DISSERTATION

COAGULATION ABNORMALITIES IN *EHRLICHIA CANIS*-INFECTED DOGS AND DETECTION AND DYNAMICS OF ANTI-PLATELET ANTIBODIES IN THROMBOCYTOPENIC DOGS

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ABSTRACT

COAGULATION ABNORMALITIES IN *EHRLICHIA CANIS*-INFECTED DOGS AND DETECTION AND DYNAMICS OF ANTI-PLATELET ANTIBODIES IN THROMBOCYTOPENIC DOGS

Vector-borne diseases affect millions of people and domestic animals worldwide resulting in significant morbidity and mortality rates. In dogs, vector-borne diseases such as *Ehrlichia canis* can cause a myriad of clinical signs (lethargy, weight loss, and epistaxis) and hematological abnormalities (hyperglobulinemia, thrombocytopenia, and anemia). It has been previously reported that *E. canis* results in systemic inflammation and vasculitis as well as the formation of immunoglobulin associated platelets or anti-platelet antibodies. It has been theorized that anti-platelet antibodies can deleteriously affect platelet function and if concurrent significant thrombocytopenia is present, signs of bleeding may manifest. However, anti-platelet antibodies and thrombocytopenia can occur in a variety of disease processes in the dog including other vector-borne diseases, neoplasia, and idiopathic primary immune syndromes. Thrombocytopenia is also one of the most common acquired hemostatic abnormality observed in dogs. Consequently, determining the underlying cause and mechanism for thrombocytopenia in dogs can represent a frequent diagnostic challenge. Additionally, inflammation is often present in dogs with thrombocytopenia due to various causes. Inflammation and immune system processes directly affect hemostasis which can lead to derangements in the coagulation system resulting in clinical signs of bleeding or thrombosis. The goals of the research described in this dissertation were to investigate the dynamic changes of anti-platelet antibodies in thrombocytopenic dogs and the

changes that occur in the coagulation system during a vector-borne infection such as *E. canis* in dogs.

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Platelet function testing in dogs

Platelets are cells which were originally thought to play only a very simple role in coagulation ("platelet plug") but it is now well established that they are dynamic cells which are integral to various processes in hemostasis, inflammation, and immunology.[1,2] Therefore, the ability to assess platelet function can be helpful in fully understanding various disease states and syndromes.

Platelet function can be evaluated via a variety of methods in veterinary medicine. Examples range from simple methods such as the buccal mucosal bleeding time (BMBT) to more complex methods requiring specialized equipment such as thromboelastography (TEG) platelet mapping.[3] Each method has limitations which must be taken into account when evaluating results in the clinical or research setting. Historically, one of the most common ways to assess platelet function in veterinary clinical practice includes the BMBT however this test is significantly affected by multiple factors such as hematocrit, platelet count, and systemic protein levels.[3] Additionally, the results of the test are also significantly affected by the device being used and who is performing the test.[4] Due to the variability of results and subjective interpretation of the BMBT, other assays have been investigated for assessing platelet function in dogs.

Platelet function can also be assessed by objectively evaluating how well platelets aggregate to various agonists and can be performed in low or high shear environments. For example, this can be evaluated in low shear environments via turbidometric light transmission aggregometry (LTA) where platelet-rich plasma (PRP) is used or in whole blood samples via impedance aggregometry (light or electrical methods).[3] Although LTA has historically been

considered the gold standard for assessment of platelet function in people, due to the use of PRP, it comes with certain limitations that are not present in whole blood assays. Because platelets are quite active and responsive to their environment, the less manipulation performed on a sample may be more reflective of their true *in vivo* function. For example, it has been previously shown that when PRP is diluted in platelet-poor plasma (PPP), results are quite variable which is thought to be related to products in PPP that have various *in vitro* effects on platelet function.[3,5] Another limitation of LTA is that platelet function is not being assessed in whole blood so the true behavior and function of platelets may not be fully represented which could have significant impacts on interpretation of results.[6] Therefore, a benefit of whole blood impedance assays is that they are performed in whole blood and little manipulation is done to the sample. However, LTA is affected by platelet count and impedance aggregometry is affected by the hematocrit, platelet count, and white blood cell count which must be taken into account when evaluating test results from either technique.[3,5,6,7,8,9,10]

Other assays to assess platelet aggregability in response to various agonists include the Platelet Function Analyzer-100 (PFA-100), Platelet Function Analyzer-200 (PFA-200), VerifyNow, and PlateletWorks.[3,11,12] At this time, the PFA-100 has been evaluated in more veterinary studies and assesses platelet aggregability in high shear environments. The main result garnered from this test is the closure time (CT) which is the time it takes for the aperture in the test cartridge to close. Like the previously mentioned light and impedance aggregometry tests, the PFA-100 is also affected by hematocrit and platelet count in addition to other factors specific to this assay.[3] The other aforementioned assays require more studies in veterinary medicine at this time.

Platelet function can also be assessed by dynamic viscoelastic testing such with TEG or TEG platelet mapping which can be evaluated in a native state, when using a specific platelet inhibitor, or when using a specific coagulation and platelet activator to evaluate the contribution of platelets to a given result.[3] However, these tests also are affected by a variety of factors such as hematocrit, platelet count, and fibrinogen but will be discussed in more detail in the next section.[13] An assessment of platelet function in dogs can also be determined by evaluating indices or markers of platelet activation. This can be performed by simply evaluating the complete blood count[14,15] or it can be done through more specialized means such as flow cytometry.[3] Additionally, particular platelet function defects or abnormalities can be assessed by specific tests such as the clot retraction test, quantitative and functional vonWillebrand factor (vWF) tests, electron microscopic analyses, and secretion and uptake assays.[3]

As a result, there are many different tests used to assess platelet function in dogs. The choice of test depends on many factors such as expertise and familiarity with the test, availability and cost of specific equipment and reagents, goals of the study, limitations of the test, etc. For example, assessing platelet function in a high shear environment with the PFA-100 may not be suitable if assessing a disease state known to occur in a low shear environment.

Vector-borne diseases such as *Ehrlichia canis* have a wide variety of effects on the body including effects on hemostasis and potentially platelet function.[16,17,18,19,20] Although it has been theorized that platelet dysfunction occurs during canine ehrlichiosis, no studies have investigated platelet function using whole blood. Because this disease is a blood-borne infection and interacts with various cells of the vascular system and immune system, a whole blood platelet function assay seems most appropriate for further investigation of how platelets are affected during canine ehrlichiosis. The Multiplate® is a whole blood electrical impedance platelet aggregometry assay

which can be used to assess platelet function in dogs. Various platelet agonists can be used for this assay such as collagen, adenosine diphosphate (ADP), and arachidonic acid (ASPI) which are used to stimulate platelets and evaluate platelet aggregability potential.[21] This type of testing is user friendly however the analysis components and reagents are expensive and can be cost prohibitive. According to the manufacturer of Multiplate®, it is recommended to only use the reagents for a short time following reconstitution but if it was determined that the reagents are viable after prolonged storage, this would greatly reduce the monetary burden of this assay. This has not be investigated previously in veterinary medicine. Therefore, a study comparing the aggregation results using reagents stored at various time periods at different temperatures in healthy dogs could help determine if prolonged reagent storage is a viable option.

However, before applying an assay to a dog in a diseased state, it is important to first determine how the assays performs in healthy dogs. This is related to multiple factors such as inter- and intra-individual variation as well as inter- and intra-assay variability.[22,23,24,25] For example, if a healthy dog's platelet function parameters vary by 50% over time (e.g. week to week), then that is significantly different than if the parameters were only to vary by 10% over time. If this variation is due to the individual rather than the assay, then this is related to biological variation and is important for interpretation of test results.[25] Several studies have previously investigated the Multiplate® in healthy dogs with regards to variability, recommended storage duration, optimal concentrations of agonists to use, and optimal duration of testing.[21,26,27,28,29] From these studies, it was determined that the overall assay variability of Multiplate® is minimal potentially due to the automated pipetting system of this technique.[21,29] Additionally, samples should be evaluated with heparin or hirudin anti-coagulated blood[21,26,27] but the biological variation has only been investigated in citrate anti-coagulated blood.[29] Determining the biological

variation in healthy dogs using the recommended anti-coagulant would be key to appropriately evaluating platelet function testing results in diseased dogs such as those infected with *E. canis*.

1.1 Thromboelastography testing in dogs

Thromboelastography (TEG) is one of the testing modalities that allows for the assessment of blood's viscoelastic properties. Examples of similar assays include rotational thromboelastometry (ROTEM) and PlateletMapping.[13,30,31,32,33,34] Essentially, TEG is considered a specialized assay that can further characterize the coagulation system with regards to clot formation and clot breakdown. Although other tests such as the one-stage prothrombin time (OSPT) and activated partial thromboplastin time (APTT) can assess aspects of clot formation, they are not considered particularly sensitive assays. Additionally, they do not assess aspects of clot breakdown or fibrinolysis. In contrast, D-dimers, fibrin-degradation products (fdps), plasminogen, and anti-plasmin are some of the components that have been previously investigated in dogs to evaluate fibrinolysis but they cannot be used for assessing clot formation.[35] A single test that is able to globally evaluate primary hemostasis (platelets), secondary hemostasis, and fibrinolysis would therefore be very beneficial and TEG can serve in this capacity.

Although TEG can garner a great deal of information about a patient's coagulation system, various pre-analytical and analytical components can affect results and should be taken into account when performing these assays. Similar to platelet function assays, TEG is known to be affected by several factors that can influence the results of the tracings. The main factors that are reported to affect TEG variables and potential interpretation include the hematocrit, platelet count, and fibrinogen concentration.[33] Additionally, blood samples for TEG should be collected in citrated tubes, can be performed with an activator such as kaolin or tissue factor (TF) or without an activator (native), and can be performed on samples such as whole blood or plasma. However,

it is recommended to use an activator such as TF or kaolin rather than performing native samples since native TEG results can be more variable.[23,32] For assessing aspects of clot breakdown or fibrinolysis, certain TEG variables can be assessed in the absence or presence of tissue plasminogen activator (tPA).[33] The various components of the TEG output can be evaluated to determine if there is evidence of hypocoagulability, hypercoagulability, hypofibrinolysis, hyperfibrinolysis, or normal test results. However, there is no consensus for what TEG variables should be used to define these situations (e.g. hypercoagulability) so most investigators define this for each respective study.

TEG has been evaluated in many different studies and diseases in human medicine and similarly, studies involving TEG in veterinary medicine have also recently dramatically increased. TEG has been investigated in dogs in a variety of scenarios that range from diseases associated with hypercoagulability such as hyperadrenocorticism[36,37,38,39,40], immune-mediated hemolytic anemia (IMHA)[41,42,43], and pulmonary thromboembolism (PTE)[44] to bleeding conditions such as acute traumatic coagulopathy (ATC).[45,46,47] However, few studies have investigated viscoelastic testing such as TEG in dogs with infectious diseases and none have been previously performed in dogs infected with *Ehrlichia canis*.

The infectious diseases that have been previously investigated via viscoelastic testing in dogs include parvovirus enteritis, *Babesia rossi, Spirocerca lupi*, *Leishmania infantum*, *Angiostrongylus vasorum*, and leptospirosis respectively.[48,49,50,51,52,53,54] ROTEM was performed in the studies involving *L. infantum* and leptospirosis and in one of the *A. vasorum* studies but TEG was performed in all of the other previously mentioned studies. There are many similarities between TEG and ROTEM but both represent ways to further assess a patient's coagulation system beyond traditional testing. The study that was performed in dogs affected by parvoviral enteritis

was pursued due to the observation that dogs affected by this virus were prone to thrombosis and phlebitis associated with catheters and thus were suspected to be hypercoagulable. Native TEG was performed in healthy dogs and parvovirus infected dogs and revealed that all of the dogs appeared hypercoagulable and several of these dogs had a venous thrombosis or phlebitis complication.[48] Another study performed in dogs infected with *S. lupi* found that most infected dogs were hypercoagulable (via TF-activated TEG) as compared to healthy dogs and also that a higher degree of hypercoagulability (as defined by a specific parameter in the study) was associated with neoplastic transformation of the associated lesions.[50] In contrast, dogs with leptospirosis and *A. vasorum* can have bleeding tendencies and the mechanisms for clinical bleeding are not fully understood. Two of the most relevant studies in dogs infected with *A. vasorum* showed that dogs appeared hypocoagulable via TEG[52] and ROTEM[54]. Additionally, the most recent study in dogs naturally infected with *A. vasorum* also showed evidence of hyperfibrinolysis (via ROTEM) in addition to probable consumptive hypofibrinogenemia. These abnormalities resolved following administration of plasma and an anti-fibrinolytic drug thus demonstrating that the significant bleeding in these dogs is likely related to hyperfibrinolysis which viscoelastic testing was able to characterize.[54] With regards to leptospirosis, some dogs appeared hypercoagulable, hypocoagulable, or normal (via ROTEM) but dogs that appeared hypocoagulable had more bleeding manifestations and had a higher mortality rate.[53]

The study in dogs infected with *L. infantum* was evaluated using ROTEM and revealed that although symptomatic dogs had some differences in ROTEM results between asymptomatic and healthy dogs, these changes were still within their reference interval. Therefore, this study did not demonstrate large abnormalities in the coagulation system in dogs infected with *L. infantum*.[51] Another study using native TEG was pursued in dogs infected with *B. rossi* since some dogs do

not exhibit clinical evidence of bleeding despite significant thrombocytopenia. This study showed that infected dogs overall had normal TEG tracings but they commented that this could have been due to a cumulative effect from the derangements seen in hematocrit, platelet count, and fibrinogen in these dogs. Alternatively, they postulated that the normal TEG tracings could have been observed due to activated platelets binding large amount of fibrinogen resulting in stronger clots and that is potentially why no bleeding is observed in these dogs despite severe thrombocytopenia.[49]

The aforementioned studies demonstrate how viscoelastic testing can be utilized in veterinary medicine and research in clinically ill dogs however like platelet function assays, it is important to first determine how these assays perform in healthy dogs. As mentioned in the platelet function testing section, it is important to understand the components of variability for TEG analyses such as inter- and intra-assay variability (related to analytical variation) and inter- and intra-individual variability (related to biological variation).[22,23,24,25] Again, for appropriate interpretation of results from TEG testing, knowledge of both analytical and biological variation is necessary. Some studies have already previously evaluated analytical and biological variation of TEG testing in healthy dogs.[23,55,56,57] These studies have evaluated the analytical variation for TF-activated and kaolin-activated TEG in dogs using plasma or whole blood and found the degree of variation to be acceptable.[23,55,56] Furthermore, the biological variation for native TEG (using whole blood) and TF-activated TEG (using plasma) has also been previously evaluated in healthy dogs.[23,57] However, the biological variation for TF-activated TEG using fresh citrated whole blood in healthy dogs has not been previously evaluated. This is important to determine since according to the recent guidelines for TEG analysis in dogs, TF-activated citrated blood samples are one of the recommended samples for clinical use and interpretation.[31] Therefore, before additional

studies are pursued in clinically ill dogs such as dogs infected with *E. canis*, the biological variation in healthy dogs should be determined to allow for accurate interpretation of results that could lead to clinically meaningful findings.

1.2 Ehrlichia canis and its effect on hemostasis

Hemostasis was often only typically discussed as being divided into two major components; primary and secondary hemostasis with secondary hemostasis being split into three easily digestible arms (intrinsic, extrinsic, and common). However, as with most things in life, the topic of hemostasis became more complicated with the cell-based model and it is now well known that the cells and components of hemostasis are also often directly involved with inflammation and immunity. These interactions are complex and occur during health and disease.[58,59,60] Different diseases have varying effects on these processes which often result in the characteristic clinical signs and presentations of the particular disease.

When considering the effects of certain vector-borne diseases on hemostasis, canine monocytotropic ehrlichiosis caused by *E. canis* is of particular interest not only due to its widespread distribution and potential ability to cause fatal infections in the dog but also because of the different hemostatic derangements that can occur. The clinical course of *E. canis* infection in dogs has been described extensively in multiple natural and experimental studies. In dogs, *E. canis* has been reported to cause hematologic abnormalities such as anemia, thrombocytopenia, leukopenia, lymphocytosis, and hyperglobulinemia in addition to bleeding tendencies.[17,61,62,63] The bleeding manifestations that are observed in dogs typically involve mucosal surface bleeding (petechiation, ecchymoses) but bleeding can occur from multiple sites and can present as epistaxis, melena, hematemesis, hyphema, prolonged bleeding from surgical sites, etc.[17,64,65,66,67] The cause of bleeding is thought to be related to multiple processes such as bleeding from significant

thrombocytopenia, platelet dysfunction, vasculitis, and hyperviscosity syndrome.[16,17,18,19,20,61,62,68,69] The severe thrombocytopenia that can be observed is thought to be related to non-immune (consumption at sites of vasculitis, decreased production from the bone marrow) and immune (increased clearance by splenic macrophages, anti-platelet antibodies) processes.[16,17,61,62,70,71,72,73,74,75,76,77] Although it is considered much more common for dogs with ehrlichiosis to show signs of bleeding, thrombotic complications have also been documented. Reports of thrombotic complications in this disease are scarce but include portal vein thrombosis, aortic thrombosis, and splenic thrombi.[78,79] The various sequelae of *E. canis* infections in dogs demonstrate a close relationship with components of hemostasis.

Although it is clear *E. canis* has dramatic effects on hemostasis, the underlying mechanisms have still not been elucidated. It is suspected that this is due to how complicated the various interactions are because cells of hemostasis, inflammation, and immunity are all likely involved. For example, factors that have been previously shown to influence the resultant manifestations of ehrlichiosis infections include how the host responds to infection and the type of *E. canis* strain causing infection. Specifically, the type of immune response (cellular immunity versus humoral immunity) and characteristic cytokine/chemokine profiles have been shown to affect the clinical course of this infection.[17,80,81,82,83,84,85] In addition, different *E. canis* strains exist and some appear to be more virulent than others which results in more dramatic clinical signs in dogs.[18,68,86,87]

Ehrlichia canis has also been shown to cause a significant inflammatory response in dogs that can be documented not only by evaluating cytokine inflammatory profiles but by evaluating certain acute phase proteins such as C-reactive protein or haptoglobin.[88,89] Additionally, ehrlichiosis caused by *E. canis* in dogs is associated with disruption of the endothelium. This appears to occur via a variety of mechanisms particularly via infiltration of the subendothelial layer

and perivascular region of multiple organs with lymphocytes, monocytes/macrophages, and plasma cells.[90,91] Of additional importance is that *E. canis* infects monocytes/macrophages which are the major components of the reticulo-endothelial system that are constantly interacting in health and disease. Therefore, this allows *E. canis*-infected cells to directly and deleteriously interact with the endothelial layer of many organs in the body.[90,91] It could also theorized that the aforementioned inflammation that occurs with ehrlichiosis could also lead to disruption of endothelial cell-to-cell junctions.[59]

Therefore, canine monocytotropic ehrlichiosis appears to be intimately involved with vital elements of hemostasis such as platelets and the endothelium. The infection is also typically quite inflammatory and inflammation has additional effects on platelets and endothelium but also on monocytes/macrophages, neutrophils, procoagulant/anticoagulant proteins, and fibrinolytic/anti-fibrinolytic proteins.[59,60] As a result, ehrlichiosis has the potential to cause bleeding or aberrant clotting tendencies in infected dogs. As previously mentioned, bleeding diatheses are more commonly reported in dogs whereas clotting complications are rarely reported. However, an additional intriguing finding clinically is that some dogs exhibit no overt signs of bleeding despite significant thrombocytopenia.

The overall characteristics of hemostasis during an *E. canis* infection in dogs (natural or experimental) have never been evaluated globally. Various components have been individually investigated but a study focusing on the multiple hemostatic constituents, how they change during infection, and how these changes could explain clinical presentations (e.g presence or absence of bleeding) has not been pursued. Investigating these changes could help to explain why clinical signs of bleeding do not always occur despite severe thrombocytopenia in dogs. For example, another vector-borne disease that can be associated with severe thrombocytopenia yet lack clinical

signs of bleeding includes *B. rossi* infections in dogs. Two previous studies have evaluated why this might occur with one study evaluating platelet indices[15] and another study evaluating dynamic viscoelastic testing (via TEG).[49] The results of both of these studies indicated that activated platelets could be the reason for the lack of bleeding and discussed how activated platelets have been shown to bind more fibrinogen thus resulting in stronger clots.[15,49] Interestingly, the TEG analyses from dogs with *B. rossi* were overall normal whereas TEG analyses from dogs with *A. vasorum* revealed that dogs are actually hypocoagulable and hyperfibrinolytic which was hypothesized to be why bleeding was observed in this type of infection.[52,54] In one of these studies[54] it was proposed that the hyperfibrinolysis could be related to release of plasminogen activators from the endothelium due to inflammation. As mentioned previously, *E. canis* also causes endothelial inflammation so it is possible that this abnormality could also be present during *E. canis* infections but this has never been evaluated. Platelet indices of activation and dynamic viscoelastic testing thus represent a way to potentially investigate why bleeding is not seen despite severe thrombocytopenia in dogs infected with *E. canis*. However as previously mentioned, traditional parameters of coagulation and fibrinolysis (e.g. clotting times, fibrinogen, D-dimers, etc.) should also be evaluated when performing viscoelastic testing to better understand changes seen in resultant tracings.[33] In addition, concurrently evaluating platelet function and presence of anti-platelet antibodies (which have been theorized to disrupt platelet function)[19] would also be important to more fully describe hemostatic changes during ehrlichiosis. Therefore, possible avenues to explore the clinical question of why bleeding does not always occur in *E. canis*-infected dogs despite severe thrombocytopenia include platelet indices of activation, measurement of traditional coagulation parameters, acute phase protein measurements, flow-cytometric analysis, platelet function testing, and dynamic viscoelastic testing.

1.3 Detection and dynamics of anti-platelet antibodies in thrombocytopenic dogs

Thrombocytopenia is one of the most common abnormalities observed in dogs and can be due to a variety of processes; immune and non-immune.[92] The main mechanisms of thrombocytopenia include decreased or lack of production, increased destruction or consumptive processes, abnormal loss, and sequestration. Thrombocytopenia can occur as a result of one of these mechanisms or a combination due to various infectious, neoplastic, inflammatory, toxic, and immune-mediated conditions.[93] As a result, determining the underlying etiology for thrombocytopenia in dogs can be challenging. However, knowing if the observed thrombocytopenia is associated with an immune process can help guide diagnostic work-ups and overall management of a patient. This can be accomplished by evaluating a patient for the presence of immunoglobulin-associated platelets where if these are detected, a potential immune-mediated process is likely contributing to the thrombocytopenia.[92,94] The detection of anti-platelet antibodies in dogs has been previously assessed by various techniques such as ELISA, immunofluorescence, immunoradiometric methods, and flow cytometry but due to various factors (e.g. large sample of blood volume required, tedious assay technique, and restricted time frames to perform the assay), none of these tests are routinely used in veterinary practice.[95,96,97,98] Therefore, an assay that requires a small amount of blood, is relatively easy to perform, and can be performed on stored samples would be of great benefit to veterinary patients. A potential candidate test for this purpose is flow cytometry done by either direct or indirect methods. Direct methods involve evaluating a patient's platelets for the presence of anti-platelet antibodies whereas indirect methods involve incubating a patient's sera with a healthy dog's platelets and evaluating for the presence of anti-platelet antibodies.[92,70]

The indirect method seems very attractive as a potential assay since sera can be evaluated as a fresh sample or after being stored for a period of time however it has been shown by some studies to be less sensitive[70,98] than the direct method while another study showed it to be more sensitive.[99] In human medicine, indirect flow cytometric are considered less sensitive and direct methods are preferred.[100] Possible reasons for the decreased sensitivity of the indirect method compared to the direct has been hypothesized to be related to the fact that few antibodies are in circulation in the plasma or sera because they are tightly associated with platelets and as a result are not detected by the assay.[70,98] With regards to the direct method, most studies have shown it to have good sensitivity and high specificity[70,98,99] but the main problem with this method in veterinary medicine is that fresh samples have historically been required for analysis. This was previously investigated and had contradictory results. One author advocated that samples should not be analyzed beyond 24 hours of storage due to increased binding and possible false positive results[94,101,102] whereas another author showed that samples could be evaluated up to 72 hours when samples were stored on ice.[98] If samples for direct flow cytometry could be stored for longer periods than 24 hours, this could potentially address the concerns of decreased sensitivity using indirect methods and also alleviate the time constrictions currently associated with direct flow cytometric assays in veterinary medicine. Future studies should be performed in dogs to develop a clinically useful direct and indirect flow cytometric assay for detection of anti-platelet antibodies.

If thrombocytopenia is subsequent to an immune process, this can be due to primary/idiopathic immune-mediated thrombocytopenia (IMTP) where no underlying cause is found or secondary IMTP where something triggered immune destruction of platelets.[93] For example, anti-platelet antibodies have been detected in various disease states in dogs ranging from idiopathic canine IMTP to various infectious diseases to different types of neoplasia. Examples

of infections where antibodies have been detected previously in dogs include *Ehrlichia canis*, *Rickettsia rickettsii, Dirofilaria immitis, Anaplasma* spp., *Leishmania infantum, Babesia* spp., and *Leptospira* spp.[71,72,73,74,75,76,77] Other types of diseases where antibodies have been detected include systemic lupus erythematosus (SLE), immune-mediated hemolytic anemia (IMHA), myelodysplasia, disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome (SIRS), chronic hepatitis, and acute pancreatitis.[96,70,76] The main types of neoplasia where antibodies have been documented include lymphoma, histiocytic neoplasms, and hemangiosarcoma.[73,76,96] As a result, the detection of anti-platelet antibodies does not indicate that a patient has idiopathic IMTP as they can form in a variety of other diseases. At this time, there is no assay available in veterinary medicine or human medicine for that matter that is able to definitively different primary IMTP from secondary IMTP.[99,103]

As mentioned above, antibodies have been detected and documented in dogs diagnosed with idiopathic IMTP but no studies have been performed to investigate the dynamics of these antibodies over time. In human medicine, anti-platelet antibodies or platelet auto-antibodies are not detected in every person with primary immune thrombocytopenia (ITP) and is reportedly only detected in about 60-70% of patients.[104,105] The reasons for this are still being investigated but possibilities include that primary ITP in people can result not only from auto-antibody formation but also can occur as a result of direct T-cell destruction and platelet desialylation.[1,104,105,106,107,108,109,110,111,112] It is unknown if these mechanisms occur in dogs with naturally occurring IMTP. Future studies evaluating thrombocytopenic dogs for the presence of anti-platelet antibodies and their final diagnosis and response to therapy should be performed.

