myxomatous valvular heart disease (the most common form of heart disease in dogs) can result in turbulent blood flow through the heart, as blood is abnormally ejected during systole through an insufficient valve. As the mitral valve degenerates, the valve leaflets become thicker (endocardiosis), which can adding to the turbulence of the blood flow (due to progressive valvular insufficiency).[49] This turbulence can potentially cause platelet activation and coagulation abnormailities.[50-52]

Platelet activation is also possible in cardiac stenotic diseases such as pulmonic stenosis. Pulmonic stenosis is a congenital abnormality characterized by thickened, asymmetric leaflets of the pulmonic valve and a narrowed valve annulus. This condition can lead to hypertrophy of the right

ventricle and eventually to right-sided heart failure, particularly when the condition is associated with tricuspid insufficiency.[53] Stenotic conditions lead to turbulent blood flow as the blood is forced with increased velocity through a narrowed opening containing abnormal valve leaflets. While no studies are available demonstrating activation of the coagulation system in patients with pulmonic stenosis, it has been shown that human patients with mitral valve stenosis have activation of their coagulation system (as demonstrated by increased soluble P-selectin, beta thromboglobulin and D-dimers) which is reversible with resolution or amelioration of the stenosis.[54,55]

Patent ductus arteriosus (PDA) is a congenital disease characterized by a failure of the closure of the embryological vessel that connects the pulmonic artery to the aorta. This causes shunting of blood, typically from the left side of the heart to the right side, due to the higher aortic pressures, although this shunting may be reversed in severe conditions.[56] Turbulent blood flow into the pulmonary artery may be documented by echocardiography in patients with PDA. Platelets have recently been found to play a central role in PDA closure in children.[57] Failure of PDAs to close in in children have been typically associated with a more significant thrombocytopenia than in patients where PDAs have closed.[58] Additionally, one study demonstrated increased levels of platelet activating factor in tracheal aspirates from children with PDAs.[59] It is reasonable to hypothesize that this increase in PAF, in addition to the turbulence found in this disease, may lead to a hypercoagulable state in patients with PDAs.

Because people and dogs with some cardiac diseases have abnormal coagulation parameters, we sought to investigate whether various cardiac diseases caused a hypercoagulable state in dogs. Furthermore, we sought to determine if improvement in the clinical and/or echocardiographic parameters of a dog's heart disease resulted in a corresponding change in any documented coagulation abnormalities. We hypothesized that dogs with cardiac disease would be relatively hypercoagulable and have increased markers of platelet activation when compared to the normal population, and that effective treatment of their cardiac disease would resolve or partially resolve these coagulation abnormalities.

Title: MATERIALS AND METHODS

This prospective, observational study examined the platelet function and coagulation status of dogs presenting to the University of Georgia's cardiology service and dogs without cardiac or other systemic disease presenting to the University of Georgia's Community Practice Clinic or belonging to faculty/staff. Two different methods were used to evaluate platelet function and coagulation status of each dog. Complete blood counts (CBCs) were performed using a commercial in house cell analyzer (Heska CBC Diff, Loveland, CO) to determine hematocrit, platelet and differential leukocyte counts. Additionally, a thromboelastagram was performed on all samples in order to characterize the global coagulation status of the animal. Flow cytometric assessment of both a platelet-rich plasma (PRP) sample (to examine the degree of platelet activation as assessed by platelet P-selectin expression) and a leukocyte sample (to determine the degree of platelet-leukocyte aggregates) was performed (see appendix).

Blood was collected from 38 client-owned dogs presenting to the University of Georgia's cardiology department for evaluation and treatment of various naturally occurring cardiac diseases. All patients were evaluated by one of two board certified cardiologists. Patients were placed into one of five categories based on their primary cardiac disease: chronic mitral valve disease (MVD), subaortic stenosis (SAS), pulmonic stenosis (PS), patent ductus arteriosus (PDA), or dilated cardiomyopathy (DCM). 2.7 mL of blood was collected from each patient via cephalic or jugular venipuncture using an 18 or 20 gage needle and syringe. The blood was then immediately transferred into a commercial blood tube containing 3.2% sodium citrate (BD vacutainer buff Na Citrate, Franklin Lakes, NJ) for a final blood:citrate ratio of 9:1. Blood was collected in an identical fashion from each patient at each recheck.

Blood was also collected from 22 healthy dogs presenting to the University of Georgia's Community Practice Clinic for routine vaccination, dental prophylaxis or elective surgical sterilization, or

dogs that were volunteered by hospital faculty or staff, or from dogs enrolled in the University of Georgia's blood donor program. All patients were deemed healthy based on physical examination, evaluation of CBC, and cardiothoracic auscultation performed by faculty clinicians. 2.7 mL of blood was collected from each patient via cephalic or jugular venipuncture and anticoagulated using 3.2% sodium citrate for a final blood:citrate ratio of 9:1 in a similar manner to the dogs with cardiac disease.

Blood samples from all dogs were processed immediately after collection in the following manner: Each sample was gently inverted in the sodium citrate tube several times to ensure even distribution of anticoagulant. 20 µL of citrated whole blood was removed and a complete blood count was performed using the same previously mentioned commercial automated impedance cell counter. Then 720 µL was removed and placed in a 1.5 mL microcentrifuge tube to be used for the TEG evaluation. The remainder of the blood was used for flow cytometric evaluation (see appendix).

Thromboelastography was performed in the following manner as previously described.[30] Citrated whole blood samples were stored at room temperature (25 ° C) for 30 minutes before analysis. For each sample, TEG analysis was performed on native as well as kaolin activated samples. Analysis was performed on 1 of 3 thromboelastographs (TEG 5000, Haemoscope, Niles, IL). For the kaolin sample, 7 µL of kaolin (kaolin, Haemoscope, Niles, IL) was added to 340 µL of citrated whole blood in a 1.5 mL microcentrifuge tube. Then 340 µL of the kaolin activated sample was added to 20 µL of 0.2 M $CaCl_2$ ($CaCl_2$, 0.2 M, Haemoscope, Niles, IL) in a plain TEG cup (Unactivated cups and pins, Haemoscope, Niles, IL) without additional activators. In addition, 340 µL of native citrated whole blood was added to 20 µL of 0.2 M $CaCl_2$ in a plain TEG cup without additional activators for the unactivated sample. All samples were allowed to run for a minimum of 1 hour. For each tracing, the variables *R* and *K* were reported in minutes, MA in millimeters, and α-angle (Ang) in degrees. LY 30 and LY 60 were reported in percent. A coagulation index (CI), which has been previously reported, was used to determine an overall coagulation status using the formula - CI=0.1227 * R + 0.0092 * K + 0.1655 * (MA) - 0.0241(α) - 5.0220.[60] When interpreting the CI, a CI > 4 was considered hypercoagulable, a CI between 4 and -4 was

considered to have normal coagulation and a CI < -4 was considered hypocoagulable.[60] G-value was calculated from the MA using the formula G = 5000 × MA/(100 – MA).[5] The 3 thromboelastographs used in this study are housed in the veterinary coagulation laboratory of the University of Georgia. They receive regular scheduled maintenance and regular quality control assessments. The University of Georgia has generated an institutional reference interval for canine whole blood assays on these machines.

Inclusion into the study as a control dog included a normal physical exam (including a normal cardiothoracic auscultation with no heart murmurs), and no clinical history of systemic disease. Patients were excluded as controls if abnormalities were detected on bloodwork or if physical exam abnormalities consistent with inflammatory processes (e.g. severe gingivitis, fever, lameness, swelling) were found.

This study design was approved by the University of Georgia's Clinical Research Committee and written owner consent was obtained for all animals participating in the study.

Statistical Analysis

All statistical analysis was performed using a commercial software package (Minitab 16, State College, PA). All continuous variables were analyzed for normality with the Shapiro-Wilk normality test. TEG variables (R, K, angle, MA, G and CI) were normally distributed and are reported as mean ± SD. One-way ANOVA analysis was used to compare normally distributed continuous variables with categorical variables (presence of pulmonary edema, presence of arrhythmias or enlarged left atria) using a Tukey family error rate with a confidence interval of 95%. Welch's t-test (which does not assume equal variances) was used to compare all continuous variables with controls. Stepwise regression analysis (using both forward inclusion and backward elimination) with α set to 0.15 for both inclusion and exclusion was used to identify predictive models for TEG variables with relationship to cardiac indices of disease severity (LA:AO, RVOT, and EVDI). Linear regression analysis was used to identity significant variables identified in the stepwise model. Significance throughout the analysis was defined as $P < 0.05$.

Title: RESULTS

Dogs with cardiac disease

A total of 38 dogs with cardiac disease and 22 control dogs were included in the study. 8 dogs had a primary diagnosis of pulmonic stenosis (PS), five dogs were diagnosed with dilated cardiomyopathy (DCM), 16 dogs were diagnosed with mitral valve disease (MVD), seven dogs had a patent ductus arteriosus (PDA), and one dog was diagnosed with sub aortic stenosis (SAS). Three control dogs were eliminated from the study due to the presence of confirmed or suspected inflammatory conditions. One dog was excluded after severe gingivitis and loose teeth were found on dental examination, one dog was eliminated due to an increased alkaline phosphatase enzyme activity, gingivitis and gingival hyperplasia, and one dog was eliminated due to gingivitis and a cranial cruciate ligament tear. This resulted in 19 dogs being used as controls in this study.

In the control population the most common dog breeds represented were Staffordshire Terriers (3), mixed-breed (3), Belgian Malinois (2), Rat terrier (2), Labrador retriever (2), Siberian Husky (2), and Great Dane (2). Other breeds included Australian shepherd (1), German shepherd (1), and Rottweiler (1). The breeds represented in group with cardiac disease included Cavalier King Charles Spaniels (5), Chihuahua (4), dachshund (3), mixed-breed (3), Labrador retriever (3), Doberman Pincher (2), coonhound (2), and Maltese (2), as well as other breeds only represented once. The median (range) age of dogs with cardiac disease was 80.26 months (2-202 months) while the median age of control dogs was 55.8 months (13 – 120 months). There was no statistically significant difference in age between the groups (P= 0.097).

The mean ± SD weight of dogs with cardiac disease was 14.3 ± 12.78 kg while the mean ± SD weight of the control dogs was 30.9 ± 17.2 kg. The control dogs were statistically heavier than the dogs

with cardiac disease (P= 0.001). Of the population of dogs with cardiac disease, there were 19 females (14 spayed, five intact) and 19 males (13 neutered and six intact) while there were 6 females (all spayed) and 13 males (10 neutered and three intact) in the control group.

Upon enrollment in the study, dogs with heart disease analyzed together showed significantly altered coagulation abnormalities as demonstrated by an increased CI (native P= 0.001 and kaolin P<0.001 – see box plots below), an increased R time (native P= 0.035 kaolin P= 0.001) and increased MA (kaolin P=0.006) compared with controls. Mean ± SD CI for dogs with heart disease was 3.85 ± 1.08 (native) and 4.21 ± 1.32 (kaolin) while the mean ± SD for controls was 2.87 ± 0.87 (native) and 2.98 ± 0.89 (kaolin). The mean ± SD R time for dogs with heart disease was 9.1 ± 13.8 minutes (native) and 4.87 ± 1.72 minutes (kaolin) while the mean ± SD for controls was 3.98 ± 1.35 minutes (native) and 3.57 ± 0.92 minutes (kaolin). The mean ± SD MA (mm) for dogs with heart disease was 60.57 ± 0.03 mm (kaolin) while the mean ± SD for controls was 54.72 ± 5.93 mm (kaolin). (See table 2) The difference in MA between dogs with cardiac disease and control dogs for the native samples did not reach statistical significance (P=0.48).

Figures 3 and 4 – Box and whiskers plot of CI for all dogs with cardiac disease versus control dogs

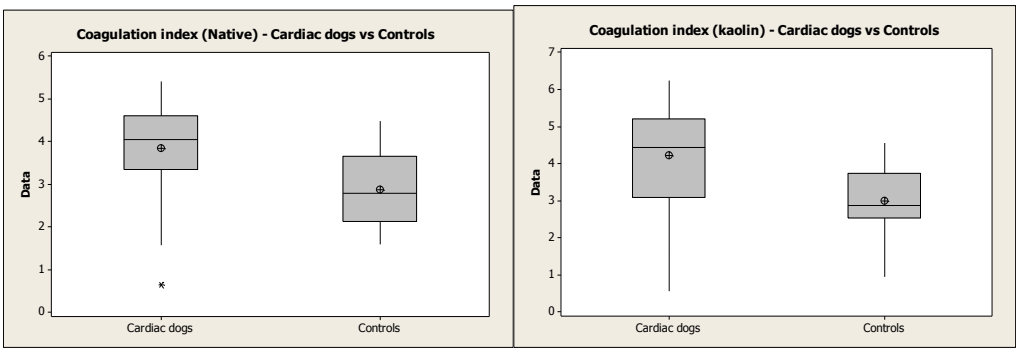


Table 1 – All dogs with cardiac disease vs. controls – indices with significance

	All Cardiac dogs	Controls	P-value
Coagulation index – Native	3.85 ± 1.08	2.87 ± 0.87	0.001
Coagulation index - Kaolin	4.21 ± 1.32	2.98 ± 0.89	0.000
R time (native) (min)	9.1 ± 13.8	3.98 ± 1.35	0.035
R time (kaolin) (min)	4.87 ± 1.72	3.57 ± 0.92	0.001
MA (kaolin) (mm)	60.57 ± 0.03	54.72 ± 5.93	0.006

The complete blood cell count indices of all dogs with cardiac disease were compared with the control population. Two samples from the study population were discarded due to probable erroneous readings from the CBC machine (both showed extremely low platelet counts - 20,000 and 22,000 with no MPV reported in the absence of any clinical bleeding). There was no significant difference in dogs with heart disease and controls in regard to hematocrit (P=0.39), white blood cell count (P=0.50) or mean platelet volume (P=0.85). However there was a significant difference in the platelet count between dogs with cardiac disease and the controls (P<0.001). All cell counts were adjusted for dilution of citrate at the time of sample collection by multiplying the cell count by 1.1. After adjustment, the mean ± SD platelet count of the control dogs was 309,400 ± 99,700 while the control population had a platelet count of 191,000 ± 57,700.

Caviler King Charles Spaniels are known to have larger but fewer platelets than other breeds.[1] Because five of the dogs with cardiac disease were of this breed, we evaluated the platelet count, mean platelet volume (MPV) and TEG indices of the King Charles Spaniels. There was no significant difference between the platelet count in the King Charles Spaniels (217,400 ± 92,300) and either the

remainder of the dogs with heart disease (P= 0.21) or the control dogs (P= 0.74). The mean platelet volume (MPV) in the Cavilers was 8.23 ± 0.25 which was significantly higher than the rest of the dogs with heart disease (P= 0.034). The difference in MPV of the Cavilers approached, but did not reach a statistically significant difference when compared to the control population (P= 0.063). There were no significant difference found between any of the TEG parameters of Cavilers and the control population.

Using a fisher's exact test, left atrial enlargement (defined as an LA:AO of greater than 1.5) was compared to the overall coagulation state (hypo-, normo- or hypercoagulable as defined by the CI).[60] No significant differences were found in either the native (P=0.49) or the kaolin (P=0.50) activated samples.

Similarly, no significant differences were noted between dogs with and without signs of heart failure (defined as the presence of pulmonary edema on thoracic radiographs). A total of 7 instances were identified in which patients presented in heart failure. One patient had SAS, one patient with a PDA, and three had MVD. One of these patients had three samples analyzed while experiencing one episode of failure and two had one sample taken while in failure. There was no difference between the CIs (native P= 0.24, kaolin P= 0.22) when dogs with pulmonary edema were compared to the remainder of dogs with heart disease.

Dogs with an arrhythmia were compared to dogs with cardiac disease without an arrhythmia. When pooled to include repeated samples (each time a patient was evaluated and samples collected was taken as one instance), 11 of 45 dogs evaluated had an arrhythmia. Arrhythmias included atrial fibrillation (8), sinus tachycardia (2) and sinus rhythm with multiple ventricular premature contractions (1). Two dogs had MVD (one patient with one sample and one patient with 5 samples), two dogs had DCM (one sample each), two patients with one sample each had PDA and one patient had SAS. No significant difference was seen in the majority of TEG parameters in dogs with an arrhythmia compared to those without. Only the CI of the kaolin activated sample was significantly correlated to the presence of an arrhythmia (P= 0.027).

A stepwise regression analysis was performed examining the relationship between left atrial size (as determined by the LA:AO ratio) and all TEG variables. Analysis revealed that R, G and CI of native samples had a significant relationship to LA:AO ratio ($R^2 = 0.507$) with P values of 0.001, 0.018 and 0.007 respectively.

Age was independently evaluated for a correlation with TEG and there was no significant correlations found between any of the TEG variables and age.

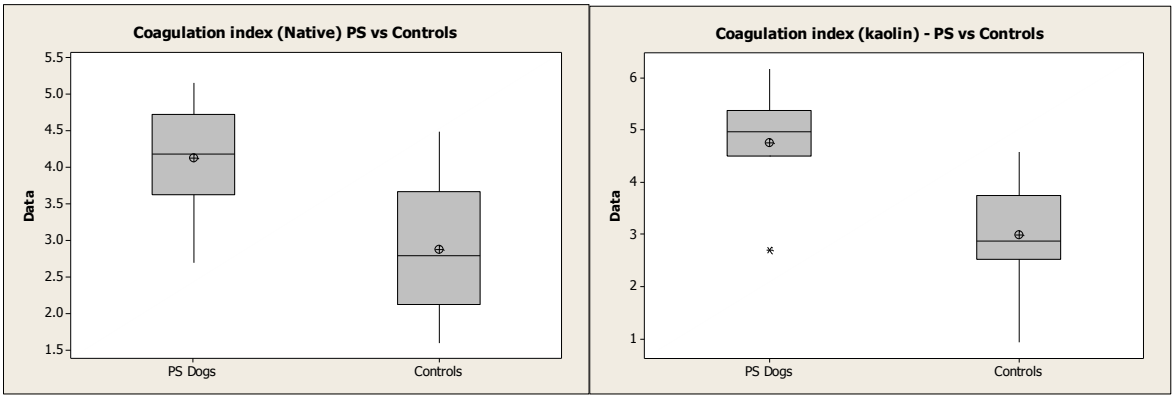
Pulmonic Stenosis

There were eight dogs in the study diagnosed with PS. One dog had inaccurate TEGs performed and these samples were not available for analysis. Therefore, seven dogs had TEGs available for analysis Overall, dogs with PS were more hypercoagulable based on both native and kaolin activated CI (see table 2). Interestingly, when taken individually, none of the TEG variables were significantly different from controls but when taken in aggregate via the coagulation index, significant differences were found. Significant correlation could not be found between RVOT and TEG variables because the number of patients was not sufficient to power the regression models.