Although it is unknown if these mechanisms occur in dogs, similar to human ITP, canine IMTP appears to be predominantly related to the presence of anti-platelet antibodies.[99] Therefore,

determining the dynamics of these antibodies during the course of disease could be of prognostic and therapeutic benefit. Recently in human medicine, there has been a call for well-designed prospective studies to investigate these auto-antibodies for that exact purpose.[112] There have been a handful of studies performed in humans to evaluate this but they have shown contradictory results.[113] However, more recent studies have shown that the dynamics (i.e. presence or absence, increase or decrease) of platelet auto-antibodies can be related to the disease severity, disease recurrence/relapse, and response to different medications.[112,113,114,115] For example, one study found that patients who had antibodies tended to have more severe disease characterized by chronic ITP, more severe signs of hemorrhage, and a more positive response to immunosuppressive drug therapy than a splenectomy.[113] Another study showed that people with antibodies also demonstrated a higher risk of recurrence of ITP (72%) as compared to people who lacked antibodies (32%).[114] Furthermore, another study showed that in people who lacked antibodies, there was a lack of response to rituximab. The mechanism of rituximab in humans with ITP is still being studied but it is thought to primarily work through B cell depletion which would theoretically inhibit platelet auto-antibody production. However, some people do not seem to respond to this therapy and it's theorized that if antibodies are not detected in a person with ITP, then rituximab may not be the best therapeutic option.[115] Another group investigated this as well and found that people responded to rituximab similarly whether they had auto-antibodies or not (it should be noted they were also receiving other standard therapy for ITP) but did however find that if auto-antibodies remained following treatment, this appeared to again be associated with more severe disease.[112]

The dynamics of anti-platelet antibodies over the course of disease and treatment have never been investigated in dogs with primary IMTP. It is unknown if some dogs with primary

IMTP demonstrate persistence of antibodies over time and whether this correlates to response to treatment or risk of relapse. Additionally, it is unknown in human and veterinary medicine if the presence of antibodies would help predict a patient's response during the tapering of medications. Canine primary IMTP is traditionally monitored through serial evaluation of complete blood counts, specifically the platelet count but it is unknown if concurrent measurement of anti-platelet antibodies would help enhance patient management. Therefore, serial monitoring of anti-platelet antibodies should be pursued in dogs diagnosed with primary IMTP to evaluate the possible clinical implications involving prognosis, response to therapy, and risk of recurrence/relapse.

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Chapter 2: Research Overview and Specific Aims

2.1 Research Overview

Vector-borne diseases frequently occur in humans and animals resulting in morbidity and mortality worldwide. *Ehrlichia canis* is one of the most common vector-borne diseases to affect dogs, can present in various phases, and is well-known to have clinically relevant effects on hemostasis; the mechanisms for which are not completely understood. Helping to elucidate the global effects on hemostasis during canine ehrlichiosis could help to determine how best to treat and manage patients with hemostatic abnormalities. Thrombocytopenia is commonly observed in canine ehrlichiosis in addition to multiple other disease states (e.g. other infectious causes, primary immune-mediated, neoplastic, etc.) and can result in anti-platelet antibodies. The detection and dynamics of such antibodies in dogs could enhance understanding of disease pathophysiology not only in canine ehrlichiosis but also in dogs affected by idiopathic immune-mediated thrombocytopenia. Therefore, the goals of the research described in this dissertation include to investigate the global effects of *Ehrlichia canis* on the hemostatic system in dogs to understand various clinical manifestations, to detect anti-platelet antibodies in dogs affected by thrombocytopenia, and to evaluate the dynamics of these antibodies over time. However, in order to properly study coagulation in canine ehrlichiosis and anti-platelet antibodies in dogs, assays must be validated and developed for this particular research. As a result, Chapter 3 involves investigating the stability of platelet reagents after prolonged storage for use in studying platelet function using whole blood impedance platelet aggregometry. Chapter 4 details the validation of specialized coagulation testing (thromboelastography) and platelet function testing (whole blood impedance platelet aggregometry) in healthy dogs so that results can be evaluated in ill dogs such

as dogs affected by canine ehrlichiosis. Chapter 5 involves the validation and development of a flow cytometric assay for the detection of anti-platelet antibodies in dogs. Chapter 6 explores the detection of anti-platelet antibodies in thrombocytopenic dogs in addition to the dynamics of such antibodies in dogs diagnosed with primary immune-mediated thrombocytopenia.

2.2 Specific Aim 1 (Chapter 3: Reagent stability for platelet aggregometry)

Platelet function can be assessed in dogs by several different techniques including via whole blood impedance platelet aggregometry. As the name would infer, this assay is performed with whole blood and the magnitude of electrical impedance generates results of interest for interpretation.[1] Different platelet agonists can be used for this test which stimulate platelets to aggregate and response to these agonists correlate with platelet function. Examples of agonists frequently used in veterinary medicine include adenosine diphosphate (ADP) and arachidonic acid (AA).[1-9] When using these agonists in whole blood impedance platelet aggregometry, the agonists must be reconstituted and can be stored in aliquots at 2-8°C for 24 hours or ≤ -20°C for 4 weeks (Multiplate 5.0 Analyzer, Diapharma Group Inc). This testing method can be cost prohibitive but if the reagents could be stored for a prolonged time yet still retain their stability, this could greatly decrease costs and allow more studies to be pursued utilizing this testing modality. Therefore, the specific aim of this chapter was to investigate the stability of the platelet agonist reagents, ADP and AA after prolonged storage in two different conditions for up to 6 months for use in whole blood impedance platelet aggregometry in dogs. This was investigated by freezing aliquots of reconstituted ADP and AA at -20°C and -80°C monthly for six months and comparing platelet aggregometry results to fresh reagents using whole blood from healthy dogs.

2.3 Specific Aim 2 (Chapter 4: Thromboelastography and platelet aggregometry validation)

Thromboelastography (TEG) and whole blood impedance platelet aggregometry are considered specialized tests for the assessment of the coagulation system because they go beyond traditional tests such as clotting times and the buccal mucosal bleeding time. TEG is a method used to evaluate the viscoelastic properties of hemostasis (clot formation and breakdown) whereas as mentioned in section 2.2, whole blood impedance platelet aggregometry is utilized to evaluate platelet function[1,10] Although both of these testing modalities can generate a wealth of data, knowing how to interpret said data is of paramount importance. Every test has variability associated with it and this variability is key to knowing what differences in testing results could equate to a clinically meaningful difference. For example, if a test has a large degree of variability within an individual tested serially over time, then large ranges can be established to account for such variability. Alternatively, if a test has a very small degree of variability within an individual serially tested over time, then small differences between measurements could be more impactful. There are different types of variability when describing tests, one of which include biological variation. Biological variation includes intra-individual variability (CV_I) and inter-individual variability (CV_G) which describe the differences in results within an individual and the differences in results between individuals respectively.[11] These factors in addition to the index of individuality (defined as CV_I/CV_G) help determine if subject-based or population based references intervals should be utilized.[11,12] This information is important to determine initially in heathy individuals before it is applied to sick individuals but is also required to define how results in healthy or sick individuals are interpreted. Although TEG and whole blood impedance platelet aggregometry have been previously investigated in multiple veterinary studies, the CV_I, CV_G, and index of individuality have not been determined in healthy dogs using reagents (i.e. activators such as tissue

factor) and specific testing parameters (i.e. heparinized blood samples) currently recommended in veterinary medicine.[1,2,4,13] Therefore, before TEG and whole blood impedance platelet aggregometry are evaluated for studies in this dissertation, validation of the assays must be performed. Consequently, the specific aim of this chapter was to assess the CV_G, CV_I, and index of individuality over time for tissue factor (TF)-activated TEG and heparinized whole blood impedance platelet aggregometry variables in healthy dogs. This was investigated by performing TF-activated TEG and whole blood impedance platelet aggregometry in healthy dogs at three different time points (day 1, day 4, day 6) to determine specific parameters of assay variability.

2.4 Specific Aim 3 (Chapter 5: Coagulation evaluation in experimental E. canis *infection)*

It is well known that dogs infected with *Ehrlichia canis* can present with a variety of presentations including manifestations of bleeding. Additionally, blood work abnormalities such as thrombocytopenia can commonly accompany each phase of infection; acute, subclinical, or chronic.[14,15] The underlying mechanisms for bleeding and thrombocytopenia are not completely understood but are theorized to involve immune and non-immune processes on platelets in addition to vasculitides.[16] Of particular interest however is the clinical conundrum of why certain dogs infected with *E. canis* do not exhibit bleeding phenotypes despite having severe thrombocytopenia.[14] A prospective study investigating the global effects of canine ehrlichiosis on hemostasis using traditional and specialized testing such as TEG and platelet aggregometry has never been pursued. Elucidating and describing these effects could help veterinarians better understand the potential clinical manifestations of this disease in dogs. Therefore, the aim of this chapter was to assess platelet indices of activation, platelet function as assessed by whole blood impedance platelet aggregometry, percentage of anti-platelet antibodies (percent IgG), and TEG measurements including velocity curve (Vcurve) variables in dogs experimentally infected with

E. canis. This was investigated by evaluating traditional and specialized aspects of hemostasis at baseline and once weekly in *E. canis* experimentally infected dogs serially over time for a total of eight weeks.

2.5 Specific Aim 4 (Chapter 6: Flow cytometry validation)

Thrombocytopenia is not only a frequent finding in dogs infected with *E. canis* but it is also one of the most common blood work abnormalities observed in dogs.[17] Thrombocytopenia can result due to a variety of processes including immune-mediated processes which can be due to primary or secondary causes. Primary causes refer to where no underlying etiology can be determined yet there appears to be immune-mediated destruction of platelets whereas secondary causes refer to where something extraneous triggered immune destruction of platelets. Examples of such triggers include infections (such as *E. canis*), neoplasms, drugs, etc.[18] Consequently, determining the underlying cause of thrombocytopenia in dogs can be challenging. However, if it is determined that an immune process could be contributing to the observed thrombocytopenia, this can help guide diagnostic steps and possible treatment options. This can be accomplished by evaluating for the presence of anti-platelet antibodies. The detection of these antibodies in dogs has been previously assessed by several different methods including ELISA, immunofluorescence, immunoradiometric methods, and flow cytometry.[19-22] Although these various methods have some potential for use in veterinary medicine, they are not frequently utilized. The reasons for this include that large volumes of blood are required for certain assays, the assays can be tedious to perform, and there are restricted time frames for when samples can be processed.[19-22] If an assay for detection of anti-platelet antibodies in dogs could be developed that requires a small amount of blood, is relatively easy to perform, and can be performed on stored samples, this would be of great benefit to veterinarians. A potential candidate assay that could meet these requirements is

flow cytometry. Therefore, the aim of this chapter was to develop a clinically useful flow cytometric assay for the detection of anti-platelet antibodies in dogs. This was investigated by performing direct flow cytometry in healthy and thrombocytopenic dogs at different times points (baseline, 24 hours, 48 hours, 72 hours) when stored at 4°C and also by performing direct and indirect flow cytometry in healthy and thrombocytopenic dogs and comparing the results.

2.6 Specific Aim 5 (Chapter 7: Flow cytometry in thrombocytopenic dogs)

As mentioned in section 2.5, thrombocytopenia is a common abnormality observed in dogs and can be related to immune and non-immune processes.[17,18] If thrombocytopenia in dogs occurs due to an immune-mediated process, this is similar to immune thrombocytopenia (ITP) that occurs in people which is purported to be primarily mediated by platelet autoantibodies.[23,24] Anti-platelet antibodies have been detected in a variety of disease states in dogs including ehrlichiosis, Rocky Mountain Spotted Fever (RMSF), dirofilariasis, anaplasmosis, leishmaniasis, babesiosis, leptospirosis, systemic lupus erythematosus (SLE), immune-mediated hemolytic anemia (IMHA), myelodysplasia, disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome, chronic hepatitis, acute pancreatitis, lymphoma, histiocytic neoplasms, and hemangiosarcoma.[19,26-32] Anti-platelet antibodies can therefore be detected in many different scenarios in dogs. Previous studies in veterinary medicine have focused on detecting these antibodies rather than investigating how they might change over time in diseases such as canine idiopathic immune-mediated thrombocytopenia (IMTP). In humans, the dynamics of these antibodies has been shown to be related to factors such as prognosis, risk of recurrence/relapse, and response to different treatments.[33-36] These types of studies have never been evaluated in veterinary medicine. Therefore, the aim of this chapter was to evaluate thrombocytopenic dogs (due to various causes) for the presence of anti-platelet antibodies and characterize the dynamics

of these antibodies over time in dogs diagnosed with idiopathic IMTP and how they relate to prognosis, risk of recurrence/relapse, and response to therapy. This was investigated by performing direct flow cytometry in thrombocytopenic dogs due to any cause and by performing direct flow cytometry serially in dogs diagnosed with primary IMTP.

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Chapter 3: Effects of storage conditions on two platelet agonists for whole blood impedance platelet aggregometry in dogs

I would like to thank Dr. Christine Olver for helping to organize this study and for allowing me to enroll her own personal dog after we had to exclude another potential study dog.

3.1 Overview

Background: Whole blood impedance platelet aggregometry can be performed with different agonists to evaluate platelet function. Although the manufacturer recommends disposal of stored reagent after 1 month at -20ºC or 24 hours at 4ºC, reagent integrity after reconstitution under different storage conditions is unknown. If reagent integrity is stable for longer periods, assay costs could decrease dramatically.

Objectives: Determine stability of reconstituted arachidonic acid (AA) and adenosine diphosphate (ADP) platelet agonists stored in -20ºC and -80ºC for up to 6 months.

Methods: Aliquots of reconstituted AA and ADP were stored at -20°C and -80°C monthly for six months. Six healthy staff-owned dogs were enrolled for the study. A physical examination, CBC, diagnostic panel, urinalysis, and baseline platelet aggregometry were performed in all dogs. Platelet aggregometry was performed on all dogs using fresh and stored aliquots of AA and ADP reagents on the same day. Area under the curve (AUC) was recorded from each platelet aggregometry analysis. Repeated measures (RM) analysis (one-way ANOVA) was performed and subsequent time points (1, 2, 3, 4, 5, 6 months) were compared to fresh AUC results.

Results: All dogs were clinically healthy and all diagnostic tests were normal. There were no differences in AUC obtained from fresh samples at any time point or at either temperature for AA or ADP.

Conclusions: Whole blood impedance platelet aggregometry reagents AA and ADP are stable for up to six months when stored at -20°C or -80°C, obviating the need to discard viable reagents, and decreasing assay cost.

3.2 Introduction

Whole blood impedance platelet aggregometry is a specialized assay that can be used to assess platelet function in dogs.[1] Over the past several years, veterinary studies in dogs have utilized this assay and it being used clinically with increasing frequency.[1-8] This method utilizes anti-coagulated whole blood and platelet agonists that stimulate platelets to aggregate. Platelet aggregation within the electrode-containing test cell result in electrical impedance which is recorded as several output measurements. The area under the curve (AUC) is the standardly reported measurement that describes platelet response to different agonists.[1] Arachidonic acid (AA) and adenosine diphosphate (ADP) are two agonists that are used frequently in veterinary medicine.[1-9] To perform the assay, reagents are reconstituted according to the manufacturer's recommendations (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA) and can be stored in aliquots at 2-8°C for 24 hours or ≤ -20°C for 4 weeks. The costs associated with the test cells and reagents needed for whole blood impedance platelet aggregometry can be substantial and could limit future use in veterinary medicine. It would be desirable if platelet reagents such as AA and ADP could be stored for prolonged periods of time beyond 4 weeks and retain viability but to our knowledge, this has not been previously investigated. Therefore, the purpose of this study was to determine the stability of reconstituted arachidonic acid (AA) and adenosine diphosphate (ADP) platelet agonists stored in two different conditions for up to 6 months for use in whole blood impedance platelet aggregometry in dogs.

3.3 Materials and Methods

Six staff-owned healthy dogs were enrolled for the study. The study was approved by the Institutional Animal Care and Use Committee at Colorado State University and all owners signed a client-consent form. Exclusion criteria for the study included documentation of any concurrent morbidities; abnormal blood work, urinalysis, or baseline platelet aggregometry; or aspirin, clopidogrel, non-steroidal anti-inflammatories (NSAIDs), or fish oil administration. Prior to the study initiation, a total of 6 mls of anti-coagulated blood and 5 mls of voided urine respectively were collected to perform a complete blood count (Siemens, ADVIA 120 Hematology System, Erlangen, Germany), diagnostic profile (Roche Cobas C501, Diamond Diagnostics, Holliston, MA), urinalysis, and platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA) to ensure each dog was healthy.

The platelet agonist reagents, AA (Diapharma Group Inc., Wester Chester, OH, USA) and ADP (Diapharma Group Inc., Wester Chester, OH, USA) were reconstituted according to the manufacturer's recommendations (1.0 ml of high purity deionized water added to powdered reagent). Once reconstituted, 60 µl aliquots of the respective reagent were stored at -20°C and -80°C monthly for six months. Platelet aggregometry was then performed on all dogs using fresh and stored aliquots of AA and ADP reagents on the same day. On the day of the study, 13 mls of heparinized anti-coagulated blood was collected from each dog in order to perform platelet aggregometry. Briefly, for the multiple channel electrical impedance platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA) heparinized blood samples were kept at room temperature and analyzed within 45 minutes of blood collection (range, 5-45 minutes) for 12 minutes. Before each analysis, the test cells were warmed to 37°C within the aggregometer and samples were analyzed using an automated pipette. Heparinized blood samples

(300 µl) were diluted with 300 µl of diluent and incubated for 3 minutes as recommended by the manufacturer. Platelet reagents (20 µl), ADP and AA were added to the respective test cells and AUC for ADP and for AA were recorded. As recommended by the manufacturer, the final concentration of ADP was 6.5 µM and the final concentration of AA was 0.5 mM.

For statistical analysis, the AUC was recorded from each platelet aggregometry analysis (fresh, 1, 2, 3, 4, 5, and 6 months). Repeated measures (RM) analysis (one-way ANOVA) was performed and subsequent time points (1, 2, 3, 4, 5, 6 months) were compared to fresh AUC results.

3.4 Results

All dogs were healthy based on normal physical examinations, blood work, and urinalyses and none were receiving concurrent medications other than monthly preventatives. Additionally, all dogs had baseline AUC platelet aggregometry measurements within the normal reference ranges previously established at our institution. The age range of the dogs was 3-11 years and the weight range was 13.6-34.7 kgs. The breeds included one beagle and one Cardigan Welsh Corgi while the other four dogs were mix breeds (Saint Bernard/pit bull, border collie/Corgi, Labrador mix, and shepherd mix).

The AUC measurements from each dog for AA fresh and when stored monthly for up to 6 months at -20°C ranged from 117-287 units (Table 3.1). The AUC measurements from each dog for AA fresh and when stored monthly for up to 6 months at -80°C ranged from 171-292 units (Table 3.2). The AUC measurements from each dog for ADP fresh and when stored monthly for up to 6 months at -20°C ranged from 146-339 units (Table 3.3). The AUC measurements from each dog for ADP fresh and when stored monthly for up to 6 months at -80°C ranged from 157-

337 units (Table 3.4). There were no statistical differences in the AUC obtained from fresh samples at any time point or at either temperature for AA or ADP (Figure 3.1).

Table 3.1: Area under the curve (AUC) measurements in healthy dogs for the platelet reagent arachidonic acid (AA) fresh and when stored at -20°C for up to six months for use in whole blood impedance platelet aggregometry

	Fresh	1 month	2 month	3 month	4 month	5 month	6 month
Dog #1	235	230	243	287	272	218	219
Dog #2	214	191	249	162	200	210	216
Dog #3	194	225	231	197	229	205	233
Dog #4	168	117	149	196	197	201	178
Dog #5	205	212	205	210	186	223	198
Dog #6	214	239	247	219	259	209	237

Table 3.2: Area under the curve (AUC) measurements in healthy dogs for the platelet reagent arachidonic acid (AA) fresh and when stored at -80°C for up to six months for use in whole blood impedance platelet aggregometry

	Fresh	1 month	2 month	3 month	4 month	5 month	6 month
Dog #1	235	230	246	292	267	290	224
Dog #2	214	202	248	197	217	208	209
Dog #3	194	262	182	204	233	207	245
Dog #4	168	129	141	196	195	171	184
Dog #5	205	215	214	209	200	216	213
Dog #6	214	229	237	235	247	243	224

Table 3.3: Area under the curve (AUC) measurements in healthy dogs for the platelet reagent adenosine diphosphate (ADP) fresh and when stored at -20°C for up to six months for use in whole blood impedance platelet aggregometry

	Fresh	1 month	2 month	3 month	4 month	5 month	6 month
Dog #1	252	215	263	328	339	251	254
Dog #2	207	243	191	155	153	181	170
Dog #3	254	266	258	220	223	217	218
Dog #4	179	176	182	183	175	158	146
Dog #5	166	166	163	170	168	160	157
Dog #6	237	249	227	221	235	222	222

Table 3.4: Area under the curve (AUC) measurements in healthy dogs for the platelet reagent adenosine diphosphate (ADP) fresh and when stored at -80°C for up to six months for use in whole blood impedance platelet aggregometry

	Fresh	1 month	2 month	3 month	4 month	5 month	6 month
Dog #1	252	278	186	296	337	329	254
Dog #2	207	248	247	165	162	177	187
Dog #3	254	236	270	220	239	200	232
Dog #4	179	179	176	166	178	175	177
Dog #5	166	177	161	163	157	172	166
Dog #6	237	240	214	236	232	219	227

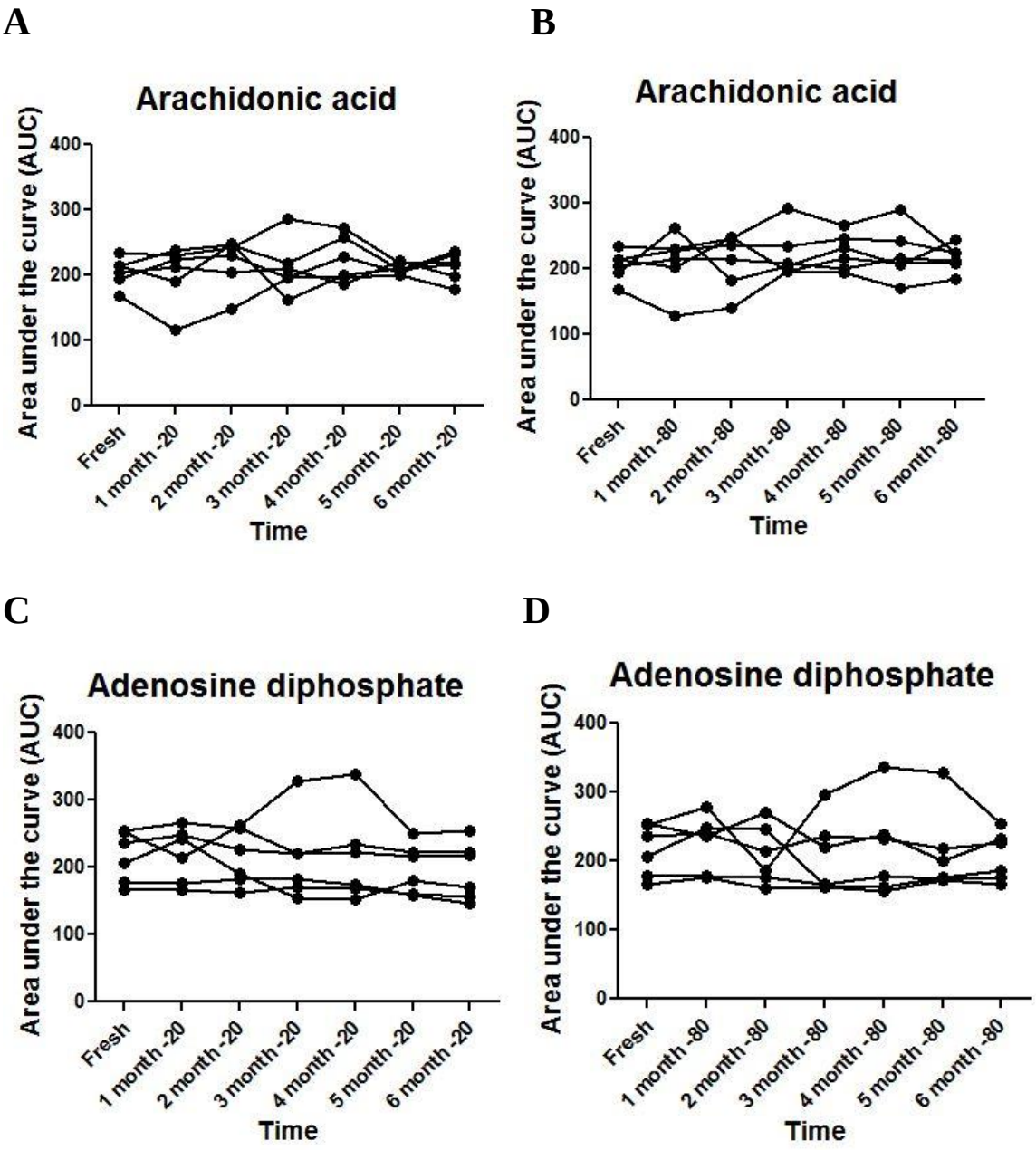

Figure 3.1: A. Area under the curve (AUC) measurements in six individual healthy dogs for the platelet reagent arachidonic acid (AA) fresh and when stored at -20°C for up to six months. **B**. AUC measurements in healthy dogs for AA fresh and when stored at -80°C for up to six months. **C**. AUC measurements in healthy dogs for the platelet reagent adenosine diphosphate (ADP) fresh and when stored at -20°C for up to six months. **D**. AUC measurements in healthy dogs for the ADP fresh and when stored at -80°C for up to six months.