Table 2 – Dogs with PS – indices with significance

	PS Dogs	**Controls**	**P-value**
Coagulation index – Native	4.13 ± 0.82	2.87 ± 0.87	0.012
Coagulation index - Kaolin	4.76 ± 1.07	2.98 ± 0.89	0.004

Figures 5 and 6 – Box and whiskers plot of CI for dogs with PS versus control dogs



Dilated Cardiomyopathy

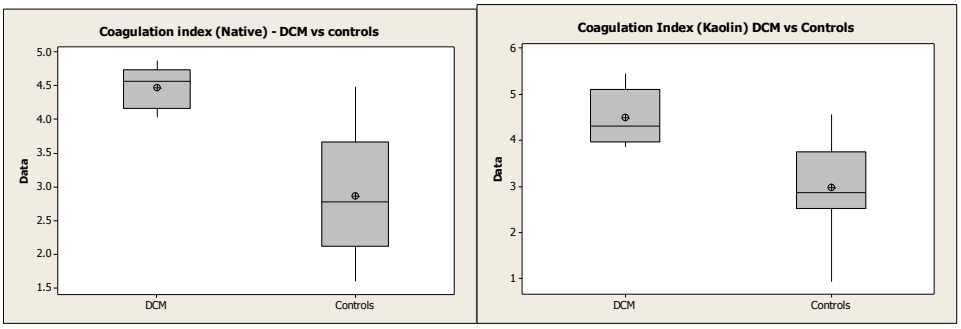
Five patients in the study had DCM. Two patients had repeated measures which were not used for analysis so as to not give additional weight to subjects with multiple samples. Patients in this study diagnosed with DCM were hypercoagulable based on increased CI, MA and G values compared to controls (both native and kaolin). No significant correlation was found between EDVI and either native (P= 0.87) or kaolin (P=0.49) coagulation index. The MPV was not significantly different between dogs with DCM and controls (P=0.072) however the platelet count was significantly higher for dogs with DCM than controls (P=0.033). The mean ± SD platelet count for the DM group was 376,000 ± 125,000 while the mean ± SD for the control group was 191,000 ± 57,700.

Table 3 – Dogs with DCM - indices with significance

	DCM dogs	Controls	P-value
Coagulation index – Native	4.48 ± 0.32	2.87 ± 0.87	0.000
Coagulation index – Kaolin	4.50 ± 0.63	2.98 ± 0.89	0.002
MA (Native) (mm)	61.62 ± 4.66	53.30 ± 5.72	0.012

MA (kaolin) (mm)	63.60± 2.80	54.72 ± 5.93	0.000
G (Native) (d/sc)	8,180 ± 1,510	5,870 ± 1,370	0.027
G (kaolin) (d/sc)	8,840 ± 1,050	6,200 ± 1,440	0.002

Figures 7 and 8 – Box and whiskers plot of CI for dogs with DCM versus control dogs



Mitral Valve Disease

There were 16 dogs diagnosed with mitral valve disease in the study. Six dogs had repeated samples. Analysis was performed on each patient's first sample when examining TEG variables against controls. Repeated samples were not compared because there was no standardization of where each patient was in their disease progression upon enrollment in the study. There was a significant difference between the CI of dogs with MVD and controls (native P= 0.030, kaolin P= 0.049). A significant difference was seen in the R value (P= 0.001 native, P= 0.002 kaolin) and angles (P= 0.028 native, P= 0.029 kaolin) in this population of dogs when compared to controls. There was no significant difference in platelet count or MPV in dogs with MVD vs controls (P=0.18 and P=0.32 respectively).

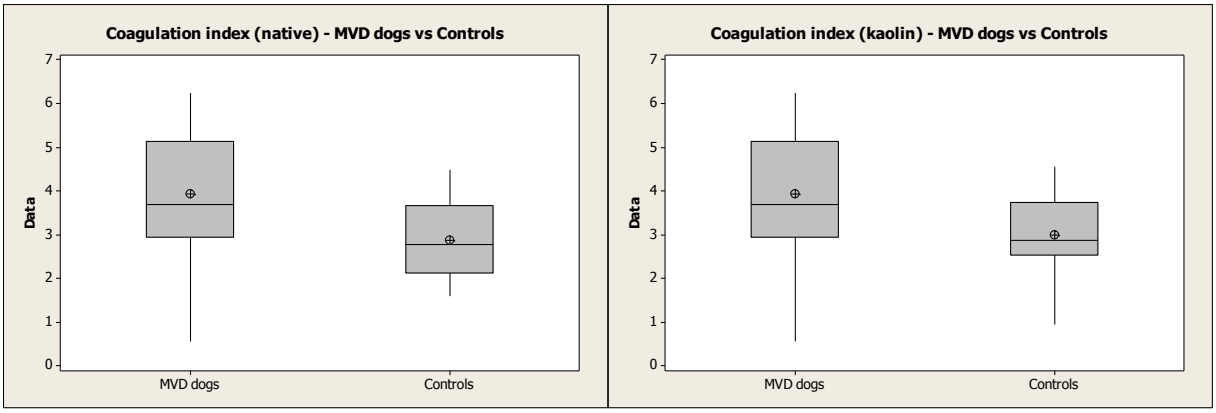
Regression analysis showed that MA (native) was significantly correlated to an increasing LA:AO ratio with p-value of 0.011. Categorical variables of increased LA size (LA:AO > 1.5) was

compared to the coagulation status (determined by CI as described earlier) and found that there was no significant association for either the native (P=0.28) or the kaolin (P= 0.29) samples.

Table 4 – Significant differences between dogs with MVD and controls

	MVD dogs	Controls	P-value
Coagulation index – Native	3.73 ± 1.27	2.87 ± 0.87	0.030
Coagulation index – Kaolin	3.92 ± 1.62	2.98 ± 0.89	0.049
R (native) (min)	7.71 ± 3.35	3.98 ± 1.35	0.001
R (kaolin) (min)	5.04 ± 1.45	3.57 ± 0.92	0.002
Angle (native) (deg)	49.6 ± 15.8	59.7 ± 6.64	0.028
Angel (kaolin) (deg)	52.8 ± 15.6	62.66 ± 6.72	0.029

Figures 9 and 10 – Box and whiskers plot of CI for dogs with MVD versus control dogs



Patent Ductus Arteriosus

There were seven dogs included in the study that were diagnosed with a PDA. Five of the seven dogs had repeated samples performed after closure of the PDA. One patient had an unsuccessful occlusion with an Amplatz canine duct occluder and subsequent surgical ligation. This patient was noted to have mild residual flow on follow up echocardiographic examination. No significant residual flow was noted on subsequent exams for any of the other patients.

Paired t-tests were used to evaluate the change in TEG variables before and after PDA closure. There was no difference between either native or kaolin coagulation indices before and after PDA closure (p-value 0.92 and 0.39 respectively).

The first sample from each patient (taken before ductus closure) was compared to controls to evaluate the TEG variables before treatment for the PDA. Analysis of each of the TEG variables (R, K, angle, MA and G) as well as CI revealed no significant differences between controls and dogs with PDAs in either the kaolin or native samples.

Table 5 – CIs for dogs with PDAs

	PDA dogs	Controls	P-value
Coagulation index – Native	3.35 ± 1.21	2.87 ± 0.87	0.40
Coagulation index - Kaolin	3.78 ± 1.10	2.98 ± 0.89	0.15

Title: DISCUSSION

This study found that there were changes in TEG values in dogs with heart disease when compared to control dogs. Specifically we found that dogs with heart disease, taken in aggregate, demonstrated a tendency toward hypercoagulation as evidenced by an increased CI and MA and hypocoagulation as evidenced by an increase in R time. Additionally, we found that some of the native TEG variables were correlated to increasing left atrial size.

There are a number of possible reasons for the disparity seen in the elevations of both the CI and MA (typically indicating a hypercoagulable state) in conjunction with the prolonged R time (typically indicating hypocoagulability). First, R time and MA measure different parameters of the coagulation system. MA is primarily influenced by fibrinogen, platelet count and function, thrombin and hematocrit while R time is primarily dependent on coagulation factors and endogenous anticoagulants.[41] There was no difference seen in hematocrit between the dogs with heart disease and controls so hematocrit is unlikely to be responsible for the difference in MA. However, the increase in MA seen in the dogs with cardiac disease may be due to the elevated platelet count in this set of dogs compared to the controls. If more functional platelets are present in the dogs with cardiac disease it is reasonable to believe that the clot formed by these platelets would be stronger and therefore have a larger MA as seen on TEG. As expected, the platelet count was significantly correlated with CI. The Pearson correlation coefficient between platelet count and CI for the native sample was 0.545 (P=0.001) and for the kaolin sample was 0.351 (P=0.042). Finally the activity of platelets may have been different between dogs with heart disease and the control population which could also have influenced the difference in MA.