3.5 Discussion

The results of the study showed that both platelet reagents, AA and ADP are viable when stored at -20°C and -80°C for up to 6 months when performing whole blood impedance platelet aggregometry in dogs. It is unknown if the reagents are viable after 6 months at either -20°C or -80°C and would require further investigation. Another commonly used agonist in veterinary medicine is collagen but it is also unknown if this reagent can be stored for prolonged periods of time so additional studies to investigate this are warranted.

In conclusion, the reconstituted reagents AA and ADP can be stored for prolonged periods of time before analysis which would dramatically decrease costs associated with performing this assay and allow for more studies to be pursued using this methodology to evaluate canine platelet function.

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Chapter 4: Inter-individual and intra-individual variability of tissue-factor activated thromboelastography and whole blood impedance platelet aggregometry in healthy dogs

I would like to thank Dr. Christine Olver for her guidance and assistance with this study and to Dr. Lappin for his mentorship in critically evaluating assays.

4.1 Overview

Objective: To assess inter-individual (CV_G) and intra-individual (CV_I) variability over time for thromboelastography (TEG) and whole blood impedance platelet aggregometry variables in healthy dogs.

Animals: Tissue factor (TF)-activated TEG and adenosine diphosphate (ADP) and arachidonic acid (AA)-induced platelet aggregometry were performed in six healthy Beagles over time.

Measurements and Main Results: TEG, platelet aggregometry, platelet count, hematocrit, and fibrinogen were recorded at three different time points (day 1, day 4, and day 6). Activated partial thromboplastin time (APTT), one-stage prothrombin time (OSPT), antithrombin activity, and D-dimer concentrations were measured on the first day of the study. For TEG, the variables reaction time (R), clotting time (K), rate of clot formation (α), and maximum amplitude (MA) were recorded. For platelet aggregometry, the area under the curve for ADP (AUC_{ADP}) and for AA (AUC_{AA}) were measured. The CV_I was lower than the CV_G over time for MA, AUC_{ADP}, and AUC_{AA} however the CV_I was higher than the CV_G for the TEG variables R, K, and α. There were no statistical differences in the platelet count, hematocrit, and fibrinogen measurements over time.

Conclusions: In healthy dogs, a subject-based reference interval for ADP- and AA-induced platelet aggregometry and the TEG variable MA provide a more sensitive method to detect relevant

changes. However, due to the high CV_I for the TEG variables R, K, and α, population-based reference intervals may be more appropriate than serial measurements in healthy dogs.

4.2 Introduction

The use of specialized assays to assess components of the coagulation system is becoming more common in veterinary medicine. Two such assays include thromboelastography (TEG) and platelet aggregometry which evaluate the viscoelastic properties of blood clotting and platelet function, respectively.[1,2] Although these assays have been used in many experimental studies, their potential for elucidating a bleeding or thrombotic phenotype in the clinical setting is still being investigated. It is well known that any test or analyte of interest can vary over time with regards to inter-individual variability (CV_G) and intra-individual variability (CV_I) in both healthy and ill individuals.[3] CV_I is used to describe differences of a variable of interest within an individual whereas CV_G is used to describe differences of a variable of interest between individuals.[3] In clinical practice, the determination of how much an analyte varies within or between individuals is important for accurate assessment of test results and optimal care of our veterinary patients. Additionally, CV_I and CV_G both reflect biological variation and are used to evaluate whether subject-based or population-based reference intervals should be utilized.[4] Unlike population-based reference intervals that include values from 95% of a healthy reference population (multiple individuals) at a single time point, subject-based reference intervals are created by measuring a parameter from the same individual over a set amount of time using the same methodology.[5] The index of individuality, a calculation which includes measures of biological (comprised of CV_I and CV_G) and analytical variation, can also be used to determine whether a subject-based or population-based reference interval should be used.[3,4] The calculation to determine analytical variation, which is a measure of the precision of a testing instrument, requires duplicate

determinations of the same analyte. Under certain testing parameters when duplicate samples are not available, it is recommended to use a simplified equation for the index of individuality (defined as CV_I/CV_G) in place of the traditional calculation. Therefore, CV_I, CV_G, and index of variability (simplified or traditional) are parameters that can be utilized in order to determine whether a subject-based or population-based reference interval should be used for an analyte of interest.[3,4]

The goal of these calculations is to determine which is more likely to identify clinically relevant abnormalities: detecting significant changes using serial evaluations or identifying a single value outside a population-based reference interval.[4] This can be approached by first determining the biological variation for the variable of interest which as mentioned above is comprised of CV_I and CV_G. If CV_G is greater than CV_I, then serial evaluation within an individual (i.e. using a subject-based reference interval) rather than single measurements (i.e. using a population-based reference interval) may provide greater sensitivity to detect clinically significant abnormalities. If the CV_I is greater than the CV_G however, then serial evaluation would not offer increased sensitivity.[4,5] For example, if a single TEG assay determines a patient to be normal based off of an established population-based reference interval, knowledge of the CV_I and CV_G of the assay are vital for appropriate interpretation of the results. If the CV_G is greater than the CV_I, then values from a hypercoagulable patient could still fall within the population-based reference interval resulting in misclassification of the patient and lack of appropriate treatment. In this case, if a baseline value (initial value) was collected and subsequent serial values were evaluated, then trends could be more meaningful and appropriate for clinical interpretation and case management. On the contrary however, if the CV_I is greater than the CV_G, than the patient is likely classified correctly based off of the established population-based reference interval. This example helps

illustrate why the CV_I and CV_G of assays such as TEG and platelet aggregometry are important to determine before accurate interpretation of results can be pursued.

Previous studies have investigated the CV_G and/or CV_I in healthy dogs for both TEG[6,7] and platelet aggregometry[8,9] however to our knowledge, the biological variation has not been assessed in healthy dogs using fresh citrated blood for tissue factor (TF)-activated TEG or heparinized whole blood for impedance platelet aggregometry. Therefore, the biological variation of TF-activated TEG using fresh citrated blood in dogs is currently unknown. This is important to determine since according to the recent guidelines for TEG analysis in dogs, TF-activated citrated blood samples are one of the recommended samples for clinical use and interpretation. TEG analysis in dogs can be performed with an activator such as kaolin or TF or without an activator (native)[10] and can be performed on samples such as whole blood or plasma.[10,6] However, it is recommended to use an activator such as TF or kaolin rather than native TEG.[10] Additionally, it is recommended that fresh citrated blood samples should be analyzed 30 minutes after blood collection rather than being held for prolonged periods (should be processed within 120 minutes[11]) or frozen for plasma assays.[10] For whole blood impedance platelet aggregometry, hirudin, heparin, and citrated blood samples can be used. However, hirudin or heparinized blood samples are recommended instead of citrated blood samples in dogs.[2,8,12] Unfortunately, the biological variation for whole blood impedance platelet aggregometry has only been previously investigated using citrated blood samples[9] so the biological variation using hirudin or heparin in dogs is also unknown. The purpose of this study was to assess the CV_G, CV_I, and index of individuality (using a simplified equation of CV_I/CV_G) over time for TF-activated TEG and heparinized whole blood impedance platelet aggregometry variables in healthy dogs. Our hypothesis was that population-based reference intervals would be appropriate to use for TF-activated TEG but that subject-based

reference intervals are more appropriate to use for heparinized whole blood impedance platelet aggregometry in healthy dogs.

4.3 Materials and Methods

This prospective study utilizing six clinically healthy beagles (Ridglan Farms, Inc.) was approved by the Institutional Animal Care and Use Committee prior to data collection. All dogs were castrated males with a weight range of 13.8-15.7 kgs and age range of 21-23 months. All dogs were housed under the same conditions, were not on any medications and had no history of previous medication administration.

The study was performed at three different time points (day 1, day 4, day 6) in all six dogs for TF-activated TEG and whole blood impedance platelet aggregometry. The dogs were fasted for 10 hours before each analysis and approximately 3.2 mls of blood from the jugular vein was drawn with a 20 gauge needle. Each blood draw was considered atraumatic with no re-direction. The appropriate volumes of blood were placed into a tube containing 3.2% sodium citrate buffered (BD Vacutainer® 3.2% sodium citrate tubes, Franklin Lakes, NJ, USA) and a heparin tube (Sarstedt lithium heparin micro tube, Numbrecht, Germany) respectively and were gently inverted five times to allow proper mixing.

For the multiple channel electrical impedance platelet aggregometry, an electrical control was performed before each analysis as recommended by the manufacturer (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA). For TEG (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation, Braintree, MA, USA), controls (Level I and Level II [Level I and II control; Haemonetic Corporation, Braintree, MA, USA]) were performed at all three time points as recommended by the manufacturer prior to

analysis of the samples. Level I and Level II are biological controls provided by the manufacturer (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation, Braintree, MA, USA) and represent normal and hypocoagulable plasma respectively. The reagents for platelet aggregometry were reconstituted according to the manufacturer's recommendations (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA) on day 1 of the study. The reagents were then stored in 60 µl aliquots at -20°C and used on day 4 and day 6 for analysis. TF was prepared before each individual TEG analysis. The heparinized blood samples for platelet aggregometry were kept at room temperature and analyzed within 29-40 minutes after blood collection for a total of 12 minutes. Before each analysis, the test cells were warmed to 37°C within the aggregometer and an automated pipette was used pipette blood into test cells to decrease pre-analytical variability. The heparinized blood samples (300 µl) were diluted with 300 µl of diluent (NaCl) and incubated for 3 minutes as recommended by the manufacturer. Adenosine diphosphate (Diapharma Group Inc., West Chester, OH, USA) (ADP) and arachidonic acid (Diapharma Group Inc., West Chester, OH, USA) (AA) were used as platelet agonists and area under the curve for ADP (AUC_{ADP}) and for AA (AUC_{AA}) were recorded. Note: the area under the curve value is an arbitrary unit. As recommended by the manufacturer, the final concentration of ADP was 6.5 µM and the final concentration of AA was 0.5 mM. To evaluate for spontaneous platelet aggregation, an identical volume of saline was used in place of the agonists. For TEG, the citrated blood samples were allowed to sit at room temperature for 30 minutes before analysis. Briefly, the cups were warmed to 37°C and 20µl of 0.2 M $CaCl_2$, 10µl of TF (Tcoag TriniCLOT™ PT Excel, Bray, Wicklow, Ireland) at a final dilution of 1:1000, and 330 µl of citrated whole blood were added to the cup and immediately analyzed. The TEG tracings were then generated for at

least 60 minutes and the variables reaction time (R), clotting time (K), rate of clot formation (α), and maximum amplitude (MA) were recorded.

On day 1 only, activated partial thromboplastin time (APTT), one-stage prothrombin time (OSPT), antithrombin activity, and D-dimer concentrations (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) were all measured in each dog from citrated blood samples. At all three time points in all dogs, the platelet count (Siemens, ADVIA 120 Hematology System, Erlangen, Germany), hematocrit (Siemens, ADVIA 120 Hematology System, Erlangen, Germany), and fibrinogen (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) (via the Clauss method) were also measured and recorded from citrated blood samples.

The statistical analysis was performed using commercially available software (R package, version 3.2.2 and GraphPad Prism 5.0, San Diego, CA, USA) For each response variable (R, K, α, MA, AUC_{ADP}, and AUC_{AA}), a one-way random effects model was used to estimate variance components corresponding to CV_G and CV_I. CV_G and CV_I were calculated by dividing their respective standard deviations by the mean of the response.[3] The index of individuality (using a simplified equation of CV_I/CV_G) was also calculated for the following variables; R, K, α, MA, AUC_{ADP}, and AUC_{AA}. Specifically, an index of < 0.6 indicates a high degree of individuality so population-based reference intervals should not be used whereas an index of > 1.4 indicates that population-based reference intervals are appropriate.[4] However, if the index is < 1.0 but not < 0.6, a population-based reference interval is not typically recommended and serial evaluations or a subject-based reference interval is preferred.[3] Normality was assessed by the Kolmogorov–Smirnov test and a repeated measures ANOVA with Bonferroni multiple comparison test was performed on the platelet count, hematocrit, and fibrinogen over the three time points with $p < 0.05$ considered significant.

4.4 Results

None of the dogs in this study had clotting times (APTT, OSPT) above the reference interval but two dogs did have OSPT and/or APTT below the lower limit of the reference interval. The mean OSPT was 7.0 seconds with a range of 6.5-7.6 seconds (reference interval 7.1-9.1 seconds) whereas the mean APTT was 10.7 seconds with a range of 8.5-11.8 seconds (reference interval 9.4-15 seconds). The antithrombin activity was normal in all dogs with a mean of 144% and ranged from 143-145% (reference interval 95-160%). The D-dimers were elevated in 5 out of the 6 dogs but fell within the ranges seen on other healthy dogs at our hospital (data not shown). The mean D-dimers was 157 ng/mL and ranged from 62.8-256 ng/mL (reference interval 15-125 ng/mL).

The platelet count, hematocrit, and fibrinogen were all determined on day 1, day 4, and day 6 of the study. The mean platelet count was 2.34 x 10^9/L (2.34 x 10^3/µL) and ranged from 1.74-3.03 x 10^9/L (2.34 x 10^3/µL) (reference interval 2.0-5.0 x 10^9/L [x 10^3/µL]). The samples with platelet counts that were below the reference range had few to moderate numbers of platelet clumps. The mean hematocrit was 0.445 L/L (44.5%) and ranged from 0.406-0.47 L/L (40.6-47%) (reference interval 0.4-0.55 L/L [40-55%]). The mean fibrinogen was 5.82 µmol/L (198 mg/dL) and ranged from 3.88-8.35 µmol/L (132-284 mg/dL) (reference interval 2.94-8.82 µmol/L [100-300 mg/dL]). Additionally, there were no statistical differences in the platelet count ($p=0.31$), hematocrit ($p=0.36$), and fibrinogen ($p=0.91$) measurements over time.

The CV_I, CV_G, index of individuality (using CV_I/CV_G), number of samples, and mean values ± standard deviation over time for the TEG variables R, K, α, MA, and platelet aggregometry variables AUC_{ADP}, and AUC_{AA} are reported in Table 4.1. For TEG, 17/18 samples are reported because one of the blood samples showed multiple errors and a repeat sample could

not be obtained due to study design limitations. A graphical depiction of the difference between CV_I and CV_G for AUC_{ADP} is shown in Figure 4.1. The coefficients of variation (σ/μ) for Level I and Level II controls at the three measured time points are reported in Table 4.2.

Table 4.1: Intra-individual variability (CV_I), inter-individual variability (CV_G), and index of individuality (CV_I/CV_G) over time for tissue-factor (TF)-activated thromboelastography (TEG) and whole blood impedance platelet aggregometry in healthy Beagles

Measurement*	Sample number	Mean ± SD	CV_I (%)	CV_G (%)	CV_I/CV_G
R (min)	17	3.19 ± 0.85	25.6	7.98	3.21
K (min)	17	5.51 ± 2.0	31.05	19.73	1.57
α (degrees)	17	37.36 ± 8.48	20.93	9.23	2.27
MA (mm)	17	46.22 ± 8.04	10.56	14.5	0.73
AUC_{ADP} (units)	18	239.22 ± 36.34	6.84	14.44	0.47
AUC_{AA} (units)	18	222.78 ± 48.95	10.43	20.59	0.51

*****R = reaction time, K = clotting time, α = rate of clot formation, MA = maximum amplitude, AUC_{ADP} = area under the curve for ADP, AUC_{AA} = area under the curve for AA, ADP = adenosine diphosphate, AA = arachidonic acid, CV_I = intra-individual variability, CV_G = inter-individual variability, CV_I/CV_G = simplified equation used to calculate index of individuality

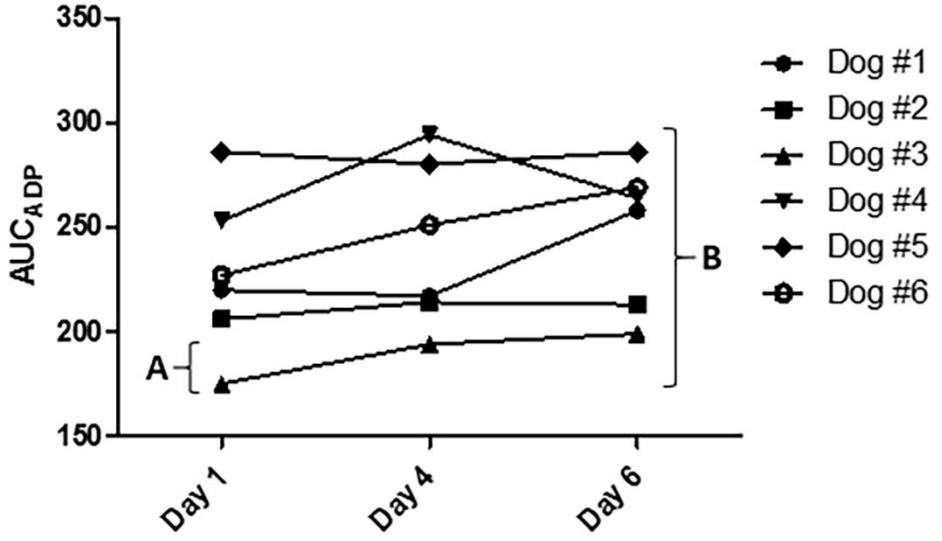

Figure 4.1: ADP-induced aggregation measured by whole blood impedance platelet aggregometry in six healthy Beagles on day 1, 4, and 6. Platelet aggregation was measured as AUC_{ADP} (area under the curve for adenosine diphosphate). A = Range of values from a single individual. B = Range of values from multiple individuals.

Table 4.2: Coefficients of variation (values given as %) for Level I and Level II controls over time for thromboelastography (TEG) in healthy Beagles

TEG variable	Level I	Level II
R (min)	7.07	7.14
K (min)	0	6.66
α (degrees)	1.10	1.34
MA (mm)	3.12	1.82

*****R = reaction time, K = clotting time, α = rate of clot formation, MA = maximum amplitude

4.5 Discussion

In this study, the CV_I was lower than the CV_G for the TEG variable MA and for both platelet aggregometry variables AUC_{ADP} and AUC_{AA}. However, for the remaining measured TEG variables, R, K, and α, the CV_I was higher than the CV_G. Based on the results of this study, serial measurements using a subject-based reference interval for ADP and AA-induced platelet aggregometry and the TEG variable MA is more likely to detect relevant changes in healthy dogs, a finding which could translate to ill dogs. However, due to the high CV_I for the TEG variables R, K, and α, serial measurements may not be superior to a single measurement and comparing it to population-based reference intervals.

The aforementioned platelet aggregometry results are similar to the recent study[9] where CV_I was also found to be lower than the CV_G in healthy dogs for the AUC using ADP- and AA-induced whole blood impedance platelet aggregometry. The authors of that study calculated the index of individuality using analytical variation (CV_A) and biological variation ($(CV_A^2 + CV_I^2)^{1/2}/CV_G$) for both of the platelet aggregometry variables and concluded that population-based reference intervals are not appropriate to use and that subject-based reference intervals (i.e. serial readings in an individual patient) are likely superior.[9] As stated previously, the index of individuality is a method used to evaluate whether population-based reference intervals are useful for a specific test or analyte and as seen by the equation, requires determination of the biological variation.[3,4] One of the limitations of that study however is that the whole blood aggregometry was performed on citrated blood samples rather than hirudin or heparinized samples.[2,8,12] When citrate is used, spontaneous aggregation can occur (when using recalcified citrated blood with no agonist)[8] and AUC readings (when using certain agonists) tend to be lower[2,12] which could lead to misinterpretation of results. We performed a saline control for each aggregometry assay in order

to ensure that spontaneous aggregation was not occurring when using heparin as the anti-coagulant.

Limiting our study was the inability to calculate the traditional index of individuality since duplicate analyses are required to calculate analytical variation. This was not able to be performed in our study due to budget limitations and lack of multiple TEG machines. However, when samples are being processed by an automatic analyzer, the analytical variation is often less than the CV_I so a more simplified equation (CV_I/CV_G) can be used to calculate the index of individuality.[4] Additionally, the controls (Level I and Level II) for TEG and electrical control for platelet aggregometry were performed before each analysis as another measure to ensure that the analytical variation was low. For platelet aggregometry specifically, the electrical control ensures that the channels are all functioning appropriately and when analyzing an actual sample; measurements that exceed the accepted coefficient of variation are flagged and discarded. As mentioned in the statistics section, an index of < 0.6 indicates a high degree of individuality whereas an index of > 1.4 indicates a lower degree of individuality allowing population-based reference intervals to be used. If we had assumed that analytical variation was $< CV_I$ in our study, the calculated index of < 0.6 for AUC_{ADP} and AUC_{AA} (Table 4.1) indicates that subject-based reference intervals are preferable to population-based intervals for these measurements.[3,4] Therefore, regardless of the differences between the Blois et al study and our study, both found that population-based reference intervals are likely to underestimate the frequency of abnormalities when performing ADP and AA-induced whole blood impedance platelet aggregometry in healthy dogs.

Previous studies[6,7] have evaluated the CV_I and/or CV_G of TEG parameters in dogs but no studies have been performed using fresh citrated whole blood for TF-activated TEG analysis. This

is important to determine since it is recommended to use fresh citrated whole blood when performing TEG analysis rather than plasma.[10] In the study by Wiinberg et al[6], where samples were from previously thawed plasma, the results showed that the CV_I was greater than the CV_G for the TEG variables R, α, and MA. Again, the index of individuality was calculated for these variables and based on the coefficients of variation data and index of individuality results, the authors concluded that population-based reference intervals were sensitive for evaluating TEG parameters.[6] Although the samples (plasma versus citrated whole blood) were different between Wiinberg's study and our study, the results for the TEG variables were similar with the exception of MA although both studies demonstrated CV_G and CV_I that were very close in magnitude for this variable. It was therefore considered possible that if we had been able to calculate the traditional index of individuality, we may have also found that MA could be evaluated using population-based reference intervals. To investigate this, if we had assumed the analytical variation was less than the CV_I for the TEG analysis, we could calculate the index of individuality using the simplified equation as discussed above. When using this equation however, we found similar results to our original analysis where the index was greater than 1.4 (Table 4.1) for the TEG variables R, K, and α showing that population-based intervals are appropriate to use and that subject-based intervals are not of additional benefit. As seen in Table 4.1, for the TEG variable MA, the index was < 1.0 but was not < 0.6 so using a population-based reference interval is not sensitive enough to detect subtle changes and serial evaluations or a subject-based reference interval may be more beneficial to use.[3] As stated previously, determining the biological variation of assays such as TEG and platelet aggregometry in healthy dogs is important for calculating the index of individuality and determining the usefulness of a population-based or subject-based reference interval.[4,5] Studies investigating these parameters could help determine when

differences in TEG or platelet aggregometry results represent a significant change in a clinical patient.

Because several factors can affect TEG results, the hematocrit, platelet count, and fibrinogen levels were reported in all of the dogs at each time point as previously recommended.[13] These values did not change significantly over time so we do not think changes in these variables affected our results. In addition to these variables, we also evaluated the clotting times (APTT and OSPT), antithrombin activity, and D-dimers on day 1 to ensure they were also within normal limits. Although the D-dimers were slightly elevated in some of the dogs, they were clinically normal, their complete blood counts showed no evidence of inflammation (data not shown), and their values fell within the ranges of other clinically healthy client-owned dogs (data not shown). D-dimers are known to become elevated during fibrinolytic activity but may not indicate the presence of disease if the elevation is mild as more significant increases in D-dimers tend to indicate disease.[14] Additionally, D-dimers can become increased in humans during different types of exercise[15] and all of these dogs were young and extremely active (had daily exercise) so it is possible this contributed to the mild elevations observed although this has not been in evaluated in dogs. Therefore, we do not think the mild elevations seen in D-dimers affected the results of our study.

Since analytical variation could not be calculated in this study, every effort was made to decrease pre-analytical variability. This was accomplished via the following methods; handling all of the Beagles in the same way, having the same investigator perform the venipuncture at each time point using the same size needle and syringe, ensuring an atraumatic venipuncture, having the same investigator perform all of the specialized hemostasis assays (TEG analysis and platelet aggregometry) and using the same reagents (same batch number) for all specialized hemostasis

assays. Additionally, in order to evaluate any variability that could arise from the TEG or aggregometry machine themselves, controls were performed which were found to have low coefficients of variation.