It is unclear why patients with cardiac disease in the present study had platelet counts that were higher than those of the control population. It is worthwhile to note that while the platelet counts were

statistically elevated in the dogs with cardiac disease compared to the control population, the dogs with cardiac disease had mean platelet counts that would be considered to be within the reference range and the control population had mean platelet counts that were slightly below the reference range with the standard deviation placing the counts well within the reference range. It has been postulated by Ly and colleagues that an increased platelet counts seen in people with cardiac disease may be due to increased levels of thrombopoietin seen in humans days after an acute myocardial infarction.[61] While no dogs in this study had coronary arterial disease, the same undefined mechanisms that may be responsible for increased thrombopoietin in the people studied by Ly may also be at play in dogs with significant cardiac disease. Sampling error and in vitro activation of platelets from dogs in the control population may have falsely lowered the platelet counts in this population. Alternatively, and more likely, the differences seen in platelet count between patients with cardiac disease and the controls is merely due to sampling variability and type 2 error secondary to the small numbers in this study and wide range of values considered to be within the reference interval for a canine platelet count.

Because we did not measure any of the clotting factors nor natural anticoagulants such as aPC in this study, it is not known if the dogs with cardiac disease had a deficiency of any of their clotting factors or an elevation in endogenous anticoagulants which could lead to a prolonged R time. There is no evidence relating cardiac disease with clotting factor deficiency. However, if dogs with cardiac disease truly had a decrease in factor activity (potentially from systemic inflammation or coagulation causing a consumptive loss of clotting factors) this would lead to prolonged R times. In addition, if significant enough inflammation accompanied their heart disease it is reasonable to believe that this could lead to increased levels of aPC (an acute phase protein) and would have contributed to a prolonged R time. We did not measure fibrinogen or thrombin which could have been elevated in dogs with cardiac disease and contributed to an increased MA. Of note is the fact that the R time factors far less into the CI formula compared to the MA. Mean R values seen in this population were between 3.5 and 9.1 with a multiplication factor of 0.1227 while the mean MA for the same population was between 54.7 and 60.6

with a correction of 0.1655. Therefore, in this population, MA would account approximately 9 fold more than R time. Consequently, CI is far more influenced by MA than R time. It would be reasonable in future studies to evaluate coagulation factors and the aPC levels in dogs with and without heart disease in order to evaluate the degree of inflammation present as well as help to delineate the contribution coagulation factors and aPC plays in the R time for this population.

There was a moderate correlation between left atrial size and three TEG parameters (R, G, CI) when patients with heart disease were taken in aggregate. When patients with heart failure were analyzed separately, there was no correlation between left atrial size and any of the measured indices. Humans with heart failure are known to have both activation of the coagulation system as well as increased platelet activation.[46] While heart failure may not be directly correlated to left atrial size (as there are many underlying etiologies for heart failure) the systemic effects on the body are similar. Increased levels of angiotensin II, epinephrine, norepinephrine and cytokines are hallmark responses to heart failure[53] all of which can also activate the coagulation or inflammatory systems. In our group of patients, there were six instances of pulmonary edema at the time of sample collection. None of the CIs were significantly different from other dogs with cardiac disease but without pulmonary edema. In humans, cardiac disease is most often complicated by vascular disease, particularly arteriolosclerosis and coronary artery disease, which have an undetermined independent effect on platelet activity.[46] However, vascular diseases are unlikely to have an impact on the dogs evaluated in the current study. It is also possible that canine platelets do not react to turbulent blood flow as seen in cardiac disease the same way as human platelets. Alternatively, a type 1 error is possible as the number of instances of patients in failure was only 4 in this study who presented with different disease (MVD – 2, PDA -1, SAS-1).

Only the coagulation index in TEG samples activated by kaolin was significantly associated with the presence of an arrhythmia. In people, a hypercoagulable state and predisposition to strokes are well documented with arrhythmias, particularly atrial fibrillation (a-fib).[62] There are many possible reasons why the dogs in this study are different from people with arrhythmias who have been shown to have

altered coagulation and platelet activation. First, the majority of people with atrial fibrillation have lone a-fib.[63] Dogs in this study had a-fib or sinus tachycardia along with other heart disease but the presence of a-fib or sinus tachycardia did not appear to make them more prone to TEG abnormalities. The fact that activation by kaolin made the coagulation index reach a significant difference is intriguing. This may be the result of an alteration in coagulation factor activity. Kaolin activates the intrinsic pathway, shortening the R time and potentially providing a more reliable tracing for the TEG.[64] It is possible that the coagulation factors are increased in dogs with arrhythmias thereby increasing the impact that kaolin has on the TEG tracing. This alteration may have been enough to change the kaolin activated CI and show significance in the results. Alternatively, the fact that uncovering a significant relationship required an activator in this group of dogs could be indicative of how tenuous the relationship is between arrhythmias and this population of dogs. Finally since there were many other covariates which were not taken into account in this analysis (type of heart disease with arrhythmia, age of dog, nature of the arrhythmia and the fact that some dogs contributed multiple times to this data set) a type 2 error could have occurred and significance was not found in the other variables.

As sub-populations of dogs with specific heart diseases were examined, dogs with pulmonic stenosis were distinguished by increased CI compared to control dogs. The pathology of PS suggests that damage to the endothelium as a result of an increased velocity of blood passing through the stenotic pulmonary valve may occur. Dogs with PS had a mean RVOT velocity of 6 m/s, substantially above the normal (< 2.5 m/s).[65] As high velocity blood moves through a stenotic artery, very high shear rates can result. This high shear stress can activate platelets and coagulation primarily through a shear induced binding of vWF and platelet GP1b-IX-V complex. This in turn results in increased cytoplasmic calcium levels in the platelet through the GP1B receptor pathway.[98,99] RVOT velocity overall was not correlated with coagulation indices. The low numbers of patients in this study resulted in insufficient power to generate a linear regression model and none of the Pearson correlation coefficients reached significance.

A total of five dogs diagnosed with DCM were evaluated. Two of these dogs had repeated measures, which were included in the analysis of ESVI. Dogs with DCM showed significant differences in TEG variables (CI, MA and G for both native and kaolin samples) from other subgroups that were analyzed. Dogs with DCM potentially have a different mechanism of activation of coagulation from the other dogs in this study with cardiac disease. In all of the other subsets of patients, turbulent blood flow is thought to be the most significant condition causing endothelial damage, direct activation of platelets and predisposing them to alterations in platelets and coagulation. In patients with DCM, the most likely culprit in the activation of coagulation is the stasis of blood within the dilated chambers.[66] In people with DCM, platelet activation, as suggested by an increase in mean platelet volume,[67] has been shown to increase in the face of DCM.[68] In this study, there was no difference in the MPV in dogs with DCM and control dogs. However the platelet count in this population of dogs was significantly higher than the controls. While the exact reasons for this disparity are unclear and may be related to a sampling error from the very small population of dogs in the DCM group, the increased platelet count likely contributed to the increase in MA, CI and G for the same reasons as delineated above. It remains unclear if there was increased platelet activation in the population of dogs studied here and further tests would be needed to determine the activation status of platelets in dogs with DCM. No correlation was found between the severity of DCM (based on ESVI) and coagulation. This could be due to the relatively minor changes in ESVI between dogs or the fact that there were very few dogs used for analysis or that activation of coagulation reached its maximum at relatively low ESVI and no further exacerbation of the dilation would generate more coagulation.

In dogs with mitral valve disease, there was a significant increase in many of the TEG parameters including CI, R and angle in both the native and kaolin activated samples. Increased platelet activity has been documented in people with mitral valve disease as determined by MPV, factor analysis and platelet aggregometry.[69,70] Walsh and colleagues noted no relationship between FV and FVIII nor PT/PTT when comparing humans with MVD. However, a dynamic test such as TEG that was used in this study may

indicate why we were able to demonstrate a difference when static tests used previously did not. Icli and colleagues identified an increase in MPV in people with MVD[69] however, we found no such relationship between dogs with MVD and control dogs.

None of the TEG indices reported here were significantly different between dogs with PDAs and the control population. Interestingly there was also no difference seen in any of the TEG variables before or after closure of the PDA. Platelets play a critical role in the spontaneous closure of PDAs in people and rats.[71] From this study it appears that the abnormal flow through the patent ductus does not cause systemic alterations in coagulation as demonstrated by significantly abnormal TEG variables. There are several potential reasons for this. One possible explanation is that the turbulence as it travels through the ductus is not sufficient to cause significant enough jet lesions on the endothelium to activate the coagulation system. Another possible explanation is that the volume of blood that encounters turbulence as it flows through the patent ductus is not sufficient to cause the systemic activation of the coagulation system that would be necessary to detect on TEG analysis. Finally the population evaluated in this study may be underpowered to identify a difference in populations and a type 2 error could have been committed.