A potential limitation for this study was the small number of dogs used. However, when evaluating CV_I and CV_G, a small number of subjects can be evaluated as long as factors such as pre-analytical and analytical variation is considered small.[3] Another possible limitation of this study was that only male dogs were used which could have affected the biological variation for the TEG and platelet aggregometry results. In human studies, females can appear hypercoagulable via TEG[16] or rotation thromboelastometry (ROTEM)[17] as compared to males. This could be due to a variety of factors including differences in hematocrit as females tend to have a lower hematocrit.[17] However, there was no difference in TEG results between sexes in dogs in a previous study.[18] With regards to platelet aggregometry, females have been reported to have higher platelet reactivity to various agonists[19] but this has not been investigated using whole blood impedance platelet aggregometry nor has this been investigated previously in dogs. Although having all male dogs could have affected the actual TEG and/or platelet aggregometry results, it is unknown how this would affect biological variation so this could be investigated in future studies. Another potential limitation includes the timing of our assays (day 1, 4, and 6). These assays could have been performed daily in the dogs to try to better reflect how these assays might be used in the clinical setting however no guidelines currently exist for optimal timing of these assays. Future studies could be performed to help determine optimal timing for serial evaluations particularly when monitoring patients.

In conclusion, population-based reference intervals are not appropriate to use for ADP- or AA-induced whole blood impedance platelet aggregometry or for the TF-activated TEG variable

MA in healthy dogs so serial measurements or subject-based reference intervals may be more sensitive. However, for the TEG variables, R, K, and α, population-based intervals are appropriate to use for interpretation and serial measurements may not be more beneficial in healthy dogs. It is important to remember that these conclusions only apply to healthy dogs using the particular blood samples, reagents, activators, and methods that were used in this study. Additional studies should be performed in clinically ill dogs with different diseases to assess the CV_I and CV_G since this has the potential to have a major impact on treatment decisions, patient management, and clinical outcome.

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Chapter 5: Characteristics of hemostasis during experimental Ehrlichia canis infection

I would like to thank Dr. Lappin for allowing me to study my favorite aspects of coagulation in experimentally infected *E. canis* beagles and for listening to me ramble excitedly about the results. I would also like to thank Dr. Olver for her guidance in evaluating the coagulation results and for always being willing to discuss the complexities of coagulation with me.

5.1 Overview

Background: *Ehrlichia canis* infection in dogs can cause thrombocytopenia and clinical evidence of bleeding. It is unknown why some dogs show signs of bleeding whereas others do not despite clinically relevant thrombocytopenia.

Hypothesis/Objectives: Activated platelets, decreased fibrinolysis or both mitigate bleeding tendency. Assess standard hemostatic variables, platelet dynamics, and specialized coagulation testing in dogs experimentally infected with *E. canis* to evaluate this clinical discrepancy.

Animals: Four healthy laboratory beagles.

Methods: Dogs were given blood infected with *E. canis* IV. Platelet indices of activation, platelet aggregometry, anti-platelet antibodies (percent IgG), complete coagulation panel, and thromboelastography (TEG) were measured before inoculation and on weeks 1-8. Dogs were treated with doxycycline at approximately 5 mg/kg PO q12h between weeks 3 and 4 (day 24). For each variable, 1-way repeated measures analysis (1-way ANOVA) with post-hoc analysis was performed with statistical significance set at $P < 0.05$.

Results: Dogs had significantly lower platelet counts, evidence of activated platelets, and anti-platelet antibodies during E. canis infection. Dogs also appeared hypercoagulable and hypofibrinolytic using TEG as compared to baseline, changes that persisted for variable amounts of time after doxycycline administration. No overt signs of bleeding were noted during the study.

Conclusions and clinical importance: Activated platelets and a hypercoagulable, hypofibrinolytic state could explain the lack of a bleeding phenotype in some dogs despite clinically relevant thrombocytopenia. Findings from our pilot study indicate that additional studies are warranted.

5.2 Introduction

Ehrlichia canis infection in dogs can manifest with clinical signs related to bleeding and also commonly causes thrombocytopenia, particularly in the acute phase of infection.[1,2] The exact mechanisms of bleeding and thrombocytopenia are unknown but thought to be related to processes such as vasculitis and immune and non-immune processes affecting platelets.[3] For example, several studies have documented anti-platelet antibodies in dogs with ehrlichiosis.[4,5,6,7,8,9] Platelet dysfunction in infected dogs with anti-platelet antibodies also has been identified and 1 study proposed that these antibodies may interfere with primary hemostasis thus contributing to bleeding events.[3,10] Despite these processes, not all dogs infected with *E. canis* show signs of bleeding.[1] Currently, it is not clear why some dogs show signs of bleeding whereas other dogs do not despite clinically relevant thrombocytopenia. We hypothesize that platelets become activated during infection, blood clots become resistant to fibrinolysis or both, factors that could prevent a bleeding phenotype. A study in dogs naturally infected with *Babesia rossi* identified the presence of large activated platelets based on hematologic platelet indices. This finding was theorized to contribute to the lack of bleeding seen in dogs despite severe thrombocytopenia.[11] Another study in dogs showed that systemic inflammation is associated with decreased fibrinolytic activity as determined by thromboelastography (TEG).[12] This situation could help prevent bleeding events in dogs affected by an inflammatory disease such as ehrlichiosis. Therefore, the purpose of our study was to assess platelet indices of activation, platelet function as assessed by whole blood impedance

platelet aggregometry, percentage of anti-platelet antibodies (percent IgG), and TEG measurements including velocity curve (Vcurve) variables in dogs experimentally infected with *E. canis*.

5.3 Materials and Methods

Experimental E. canis *infection.* This prospective study was approved by the Institutional Animal Care and Use Committee and utilized 4 healthy purpose-bred beagles and 1 client-owned dog that was clinically normal, but positive for *E. canis* DNA in blood[13] and *Ehrlichia* spp. antibodies in serum.[a] The beagles were castrated males with a weight range of 13.8-15.7 kg and age range of 21-23 months at the start of the study. The beagles were housed under the same conditions, were not receiving any medications, and did not have a history of previous medication administration. Samples from all 4 dogs were tested initially and at each week (week 1-8) for antibodies against *Anaplasma* spp., *Borrelia burgdorferi*, and *E. canis/E. ewingii*, antigens of *Dirofilaria immitis*, and DNA of *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Ehrlichia* spp., the hemoplasmas, *Neorickettsia* spp., and *Rickettsia* spp (SNAP® 4Dx® Plus, IDEXX Laboratories Inc., Westbrook, ME, USA and Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO, USA.[13]

A total of 8 ml of anti-coagulated blood was collected from the client-owned *E. canis*-infected dog and all 4 of the *E. canis*-naïve dogs were each given 2 ml of this blood IV via the cephalic vein. After inoculation, the dogs were observed daily for clinical abnormalities and blood samples were scheduled to be collected on weeks 1 – 8. In addition to testing for infection, other assays performed included CBC (Siemens, ADVIA 120 Hematology System, Erlangen, Germany), plasma fibrinogen concentration (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland), tissue factor (TF)-activated TEG (TF-TEG) (TEG 5000 Thromboelastograph Hemostasis Analyzer,

Haemonetics Corporation, Braintree, MA), TF-activated TEG (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation, Braintree, MA) with tissue plasminogen activator (tPA) added (TF + tPA-TEG), whole blood impedance platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA), and direct flow cytometry detecting percent of immunoglobulin-associated platelets (percent IgG) and are described below. The dogs were fasted for 10 hours before each analysis and approximately 12 ml of blood from the jugular vein was drawn with a 20 gauge needle using a vacutainer (BD Vacutainer® Single Use Needle Holder, Franklin Lakes, NJ, USA). The blood for TEG and platelet aggregometry assays was drawn from the other jugular vein with a 20 gauge needle. For the TEG and aggregometry assays, each blood collection event had to have a single penetration of the vein with no re-direction. The appropriate volumes of blood were placed into the following tubes: EDTA (BD Vacutainer® K2 EDTA 3.6 mg 2.0 ml tubes, Franklin Lakes, NJ, USA, red top (Covidien Monoject™ Blood Collection Tube No Additive 5.0 ml tubes, Minneapolis, MN, USA) (serum), 3.2% sodium citrate buffered (BD Vacutainer® 3.2% sodium citrate tubes, Franklin Lakes, NJ, USA) and a heparin tube (Sarstedt lithium heparin micro tube, Numbrecht, Germany), respectively, and were gently inverted 5 times to allow proper mixing.

Dogs that developed findings suggesting clinical ehrlichiosis were to be treated with doxycycline at 5 mg/kg PO, q12h for 4 weeks and supportive care as indicated.

Complete blood cell count and standard coagulation tests. A CBC was performed on each sample collection day and the following variables were recorded: hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV), plateletcrit (PCT), platelet volume distribution width (PDW), mean

platelet mass (MPM), mean platelet component concentration (MPC) and platelet component distribution width (PCDW). Activated partial thromboplastin time (APTT) (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland), 1-stage prothrombin time (OSPT) (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) and antithrombin activity (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) were measured at week 0 and weeks 2-6 in all dogs. D-dimer concentrations (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) were measured at week 0 and weeks 2-8 in all dogs. Plasma fibrinogen concentration (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland) was measured at all time points (weeks 0-8).

Thromboelastography. For TF-TEG, citrated blood samples were allowed to sit at room temperature for 30 minutes before analysis. Briefly, the cups were warmed to 37°C and 20 µl of 0.2 M $CaCl_2$, 10 µl of TF (Tcoag TriniCLOT™ PT Excel, Bray, Wicklow, Ireland) at a final dilution of 1:1,000, and 330 µl of citrated whole blood were added to the cup and analyzed. Tissue factor was prepared before each individual TEG analysis. The TEG tracings then were generated for at least 60 minutes and the variables reaction time (R), clotting time (K), rate of clot formation (α), and maximum amplitude (MA) were recorded.

For TF + tPA-TEG, citrated blood samples were allowed to sit at room temperature for 30 minutes before analysis. Briefly, the cups were warmed to 37°C and 20 µl of 0.2 M $CaCl_2$ and 10 µl of TF (Tcoag TriniCLOT™ PT Excel, Bray, Wicklow, Ireland) at a final dilution of 1:1,000 were added to the cup. To prepare tPA (Cathflo® Activase® (Alteplase), 2 mg Vial, Carroll, OH, USA), the vial was reconstituted with sterile water resulting in 1.08 million units/ml. Then, 4.1 µl of the reconstituted tPA was added to 996 µl of a phosphate-buffered solution (PBS) to make the stock tPA solution. The stock solution (10 µl) was added to 400 µl of citrated whole blood, mixed

gently, and 330 µl of this mixture was added to the cup and analyzed. The TF was prepared before each individual TEG analysis and the tPA solution was kept on ice between analyses but was discarded after each testing period. The TEG tracings then were generated for at least 60 minutes and the variables R, K, α, MA, percent of clot lysis 30 minutes after MA is reached (LY30), amount of clot lysis 30 minutes after MA is reached (CL30), percent of clot lysis 60 minutes after the MA is reached (LY60), and amount of clot lysis 60 minutes after MA is reached (CL60) were recorded. Additionally, velocity curve variables were recorded denoted as the maximal rate of thrombus generation (MRTG), time to maximum rate of thrombus generation (TMRTG), total thrombus generated (TG), maximum rate of lysis (MRL), time to maximal rate of lysis (TMRL), and total lysis (L). Controls (Level I and Level II [Level I and II controls; Haemonetics Corporation, Braintree, MA, USA]) were performed at each time point as recommended by the manufacturer (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation, Braintree, MA, USA) before analysis of the samples.

Hypercoagulability, using TF-TEG, was defined as a statistically higher MA value as compared to baseline (week 0). Hypofibrinolysis, using TF + tPA-TEG was defined as an LY30 or LY60 statistically lower or a CL30 or CL60 statistically higher than the baseline (week 0) result. From the velocity curve variables, hypercoagulability was defined as an MRTG or TG statistically higher or a TMRTG statistically lower than the baseline (week 0) result. From the velocity curve variables, hypofibrinolysis was defined as an MRL statistically lower or a TMRL statistically higher than the baseline value.

Whole blood impedance platelet aggregometry. For the multiple channel electrical impedance platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA),

heparinized blood samples were kept at room temperature and analyzed within 40 minutes of blood collection (range, 29-40 minutes) for 12 minutes. Before each analysis, the test cells were warmed to 37°C within the aggregometer and an automated pipette was used to decrease pre-analytical variability. Heparinized blood samples (300 µl) were diluted with 300 µl of diluent and incubated for 3 minutes as recommended by the manufacturer. Adenosine diphosphate (ADP) (Diapharma Group Inc., West Chester, OH, USA) and arachidonic acid (AA) (ºDiapharma Group Inc., West Chester, OH, USA) were used as platelet agonists and area under the curve (AUC) for ADP (AUC_{ADP}) and for AA (AUC_{AA}) were recorded. Note that: the AUC value is an arbitrary unit. As recommended by the manufacturer (Multiplate 5.0 Analyzer, Diapharma Group Inc., West Chester, OH, USA), the final concentration of ADP was 6.5 µM and the final concentration of AA was 0.5 mM. To serve as a control and to evaluate for spontaneous platelet aggregation, an identical volume of saline was used in place of the agonists (AUC_{saline}). The reagents were reconstituted and stored according to the manufacturer's recommendations (Diapharma Group Inc., West Chester, OH, USA) in 60 µl aliquots.

Direct flow cytometry assay for platelet-associated immunoglobulin. The protocol used for our study was modified from other protocols previously described in the literature.[13,14] Blood (700 µl) anticoagulated with EDTA was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200 · g for 1 minute 30 seconds at 20°C to generate platelet-rich plasma (PRP). Platelet-rich plasma was removed from the erythrocyte layer and placed into an Eppendorf tube (Light Labs SNAPLOCK Microcentrifuge Tubes, Dallas, TX, USA). Each PRP sample was adjusted to $2 \cdot 10^6$ cells/ml using a manual hemocytometer to provide a standard volume of PRP that then was pelleted by centrifugation at 1000 · g for 5 minutes at 20°C. The platelets were re-

suspended and washed 3 times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature with 50 µl of a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled rabbit anti-dog IgG (FITC-conjugated AffiniPure rabbit anti-dog IgG (H + L) Jackson ImmunoResearch Labs, 304-095-003, West Grove, PA, USA) for 30 minutes. After incubation, the platelets were washed 3 times with PBS-EDTA-BSA solution and re-suspended in 200 µl of PBS-EDTA-BSA solution for flow analysis. Gate settings used for our study were previously established with PE-labeled mouse anti-human CD61 (Anti-human CD61 (Integrin beta 3) PE, VI-PL2, 12-0619-42, eBioscience, San Diego, CA, USA) using healthy dog samples. Platelets from a healthy beagle served as a negative control at each time point. Samples were considered positive if the percent IgG was > 2 standard deviations (SD) above the reference range determined from the negative control and the 4 healthy beagles before inoculation with *E. canis*. All samples were analyzed using a Cyan ADP instrument (Cyan ADP instrument, Beckman Coulter, Miami, FL, USA) and the generated data was analyzed using FlowJo software (FlowJo software, Tree Star, Ashland, OR, USA).

Statistical Analysis. For each response variable, a repeated measures analysis (1-way ANOVA) was performed using the lme4 and lmerTest packages in R (R package, version 3.2.2). To account for repeated measures, dog was included as a random effect. Time points were compared to week 0 using Dunnett's method with the lsmeans package in R (R package, version 3.2.2). In order to investigate the effects of doxycycline on the measured variables, an additional analysis was performed where time points were compared to week 3 using Dunnett's method with the lsmeans package in R (R package, version 3.2.2). For all tests, a $P < 0.05$ was considered significant.

5.4 Results

No complications were observed after the inoculations. All dogs inoculated from the client-owned donor dog became persistently positive for *E. canis*/*E. ewingii* antibodies in serum (SNAP® 4Dx® Plus, IDEXX Laboratories Inc., Westbrook, ME, USA) and were positive by PCR (Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO, USA) for *E. canis* by week 2. At week 3 after inoculation, all 4 of the dogs had lost weight, were hyporexic and subjectively lethargic. Thus, all dogs were treated with doxycycline at approximately 5 mg/kg, PO, q12h for 4 weeks starting between weeks 3 and 4 (day 24) of the study. All 4 dogs became clinically normal within 4 days of doxycycline administration and all dogs became PCR (Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO, USA) negative for *E. canis* at week 5 and remained PCR negative for the duration of the study. None of the dogs developed positive test results for any other tested infectious agent during the course of the study.

The repeated measures 1-way ANOVA for the TF-TEG, TF + tPA-TEG and whole blood impedance platelet aggregometry identified statistical differences for all variables with the exception of the fibrinolysis value MRL and the platelet aggregometry value, AUC_{saline}. Note that: TEG in dogs can be performed using an activator (e.g. TF, kaolin) or without an activator (native) however it is recommended to use an activator such as TF or kaolin.[15] Tissue factor was chosen as the activator for our study because the reference ranges used at our institution are based on TF-activated TEG analyses.

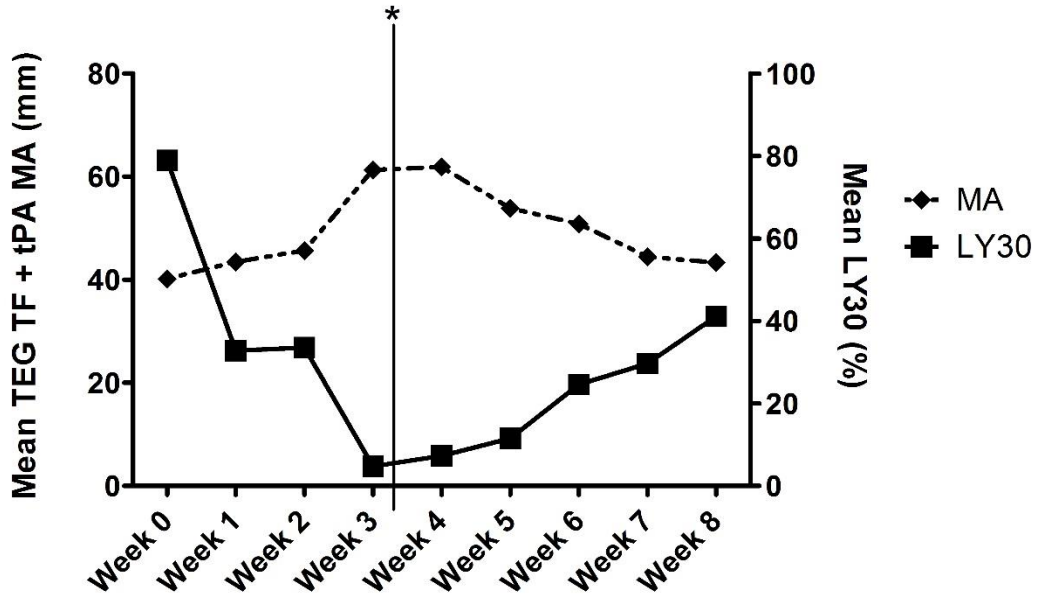
Several statistically significant differences from baseline were identified in all dogs for TEG and platelet aggregometry at multiple time points (Table 5.1) with the exception of only 4 variables: TF + tPA-TEG variables R, TMRTG, and MRL and platelet aggregometry variable AUC_{saline}. Similarly, Dunnett's method identified multiple statistically significant differences

from week 3 in all dogs at multiple time points (Table 5.1) with the exception of 4 variables: TF + tPA-TEG variables K, TMRTG, and MRL and platelet aggregometry variable AUC_{saline}. For TF-TEG, dogs had shorter R times, smaller K values, larger α values, and higher MA values as compared to baseline at the respective time points (Table 5.1). For TF + tPA-TEG, dogs had smaller values for K, LY30, LY60 and higher values for α, MA, CL30, CL60, MRTG, TG, TMRL and L as compared to baseline at the respective time points (Table 5.1). After doxycycline administration on day 24, several statistical differences were noted as compared to week 3 (Table 5.1). Overall, for TF-TEG, at week 3, dogs had shorter R times, smaller K values, larger α values, and larger MA values as compared to other time points. Overall, for TF + tPA-TEG, at week 3, dogs had smaller values for R, K, LY30, LY60, and TMRL and higher values for α, MA, CL30, CL60, MRTG, TG, and L as compared to the respective time points. The average MA values and average LY30 values over time in relationship to doxycycline administration are presented in Figure 5.1. The average MRTG and average TMRL values over time in relationship to doxycycline administration are presented in Figure 5.2.

Table 5.1: Dunnett's test results for the tissue factor (TF)-activated thromboelastography (TEG), TF + tissue plasminogen activator (tPA) TEG and whole blood impedance platelet aggregometry variables in beagles experimentally infected with *Ehrlichia canis*

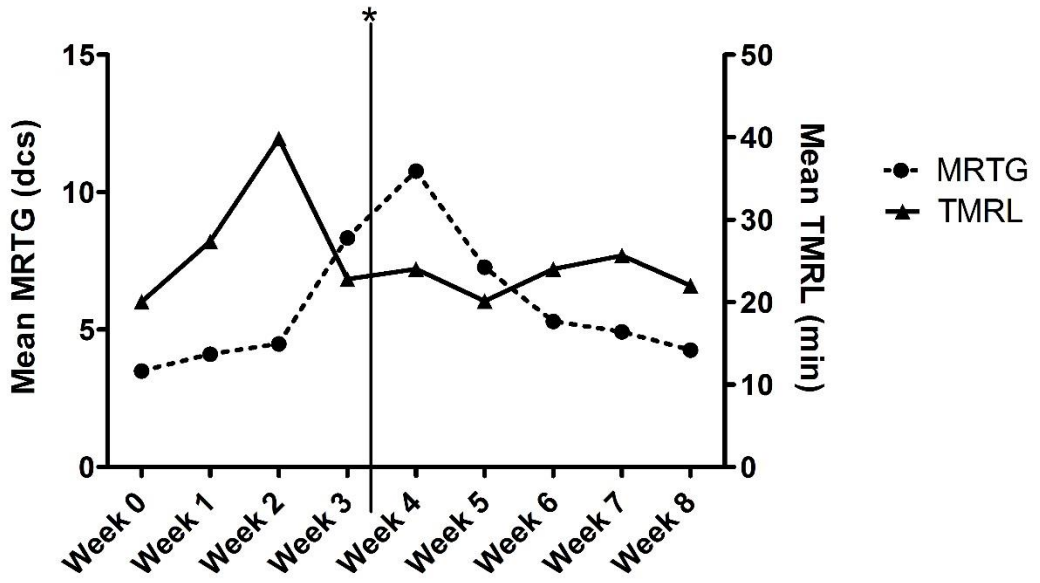
Measurement*	Time different from Week 0	Time different from Week 3
TF-activated R (min)	Week 2, 3, 4, 5	Week 0, 8
TF-activated K (min)	Week 1, 2, 3, 4, 5	Week 0, 2, 8
TF-activated α (degrees)	Week 1, 2, 3, 4, 5	Week 0, 1, 2, 7, 8
TF-activated MA (mm)	Week 1, 2, 3, 4, 5	Week 0, 1, 2, 6, 7, 8
TF + tPA R (min)	None	Week 1, 8
TF + tPA K (min)	Week 3	None
TF + tPA α (degrees)	Week 3, 4	Week 0, 1
TF + tPA MA (mm)	Week 3, 4, 5	Week 0, 1, 2, 7, 8
TF + tPA LY30 (%)	Week 1, 2, 3, 4, 5	Week 0
TF + tPA CL30 (%)	Week 1, 2, 3, 4, 5	Week 0, 8
TF + tPA LY60 (%)	Week 1, 2, 3, 4, 5	Week 0, 1, 2, 8
TF + tPA CL60 (%)	Week 3	Week 0, 1
MRTG (dcs)	Week 3, 4, 5	Week 0, 1, 2, 8
TMRTG (min)	None	None
TG (dcs)	Week 3, 4, 5	Week 0, 1, 2, 5, 6, 7, 8
MRL (dcs)	None	None
TMRL (min)	Week 2	Week 2
L (dcs)	Week 3, 4	Week 0, 1, 2, 7, 8
AUC_{saline}	None	None
AUC_{ADP}	Week 2, 3	Week 5, 6, 7, 8
AUC_{AA}	Week 2, 3	Week 0, 4, 5, 6, 7, 8

*R = reaction time, K = clotting time, α = rate of clot formation, MA = maximum amplitude, LY30 = percent of clot lysis 30 minutes after MA is reached, CL30 = amount of clot lysis 30 minutes after MA is reached, LY60 = percent of clot lysis 60 minutes after the MA is reached, CL60 = amount of clot lysis 60 minutes after MA is reached, MRTG = maximal rate of thrombus generation, TMRTG = time to maximum rate of thrombus generation, TG = total thrombus generated, MRL = maximum rate of lysis, TMRL = time to maximal rate of lysis, L = total lysis, AUC_{saline} = area under the curve for saline, ADP = adenosine diphosphate, AA = arachidonic acid, AUC_{ADP} = area under the curve for ADP, AUC_{AA} = area under the curve for AA



*The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline.

Figure 5.1: Mean maximum amplitude (MA) values and mean percent clot lysis 30 minutes after MA (LY30) from tissue-factor (TF) + tissue plasminogen activator (tPA) thromboelastography (TEG) over time in beagles experimentally infected with *Ehrlichia canis*.



*The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline.

Figure 5.2: Mean maximal rate of thrombus generation (MRTG) and mean time to maximal rate of lysis (TMRL) from tissue-factor (TF) + tissue plasminogen activator (tPA) thromboelastography (TEG) over time in beagles experimentally infected with *Ehrlichia canis*.

For platelet aggregometry, no significant differences were identified in the values for AUC_{saline} as compared to baseline or week 3. However, the AUC_{ADP} and AUC_{AA} were significantly lower than baseline at the respective time points (Table 5.1). To further evaluate the effect of doxycycline administration, for AUC_{ADP}, at week 3, dogs had significantly lower values as compared to other time points (Table 5.1). Specifically, the AUC_{ADP} was statistically higher at weeks 5 (difference, 106.50; p-value, 0.042), 6 (104; 0.049), 7 (166.75; 0.001), and 8 (149.00; 0.002) as compared to week 3. Similarly, for AUC_{AA}, at week 3, dogs also had significantly lower values as compared to other time points (Table 5.1). Specifically, the AUC_{AA} was statistically

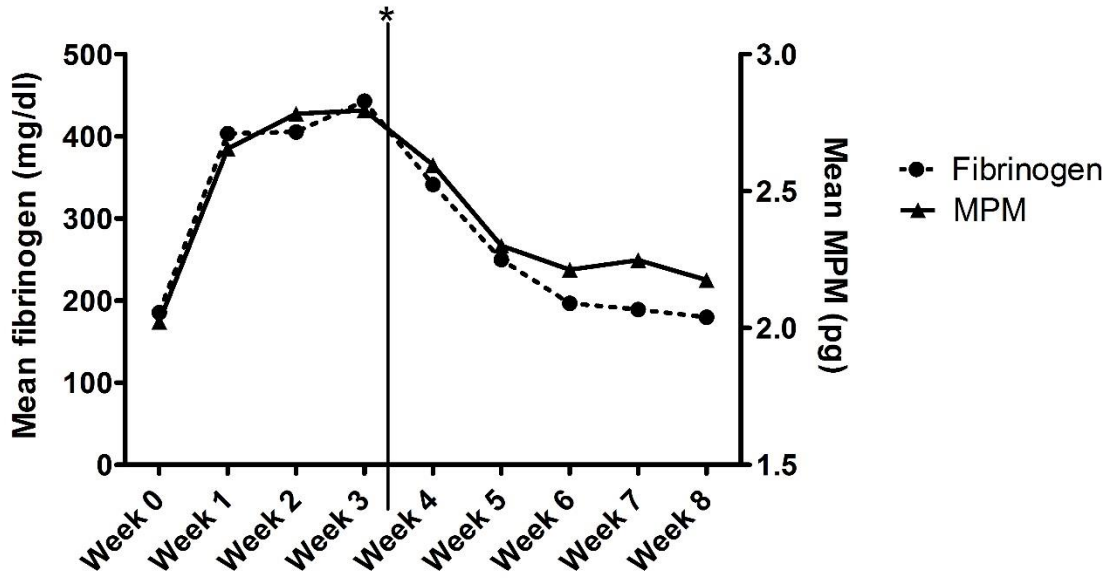
higher at weeks 0 (difference, 98.00; p-value, 0.009), 4 (86.00; 0.026), 5 (100.25; 0.007), 6 (115.25; 0.002), 7 (150.00; <0.001), and 8 (149.25; <0.001) as compared to week 3.

Results from the repeated measures 1-way ANOVA for the hematology and flow cytometry were significantly different for all variables with the exception of PDW. Multiple statistically significant differences from baseline in all dogs for the hematology and flow cytometry variables at multiple time points (Table 5.2), with the exception of only 4 variables: OSPT, PDW, MPC, and PCDW, were observed. Similarly, Dunnett's method identified multiple statistically significant differences from week 3 in all dogs at multiple time points (Table 5.2) with the exception of PDW. Overall, dogs had lower antithrombin concentrations, platelet counts, PCT, HCT, and MCHC as compared to baseline at the respective time points (Table 5.2). In contrast, dogs had higher D-dimers, OSPT, plasma fibrinogen concentration, MPV, MPM, MCV, and percent IgG as compared to baseline at the respective time points (Table 5.2). The average plasma fibrinogen concentrations and average MPM values over time in relationship to doxycycline administration are presented in Figure 5.3. After doxycycline administration on day 24, several statistical differences were noted as compared to week 3. Overall, at week 3, dogs had lower antithrombin concentrations, platelet counts, PCT, MPC, HCT, and MCHC as compared to other time points (Table 5.2). In contrast, at week 3, dogs had higher D-dimers, OSPT, APTT, plasma fibrinogen concentrations, MPV, MPM, PCDW, MCV, and percent IgG as compared to the respective time points (Table 5.2). The average platelet counts and average percent IgG over time in relationship to doxycycline administration are presented in Figure 5.4.

Table 5.2: Dunnett's test results for hematology and flow cytometry variables in beagles experimentally infected with *Ehrlichia canis*.

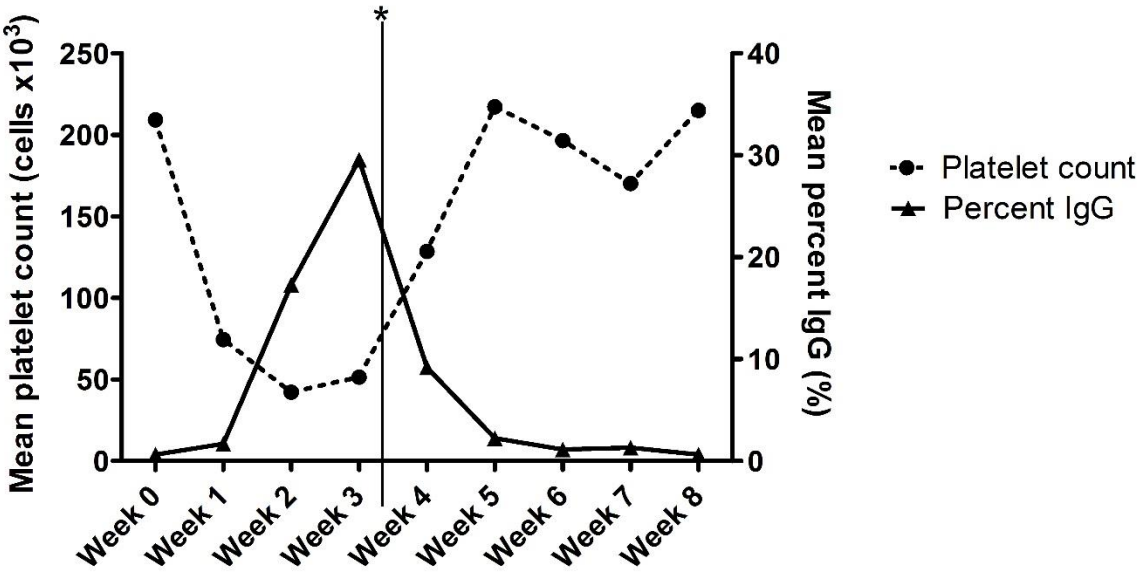
Measurement*	Time different from Week 0	Time different from Week 3
Antithrombin activity (%)	Week 3, 4	Week 0, 2, 4, 5, 6
D-dimers (ng/ml)	Week 3	Week 0, 2, 4, 5, 6, 7, 8
APTT (sec)	Week 3	Week 0, 2, 4, 5, 6
OSPT (sec)	None	Week 2, 4
Fibrinogen (mg/dl)	Week 1, 2, 3, 4, 5	Week 0, 4, 5, 6, 7, 8
Platelet count (x 10^3cells/µl)	Week 1, 2, 3	Week 0, 5, 6, 7, 8
MPV (fL)	Week 2, 3, 4	Week 0, 1, 5, 6, 7, 8
PCT (%)	Week 1, 2, 3	Week 0, 4, 5, 6, 8
PDW (%)	None	None
MPM (pg)	Week 1, 2, 3, 4	Week 0, 5, 6, 7, 8
MPC (g/dL)	None	Week 1, 8
PCDW (g/dL)	None	Week 1
Hct (%)	Week 2, 3, 4, 5, 6, 7, 8	Week 0, 1, 2, 6, 7, 8
MCV (fL)	Week 4, 5, 6	Week 2, 4
MCHC (g/dL)	Week 3, 4, 5, 6	Week 0, 2
Percent IgG (%)	Week 3	Week 0, 1, 4, 5, 6, 7, 8

*APTT = activated partial thromboplastin time, OSPT = one-stage prothrombin time, MPV = mean platelet volume, PCT = plateletcrit, PDW = platelet volume distribution width, MPM = mean platelet mass, MPC = mean platelet component concentration, PCDW = platelet component distribution width, Hct = hematocrit, MCV = mean corpuscular volume, MCHC = mean corpuscular hemoglobin concentration, Percent IgG = percent of anti-platelet antibodies



*The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline.

Figure 5.3: Mean fibrinogen and mean platelet mass (MPM) over time in beagles experimentally infected with *Ehrlichia canis*



*The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline.

Figure 5.4: Mean platelet counts and mean percentage of platelet associated immunoglobulins (percent IgG) over time in beagles experimentally infected with *Ehrlichia canis*.

5.5 Discussion

Ehrlichia canis infects dogs worldwide and clinical manifestations can include bleeding but it is unknown why some dogs do not exhibit signs of hemorrhage despite clinically relevant thrombocytopenia.[1] Overall, we found using coagulation testing that the dogs became hypercoagulable and hypofibrinolytic based on TEG after becoming infected with *E. canis*. Additionally, certain measures of platelet activation increase during infection, including during the thrombocytopenic phase.

The results from TF-TEG and TF + tPA-TEG showed that experimentally-inoculated dogs appeared hypercoagulable as compared to baseline as defined by a higher MA value and

hypofibrinolytic as defined by a lower LY30 and LY60 and a higher CL30 and CL60.[16] Additional analyses were performed to compare week 3 results to those from subsequent time points, because it was unknown if doxycycline administration would affect TEG parameters. The time point week 3 was chosen for comparison because the dogs were most clinically affected at this time and because doxycycline administration was started on day 24 of the study. After doxycycline administration, TF-TEG MA and TF + tPA-TEG MA did not statistically differ from week 3 until week 6 or week 7, respectively. After doxycycline administration, it took 2 weeks (week 6) for TF-TEG MA and 3 weeks (week 7) for TF + tPA-TEG MA to return to baseline. Before doxycycline administration, the fibrinolysis variables LY30, CL30, LY30, and CL60 all were statistically different from week 3 but did not return to baseline until week 8 with the exception of CL60. Thus, the results suggest that the dogs were hypofibrinolytic until week 8 (Figure 5.1) approximately 5 weeks afer doxycycline administration. The velocity curve variables are generated from the TEG tracing and represent the first derivative of the associated waveform. These variables provide information regarding clot formation and clot breakdown.[16] The MRTG reflects the maximal velocity of increase in clot strength and observed clot growth whereas TMRTG reflects the time interval required to reach maximal rate of clot formation.[17,18] Total thrombus generated reflects amount of clot strength produced and represents total area under the velocity curve during clot formation.[18] As a result, an increased MRTG, decreased TMRTG, and increased TG could represent a hypercoagulable state.[17,19] With regards to clot breakdown, MRL, TMRL, and L can be evaluated. The MRL reflects maximal velocity of clot breakdown, TMRL reflects the time interval required to reach maximal rate of clot lysis, and L represents total amount of clot lysis. Therefore, an increased MRL, decreased TMRL, and increased L could represent a hyperfibrinolytic state and vice versa for a hypofibrinolytic state.[20] Overall, velocity curve results

aligned with traditional TEG results in that the dogs appeared hypercoagulable and hypofibrinolytic. When evaluating the results from the dogs as compared to baseline, the dogs appeared hypercoagulable based on significantly larger MRTG and TG values (at weeks 3, 4, and 5) and also demonstrated hypofibrinolysis based on a longer TMRL value (at week 2). It also was observed that the dogs appeared hypercoagulable by week 3 (increased MRTG) and week 5 (increased TG) and these changes persisted and did not return to baseline until week 8. The cumulative findings from both TEG analyses and from velocity curve results indicated that dogs were hypercoagulable and hypofibrinolytic at certain time points over the course of infection and that these changes persisted for variable amounts of time after doxycycline administration.[16] A hypercoagulable and hypofibrinolytic state could result in a hemostatic phenotype rather than a bleeding phenotype which could help explain why bleeding may not be observed in dogs infected with *E. canis* despite severe thrombocytopenia.

The overall function of platelets also could explain why bleeding is or is not observed in *E. canis* infection. If platelets are in an activated state, they will function in primary hemostasis to prevent bleeding.[21] However, if a thrombocytopathy is present, bleeding may be observed regardless of the platelet count, but particularly if the platelet count is low. We investigated platelet function using both whole blood impedance aggregometry and parameters measured on the Advia 120 hematology analyzer. The platelet aggregometry was normal except for time periods when the dogs were thrombocytopenic. Significant decreases in platelet count were observed by week 1 and remained statistically lower than baseline until week 4 and similarly, AUC_{ADP} and AUC_{AA} were statistically lower than baseline at weeks 2 and 3. Additionally, the initiation of doxycycline administration between weeks 3 and 4 likely affected the platelet count and resultant AUC readings.[25] The results show that platelet count returned to baseline by week 5 (Figure 5.2) and

AUC returned to baseline by week 4 and 5, approximately 1-2 weeks after doxycycline administration. Therefore, no evidence of platelet dysfunction was detected before thrombocytopenia was documented or once the platelet count started to rebound and normalize. Additionally, platelet activation parameters were increased. Therefore, another reason why dogs infected with *E. canis* may not show evidence of bleeding despite severe thrombocytopenia is if platelets are in an activated state. Variables indicating increased platelet activity include increased MPV, MPM, and PCDW and decreased MPC.[11] These changes occur in the platelet because of release of intracellular components such as dense granules in addition to morphology changes in their integral shape.[11] Based on our results and aforementioned indices, there was evidence of platelet activation at weeks 2, 3, and 4 (increased MPV) and at weeks 1, 2, 3, and 4 (increased MPM) as compared to baseline. After doxycycline administration, MPV and MPM returned to baseline and were statistically different when results from week 3 and week 5 were compared. The dogs were noted to have significant decreases from their baseline platelet counts during weeks 1, 2, and 3 and had platelet counts that ranged from $20 – 100 \cdot 10^3/\mu l$. Therefore, there was evidence of platelet activation during the periods of most severe thrombocytopenia, but it is also plausible that these changes could have been related to increased platelet release from the bone marrow as a response to thrombocytopenia. The differentiation between these 2 possibilities was not possible in our study. Because there was evidence of potential platelet activation during these time periods, such a situation could have contributed to why no bleeding events were observed despite the dogs having significant thrombocytopenia.

Possible causes for thrombocytopenia and subsequent bleeding in *E. canis* infected dogs include destruction of platelets by platelet-directed antibodies, increased clearance of platelets from circulation by the spleen and sequestration as a result of vasculitis.[4,5,6,7,9,26] The percent IgG

was statistically higher than baseline at week 3 but statistically significant thrombocytopenia also was identified at weeks 1, 2, and 3. This finding is in agreement with other studies in which the thrombocytopenia observed in *E. canis* infections likely was caused by several mechanisms and not solely platelet-directed antibodies. Interestingly, the percent IgG decreased after administration of doxycycline in all dogs and all were considered negative by week 5 (Figure 5.4). This finding was surprising because the half-life of most of the immunoglobulin G (IgG) subclasses in dogs is estimated to be similar to that of humans, which is approximately 20-21 days. Four subclasses of IgG occur in both humans and dogs, but they are categorized differently.[27] A previous study reported that natural and experimental *E. canis* infections in dogs are associated with a predominance of IgG2 rather than IgG1.[28] Immunoglobulin G2 is the functional human analog to the canine IgG subclass A which is not thought to be involved in antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity, but research is ongoing to better characterize the canine IgG subclasses.[27] The rapid decrease in percent IgG after doxycycline may be related to other non-antimicrobial effects of this drug. For example, it has been previously documented that dogs infected with *E. canis* and healthy dogs both had an increase in platelet counts suggesting that doxycycline may result in platelet proliferation.[25] Additionally, doxycycline has been shown to decrease antibody production and inhibit proliferating lymphocytes.[25,19] It also has been shown to decrease the expression of IgG on granulocytes, but it is unknown if this occurs with platelets.[30] Therefore, if the platelets have decreased survival in circulation because of antibody binding but doxycycline causes proliferation of platelets and decreased antibody production, this could explain why the percent IgG decreased so rapidly. It is currently unknown if doxycycline decreases IgG expression on platelets similar to granulocytes or if it interferes with antibody-antigen binding on the platelet surface. Further studies are needed to

determine the relationship between doxycycline and the percent IgG response in thrombocytopenic dogs.

Another notable finding observed during our study was that all of the dogs were strongly positive on serology[a] testing for *Ehrlichia* spp. at week 1 but were PCR negative. At week 2, all dogs exhibited a weak positive result by serology[a] testing but all were PCR positive. Then, at week 3, 2 of the dogs were negative and 2 dogs were positive on serology testing. Not until week 6 were all dogs noted to be serology positive again. Experimentally-infected dogs can become seropositive to *E. canis* as early as 7 days post-inoculation, but seropositivity may not be seen for 28 days post-inoculation.[1] We suspect that the strong positive results initially seen in all 4 dogs were actually antibodies transferred passively from the *E. canis* positive donor blood. This would also explain why the subsequent weeks showed a weak positive result which transitioned into a negative result in 2 of the dogs. Because we suspect the dogs were not only inoculated with *E. canis* positive blood but also with antibodies against *E. canis* from the donor dog, this also could explain the relatively delayed conversion to seropositivity in some of the dogs.

Dogs infected with *E. canis* have been shown previously to have decreased platelet function based on several platelet aggregation studies,[3,10] but to our knowledge, no studies have been performed using multiple channel electrical impedance platelet aggregometry. However, when using impedance platelet aggregometry, decreased platelet counts and a high HCT can cause *in vitro* effects on platelet aggregometry resulting in lower AUC readings.[22,23,24] As a result, AUC readings may appear erroneously low due to thrombocytopenia rather than reflecting true platelet dysfunction. To address this, an alternative technique is to standardize the platelet count in all samples before performing aggregometry. Doing so requires manipulation of the platelets (centrifugation) and the test is not performed in whole blood which may not reflect the true changes

occurring *in vivo* (in whole blood).[3,10] Our testing was performed using whole blood, and the platelet count was not adjusted. As a result, the observed AUC readings likely were affected by the significant changes in platelet count and HCT observed at certain time points, which is a limitation of the study.

There were several other limitations to this study not previously mentioned. The primary limitation was use of blood from a naturally-infected dog to initiate *E. canis* infection rather than tick infestation. It currently is unknown whether factors imparted by *Rhipicephalus sanguineus* could result in different findings to those obtained by inoculation of whole blood from a naturally-infected dog or cell culture-derived *E. canis*. However, evaluation of the coagulation system using TEG has not previously been performed in dogs experimentally or naturally-infected with *E. canis*, and thus an experimental model initially was investigated. In addition, the clinical, laboratory, and treatment responses to doxycycline occurred in all dogs and were similar to those findings reported with naturally-occurring infection, suggesting that our findings are valid.[1] However, our results should be confirmed in naturally-infected dogs or dogs infested experimentally with *R. sanguineus* that carry *E. canis*. Another potential limitation is that the naturally-infected donor dog blood used to initiate *E. canis* infection could have contained other infectious agents that could have affected the results. However, we believe this is unlikely because the dogs tested negative several times for other known causes of thrombocytopenia. This limitation also could occur when naturally-infected dogs are studied or wild-caught *R. sanguineus* are used to initiate infections. A final limitation is the small number of dogs that were used. Thus, whether these findings are clinically relevant is unknown and additional studies are warranted.

Although the mechanisms of thrombocytopenia and bleeding are not completely understood in dogs infected with *E. canis*, our results showed that activated platelets and a

hypercoagulable, hypofibrinolytic state may explain the lack of a bleeding phenotype in some dogs despite substantial thrombocytopenia. Additionally, further studies are needed to investigate the relationship between doxycycline administration and platelet dynamics in thrombocytopenic dogs.

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Chapter 6: Validation of a Flow Cytometric assay for detection and quantitation of anti-platelet antibodies in dogs

I would like to thank Dr. Dow and Dr. Lappin for helping me with development of this assay. I had not performed flow cytometry for years so it took some time to get used to working with flow cytometers again but Dr. Dow was always patient and willing to talk about my results. Dr. Lappin is also due a big thank you for allowing me to perform so many analyses with the support of the Center for Companion Animals.

6.1 Overview

Thrombocytopenia is a common clinical condition in dogs. Causes include infectious, neoplastic, inflammatory, toxic, and immune-mediated conditions. Elucidating the underlying cause for thrombocytopenia can therefore represent a challenge to veterinary practitioners. Additionally, determination of whether an immune process could be contributing to a patient's thrombocytopenia is important for refining differentials and enhancing understanding of a particular disease process. A reliable and easily interpretable clinical test for the detection and quantification of anti-platelet antibodies in dogs has the potential to greatly improve the management of thrombocytopenia in dogs. Therefore, the purpose of this study was to develop a clinically practical flow cytometric assay for the detection of anti-platelet antibodies in dogs. Initial assay development and optimization was performed using blood and serum from 9 healthy beagles and 12 dogs with naturally-occurring thrombocytopenia. Platelet-bound antibodies (percent IgG) were analyzed from blood samples stored for 0, 24, 48, or 72 hours. For healthy non-thrombocytopenic dogs, there was no significant difference between fresh and 24 and 48 hour samples but there was a significant difference in the percentage of antibody-positive platelets between fresh and 72 hour blood samples. There was no significant difference in the percentage of antibody-positive platelets between fresh and 24, 48, or 72 hour blood samples in the

thrombocytopenic dogs. A cut-off value of ≤ 10% antibody binding was defined to differentiate negative and positive classifications and was determined by serial direct flow evaluations in a healthy dog. Based on this cut-off value, healthy and thrombocytopenic dogs were consistently categorized at every time point. The average intra-assay coefficient of variation for the thrombocytopenic dogs was 4.32%. The indirect flow cytometric methods of detecting anti-platelet antibodies did not provide reliable or repeatable results in either healthy or thrombocytopenic dogs. Based on these results, we conclude that direct flow cytometry represents a clinically practical assay and is valid up to 72 hours for the detection of anti-platelet antibodies in dogs.

Keywords

Thrombocytopenia, flow cytometry, anti-platelet antibodies, percent IgG

6.2 Introduction

Thrombocytopenia is a common blood work abnormality observed in dogs that can be related to artefactual pseudothrombocytopenia (e.g. sample handling, anti-coagulant induced) but can also be due to immune and non-immune processes on platelets. It is also the most common acquired hematologic abnormality observed in dogs (Brooks and Catalfamo, 2010). The major mechanisms of thrombocytopenia include decreased or lack of production, increased destruction or consumptive processes, abnormal loss, and sequestration (Neel et al, 2014). Thrombocytopenia can occur as a result of one of these mechanisms or a combination due to infectious, neoplastic, inflammatory, toxic, and immune-mediated conditions. Determining the underlying cause for thrombocytopenia in dogs can therefore be challenging. However, establishing if an immune process is contributing to the observed thrombocytopenia can help further characterize a patient's

disease and guide future diagnostic testing. Specifically, for immune processes, anti-platelet antibodies can form that are directed against platelets which can contribute to a shortened platelet life span and resultant abnormally low platelet number (Wilkerson et al, 2001; Brooks and Catalfamo, 2010). The detection of anti-platelet antibodies in dogs has been previously assessed by various techniques such as ELISAs, immunofluorescence, immunoradiometric methods, and flow cytometry but none of these tests are commonly used in veterinary practice (Kristensen et al, 1994a, Kristensen et al, 1995b, Lewis et al, 1995, Scott et al, 2002).

The development of a clinically applicable for detection of anti-platelet antibodies would be of great benefit to veterinary patients. In order to be clinically applicable and have a more widespread impact, the test must not only provide repeatable and reliable results but also must be viable after several days of storage. This requirement is a realistic expectation since many private practices and veterinary academic institutions would not have the capability to perform this type of test on-site. Previous studies have shown varying results regarding the stability of stored samples for flow cytometry; one study showed that samples could be reliably evaluated when stored appropriately up to 72 hours after collection (Lewis et al, 1995) whereas another author reported that samples should not be evaluated after 24 hours (Wilkersen and Shuman, 2001a; Wilkersen et al, 2001b; Wilkersen MJ, 2012). If blood samples must be evaluated within 24 hours of collection, this would increase costs of shipping to owners and veterinary practitioners in addition to greatly restricting when samples could be received. However, if a blood sample could be evaluated after up to 72 hours of appropriate storage, this would allow patients to be evaluated from distant locations and would widen the acceptable test receiving timeframe.

A possible candidate test for the development of a clinically applicable assay in dogs is flow cytometry. Flow cytometry can be performed using direct or indirect methods and can

provide the percentage of anti-platelet antibodies (percent IgG). The direct method entails using a patient's platelets and directly screening for antibodies whereas the indirect method involves using a patient's sera and a healthy dog's platelets to indirectly screen for antibodies (Kristensen et al, 1994b; Lewis and Meyers, 1996). Flow cytometry could therefore be a possible test used in practice to help determine if an immune process is contributing to a patient's thrombocytopenia but would not be able to distinguish between causes of thrombocytopenia. To date, there is not an assay that has been able to definitively differentiate the etiology of thrombocytopenia in a dog such as whether it is due to infectious, neoplastic, or primary immune-mediated causes (Bachman et al, 2015). However, regardless of the etiology, determining that an immune process is present can help narrow the differential list and lead to more directed diagnostics.

Therefore, the detection of anti-platelet antibodies in dogs is important and can enhance understanding of a patient's disease. The purpose of this study was to develop and evaluate direct and indirect flow cytometric assays for the detection of anti-platelet antibodies in dogs. We hypothesized that the direct assay would provide reliable results out to 72 hours when stored appropriately but that the indirect assay would not provide reliable results and was unlikely to be clinically applicable.