Throughout this study, TEG parameters, particularly CI, were higher in dogs with various forms of heart disease (all dogs with cardiac disease, PS, DCM, MVD, and kaolin activated PDA) than in the control population. However, the clinical significance of this finding is questionable. A dog can be considered hypercoagulable if the CI is above 4.[60] All dogs with cardiac disease, PS and DCM dogs had mean CI values over 4. However, all the mean values were only slightly over 4.0 and the SD placed the CIs well within the normocoagulable range. In fact, the highest mean CI was 4.76 for the kaolin activated PS samples with the low end of the SD at 3.69. So while there were statistically significant elevations in CIs among the population with cardiac disease, this difference may very well not translate into a clinically relevant abnormality.

There are a number of potential limitations to this study. First and foremost the limited number of patients in this study potentially led to both type 1 and type 2 errors. There were only five patients in the DCM group, five in the PDA group, and eight in the PS group. This lead to a small patient population and large variances in the sample groups. These large variances and small numbers are reflected in the high SD and broad ranges that were found in the patient population. A larger patient pool would allow for not only more robust statistical analysis of the cardiac diseases that were analyzed but also potentially stratification of disease severity.

It is worth noting that the control population and the sample population were not uniform. Dogs in the sample pool were significantly smaller than dogs in the control population. This is likely due to the fact that most of the dogs in the control population were blood donors for the UGA CVM blood bank. Dogs that are enrolled in this program must weigh at least 25 kg and therefore this bias would select for a population of larger dogs. No such restriction was placed on the sample population and so it is reasonable that this population of dogs would have a larger variation in patient size.

Also, while not statistically significant, dogs in the control population were older than those in the control population. This is in part due to the restrictions in the blood donor program. Dogs older than 9 are not allowed to participate in the program as so a greater percentage of dogs in the control group who are also part of the blood donor program would be more likely to be younger. Additionally, dogs with heart diseases (e.g. DCM and MVD) that are progressive in nature would be more likely to seek specialty veterinary care (and thus be eligible for inclusion in this study) when they are older. This would increase the average age of dogs in the sample population. On the other hand, the dogs in this study with congenital heart diseases (PS and PDA) were younger and thereby decreased the overall age of the patients in this pool. There is some evidence to suggest that increasing age is weakly correlated to a hypercoagulable state (as defined by TEG analysis) in people[72] and potentially in male macaques.[73] However, a correlation between increasing age and TEG variables had not been documented in dogs. There was no significant correlation between age and TEG variables found in this population of dogs.

Secondly, the control population was likely not a uniform population of normal dogs without inflammatory disease. Recruitment of a new control population would be advantageous to ensure that the patient population is compared to a truly normal population. Various techniques can be utilized to ensure a normal population. One test that could easily be performed is an echocardiogram on each control dog to endure occult heart disease is not present in control dogs. While perspective control dogs had a thorough auscultation by a veterinary cardiologist, the possibility exists that some of the dogs in the control group had mild cardiac disease that was not detectible with auscultation. Also testing for abnormal values of inflammatory biomarkers such as activated Protein C activity, antithrombin activity, TNF-α, IL-10 IL-13 or IL-17A could help to screen out patients with occult inflammation which could potentially alter their coagulation indices.[74,75] Finally, analysis of a CBC, serum biochemistry and urinalysis could help to eliminate potential control dogs who have other systemic illness. One such dog was identified and eliminated from the control dog pool because it had a routine pre-anesthetic chemistry screening which suggested this patient could have cholestatic disease and hence not be considered a "normal" dog.

There was a trend found in this study towards increased CI in older canine patients. This observation deserves further study as if this trend is true, an age-matched and perhaps breed matched controls may be necessary for future studies. Additionally, further examination into the correlation between age and coagulation or platelet activity may generate normal ranges for specific age groups.

Finally, while this study did not definitively answer the question of how platelet activation and coagulation parameters change after correction or improvement of heart disease, it does give direction into further study. Specifically, dogs in this study with PS had evidence of significantly increased CIs. There were no repeated studies of these dogs after treatment (typically balloon valvuloplasty), but since the measured indices were substantially abnormal before treatment it is reasonable to wonder if these values improve or even return to baseline after their stenosis is improved. Based on this study, it is less likely that dogs with PDAs would have significant changes to their CI before and after treatment.

Title: CONCLUSION

This study demonstrates that there are differences in coagulation in dogs with heart disease as compared to control dogs. Specifically, altered coagulation was seen in dogs with PS, DCM and MVD but not in dogs with PDAs. While this data is preliminary it indicates that alterations in the coagulation system are present in dogs with turbulent blood flow.

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Title: APPENDIX 1

Introduction

An additional way to measure changes in the coagulation system that occurs in patients with heart disease is to examine the activation status of circulating platelets. Increased levels of activated circulating platelets have been shown to predispose individuals to an increased risk of pathologic thromboembolism.[76] Numerous tests have been used to attempt to quantify the activation state of platelets in animals. These include platelet aggrogometry, detection of thromboglobulin, soluble P-selectin and thromboxin metabolites (an in vitro marker of platelet thromboxin release and therefore platelet activation)[77] as well as with the use of flow cytometry to detect membrane P-selectin, P-selectin expression on microparticles, ligand-induced binding sites in GP IIb/IIIa, fibrinogen, dense granule membrane proteins and thrombospondin.[78] In people, P-selectin expression detected by flow cytometry has been the most widely used test for quantifying activated platelets.[78] Membrane bound P-selection expression is upregulated by a number of disease states in people, including peripheral vascular disease, myocardial infarction, and arteriosclerosis.[43,44]

Flow cytometry characterizes cells by identifying specific cell surface proteins, sugar clusters, glycolipid and glycoprotein structures. The technology of flow cytometry has existed since the 1930s when scientists constructed a microscope that allowed cells to flow past the objective one cell at a time.[79] Since then the technology has improved to allow flow cytometers to use both the intrinsic properties of cells (size and complexity/granularity) as well as fluorescent labeling to identify and even sort specific populations of cells.

A flow cytometer works by passing cells individually in a sheath of fluid past a beam of light and a number of laser emitters of specific (and multiple) wavelengths. The light reflected off or emitted by individual cells is measured by photomultiplier detectors that results in an electrical signal that is captured by a computer. Through the use of fluorescent antibodies that can bind to specific cell surface proteins, flow cytometry can recognize specific protein expression on the cells of interest.[80]

Flow cytometry has been used in small animal veterinary medicine to evaluate platelet physiology and pathophysiology. The specific antibodies and conditions for these studies are listed in Table 6.

Table 6 - – Flow cytometric studies of platelets in dogs

Study	**Study type**	**Antibodies used**	**Methodology**	**Results**
Weiss et al 2006 JVIM[81]	Prospective clinical trial	CD-62P	20 healthy controls, 20 clinical dogs with IMHA. Erythrocyte depleted blood with P-selectin analyzed via flow. Detected median fluorescence of platelets.	P-selectin expression was 8.1 fold greater for patients with IMHA than controls. 7 dogs had evidence of thromboembolic disease. 75% of IMHA dogs had abnormal P-selectin levels
Tarnow et al. Vet Immuno & Immunopath 2008[82]	Experimental evaluation of platelet-leukocyte aggregation and platelet activation in healthy dogs	CD-14, CD-61, CD-62P, CAP-1	Citrated blood, marked with specific antibodies, was evaluated using physiologic activators (ADP, epinephrine, collagen, α and γ thrombin) at varying concentrations.	Individual physiologic agonists (ADP, collagen, and epi) increased LPAs and platelet activation (as determined by P-selectin and CAP-1 expression) modestly. Combinations of

				agonists (ADP/epi, ADP/collagen and epi/I-BOP (a thromboxane prostinoid receptor agonist) increased LPAs to a greater degree. Both α and γ thrombin activated canine platelets significantly.
Moritz et al. Vet Clin Path 2003[78]	Experimental examination of activated canine platelets	CD-62P	EDTA and citrated blood from 12 normal staff owned dogs. P-selectin was evaluated to determine platelet activation. Forward and side scatter were used to identify platelet aggregates and microparticles. Platelets were stimulated with PMA (phorbol myristate acetate).	PMA stimulated platelets had increased MFI and slight increase in the percentage of platelet aggregates and microparticles (citrate > EDTA). No effect seen on addition of 0.5% paraformaldehyde. No change in platelet activation seen after incubation for 4 hours at 4ºC.
Brooks et al. Blood 2002[83]	Evaluation of hereditary Scott syndrome in family of 5 dogs	Annexin V and CD-61	Evaluated numerous parameters (platelet function, vWF analysis, ACT, triglyceride, cholesterol, platelet factor 3 availability, prothrombin consumption, platelet phospholipid composition, prothrombinase activity) including flow cytometry.	Affected dogs had decreased expression of surface phosphatidylserine confirming the presence of a heritable Scott Syndrome-like condition in dogs.

Lucidi et al. Vet Clin Path 2011[84]	Experimental evaluation of erythrocytes and platelets	DEA 1.1 antibodies	172 blood samples were collected in EDTA. Flow cytometry was used to try to detect DEA 1.1 antigen on platelets and erythrocytes . Platelets and erythrocytes were evaluated separately.	DEA 1.1 was not found on canine platelets but was found on erythrocytes.
Ridyard et al. JSAP 2010[85]	Clinical evaluation of 14 cases of dogs with idiopathic IMHA and 14 control dogs.	CD-61, antihuman fibrinogen antibody and CD 62P. PLAs were evaluated with CD-61 and either CD-45 or CD-14.	EDTA and citrated blood was obtained. Platelets were stimulated with thrombin in the presence of GPRP. Compared the proportion of activated platelets and PLAs in dogs with IMHA and control dogs.	There was a lack of global activation of platelet activation as measured by P-selectin expression in dogs with IMHA. Platelet activation was seen in severely thrombocytopenic dogs.
Sharpe, et al. AJVR 2010[86]	Experiment designed to evaluate the effect of ultra low dose aspirin on platelet activation, aggregation and PLAs.	CD 62P and CD 61 for activated platelet detection. PLA were detected using CD 61 and CD 18.	EDTA anticoagulated whole blood from 18 clinically normal dogs was collected before and after treatment with low dose aspirin (0.5 mg/kg). Whole blood aggregometry was performed. Platelet activation was measured with flow to detect presence of P-selectin.	Ultra low dose aspirin administration did not significantly increase CD 62P expression. Whole blood impedance aggregometry was delayed in aspirin treated animals when subjected to ADP and collagen. Up to 30% of treated dogs failed to have an effect. Thrombin caused an

				increased expression of P-selectin from basal levels. Aspirin administration did not affect this expression.
Dircks et al. JAVMA 2009[87]	Retrospective case series of 83 thrombocytopenic dogs	CD-9 to identify platelets and goat anti-dog IgG	EDTA blood was collected to look for platelet-bound antibodies in dogs with thrombocytopenia. Used FSA and SSC on PRP plus CD-9 to identify platelets. Platelet count, MPV and some bone marrow aspirates were also collected.	Platelet bound IgG was found in 37/83 (45%) of thrombocytopenic dogs. MPV was increased in dogs without platelet bound antibodies.
Moritz et al. AJVR 2005[88]	Evaluation of platelets in 9 septic, 11 non-septic inflammatory and 20 control dogs.	CD-62P	PRP was obtained for EDTA collected whole blood. Activated platelets were identified with P-selectin. 20 dogs were used as controls to make reference ranges.	12 dogs with inflammatory disease had P-selectin expression outside of the reference range (60%). 16 dogs with inflammatory disease had mean platelet component concentrations below the reference range.
Willis et al. AJVR 2006[89]	Experimental design to identify activated platelets in normal dogs	CD-61 to identify platelets. Platelet activation - CD-62P and	Blood samples from 10 dogs were obtained in citrated tubes. Micropartices were also detected based on size and complexity as well as 1 µm	A significant difference in platelet microparticle formation, P-selectin events, PLA and phosphatidylserine exposure was found between

		Annexin-V. PLAs - CD-61 and CD-45.	latex beads. ADP and PAF were used for stimulation.	stimulated and unstimulated platelets.
Thomason et al. JVIM 2011[90]	Experimental design to recognize COX-1 and COX-2 expression on platelets before and after the administration of aspirin.	Anti-ovine COX-1, and CD-9 for the COX-1 studies. Mouse anti-human COX-2 antibodies and CD-61 for the COX-2 studies.	Citrated blood was collected for 8 normal dogs. Isotype control of IgG was run in parallel. PFA-100 and urinary 11-Dihydro-thromboxane B2 were also analyzed.	COX-1 and COX-2 were identified in canine platelets. The expression of COX-1 increased by an average of 250% in patients treated with aspirin while the expression of COX-2 did not change.
Knudsen et al. AJVR 2011[91]	Experimental study to detect coated platelets (those with high concentrations of α granule proteins) and evaluate the binding of recombinant FVII to platelets. 4 dogs with hemophilia A, 4 dogs with hemophilia B, 4 dogs with vWD and 6 clinically	CD-61, CD-62P and fibrinogen antibodies	PRP was prepared from each dog. Platelets were triple labeled and samples were stimulated with canine thrombin, convulxin or both. Recombinant human VII was labeled with FITC to determine the binding of FVII to platelets. Formation of coated platelets was measured based on the presence of fibrinogen externalization.	CD-61 expression increased over baseline in all dogs stimulated with both thrombin and convulxin. Coated and non-coated platelet populations could be differentiated using three markers with fibrinogen identifying specific populations and P-selectin identifying general activation. Dogs with hemophelia and vWD can generate coated platelets. Human recombinant FVIIa

	normal dogs were evaluated.			bound preferentially to coated platelets.
Tanaka et al JVIM 2002[50]	Experimental study to determine the life span of platelets before and after the introduction of mitral regurgitation	NHS-biotin	6 healthy dogs. Platelet life span was measured before and after experimentally induced MR via transection of chordae tendineae. Biotin was conjugated with the platelets and platelets were re-infused into the subject. Flow was used to detect platelets over 4 days.	Platelet life span was significantly shorter after mitral valve disease was induced in the six dogs.
Dircks et al AJVR 2012[92]	Experimental study to determine platelet neutrophil aggregates and neutrophil shape in dogs with inflammatory diseases	CD 61 or CD 163	20 dogs with SIRS (Sepsis = 6 or DIC=12) and 10 control dogs. CD 61+ neutrophils and neutrophil size and complexity were measured initially and after additions of agonists (PMA, collagen, ADP, epi, LPS and AA – as well as combinations)	Increased PNA in dogs with SIRS than controls. Mean size of neutrophils was grater and complexity was lower in dogs with SIRS than controls. No difference when sepsis was compared to DIC. Increased percentage of PNA seen with addition of all agonists except epi with the greatest increase seen with the addition of PMA.

More specifically, we sought to determine if platelet activation (as assessed by platelet P-selectin expression and the presence of platelet-neutrophil aggregates) was present to a greater degree in dogs with naturally occurring heart disease, compared to control dogs without heart disease.

Materials and Methods

In addition to the TEG analysis and CBC, flow cytometric assessment of both a platelet-rich plasma (PRP) sample (to examine the degree of platelet activation as assessed by platelet P-selectin expression) and a leukocyte sample (to determine the degree of platelet-leukocyte aggregates) was performed.

After the removal of sample for CBC and TEG analysis, 900 µL of citrated whole blood was removed and placed into a 1.5 mL microcentrifuge tube to be processed for the platelet-leukocyte fraction. The remainder of the blood (approximately 1 mL) was processed in a centrifuge at 400 x g for 10 minutes to create PRP. 300 µL of the resulting PRP was placed in a 1.5 mL microfuge tube.

The leukocyte fraction was prepared in duplicate in the following manner:[86] 100 µL of 4% paraformaldehyde was added to 900 µL of citrated whole blood in a 1.5 mL microcentrifuge tube and incubated at room temperature for 30 minutes. The paraformaldehyde fixing step is used to cross link proteins on platelet as well as platelet-leukocyte aggregates in order to prevent any further processing-induced activation of platelets.[86] In addition, this fixing step prevents any further biological or biochemical changes thereby essentially locking the platelet-leukocyte aggregates in time. 500 µL of the resultant sample was placed into two 15 mL polypropylene conical tubes. Each solution was lysed with 4 mL of RBC lysis solution (0.5M EDTA, 0.5M $NaHCO_3$, and 0.5M NH_4Cl). The tubes were then incubated for 5-7 minutes at room temperature on a platform rocker. Next the tubes were centrifuged at 400 x g for 10 minutes. The supernatant was decanted and the lysis procedure was repeated. After the second decanting, the resultant pellet was washed twice with platelet wash buffer (137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl{\cdot}6H_20$, 0.5 mM Na_2HPO_4, 10 mM Hepes, 0.1% glucose and 0.1% Bovine Serum Albumin – adjusted to pH of 7.4) A physiologic wash buffer was chosen in this step for consistency as it is the same buffer that was used in the processing of the PRP. The final pellet was suspended in 1.5 mL of platelet wash buffer and mixed thoroughly. 100 µL of the resultant solution was placed into a 1.5 mL

microcentrifuge tube. 2 µL of CD 18-PE (AbD Serotec, clone YFC118.3, Raleigh, NC) and CD 61-FITC (BD Pharmogen, clone VI-PL2, Franklin Park, NJ) conjugated antibodies were then added to the leukocyte fraction. The tubes were incubated at room temperature, in the dark, for 30 minutes. Each sample was diluted with 1 mL of platelet wash buffer prior to analysis in order to make particles easier to resolve by the flow cytometer.

The platelet fraction was prepared in duplicate similar to a previously described method.[86] 90 µL of PRP was placed into each of four 1.5 mL microcentrifuge tubes. 2 µL of 20 mM Gly-Pro-Arg-Pro amide (Sigma Chemicals, St. Louis, MO) was added to each tube and incubated at room temperature for 10 minutes in order to inhibit fibrin formation. In two of the tubes, 10 µL of 100 U/mL of bovine thrombin was added to induce maximal platelet activation. These tubes were incubated at room temperature for 10 minutes. Into each tube, 2 µL of CD 61-FITC and CD-62P-PE (BD Pharmagen, clone AC1.2, Franklin Park, NJ) antibodies were added and the tubes were further incubated in the dark for 30 minutes. Finally 10 µL of 4% paraformaldehyde was added to each tube to cross link proteins and fix the samples. The samples were incubated with the paraformaldehyde in the dark at room temperature for 30 minutes. Each sample was then diluted with 1 mL of platelet wash buffer prior to sampling in the flow cytometer.