6.3 Materials and Methods

Animals and samples

To provide anti-platelet antibody negative samples, clinically healthy beagles were prospectively enrolled into the study which was approved by the Institutional Animal Care and Use Committee at Colorado State University prior to blood collection. All of the dogs were housed in the same conditions and were not receiving any medications. A total of 3 ml of blood were

collected via jugular venipuncture and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

One of the healthy beagles was chosen in order to establish a cut-off value for serial flow assessments at our institution which was also approved by the Institutional Animal Care and Use Committee prior to blood collection. Samples were collected from this dog once a week for 10 weeks and were processed for direct flow cytometry within 4 hours. For every blood collection, a total of 2 ml were collected via jugular venipuncture and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

To provide blood samples that would potentially be positive for anti-platelet antibodies for experiments with the direct method, client-owned dogs presenting to the Colorado State University (CSU) Veterinary Teaching Hospital between September 2016 and September 2017 were prospectively enrolled into the study. The study was approved by the institutional Clinical Review Board and all owners signed a client-consent form at the time of enrollment. To be eligible for the study, the platelet count had to be less than or equal to 75,000 platelets/µl (ADVIA 120 Hematology System, Germany) with few to no clumping observed and each dog had to have a final diagnosis of presumptive canine immune-mediated thrombocytopenia (IMTP). A total of 3 ml was collected via the cephalic vein or lateral saphenous vein and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

To provide anti-platelet antibody negative sera for the indirect method, sera was used from healthy beagles not enrolled in the current study but that had been previously shown to have a normal platelet count and were considered negative via direct flow cytometry. To provide samples that would potentially be positive for anti-platelet antibodies for use in experiments with the indirect method, sera was used from unhealthy dogs that had been previously documented to have

thrombocytopenia, were considered positive via direct flow cytometry, and were diagnosed with IMTP. All sera was stored at -80°C until processing.

Storage of EDTA anti-coagulated whole blood

In order to assess the direct flow cytometry assay at different storage time points, after each blood collection, the whole blood samples were stored at 4°C before processing. Each sample was individually processed at four separate time points; fresh, 24 hours, 48 hours, and 72 hours after blood collection. For the fresh sample, all of the samples were processed within 4 hours of blood collection.

Direct flow cytometry for anti-platelet antibodies

The protocol used for this study was modified from other protocols previously described in the literature (Kristensen et al, 1994b; Terrazzano et al, 2006). EDTA (BD Vacutainer®, USA) anticoagulated blood (500 µl) was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200 x g for 1 minute 30 seconds at 20°C to generate platelet-rich plasma (PRP). PRP was removed from the erythrocyte layer and placed into an Eppendorf tube (Light Labs SNAPLOCK Microcentrifuge Tubes, USA). Each PRP sample was adjusted to 2 x 10^6 cells/ml using a manual hemocytometer. Platelets were then pelleted by centrifugation at 1000 x g for 5 minutes at 20°C. The platelets were re-suspended and washed three times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature protected from light with 50 µl of a 1:200 dilution of FITC-labeled rabbit anti-dog IgG (Jackson ImmunoResearch Labs, USA) for 30 minutes. After incubation, the platelets were washed three times with PBS-EDTA-BSA solution and re-suspended in 200 µl of PBS-EDTA-BSA solution for flow analysis. The percentage of IgG positive cells (percent IgG)

in duplicate was recorded from each sample. Gate settings used for this study were previously established with PE-labeled mouse anti-human CD61 (eBioscience, USA) using healthy dog samples. All samples were analyzed in duplicate using a Cyan ADP instrument (Beckman Coulter, USA) and the generated data was analyzed using commercial software (FlowJo, USA).

Indirect flow cytometry for anti-platelet antibodies

Several protocols were investigated for this study that were modified from other protocols previously described in the literature (Kristensen et al, 1994b; Waner et al, 1995; Harrus et al, 1996; Terrazzano et al, 2006; Bachman et al, 2015). The platelet suspensions generating PRP were prepared as described above in section 2.3 but several PRP suspensions (2 x 10^6 cells/ml, 3 x 10^6 cells/ml, and 4 x 10^8 cells/ml) were utilized for the various protocols. Briefly, various volumes (10 µl, 20 µl) and dilutions of sera from abnormal dogs (1:10, 1:50, 1:100, 1:200) were incubated with various volumes (10 µl, 20 µl) of platelet suspensions from healthy dogs at various temperatures (room temperature, 37°C) for 30 or 60 minutes. Note: Fresh sera not previously frozen and aliquots of frozen sera stored at -80°C were used. Following incubation, the mixture was washed three times with PBS-EDTA-BSA solution as described above. Then, each sample was incubated at room temperature protected from light with 50 µl of a 1:200 dilution of FITC-labeled rabbit anti-dog IgG (Jackson ImmunoResearch Labs, USA) for 30 minutes. After incubation, the platelets were washed three times with PBS-EDTA-BSA solution and re-suspended in 200 µl of PBS-EDTA-BSA solution for flow analysis. The percentage of IgG positive cells (percent IgG) in duplicate was recorded from each sample. All samples were analyzed in duplicate using a Cyan ADP instrument (Beckman Coulter, USA) and the generated data was analyzed using commercial software (FlowJo, USA).

Data analysis

In order to have 80% statistical power to detect an effect size of 0.8 between the two groups compared, at least 8 dogs were needed in each group to provide a sufficient sample. Software was used to obtain a sample size using a one-way ANOVA model (R package, version 3.2.5). For the response variable percent IgG, a repeated measures analysis (one-way ANOVA) was performed using statistical software (GraphPad Prism, USA). Storage time points (24 hours, 48 hours, and 72 hours) were compared to the respective fresh sample using Dunnett's method (GraphPad Prism, USA). The average coefficient of variation (CV) was calculated for the samples from the thrombocytopenic dogs to evaluate intra-assay variability. For all tests, $P < 0.05$ was considered statistically significant.

6.4 Results

Study participant demographics

Nine healthy beagles and twelve client-owned thrombocytopenic dogs were enrolled into the study. The beagles ranged in age from 14-15 months and there were six male intact dogs and three female intact dogs. All of the beagles were deemed healthy by a normal physical examination and normal blood work (complete blood count and serum biochemistry panel). The platelet count was over 200,000 platelets/µl in all beagles. The age, sex, breed, and platelet count for the twelve client-owned thrombocytopenic anti-platelet antibody positive dogs are listed in Table 6.1. The thrombocytopenic dogs ranged in age from 3-11 years and there were four male castrated dogs and eight female spayed dogs. No thrombocytopenic dogs were sexually intact. Nine out of the twelve dogs were purebred and no breeds had more than one representative in the study. The platelet count in the thrombocytopenic dogs ranged from 0 – 60,000 platelets/µl. Eleven of the twelve

dogs were diagnosed with presumptive primary canine IMTP based on a combination of the following; the exclusion of underlying infectious diseases, exclusion of an underlying neoplastic condition, response to immunosuppressive therapy, and/or post-mortem examination. One of the twelve dogs was thought to have developed secondary IMTP following a recent vaccination due to patient history, rapid response, and because the patient did not require long-term immunosuppressive therapy. At the time of this writing, one dog had been euthanized secondary to complications from IMTP (melena, hematochezia, and mesenteric torsion) and two dogs were lost to follow-up.

Table 6.1: Age (years), sex*, breed, and platelet count (cells/µl) at time of study enrollment in client-owned thrombocytopenic dogs.

Age	Sex	Breed	Platelet count
4.5	MC	Cocker Spaniel	16,000
6	FS	Boxer	12,000
8	FS	Border Collie	15,000
11	FS	Old English Sheepdog	8,000
10	MC	Standard Poodle mix	4,000
10	FS	Catahoula mix	14,000
8	MC	English bulldog	8,000
3	FS	Terrier mix	None observed
8	FS	Lhasa Apso	2,000
7	FS	Great Dane	4,000
9	MC	Labrador retriever	60,000
5	FS	Irish Wolfhound	7,000

***FS (female spayed), MC (male castrated)**

Serial direct flow cytometry in a healthy dog

The percent IgG in the healthy dog chosen for serial flow analysis ranged from less than 1% to approximately 7%. An example is presented in Figure 6.1. All weekly samples were performed in duplicate which resulted in an average percent IgG of 1.5% with a standard deviation of 2.1 for

all analyses. To further evaluate the overall performance of the direct flow cytometric assay to differentiate positive from negative dogs, a receiver operator characteristics (ROC) analysis, data not shown, (GraphPad Prism, USA) showed an area under the curve of 0.95. Specifically, at a cut-off of 10%, the sensitivity was 98% and the specificity was 100%. Therefore, samples were considered positive if the percent IgG was greater than or equal to 10%.

A. **B.**

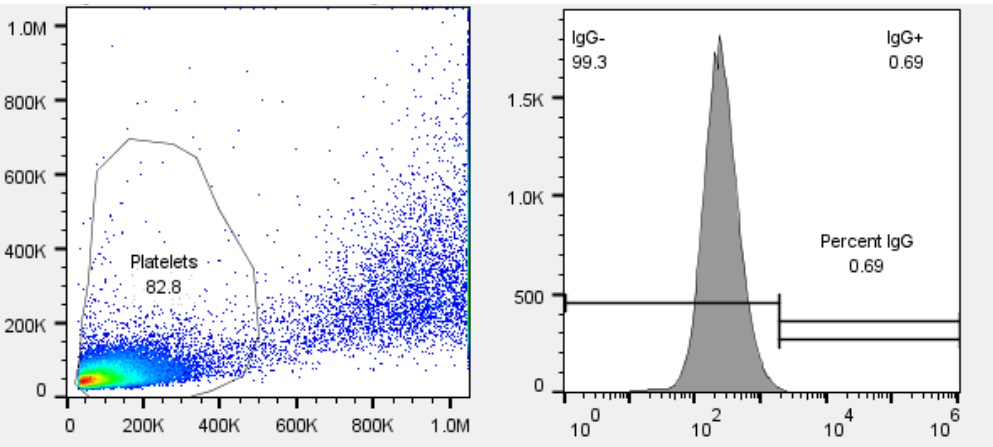

Figure 6.1: Flow cytometric results for percent of anti-platelet antibodies (percent IgG) in a healthy dog. A. Scatterplot with delineated platelet population with the y-axis representing side scatter and the x-axis representing forward scatter. B. Results are presented as a frequency histogram with the y-axis representing cell counts (platelets) and the x-axis representing the intensity channel of fluorescence (FL-1) on a logarithmic scale. The percent of IgG negative platelets is represented by IgG- and the percent positive platelets is represented by IgG+. The percent IgG is in this sample is 0.69%.

Direct flow cytometry results at different storage times in healthy dogs

Overall, there was a statistical difference between storage time points for percent IgG in the healthy dogs ($P < 0.0001$). Using Dunnett's method, the difference in percent IgG between fresh samples and 24 hour or 48 hour samples was not statistically significant. In contrast, the difference in percent IgG between fresh and 72 hours was significant. The percent IgG increased over time in all of the dogs as seen in Figure 6.2 but all of the dogs had a percent IgG of less than 10% at all time points. Therefore, none of the dogs would have been considered positive at any time point using this cutoff value.

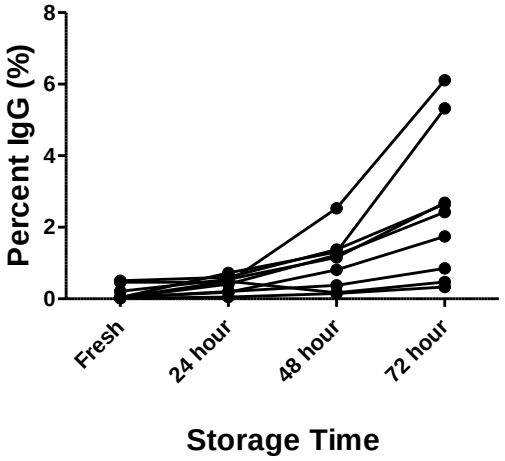

Figure 6.2: Percentage of anti-platelet antibodies (percent IgG) via direct flow cytometry at four different storage time points (fresh, 24 hours, 48 hours, and 72 hours) in healthy dogs.

Direct flow cytometry results at different storage times in thrombocytopenic dogs

An example of a thrombocytopenic dog is presented in Figure 6.3. Overall, there were no statistical differences between storage time points for the percent IgG in the thrombocytopenic dogs ($P = 0.1742$). Using Dunnett's method, the difference in percent IgG between the fresh

samples and 24 hour, 48 hour, and 72 hours was not significant. Some dogs exhibited a decrease in percent IgG over time, others exhibited a mild increase in percent IgG over time, and other dogs had relatively stable percent IgG values over time but the percent IgG for all dogs was greater than 10% at all time points (Figure 6.4). Therefore, none of the dogs would have been considered negative at any time point. The exact values of percent IgG are presented in Table 6.2.

To evaluate intra-assay variability in the thrombocytopenic dogs, an average coefficient of variation (CV) was calculated for each dog at each time point resulting in an overall average CV of 4.32%.

A. **B.**

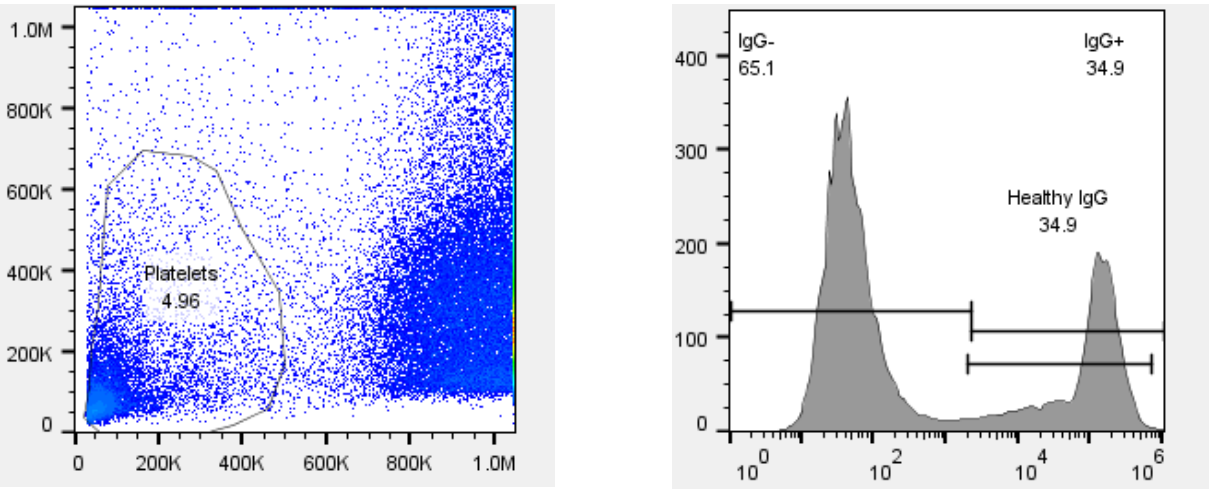

Figure 6.3: Flow cytometric results for percent of anti-platelet antibodies (percent IgG) in a thrombocytopenic dog. A. Scatterplot with delineated platelet population with the y-axis representing side scatter and the x-axis representing forward scatter. B. Results are presented as a frequency histogram with the y-axis representing cell counts (platelets) and the x-axis representing the intensity channel of fluorescence (FL-1) on a logarithmic scale. The percent of IgG negative platelets is represented by IgG- and the percent positive platelets is represented by IgG+. The percent IgG is in this sample is 34.9%.

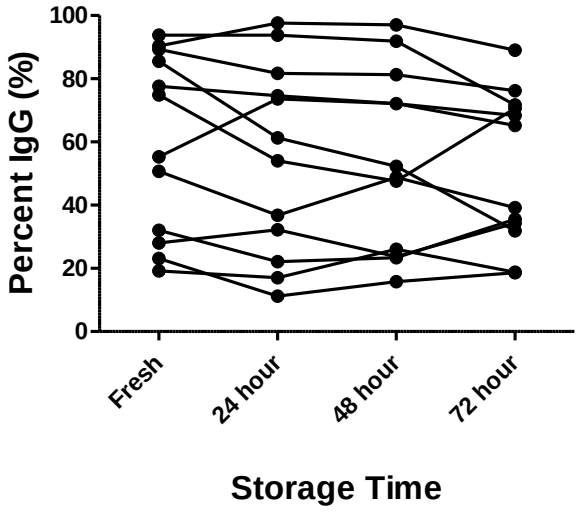

Figure 6.4: Percentage of anti-platelet antibodies (percent IgG) via direct flow cytometry at four different storage time points (fresh, 24 hours, 48 hours, and 72 hours) in client-owned thrombocytopenic dogs.

Table 6.2: Mean percentage anti-platelet antibodies (percent IgG) via direct flow cytometry at different storage time points (fresh, 24 hours, 48 hours, and 72 hours) in client-owned thrombocytopenic dogs

	Fresh (%)	**24 hours (%)**	**48 hours (%)**	**72 hours (%)**
Cocker Spaniel	89.3	81.7	81.3	76.2
Boxer	74.9	54.0	47.6	70.6
Border Collie	28.0	32.2	23.7	34.3
Old English Sheepdog	19.2	17.0	26.0	18.8
Standard Poodle mix	32.0	22.1	23.4	35.5
Catahoula mix	85.5	61.3	52.3	31.9
English bulldog	55.3	73.6	72.1	65.2
Terrier mix	77.6	74.6	72.1	68.4
Lhasa Apso	90.4	97.6	97.0	89.0
Great Dane	23.1	11.2	15.8	18.6
Labrador retriever	93.8	93.8	91.9	71.7
Irish Wolfhound	50.7	36.8	48.8	39.2

Indirect flow cytometry in healthy and thrombocytopenic dogs

None of the assays provided consistent results for healthy or thrombocytopenic dogs. A subset of healthy dogs were considered positive (percent IgG > 10%) via indirect flow testing despite having no evidence of systemic disease, documented thrombocytopenia, and had repeatable negative direct flow cytometry results.

All of the thrombocytopenic dogs that were tested were commonly classified as negative (percent IgG < 10%) despite having repeatable thrombocytopenia and positive direct flow cytometry results (percent IgG ≥ 10%). Specific examples are listed in Table 6.3. Additionally, when a previously documented positive dog's sera was tested on multiple occasions (from frozen aliquots from the same initial time point), the results were inconsistent. Specifically, the sample would be considered positive at one time point and then negative when tested at a later date. This inconsistency did not allow for the use of a positive control using indirect flow cytometry and also occurred when using fresh or frozen sera.

Table 6.3: Comparison of mean percentage anti-platelet antibodies (percent IgG) via direct and indirect flow cytometry in thrombocytopenic and healthy dogs.

	Direct percent IgG	**Indirect percent IgG**
Dog 1*	20.1%	8.4%
Dog 2*	19.8%	4.1%
Dog 3*	39.2%	1.2%
Dog 4+	0.5%	19.1%
Dog 5+	0.3%	13%
Dog 6+	0.4%	14.2%

*Thrombocytopenic dog, +Healthy dog

6.5 Discussion

The results of our study show that direct flow cytometry for anti-platelet antibodies can be reliably performed and interpreted in dogs after up to 72 hours of storage in EDTA blood at 4°C. In contrast, the indirect method used in our study did not provide reliable results and inconsistently classifies patients which would result in a high proportion of false positives and false negatives.

In order to establish a cut-off value for what would be considered positive or negative, a healthy dog was serially evaluated with direct flow cytometry. Although the average percent IgG was only 1.5%, at one point the percent IgG was 7% and this was from a freshly processed sample. If we used the cut-off of 2 standard deviations from the data, a cut-off value of approximately 5% would have been determined. This is a reasonable cut-off but when evaluating this assay, it was desirable to have a conservative cut-off to avoid the possibility for false positives and inappropriate classification of patients. Therefore, because a percent IgG of up to 7% was documented in a healthy dog, we chose a cut-off value of ≥10% rather than the less conservative 5%.

As mentioned previously, there are conflicting reports in the literature about acceptable storage times for flow cytometry when detecting anti-platelet antibodies in dogs. In 2 previous studies (Wilkersen and Shuman, 2001a; Wilkersen et al, 2001b), the percent IgG was reported to increase by 6- to 9-fold and 3- to 7-fold respectively which the authors felt could result in false positive classifications so it was recommended to perform the assay only on samples that could be processed within 24 hours. In contrast, a second study (Lewis et al, 1994) reported that the amount of platelet-bound IgG did not significantly increase after 72 hours of storage on ice and recommended that the assay could be performed within this timeframe. The protocols that were used in these three studies were similar to the protocol in our study for the direct method. Similar to our study, the amount of platelet-bound IgG or percent IgG increased over time in healthy dogs.

The first two studies did not perform statistical analysis to compare the different storage times but the third study did not find any statistical differences between storage times. Our study did not find a statistical difference between fresh and 24 or 48 hour samples but showed a statistical difference between fresh and 72 hour samples in healthy dogs. Although there was a statistical difference between fresh and 72 hours, no dogs had binding ≥ 10% so none of the dogs would have been classified as positive dogs even after 72 hours of storage at 4°C.

The percent IgG or platelet-bound IgG was only evaluated in healthy dogs in these previous studies so comparisons involving the thrombocytopenic (positive) dogs in our study is not possible. In our study, there was no significant difference in percent IgG between freshly prepared samples and samples that had been stored at 4°C for 24, 48, or 72 hours. In contrast to the healthy or negative dogs, the percent IgG in the thrombocytopenic dogs did not have an obvious pattern. The percent IgG stayed relatively similar in some dogs while there was a gradual increase or decrease in other dogs. Regardless of the pattern each individual thrombocytopenic dog demonstrated, no dogs had binding < 10% so none of the dogs would have been classified as negative dogs at any time point including after 72 hours of storage at 4°C.

In order to further characterize the direct method, an average CV was calculated in the thrombocytopenic dogs to estimate the overall intra-assay variation. For example, if the percent IgG between duplicate samples varied greatly, this could lead to uncertainty regarding how to classify the patient (i.e. positive or negative). An example would include if a patient's percent IgG was 5% in one sample and 50% in the second sample when run in duplicate. This magnitude of variability would be considered unacceptable for a clinically useful test. Our study found that the average CV in thrombocytopenic dogs was <5% which was considered an acceptable amount of variation when evaluating intra-assay variability (Reed, 2002).

The increase in percent IgG that was documented in the healthy dogs over time is likely related to several processes that have been previously described. As platelets age in circulation, the amount of immunoglobulin increases on their surface (Kelton and Denomme, 1982) whereas healthy young platelets in humans have previously been documented to have approximately 1% binding on their surface (George, 1990). Platelets stored in EDTA are also aging over time and it is possible that the normal aging process contributed to the gradual increase in percent IgG observed in our study. Additionally, it is possible that the platelet surface changes during storage which could result in additional sites for antibody attachment which could also explain the increase in percent IgG over time (George, 1990). Our study was therefore in agreement with other previously mentioned studies where there was an increase in percent IgG as storage time increased in healthy dogs.

The changes in percent IgG in the thrombocytopenic dogs were more variable at different storage times. The same theories hold true as previously mentioned for why some of the dogs had an increase in binding at longer storage times. Possible reasons for why some dogs demonstrated a decrease in percent IgG at 72 hours could include the possibility that a portion of the IgG was being transported intracellularly into the platelet as endocytosis of surface IgG has been previously documented and described (George, 1990). If IgG was being transported intracellularly, this would decrease the amount of percent IgG that is detected by the assay. Alternatively, another possibility is that because the platelet membrane is so dynamic (Ghoshal and Bhattacharyya, 2014) in health and disease states, changes in the membrane could result in less adhered antibodies that are washed away during the assay preparation. Another theoretical possibility is that IgG bound to the surface of platelets decreases over time due to the loss of IgG onto platelet-derived microparticles that are known to increase with storage (Wilkersen et al, 2001; Saito et al, 2016).

To our knowledge, no other study has assessed the change in percent IgG in thrombocytopenic dogs at various storage times using direct flow cytometry. Variability in binding for percent IgG was anticipated in both the healthy and thrombocytopenic dogs. However, in order for the direct flow method to be clinically useful for up to 72 hours of storage, the classification of dogs needed to be reliable. Although variability in percent IgG was observed in both groups of dogs (healthy and thrombocytopenic), none of the dogs were incorrectly classified based on our established cut-off value at any time point.

In contrast to the direct method, the indirect method classified healthy dogs as having percent IgG $\geq$ 10% and thrombocytopenic dogs has having percent IgG < 10%. This would not be a desirable test to use in a clinical setting since the results were not accurate, reliable, or repeatable using the methods evaluated here. It has been previously reported that the indirect flow method for detection of anti-platelet antibodies is less sensitive than the direct method (Lewis et al, 1995; Scott et al, 2002). A possible explanation for why the indirect method may not be as sensitive is because the majority of the antibodies are associated with the platelets rather than freely circulating out in the sera (Kristensen et al, 1994b). Another possibility could be related to an *in vitro* problem with the assay in that the protocols used did not provide an appropriate environment for optimal sera antibodies and platelets to interact in a consistent manner (Orsini et al, 1995). However, due to the inconsistency in results observed for the indirect method, this test using the aforementioned protocols in our study does not appear clinically useful.

There were several limitations to this study. A single breed of dog (beagle) from a homogenous source was used in this study to enroll healthy dogs and to establish the cut-off for what constitutes a negative or positive sample. It could be possible that beagles from a homogenous source exhibit differences in binding or amounts of percent IgG associated with

platelets as compared to a more heterogeneous population of dogs. However, this assay was also performed in 10 clinically healthy client-owned dogs of various breeds (data not shown) and the percent IgG was within the same ranges observed in the beagles and none of these dogs exhibited binding of ≥ 10%. Additionally, to our knowledge, it has never been documented that healthy people of different ethnicities exhibit different levels of percent IgG via flow cytometric analysis. Therefore, it seems unlikely that breed would have a significant effect on the percent IgG observed in healthy dogs and as such it was considered reasonable to only use healthy beagles in our study. However, future studies could be performed to investigate if there are any differences in percent IgG between healthy dogs of different breeds. Another limitation was that platelet microparticles were not measured which could have helped investigate why some of the thrombocytopenic dogs had percent IgG decrease over time. However, this did not appear to be clinically relevant to our study since none of the dogs were incorrectly classified despite some having a decrease in percent IgG at longer storage times. Additionally, a third group could have been included in this study such as dogs with thrombocytopenia due to reasons other than IMTP. Dogs with presumptive canine IMTP were chosen for validation of the assay because dogs with this disease would be suspected to have anti-platelet antibodies and would therefore help create cut-off values. Dogs with thrombocytopenia due to other etiologies may or may not have anti-platelet antibodies but if they are present, our laboratory has found the same trends for variability over time regarding stability of the assay (data not shown). However, future studies could be pursued in dogs with thrombocytopenia due to etiologies other than IMTP to confirm these findings.

The results of this study demonstrate that the direct flow cytometric method for the detection of anti-platelet antibodies is valid for up to 72 hours of storage at 4°C in both healthy and thrombocytopenic dogs. Although variability was observed for the percent IgG in both groups

of dogs (healthy and thrombocytopenic), none of the classifications (positive or negative) changed for any of the dogs at any time point. Therefore, the direct method represents a practical assay to be utilized in clinical veterinary practice. The indirect flow cytometric method for the detection of anti-platelet antibodies using the protocols described in our study is not a clinically applicable test due to the inconsistent classifications for both healthy and thrombocytopenic dogs. Additional studies will be required to determine how best to utilize the direct flow cytometric method in clinical practice.

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Conflict of Interest

There are no conflicts of interest to report.

Acknowledgement

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Chapter 7: Detection and dynamics of anti-platelet antibodies in thrombocytopenic dogs and in dogs diagnosed with idiopathic immune-mediated thrombocytopenia

I would like to thank Dr. Dow and Dr. Lappin for their encouragement and support regarding this subject. This quickly became one of my favorite topics of the PhD to investigate and I would not have been able to do it without their willingness to evaluate how these antibodies change over time. I would also like to thank Dr. Olver for allowing me to enroll her own personal dog (the incredibly handsome Toby) into the study.

7.1 Overview

Background: Anti-platelet antibodies can be detected in multiple disease states in dogs including idiopathic immune-mediated thrombocytopenia (IMTP). The dynamics of how these antibodies change over time in dogs with idiopathic IMTP is unknown.

Hypothesis/Objectives: The primary hypotheses are that anti-platelet antibodies (percent IgG) will be detected in thrombocytopenic dogs with multiple different etiologies and the dynamics of percent IgG in dogs with idiopathic IMTP can be used to evaluate prognosis, response to therapy, and risk of relapse. The primary objectives are to determine the percent IgG bound to platelets via flow cytometry in thrombocytopenic dogs and serially in dogs diagnosed with idiopathic IMTP.

Animals: Seventy-one client-owned thrombocytopenic dogs and twenty-seven client-owned dogs diagnosed with idiopathic IMTP.

Methods: Direct flow cytometry was performed in all thrombocytopenic dogs at initial evaluation. Flow cytometry for detection of percent IgG was performed serially at multiple time points in dogs diagnosed with suspected idiopathic IMTP. In dogs with idiopathic IMTP, a two-tailed Fisher's exact test was performed to compare survival to discharge between dogs with and without melena

and to compare prognosis, response to therapy, and risk of relapse with or without specific parameters related to changes in percent IgG.

Results: Twenty-one percent of thrombocytopenic non-IMTP dogs due to infectious, neoplastic, or toxic etiologies and all dogs with idiopathic IMTP were positive for percent IgG. Melena at initial evaluation was associated with a decreased survival to discharge (P = 0.02). The persistence of antibodies was not associated with prognosis or response to therapy but recurrence of antibodies was associated with risk of relapse (P = 0.005).

Conclusions and clinical importance: In this study, thrombocytopenic dogs without anti-platelet antibodies as detected by direct flow cytometry were unlikely to be related to an immune process. In this sample set of dogs, serial monitoring of antibodies in dogs with suspect idiopathic IMTP appeared to beneficial for determining risk of relapse.

7.2 Introduction

Anti-platelet antibodies have been detected in dogs with infectious, toxic, inflammatory, neoplastic, and immune-mediated etiologies.[1] This is also true in human medicine when evaluating thrombocytopenic patients.[2] Primary or idiopathic immune-mediated thrombocytopenia (IMTP) which can be associated with anti-platelet antibodies in both human and veterinary medicine is a diagnosis of exclusion when all known other possible secondary causes of immune-mediated thrombocytopenia have been attempted to be excluded.[1,2] Therefore, the detection of these antibodies does not confirm that an idiopathic immune-mediated condition exists and there is currently no test that is able to reliably differentiate primary from secondary causes of immune-mediated thrombocytopenia.[3,4] Historically, studies regarding anti-platelet

antibodies in dogs have focused on the detection of these antibodies by various methods and in various disease states.[3,5-17] In human medicine however, multiple studies have been performed to further investigate the characteristics of these antibodies. For example, anti-platelet antibodies are documented in approximately 60-70% of people diagnosed with primary immune thrombocytopenia (ITP).[2,18] It is therefore theorized that non-antibody dependent mechanisms such as direct cell-mediated destruction contribute to this disease in humans.[2,19-20] It is currently unknown if some dogs with idiopathic IMTP lack antibodies. Of additional interest is that there has recently been a call in human medicine to investigate antibodies in humans diagnosed with primary ITP and to see if the presence of these antibodies relate to important clinical parameters such as prognosis, response to therapy, or risk of recurrence/relapse.[21] Several studies have previously been performed in human medicine investigating these topics but have either not been prospectively designed or were performed in small cohorts of people.[22] However, more recent human studies have shown a possible benefit of detecting antibodies in relationship to severity of disease such as bleeding risk, response to therapy, and risk of recurrence.[20-24] To our knowledge, no such studies have been pursued in veterinary medicine. As a result, it is unknown how these antibodies change in dogs diagnosed with idiopathic IMTP over time and if these changes would be considered clinically relevant. Therefore, the objectives of this study were to evaluate thrombocytopenic dogs for the presence of anti-platelet antibodies and characterize the dynamics of these antibodies over time in dogs diagnosed with idiopathic IMTP and how they relate to prognosis, response to therapy, and/or risk of relapse/recurrence.

7.3 Materials and Methods

Client-owned thrombocytopenic dogs (non-idiopathic IMTP). EDTA (BD Vacutainer® K2 EDTA 3.6 mg 2.0 ml tubes, Franklin Lakes, NJ, USA) anti-coagulated whole blood previously used for a

CBC (Siemens, ADVIA 120 Hematology System, Erlangen, Germany) or submitted for testing for anti-platelet antibodies (Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO, USA) at the Colorado State University Veterinary Teaching Hospital was prospectively collected from thrombocytopenic dogs and used for evaluation of anti-platelet antibodies (percent IgG). Blood samples were collected between April 2016 – May 2018. Blood was collected and processed within 72 hours which is within the known range of accuracy of the test (see Chapter 6). To increase the number of samples positive for infectious agents, thrombocytopenic blood samples from IDEXX that were positive via PCR (Fever of Unknown Origin RealPCR^{TM} Panel (Comprehensive), IDEXX Laboratories Inc., Westbrook, ME, USA) for the infectious agents *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., *Babesia* spp., and *Leptospira* spp. were submitted for analysis. All samples were shipped on ice packs and were processed within 72 hours of blood sample collection for flow cytometry. Results for percent IgG (positive or negative) were recorded for each dog as previously described (see Chapter 6). Thrombocytopenia was defined as a platelet count less than 200,000 platelets/µl without many platelet clumps as determined by manual review by a clinical pathology technician or board certified clinical pathologist. Dogs where a secondary cause for thrombocytopenia such as neoplasia (diagnosed via cytology and/or histopathology) or an infectious etiology (diagnosed via serology and/or PCR testing) was documented were classified as non-idiopathic IMTP patients. Dogs were placed into one of several categories; infectious, neoplastic or other.

Client-owned idiopathic IMTP dogs. This prospective study was approved by the institutional Clinical Review Board and all owners signed a client-consent form at the time of enrollment. Inclusion criteria included a platelet count less than or equal to 60,000 platelets/µl with few to no platelet clumping observed and each dog had to have a final diagnosis of presumptive canine

idiopathic IMTP. Additionally, to be eligible for study enrollment, each dog had to be negative for antibodies against *Anaplasma* spp., *Borrelia burgdorferi*, and *Ehrlichia* spp., antigens of *Dirofilaria immitis*, and DNA of *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Ehrlichia* spp., the hemoplasmas, *Neorickettsia* spp., and *Rickettsia* spp. (Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, CO, USA and SNAP® 4Dx® Plus, IDEXX Laboratories Inc., Westbrook, ME, USA). Exclusion criteria included the documentation of infectious, neoplastic, or toxic conditions. EDTA (BD Vacutainer® K2 EDTA 3.6 mg 2.0 ml tubes, Franklin Lakes, NJ, USA) anti-coagulated whole blood previously used for a CBC (Siemens, ADVIA 120 Hematology System, Erlangen, Germany) at the Colorado State University Veterinary Teaching Hospital was collected from all dogs at initial evaluation and subsequent recheck appointments and used for evaluation of percent IgG. The recheck appointments were determined at the discretion of the attending clinician. Blood was collected at the initial evaluation in all dogs and was processed within 72 hours. Blood samples were collected between September 2016 – May 2018. For all dogs at each time point, the percent IgG was recorded (positive or negative). Whether there was a persistence of antibodies or recurrence of antibodies documented (yes or no) was also recorded. The persistence of antibodies was defined as the presence of antibodies 4 weeks after initiation of therapy for idiopathic IMTP. Recurrence of antibodies was defined as the presence of antibodies after a patient had been previously documented to be negative for antibodies. The prognosis (good or poor), response to therapy (yes or no), and risk of recurrence/relapse (yes or no) were also recorded for each dog. The prognosis was defined as poor if the patient had evidence of melena at initial evaluation as that has been previously reported to be a poor prognostic marker in idiopathic IMTP in dogs.[25] Response to therapy was defined as the platelet count returned to normal levels (defined as $\geq$ 200,000 platelets/μl) within four weeks of

initial diagnosis. Relapse/recurrence of IMTP was defined as a patient who had previously been documented to have platelet counts return to normal (defined as ≥ 200,000 platelets/µl) after immunosuppressive therapy was initiated but on subsequent rechecks was observed to have a platelet count of less than or equal to 100,000 platelets/µl with few to no platelet clumps present.

Direct flow cytometry assay for anti-platelet antibodies (percent IgG). The protocol used for our study was modified from other protocols previously described in the literature and was validated at our institution (also see Chapter 6).[6,15] Briefly, blood (500 µl) anticoagulated with EDTA was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200 · g for 1 minute 30 seconds at 20°C to generate platelet-rich plasma (PRP). Platelet-rich plasma was removed from the erythrocyte layer and placed into an Eppendorf tube (Light Labs SNAPLOCK Microcentrifuge Tubes, Dallas, TX, USA). Seventy microliters of PRP was then pelleted by centrifugation at 1000 · g for 5 minutes at 20°C. The platelets were re-suspended and washed 3 times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature with 50 µl of a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled rabbit anti-dog IgG^g for 30 minutes. After incubation, the platelets were washed 3 times with PBS-EDTA-BSA solution and re-suspended in 200 µl of PBS-EDTA-BSA solution for flow analysis. Gate settings used for our study were previously established with PE-labeled mouse anti-human CD61 (Anti-human CD61 (Integrin beta 3) PE, VI-PL2, 12-0619-42, eBioscience, San Diego, CA, USA) using healthy dog samples. Platelets from a healthy beagle served as a negative control at each time point. Samples were considered positive if the percent IgG was ≥ 10%. All samples were analyzed using a flow cytometer instrument (Cyan ADP instrument, Beckman Coulter, Miami, FL, USA) and the generated data was analyzed using standard software (FlowJo software, Tree Star, Ashland, OR, USA).

Statistical analysis. A two-tailed Fisher's exact test was performed to compare survival to discharge (yes or no) between dogs with and without melena. Dogs were defined as having melena if melena was observed at presentation. A two-tailed Fischer's exact test was also performed to compare prognosis (good or bad) and dogs with persistence of percent IgG (yes or no); response to therapy (yes or no) and persistence of percent IgG (yes or no); and relapse (yes or no) and persistence or recurrence of percent IgG (yes or no). The statistics were performed with commercially available software (GraphPad Prism 5.0, San Diego, CA, USA) and a $P > 0.05$ was considered significant.

7.4 Results

Client-owned thrombocytopenic dogs (non-idiopathic IMTP).

Animals. Seventy-one dogs with thrombocytopenia due to causes other than idiopathic IMTP were evaluated. These dogs were further divided into the following categories; infectious (n = 13), neoplastic (n = 23), and other (n = 36). One dog was placed into the other category in 2015 and then was later also included into the neoplastic category in 2018 resulting in a total of 71 dogs rather than 72 dogs. The age range of all dogs was 9 weeks – 15 years.

Thrombocytopenia due to infectious diseases. Note: The signalment of most of the dogs in the infectious category were not known because the majority of samples were provided by IDEXX. In addition, the details of the case from samples from IDEXX were unknown but the platelet count was recorded in each case. For the infectious agents *Bartonella* spp., *Babesia* spp., or *Leptospira* spp., no thrombocytopenic PCR positive samples were obtained for the study. The median platelet count was 62,000 platelets/µl (range 22,000 – 180,000). The signalment was known for two of thirteen dogs (2/13, 15%) infectious cases; a 9 week old FI Husky with acute ehrlichiosis and a 6 year old MC German Shepherd with disseminated *Emmonsia* spp. The specific infectious disease

etiologies and categories of positive or negative for percent IgG is presented in Table 7.1. Eight out of thirteen dogs (8/13, 62%) were positive for percent IgG. For the 9 week old FI Husky with acute ehrlichiosis, a sample at initial evaluation in addition to one serial sample was available for analysis. At initial diagnosis, the platelet count was 61,000 platelets/µl with an elevated mean platelet volume (MPV) and no clumps and the percent IgG results were positive. A course of doxycycline was initiated; 5 mg/kg PO every 12 hours for 4 weeks. Thirteen days after initial presentation, the recheck platelet count was 260,000 platelets/µl with a normal MPV and few clumps and the repeat flow cytometry was negative for percent IgG. For the 6 year old MC German Shepherd, the platelet count at initial evaluation was 76,000 platelets/µl with an elevated MPV and rare clumps and the percent IgG results were positive. Humane euthanasia was performed shortly after initial presentation so serial flow cytometry could not be performed.

Table 7.1: Anti-platelet antibodies (percent IgG) in thrombocytopenic dogs due to infectious causes.

Infectious etiology	Number of dogs positive for percent IgG	Number of dogs negative for percent IgG
Ehrlichiosis	2	
Anaplasmosis	5	5
Babesiosis		
Bartonellosis		
Leptospirosis		
Fungal disease	1	

Thrombocytopenia due to neoplastic causes. For dogs in the neoplastic category, 13 were female spayed and 10 were male neutered. None of the dogs in this category were sexually intact. The median age for the dogs was 10 years (range 5 to 15 years). Sixteen out of the twenty-three (16/23, 70%) dogs were purebred and represented 12 breeds. Breeds with more than one representative

included Golden Retriever (3/22, 14%), German Shepherd Dog (2/22, 9%), and Labrador Retriever (3/22, 14%) however there was one additional Labrador Retriever mix (1/22, 5%). There were 10 different neoplasms diagnosed. Four out of the twenty-two (4/22, 18%) dogs had two concurrent neoplasms. Specifically, the concurrent neoplasms included mast cell tumor (MCT) and soft tissue sarcoma (STS); malignant pheochromocytoma with vascular invasion and maxillary osteosarcoma (OSA); lymphoma and metastatic appendicular OSA; and metastatic apocrine gland anal sac adenocarcinoma (AGASACA) and a myelodysplastic disorder. Neoplasms that were diagnosed in multiple dogs included lymphoma (n = 6), OSA (n = 4), hemangiosarcoma (HSA) (n = 5), MCT (n = 3), transitional cell carcinoma (TCC) (n = 2), and STS (n = 2). Four out of the twenty-three (4/23, 17%) dogs with neoplasia were positive for percent IgG and nineteen out of the twenty-three (19/23, 83%) dogs were negative for percent IgG. The specific neoplasms that were associated with a positive percent IgG result included MCT, disseminated carcinoma, concurrent lymphoma and OSA, and concurrent metastatic AGASACA and acute megakaryocytic leukemia. The median platelet count in dogs in the neoplastic category was 43,000 platelets/µl (range 8,000 – 160,000 platelets/µl). Three of the four dogs that were positive for percent IgG were tested for concurrent infectious disease via serology[e] (n = 3) and/or PCR[c] (n = 2). The one dog that was not tested for concurrent infectious disease was the dog diagnosed with concurrent metastatic AGASACA and acute megakaryocytic leukemia.

Thrombocytopenia in dogs due to other causes. For dogs in the other category, 13 were female spayed, 2 were female intact, 18 were male neutered, and 3 were male intact. The median age for the dogs was 6.5 years (range 1 to 12 years). Thirty-one out of the thirty-six (31/36, 86%) dogs were purebred and represented 26 breeds. Breeds with more than one representative included Weimaraner (n = 2), Standard Poodle (n = 2), Labrador Retriever (n = 2), German Shepherd Dog

(n = 2), and miniature Dachshund (n = 2). Dogs in the other category had multiple final diagnoses (Table 7.2) but classifications with more than one representative included apparently healthy (n = 5), pancreatitis (n = 2), gastric ulceration (n = 2), pyelonephritis (n = 3), and rattlesnake bite (n = 3). The median platelet count in dogs in the other category was 96,500 platelets/µl (range 2,000 – 198,000 platelets/µl).

Table 7.2: Breed, final diagnosis, and platelet count in thrombocytopenic dogs due to causes other than idiopathic, infectious, or neoplastic.

Dog	Breed	Final Diagnosis	Platelet count (x 10^3)
1	Bichon Frise	Chronic pancreatitis	101
2	Mix breed	Apparently healthy	156
3	Weimaraner	Mitral valve endocarditis	139
4	West Highland White terrier	Rodenticide ingestion	198
5	Papillon	Chronic kidney disease	40
6	Yorkshire terrier	Pyelonephritis	40
7	German Shepherd dog	Apparently healthy	169
8	Standard Poodle	Hypoadrenocorticism	152
9	Mix breed	Trauma, dog fight	169
10	Mix breed	Apparently healthy	81
11	Corgi	Pyelonephritis	39
12	Doberman Pinscher	Gastric ulceration	76
13	Pitbull	Chronic hepatitis	147
14	Cavalier King Charles Spaniel	Macrothrombocytopenia, nephritis	11
15	Pitbull mix	Apparently healthy	198
16	Airdale terrier	Urinary tract infection	127
17	Miniature Dachshund	Erythema multiforme	103
18	Labrador retriever	Chronic enteropathy	63
19	Standard Poodle	Copper hepatopathy	143
20	Samoyed	Bilateral trigeminal neuropathy	71
21	Weimaraner	Pyelonephritis, acute pancreatitis	21
22	Mix breed	Lymphedema, vasculitis	136
23	Exotic canine	Ventricular septal defect	138
24	Great Dane	OHE* complications, DIC+	39
25	Miniature Dachshund	Coagulopathy, myelopathy	18
26	Rottweiler	Gastric ulceration	146
27	Basset Hound	Apparently healthy	39

28	Border Collie	IMHA[^]	107
29	Saint Bernard	Iatrogenic bone marrow suppression	2
30	English Setter	Rattlesnake bite	39
31	Irish Setter	Rattlesnake bite	28
32	Toy Poodle	Immune-mediated neutropenia	92
33	Xoloitzcuintli	Urelolith, urethral tear	42
34	Goldendoodle	Septic abdomen, foreign body surgery	108
35	German Shepherd	IMHA^	59
36	Labrador retriever	Rattlesnake bite	141

[*]OHE = ovariohysterectomy, [+]DIC = disseminated intravascular complications, [^]Immune-mediated hemolytic anemia

Three out of the thirty-six dogs (3/36, 8%) were positive for percent IgG and thirty-three out of thirty-six dogs (33/36, 92%) were negative for percent IgG. The specific diseases that were associated with a positive percent IgG included IMHA (n = 2), immune-mediated neutropenia (IMN), and bone marrow suppression from chlorambucil. All of the dogs that were positive for percent IgG were tested for concurrent infectious disease via serology[e] and/or PCR[c] (n = 1).

Client-owned idiopathic IMTP dogs.

Animals. Twenty-seven client-owned dogs were diagnosed with idiopathic IMTP and were enrolled in the study. There were 15 female spayed and 12 male neutered dogs. None of the dogs were sexually intact. The median age of the dogs was 8 years (range 2 to 13 years). Twenty-three out of the twenty-seven dogs (23/27, 85%) were purebred dogs and represented 21 breeds. Breeds with more than one representative included Lhasa Apso (n = 2), miniature Dachshund (n = 2), and Border Collie (n = 2).

Case presentation, prognosis, and follow-up. Clinical signs of bleeding at presentation included petechiation, ecchymoses, gingival bleeding, hematemesis, melena, and hematuria however thrombocytopenia was an incidental finding during a wellness examination in five dogs (5/27,

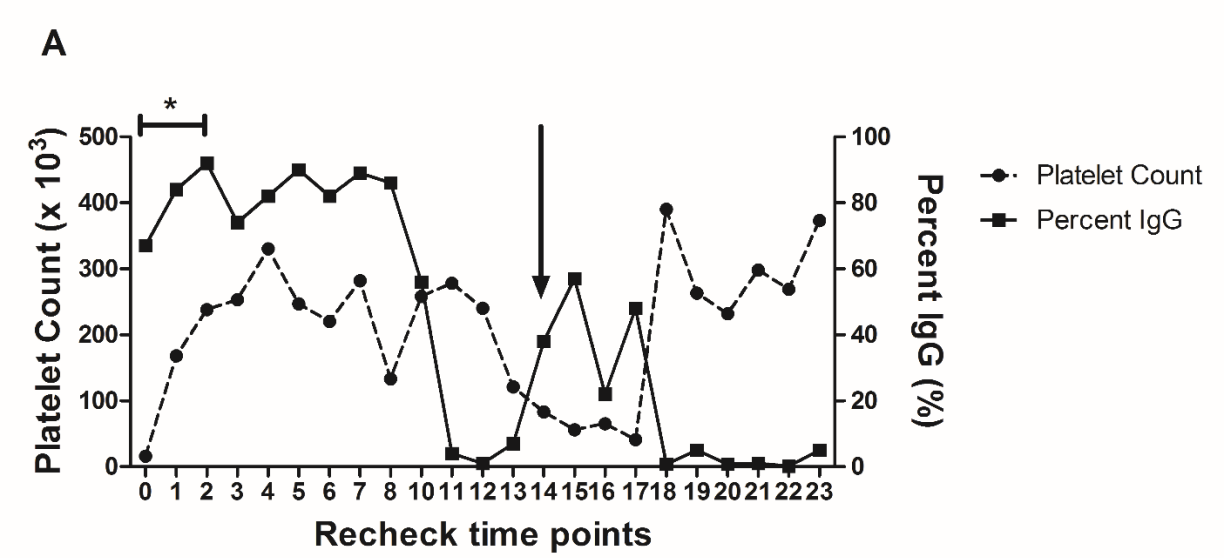
19%). Ten out of the twenty-seven dogs (10/27, 37%) presented with melena and five of these dogs (5/10, 50%) were euthanized within 48 hours of presentation. The reasons for euthanasia included acute onset of a concurrent mesenteric torsion, financial constraints, and owner concern for suffering and quality of life. In total, six out of the twenty-seven (6/27, 22%) dogs were euthanized (n = 5) or died at home (n = 1) within 48 hours of presentation and five of these dogs (5/6, 83%) presented with melena. The presence of melena at initial evaluation was associated with a decreased probability for survival to discharge (P = 0.02). Dogs with melena at initial evaluation were less likely to survive to discharge (OR = 0.06; 95% CI, 0.01, 0.67).

Twenty of the twenty-seven dogs (20/27, 74%) were able to be evaluated for prognosis as defined by this study. Seven dogs could not be evaluated for prognosis because they were not evaluated beyond 4 weeks of study due to being lost to follow-up (n = 1), humane euthanasia (n = 5), or died at home (n = 1). There was no statistical difference in prognosis between dogs with or without persistent antibodies.

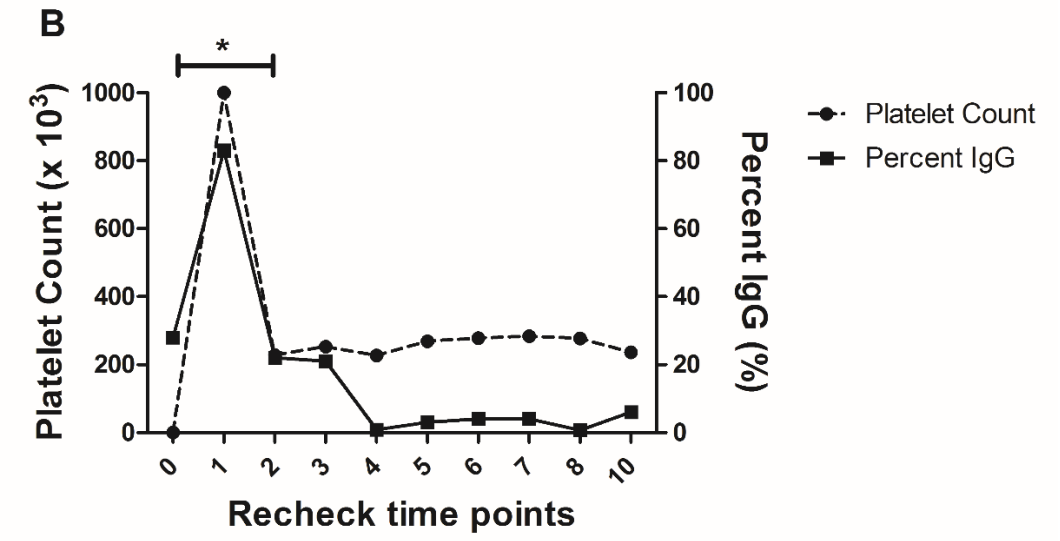
The remaining 20 dogs were followed serially for various periods of time (range 2 to 20 months) depending on time of enrollment, clinician preference, and availability of patient. One additional dog was euthanized within 2 months of diagnosis due to suspected idiosyncratic mycophenolate toxicity resulting in cutaneous lesions, increased liver enzymes, and severe bone marrow suppression. Three out of the twenty-seven (3/27, 11%) dogs were lost to follow-up at various time points after initial presentation (1 month, 2 months, and 6 months respectively). Reasons for this included non-compliant owners (n = 2) and pet relinquishment to a shelter due to financial constraints (n = 1).