One commercial flow cytometer and accompanying software was used for all samples in this study (Accuri C6, BD Biosciences, San Jose, CA). Optimization and compensation were established on each sample by one investigator. Flow cytometer calibrations were performed monthly with specific calibration beads. For the leukocyte fraction, gates were established based on forward scatter and side scatter to identify leukocyte populations of cells. Samples were gated based on CD-18 expression and 10,000 CD-18 positive events were collected. Samples were further gated to identify CD-41/61 positive events within the CD18+ gate (see figures 3-7 for an example of the gating logic). For the PRP fraction, platelets were identified and a gate was established to capture >95% of the CD-61 positive events. 10,000 CD-61 positive events were collected and analyzed.

Initial trials were conducted in order to validate each assay technique and attempt to optimize the recovery of cells, particularly leukocytes and platelets. The first experiment investigated leukocyte fraction preparation for yield of WBC and platelets. Four different methods were tested. The first method used a prepared RBC lysis solution, lysing whole blood samples twice and washing the cells twice (see above for specific technique). The second method involved lysing the whole blood once and washing the cells once. This method was investigated to determine if two wash and lysis steps were superior to one in order to justify the increased time and materials required for the two step procedure. The third method involved lysing the cells once and washing with an approximately 500 fold wash with a buffered solution. This method was examined to determine if two separate wash steps versus one larger wash step yielded a greater number of platelets and leukocytes. The fourth method involved using a whole blood preparation without lysis. The whole blood preparation is the simplest method and would reduce the time needed to prepare the samples as well as eliminate the lysis reagent from the protocol. Cell counts from the automated cell counter as well as mean fluorescent intensities for each conjugated antibody were recorded and compared.

A comparison of the platelet and leukocyte yield from blood with potassium EDTA or sodium citrate as anticoagulant was tested under the separation protocol described above. Samples were collected from a normal dog in 3.2% sodium citrate as well as 7.5% K3 EDTA tubes. The assays used in this study described above were used on both sets of samples. Data from both cell counts as well as results of mean fluorescent intensity were recorded for blood collected in both types of anticoagulants. Various concentrations of antibodies were also tested in order to determine the optimal amount of each reagent for use in the main experiment. After obtaining normal dog blood in 3.2% sodium citrate tubes, concentrations of 1, 2 and 3 µL/sample of antibody were compared in both the leukocyte and PRP fractions. Flow cytometric and cell counts were conducted as described above. This experiment was performed in order to find the saturating concentration of antibody for both platelets and leukocyte populations.

Statistical Analysis

All statistical analysis was performed using a commercial software package (Minitab 16, State College, PA). All continuous variables were analyzed for normality with the Shapiro-Wilk normality test. The percent of events that were positive for both CD 18 and CD 61, the mean fluorescent index (MFI) of FITC for these CD61 positive events, and the percent of CD61 positive events that were CD 18+ (henceforth when these three measures are taken together they will be referred to simply as flow cytometric indices or variables) were not normally distributed and are reported as median and range. Significance throughout the analysis was defined as $P < 0.05$.

Results

Preliminary experiment results:

Initially, four different methods of isolating platelets and platelet leukocyte aggregates (PLAs) (as described above) were evaluated. Method 1 involved two lysis steps with two wash steps was found to be the superior method. This method provided the highest recovery of platelets and leukocytes with the lowest number of red blood cells. Method 2 which involved one wash step and one lysis step had higher red blood cell count, while method 3 had substantially lower yield of platelets and leukocytes (see table 7). Samples were used from two individuals (one to compare methods 1&2 and another to prepare sample 3. Ratios of counts before and after sample preparation were used to account for variability between samples. Method 4 which did not use an RBC lysis step but instead involved a whole blood preparation was determined to be inferior as it yielded poor discrimination between leukocytes and background in the FSC vs SSC flow cytometry plot.

Table 7 – Comparison of methods of RBC lysis

Sample	WBC	PLT	HCT
Control (before preparation)	23.2	229	40.7
Method 1, Sample 1	13.2	100	2.9
Method 1, sample 2	16.0	99	4.4
Method 2, sample 1	0.6	21	5.1
Method 2, sample 2	8.6	73	9.5
Control (before preparation)	6.3	171	45.2
Method 3, sample 1	0.4	13	1.3
Method 3, sample 2	0.6	11	2.2

Additional preliminary experiments were conducted to determine if there was a difference between K3 EDTA and citrate anticoagulated blood for recovery of platelets. Using method 1 described above, there was no difference between EDTA and citrated whole blood as determined by either cellular recovery or a significant difference in flow cytometric indices. Therefore citrated whole blood was used in this experiment because citrated blood was needed for TEG analysis and it was determined that one 2.7 mL tube would be sufficient for all procedures in this experiment.

The final preliminary experiment involved a determination of the saturating concentration of the fluorescent antibodies. 1, 2, and 3 fold dilutions of each antibody (CD 18, CD 41/61 and CD 62P) were used in both the PRP and leukocyte fractions. There was a substantial increase in MFI for all stains when the concentration was increased from 1 µL to 2 µL but only a very modest increase in CD 18 and CD 62P

when the concentration was increased to 3 µL. It was determined that the saturating concentration for all antibodies was therefore 2 µL (in both the PRP fraction and the leukocyte fraction) for this protocol.

All samples for flow cytometric evaluation were run in duplicate. Voltage, angles and amplitudes of each laser is permanently set for the cytometer used in this experiment and was therefore not adjusted throughout this experiment. Color compensation was set on each individual sample to adjust for "bleedover" of one fluorescent color to another. This adjustment was typically between 5-8% for each color.

Results

Thirty two dogs were included for flow cytometric analysis of PLAs in this part of the study. Six samples were excluded from analysis along with the control population when it was discovered that the incorrect CD61 antibody was utilized. Because the flow cytometric variables for the control population were unreliable, statistical comparisons could not be made between dogs with cardiac disease and normal dogs. Therefore, only descriptive statistics are reported here.

Samples were gated for size and complexity (forward and side scatter respectively) and denoted as the leukocyte gate (P6 in figure 12) to identify leukocytes. Those events that fell within the leukocyte gate were then evaluated for their expression of CD 18 or CD 41/61 (see figures 13-15). CD 18+ events in the leukocyte gate were analyzed for both the percentage as well as the MFI of CD 41/61 expression. (See figures 14-15).

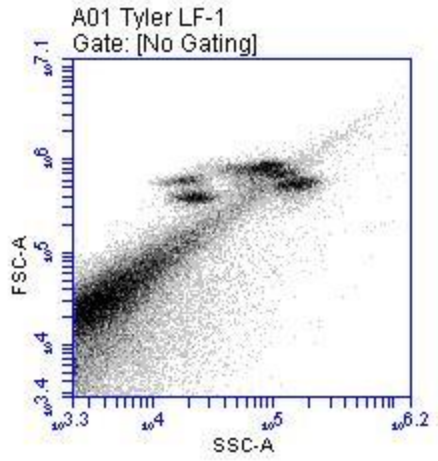

Figure 11 – Leukocytes identified by size (FCS) and complexity (SSC)

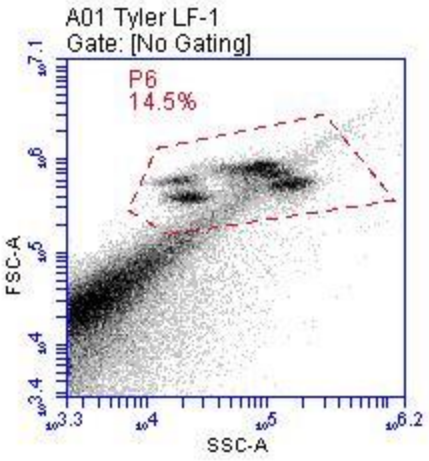

Figure 12 – Leukocyte gate – P6

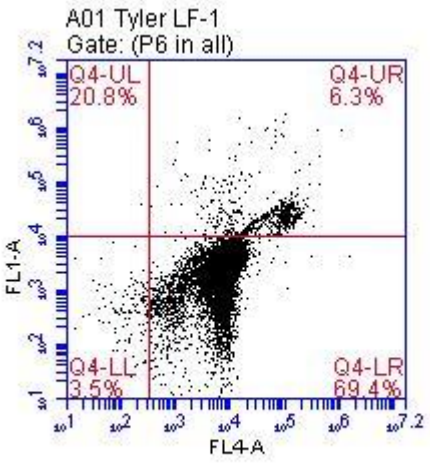

Figure 13 – Events in Q4 double positive for CD 61 and CD 18

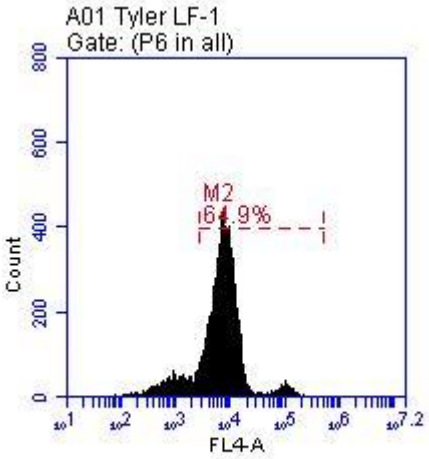

Figure 14 – Events in P6 gate which stain positive for CD 18

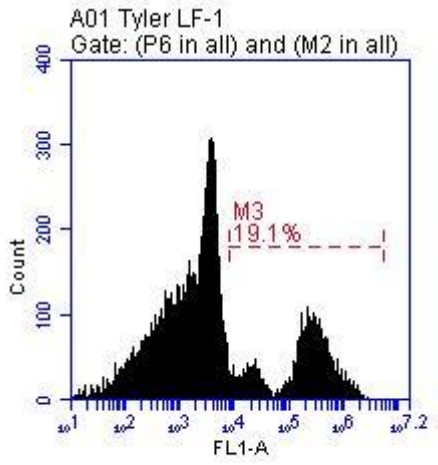

Figure 15 – Events in P6 - Events in M3 are positive for CD 18 and CD 41/61

Overall there was poor recovery of platelets from the PRP samples once the acquisition of sample data began. The mean platelet count from the dogs included in this portion of the study was 23,395. The mean platelet count from the unprocessed blood was 265,020, giving a recovery rate of only 8.8%. Because of poor sample recovery as well as inconsistent flow cytometric results from the PRP fraction, this portion of the experiment was discontinued prior to completion as the data was deemed to be unreliable. The leukocyte fraction of the experiment continued through September 2013.

The median (range) MFI of CD 61 positive events in the leukocyte gate for dogs with cardiac disease was 308,052 (59,115 - 6,887,566). The median (range) for the percentage of cells double stained strongly for CD41/61 and CD 18 in the leukocyte gate was 22.7% (2.1-79.2). The median (range) for the percentage of cells that were CD61 positive from the population of CD 18 cells was 3.5% (0.75 – 71.6).

No flow cytometry variables were significantly associated with left atrial size from the step-wise regression model. Additionally no flow cytometric indices were found correlate with left atrial size. No significant correlations were found between platelet count and the MFI of CD 61 positive events in the leukocyte gate (P=0.92), the percentage of cells double stained strongly for CD41/61 and CD 18 in the

leukocyte gate (P=0.62) and the percentage of cells that were CD61 positive from the population of CD 18 cells (P=0.64).

There was no significant difference between flow cytometric indices [percent of events in the leukocyte gate double positive for CD 18 and CD 61 (P=0.60), MFI of CD61 for events in the leukocyte gate - CD 18 + (P=0.81) and percent of CD61 positive events in the leukocyte gate - CD 18+ (P=0.25)] before and after PDA closure.

Discussion - Preliminary Experiments:

The preliminary experiments detailed above allowed us to validate the leukocyte fraction assay. It also proved that similar results could be obtained using either EDTA or citrated blood. Citrated blood was necessary for the TEG portion of this analysis but similar studies have used both EDTA as well as citrated whole blood for flow cytometric analysis.[81,86] The ability to use citrated blood for all parts of this experiment was important as it permitted a small volume of blood to be used and since only one tube needed to be collected it likely resulted in less venipuncture. Using only citrated blood for all aspects of this experiment was therefore considered to be better for the patients who participated in this study.

Initially, in addition to a CBC, TEG analysis and CBC of the PRP, both a PRP fraction as well as a leukocyte fraction was performed and analyzed with the flow cytometer. Inconsistent results were found in the PRP fraction. Specifically, two peaks representing background fluorescence and increased fluorescence seen with binding of conjugated antibodies to cells, were not routinely seen on the PRP fraction. This occurred with both the CD 61 and the CD 62P peaks but more often with the CD 62P. The loss of both peaks is interpreted as a poor sample and leads to very small MFIs in the sample.

Actions that were taken to rectify this problem included increasing the amount of antibodies which were added to the sample. The rationale behind this step was that if the antibodies were aging and their affinity to bind to the antigen was diminished then the addition of more antibody may lead to more saturation of available antigen. However this solution did not consistently improve the assay. In addition

the increased concentration of fluorescent antibodies may have caused an increase in bleedover between the orange and green color. More color compensation was needed to separate the colors which likely lead to loss of specificity in identification of positive events.

The second step which did improve the assay to some degree was in adjusting the way in which the sample was prepared. The PRP was spun initially at 400 x g based on a similar protocol.[88] The platelet counts on the PRP CBC were severely decreased from the whole blood sample. However, since the flow cytometer was set to detect a specific number of events, the platelet recovery seemed irrelevant. In retrospect, this loss of platelets likely played a major role in the loss of signal. It is possible that excessively high centrifuge speeds not only caused high numbers of platelets to settle out of the PRP and into the pellet removing them from potential analysis. Additionally this excessive centrifugation may have caused in vitro activation of platelets, leading to platelet aggregates and subsequent the loss of these platelets in the sample. When the RPMs were decreased in the centrifuge to 150 x g the recovery of platelets increased substantially. If platelets that were lost during centrifugation they were potentially the heaviest as centrifuges separate cells based on mass. Therefore activated platelets and platelet aggregates were the most likely cells that were lost and these were specifically the cells we were most interested in studying. However, because the PRP assay was unreliable for a large portion of this experiment, the data that was available at the conclusion of the experiment was not sufficient to perform meaningful analysis. Therefore, the PRP fraction was not included in the results that are reported here.

Interestingly there was also no difference seen in any of the flow cytometric variables before or after closure of the PDA. From this study it appears that the abnormal flow through the patent ductus does not cause systemic platelet activation or alterations in coagulation as demonstrated by increased PLAs or TEG variables.

There are many potential reasons for the lack of significance found in the PDA dogs. The first is that while the turbulence is through the ductus is significant, the young age of the patients in this group

may cloud the results. In humans, younger patients generally have less reactive platelets as demonstrated by flow cytometry[93] while other reports show an increase in platelet–monocyte aggregates with a decrease in P-selectin expression in younger patients.[94] While it is unclear if platelets are less reactive in younger dogs as they appear to be in people, if true then the decreased age of patients in this population with their corresponding hyporeactive platelets may have masked platelets that were activated by turbulence with the measurable result being essentially normal platelets. However, this study indicates that if anything, advancing age is associated with increased platelet activation.

The correlation between flow cytometric variables and age was very weak in this study and was therefore unlikely to significantly confound the results of the study. In people, there are conflicting reports regarding platelet activation and increasing age. One of the most convincing studies showed a decreased platelet response (using flow cytometry to detect P-selectin expression and GPIIb-IIIa expression) with increasing age,[95] while another study showed increased platelet aggregation (via the use of platelet aggregometry) in men with increasing age.[96] This disparity could be due to the different measurements of platelet function and the varying degree of coronary and other vascular diseases in the different population pools. Neither of these studies is likely to be directly applicable to dogs and further studies are needed in this area for canines.

The major limitation to this study was the lack of a control population of dogs. A control population was identified for the study but erroneous labeling of platelets prevented meaningful evaluation of the control population. Without a population of normal dogs it is difficult to draw strong conclusions about the changes in PLAs seen in the dogs with cardiac disease. Future studies should identify a robust control population who can be deemed normal based on CBC, serum biochemistry, urinalysis, normal echocardiogram and electrocardiogram and normal makers of inflammation (aPC, AT, interlukins and TNF-α levels. Additionally it would be helpful to have coagulation factor activity levels on normal dogs in order to compare any abnormalities in R time with subject dogs. Additionally future studies should ensure that the correct antibodies are used on all samples.

Another potential limitation in this study was the inconsistency in diagnostic equipment. As this experiment lasted over two years, 3-4 different flow cytometers were used to capture data. Each was the same brand and model but subtle difference can exist between machines. However, since the lasers and detection systems in this model of flow cytometer are solid state, minimal variation would occur between with regard to frequency, laser intensity and angle of detection. Furthermore, a software upgrade was performed in October 2012. This software package increased the MFI in all samples. This led to the need to multiply the MFI in samples acquired before October 2012 by a factor of 16. While this minimized the differences in samples obtained before and after October 2012, it must be allowed that other small variations occurs in data capture and processing with the different software, complicating comparisons.

This study provides many future directions for further studies into the investigation of platelet function and coagulation status of dogs with heart disease. First the assay used here has potential applications into the study of feline platelets. Thromboembolism (particularly arterial) is a significant problem in cats with heart disease. Feline platelets are difficult to assess due to the relatively small total volume of blood in feline patients, difficulty with venipuncture in the species and an increased propensity to activate compared to other species.[97] The PLA assay utilized in this study has the potential to work in cats as platelets are fixed in the first step after collection, potentially minimizing ex vivo activation of platelets and allowing them to be studied with flow cytometry.

Secondly, studies using the principles examined here could be used to examine the coagulation status of patients admitted to the ICU. The repeated measures here could be used evaluate how the coagulation status of dogs changes as their diseases either progresses or resolves. Subsets of critical patients with varying degrees of inflammation (e.g GDV, pancreatitis, trauma, sepsis) could be examined using these methods.

Finally a significant limitation of this project was the time necessary to prepare and run each PRP and leukocyte sample. Investigation into the maximum time that samples can be preserved before

analyzing without compromising the results would improve the ability to batch prepare samples and reduce the need for on demand processing. Fixing samples early in the process and allowing the samples to rest either at room temperature or chilled may allow more liberal processing time and make the assay more amenable to future studies.