Response to therapy. Twenty-one dogs were able to be evaluated for response to therapy as defined by the study; seventeen dogs (17/21, 81%) were classified as having a response to therapy

whereas four dogs (4/21, 19%) did not. Four of the seventeen dogs (4/17, 24%) that had a response to therapy (defined as occurring within 4 weeks of diagnosis) remained positive for percent IgG during this time period despite having normal platelet counts. Specifically, one dog that was persistently positive for percent IgG that had demonstrated a response to therapy (platelet count 300,000 platelets/µl with few clumps) was lost to follow-up on the 5th week following initial diagnosis so it unknown if this dog would have become negative for percent IgG. The remaining three dogs were not lost to follow-up and remained positive for percent IgG for 5 weeks, 3 months, and 7 months respectively. Figure 7.1 illustrates the dynamics of the percent IgG and platelet count in two of these dogs. Two of the four dogs (2/4, 50%) that were classified as not having a response to therapy during this time frame (within 4 weeks of initial diagnosis) remained positive for percent IgG the entire time. However, one of the four dogs (1/4, 25%) was intermittently weakly positive during this time frame. Finally, the dog that was classified as not having a response to therapy and was negative for percent IgG within 4 weeks of diagnosis had a platelet count of 154,000 platelets/µl with few clumps. It is possible this dog truly did have a response (platelet count of $\geq$ 200,000 platelets/µl) but this was not possible to determine because of platelet clumping. Figure 7.2 illustrates the dynamics of percent IgG and platelet count in these four dogs over time. There was no statistical difference in response to therapy between dogs with or without persistent antibodies.



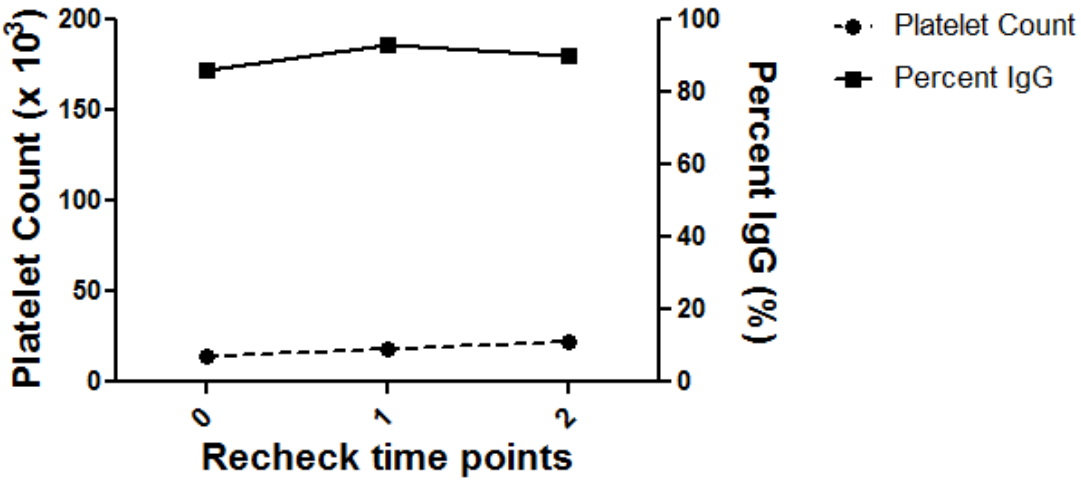
*Indicates response to therapy. Response to therapy was defined as the platelet count returned ≥ 200,000 platelets/µl with clumps within four weeks of initial diagnosis. The large black arrow indicates time of relapse. Relapse/recurrence of IMTP was defined as a platelet count dropping to ≤ 100,000 platelets/µl with few to no platelet clumping present.



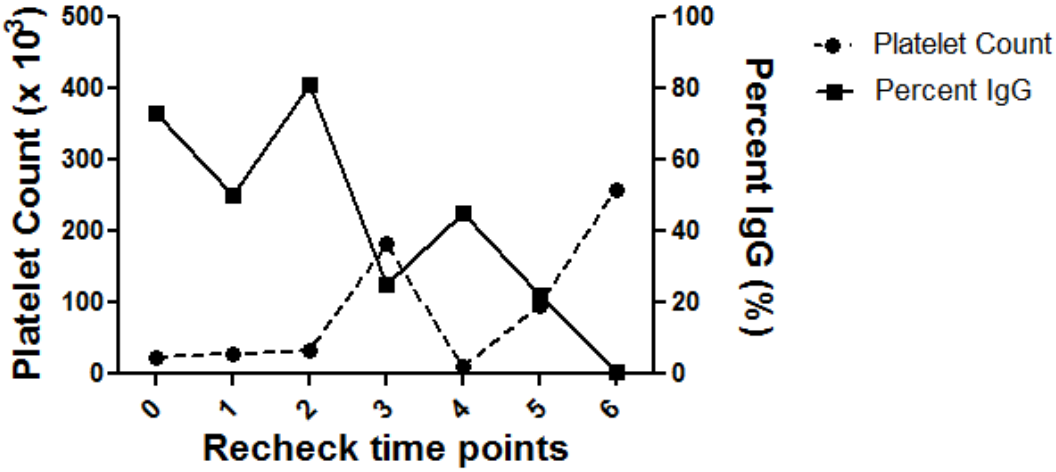
*Indicates response to therapy. Response to therapy was defined as the platelet count returned ≥ 200,000 platelets/µl within four weeks of initial diagnosis.

Figure 7.1: The platelet count and anti-platelet antibodies (percent IgG) at multiple recheck time points during study enrollment in two client-owned dogs that demonstrated a response to therapy and were diagnosed with idiopathic immune-mediated thrombocytopenia (IMTP). A percent IgG < 10% is considered negative. A = Dog that demonstrated a response to therapy and also experienced a relapse. B = Dog that demonstrated a response to therapy but did not experience a relapse. Recheck time points are 1-4 weeks apart.

A


Platelet Count (x 10^3)
200
150
100
50
0
100
80
60
40
20
0
Percent IgG (%)
0
1
2
Recheck time points
Platelet Count
Percent IgG

B


Platelet Count (x 10^3)
500
400
300
200
100
0
100
80
60
40
20
0
Percent IgG (%)
0
1
2
3
4
5
6
Recheck time points
Platelet Count
Percent IgG

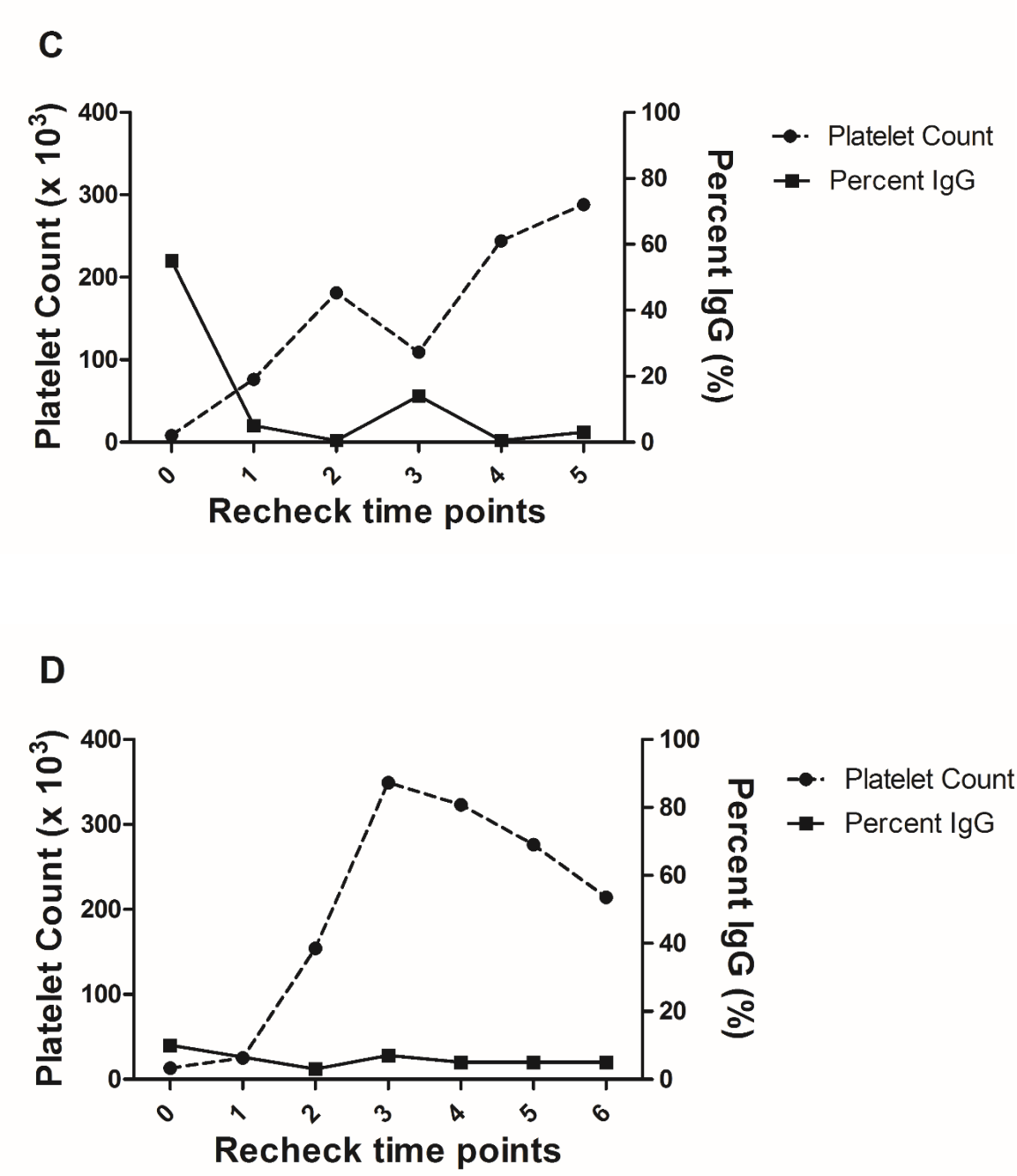


Figure 7.2: The platelet count and anti-platelet antibodies (percent IgG) at multiple recheck time points during study enrollment in four client-owned dogs that did not demonstrate a response to therapy and were diagnosed with idiopathic immune-mediated thrombocytopenia (IMTP). Response to therapy was defined as the platelet count returned ≥ 200,000 platelets/µl with clumps within four weeks of initial diagnosis. A percent IgG < 10% is considered negative. A = Dog that remained positive for percent IgG but was lost to follow-up due to pet relinquishment. B = Dog that remained positive for percent IgG for 5 weeks following initial diagnosis. C = Dog that was intermittently positive during the study period. D = Dog that became quickly negative and remained negative throughout the study period. Recheck time points are 1-2 weeks apart.

Relapse. Twenty-one dogs were able to be evaluated for relapse as defined by the study; two of the twenty-one dogs (2/21, 10%) experienced a relapse but nineteen dogs (19/21, 90%) did not. The dogs that relapsed were both historical idiopathic IMTP dogs that had already experienced a previous relapse. As a result, both dogs were enrolled into the study at the time of their second relapse. In one dog, the first relapse occurred seventeen months after the initial diagnosis of idiopathic IMTP and the second relapse occurred exactly one year after the first relapse. In the second dog that relapsed, the first relapse occurred ten months after the initial diagnosis of idiopathic IMTP and the second relapse occurred eleven months after the first relapse. In the two dogs that relapsed, both had been previously negative for percent IgG but were positive for percent IgG (recurrence of antibodies) at the time of relapse. One of these dogs was also the dog that demonstrated persistence of antibodies for 7 months before becoming negative for percent IgG (see Figure 7.1 A).

Due to the definitions of what classified a relapse in this study (relapse occurring during study period), two additional dogs did experience a relapse but could not be classified as a relapsing dog. Specifically, one dog was also historically diagnosed with idiopathic IMTP and was enrolled into the study at the time of the first relapse which occurred exactly one year after initial diagnosis. This dog was followed for 14 months and did not experience a second relapse during the study as of the time of this writing. The second dog had been lost to follow-up at 6 months due to moving out of state but later was determined to have relapsed approximately ten months following initial diagnosis (owner communication) but no blood samples were available for analysis. This dog was subsequently euthanized due to owner concern for quality of life and no additional treatment was pursued. The nineteen dogs that did not experience a relapse were followed for various amounts of time as a result of when they were enrolled into the study (range

2 months – 15 months). The risk of relapse was associated with a recurrence of antibodies (P = 0.005). Dogs that experienced a relapse were more likely to have a recurrence of antibodies (OR = 195; 95% CI, 3.1, 12200).

Treatment. At initial evaluation, twenty-five out of the twenty-seven dogs (25/27, 93%) were started on either intravenous (n = 15) or oral (n = 10) glucocorticoids (range 0.8 – 2.6 mg/kg/day) which were given either once or twice daily. Two dogs were initially not started on glucocorticoids due to concurrent conditions that could be exacerbated by glucocorticoids; diabetes mellitus and urethral sphincter mechanism incompetence. Instead of glucocorticoids, one dog was started on azathioprine and cyclosporine while the other dog was started on mycophenolate. Fifteen dogs (15/27, 56%) were administered vincristine (0.02 mg/kg IV once) at initial evaluation. Nine dogs (9/27, 33%) were administered one or more blood transfusions; six dogs received one transfusion, two dogs received two transfusions, and one dog received three transfusions. A second immunomodulatory drug in addition to glucocorticoids was started at initial evaluation in twenty dogs (20/27, 74%). Specifically, fifteen dogs (15/20, 75%) were started on either intravenous (n = 2) or oral (n = 13) mycophenolate (range 13.2 – 28.1 mg/kg/day) which was given twice daily; two dogs (2/20, 10%) were started on cyclosporine (range 4.5 – 12.1 mg/kg/day) which was given once to twice daily; one dog (1/20, 5%) was started on cyclosporine (4.5 mg/kg/day) and azathioprine (1.1 mg/kg/day); one dog (1/20, 5%) was started on azathioprine (2.3 mg/kg/day); and one dog (1/20, 5%) was started on leflunomide (0.89 mg/kg/day). Eleven dogs (11/27, 41%) received doxycycline (range 5.1 – 8 mg/kg twice daily or 10.1 – 11.7 mg/kg once daily) for 14 – 28 days.

Reported side effects from glucocorticoid administration included polyphagia, polydipsia, polyuria, urinary incontinence, joint laxity, weight gain, increased liver enzymes, worsening of

historical tracheal collapse, and suspected gastrointestinal bleeding resulting in anemia which resolved upon discontinuation of glucocorticoids. There were no reported side effects from mycophenolate in the majority of dogs with the exception of two dogs; one dog developed severe diarrhea necessitating discontinuation of the medication and one dog presented with a hemoabdomen and was suspected to have suffered a severe idiosyncratic toxicity from the drug resulting in cutaneous lesions (patchy erythema), elevated liver values, and severe bone marrow suppression. There were no reported side effects from cyclosporine administration. In the one dog that was administered leflunomide, significant diarrhea was observed within 12 hours of starting this medication resulting in discontinuation however it was later re-instituted with no diarrhea observed; therefore it is suspected the diarrhea was not related to leflunomide.

Percentage binding for anti-platelet antibodies. In order to investigate whether the percentage binding helped differentiate etiologies of thrombocytopenia in dogs that were considered positive for anti-platelet antibodies (percent IgG > 10%), the percent IgG was compared for dogs with idiopathic IMTP and non-idiopathic IMTP (including infectious, neoplastic, and other causes). Note: For this comparison, only dogs who had not any therapy for suspected IMTP were evaluated to eliminate the possible effect of previous drug administration. The magnitudes of percentage binding varied greatly and had varying amounts of overlap between the different etiologies as demonstrated in Table 7.3 from a subset of patients in this study.

Table 7.3: Anti-platelet antibodies (percent Ig) or magnitude of binding in a subset of dogs classified as positive for percent IgG (> 10%) with idiopathic immune-mediated thrombocytopenia (IMTP) and non-idiopathic IMTP due to infectious, neoplastic, and other causes

Idiopathic IMTP	Infectious	Neoplastic	Other
51%	63%	15%	18%
28%	33%	37%	52%
86%	68%	25%	35%
73%	21%	46%	
55%	11%		
10%	18%		
27%	61%		
44%	17%		
22%			
93%			
49%			
18%			

Fluorescent intensity magnitude. In order to investigate whether the fluorescent intensity helped differentiate etiologies of thrombocytopenia in dogs that were considered positive for anti-platelet antibodies (percent IgG > 10%), the fluorescent intensity (represented as geometric mean fluorescent intensity; GMFI) was compared for dogs with idiopathic IMTP and non-idiopathic IMTP (including infectious, neoplastic, and other causes). Note: For this comparison, only dogs who had not any therapy for suspected IMTP were evaluated to eliminate the possible effect of previous drug administration. The magnitude of fluorescent intensity varied greatly and had varying amounts of overlap between the different etiologies as demonstrated in Table 7.4 from a subset of patients in this study.

Table 7.4: Magnitude of fluorescent intensity (represented as geometric mean fluorescent intensity; GMFI) in a subset of dogs classified as positive for anti-platelet antibodies (percent IgG > 10%) with idiopathic immune-mediated thrombocytopenia (IMTP) and non-idiopathic IMTP due to infectious, neoplastic, and other causes

Idiopathic IMTP	Infectious	Neoplastic	Other
1606.5	7742	1365	360
259	697	475	3695
3157	2568	1241	736
9172	1132	1137	
648	1494		
86.7	888		
796.5	5368		
2350.5	3158		
307			
2389			
2096.5			
248			

7.5 Discussion

In this study, anti-platelet antibodies were primarily documented in dogs diagnosed with idiopathic IMTP but were also documented in thrombocytopenic dogs with infectious, neoplastic, toxic, or other immune-mediated conditions. All dogs diagnosed with idiopathic IMTP were positive for anti-platelet antibodies. Therefore, in this study the absence of anti-platelet antibodies in thrombocytopenic dogs was unlikely to be related to an immune process. In dogs with idiopathic IMTP, the prognosis and response to therapy were not associated with persistence of antibodies however the risk of relapse was associated with recurrence of antibodies. Serial evaluation of antibodies in conjunction with platelet count in dogs with idiopathic IMTP could be beneficial for determining risk of relapse.

Anti-platelet antibodies (percent IgG) were documented in thrombocytopenic dogs with evidence of a number of different primary causes including idiopathic IMTP which is in accordance with previous studies[3,5,6,8-17] further emphasizing the fact that these antibodies should not be used to differentiate primary from secondary IMTP in dogs. For infectious disease etiologies, it has been previously documented that dogs with ehrlichiosis and anaplasmosis can be positive for antibodies but these antibodies have never been reported in a disseminated fungal disease in a dog.[8-17] This dog was referred for a second opinion for evaluation of a persistent fever, tachypnea, elevated liver values, thrombocytopenia, and a diffuse miliary lung pattern. The dog had previously received a 4 week course of doxycycline in addition to a short course (5 days) of immunosuppressive glucocorticoids. The platelet count on initial presentation was 76,000 platelets/µl with an elevated MPV and few clumps. Humane euthanasia was pursued due to rapid clinical deterioration and the final diagnosis was determined at necropsy. To our knowledge, this is the first study to document antibodies in a thrombocytopenic dog affected by a disseminated *Emmonsia* spp. infection. For neoplastic etiologies, it has been previously documented that dogs with a variety of neoplasms can be positive for percent IgG but our study also documented antibodies in dogs with MCT, disseminated carcinoma, OSA and AGASACA which has not been previously documented.[5,8,14,16]

For thrombocytopenic dogs in the other category, only a small number were documented to be positive for percent IgG. Previous studies have documented these antibodies in IMHA and IMN which was in accordance with our study.[5,6] Additionally, these antibodies have also been detected in dogs with suspected secondary IMTP from trimethoprim/sulfadiazine drug administration[8] but to our knowledge, this has not been previously documented in a dog suffering from bone marrow suppression after chlorambucil administration. This dog was being

administered chlorambucil for a previous diagnosis of a chronic enteropathy but was subsequently noted to be severely leukopenic and thrombocytopenic. In humans, a large number of drugs have been implicated in drug-induced thrombocytopenia and can be associated with the formation of immunoglobulin-associated platelets. The mechanisms behind drug-induced thrombocytopenia are complex but include processes such as hapten, neoepitope, and/or autoantibody mechanisms.[26,27] As mentioned previously, only a small number of thrombocytopenic dogs in the other category were positive for percent IgG. None of these dogs were subsequently diagnosed with idiopathic IMTP and none required immunosuppression. Therefore, the lack of antibodies in thrombocytopenic dogs detected via direct flow cytometry is unlikely to be related to an immune process; primary or secondary. This finding is clinically relevant because anecdotally, the lower the platelet count, the more likely the process is to be immune-mediated and potentially a primary immune-mediated process. Although the median platelet count for the dogs in other category was higher than the median platelet count in dogs with idiopathic IMTP, it is important to note that several of these dogs had severe thrombocytopenia and were initially thought to be idiopathic IMTP patients by the primary clinicians. This misclassification could have resulted in additional financial burden to clients and potential negative outcomes if aggressive immunosuppression had been pursued. For example, one dog was referred for evaluation of persistent thrombocytopenia due to an unknown cause. Thrombocytopenia (32,000 - 40,000 platelets/µl) was initially documented as an incidental finding during a wellness examination for this patient. Infectious disease testing was negative in addition to thoracic radiographs and abdominal ultrasound were normal so idiopathic IMTP was suspected. Consequently, the dog was placed on doxycycline, glucocorticoids and azathioprine. However, there was no response noted and the patient was eventually tapered off of all medications within a year. The patient was then referred for further

evaluation approximately one year after thrombocytopenia had originally been documented. At initial evaluation, the platelet count was 65,000 platelets/µl with an elevated MPV and no clumps. Repeat infectious disease screening, thoracic and abdominal imaging, and genetic platelet testing were all normal. At this time, the percent IgG was negative. It was then recommended to monitor the platelet count but no treatment was recommended. For two years, the patient did clinically well with no bleeding episodes, did not receive any immunosuppressive medications, was negative for percent IgG via serial testing, and the platelet count ranged between 45,000 – 60,000 platelets/µl. Approximately three years after the very first initial documentation of thrombocytopenia, the patient was again evaluated for a wellness examination and a platelet count of 27,000 platelets/µl was observed. The patient was again placed on doxycycline and steroids but continued to remain thrombocytopenic and also began exhibiting lethargy. The patient was referred again and was thrombocytopenic at 32,000 platelets/µl with an elevated MPV and moderate clumps but the percent IgG was now positive. Repeat thoracic and abdominal screening were pursued again and revealed a peribronchial interstitial to alveolar lung pattern, hepatomegaly, and multiple hepatic and splenic nodules. Humane euthanasia was later pursued due to quality of life concerns and the post-mortem examination revealed diffuse carcinoma affecting the lungs, liver, spleen, adrenal glands, lumbar vertebrae, and bone marrow. In this patient, flow cytometry for detection of percent IgG was beneficial in several ways. Initially, the flow results did not indicate an immune process which stopped the use of unnecessary immunosuppressive medications and decreased the monetary costs and veterinary visits which reportedly greatly enhanced the owner's and patient's quality of life. Then, three years later, the patient was re-evaluated and found to be positive which was a clear change from previous testing. Additional diagnostics were pursued and it was suspected that the patient likely had a secondary IMTP related

to an underlying neoplastic process which also helped to inform the client that the thrombocytopenia was now likely clinically relevant. Therefore, the initial lack of antibodies and subsequent appearance of these antibodies was very helpful in the overall management of this patient and represents how this test could be utilized in clinical practice.

The management of two additional cases was also aided by documenting the lack of antibodies. Specifically, one dog had been diagnosed with idiopathic IMTP after presenting for lethargy and a platelet count of 81,000 platelets/µl was documented in addition to azotemia. He was administered immunosuppressive doses of glucocorticoids, doxycycline, and later azathioprine due to a recheck platelet count of 25,000 platelets/µl. The dog was then referred for a second opinion due to vomiting, persistent lethargy and azotemia in addition to significant weight loss. At initial evaluation, the platelet count was 160,000 platelets/µl, repeat infectious disease screening was negative, thoracic radiographs were normal, and abdominal ultrasound revealed an aortic thrombus with bilateral chronic renal infarcts. Azathioprine was discontinued, clopidogrel was added to address the aortic thrombus, and glucocorticoids and doxycycline were continued. However, several days later, epistaxis was noted so the clopidogrel dose was halved but significant epistaxis continued. The patient was then re-evaluated at this time and epistaxis and melena were noted and a recheck platelet count was 139,000 platelets/µl. Due to the azotemia, aortic thrombus, mild thrombocytopenia, and bleeding manifestations, vasculitis was suspected in addition to disseminated intravascular coagulation. Direct flow cytometry was performed and was negative for percent IgG. An echocardiogram was then pursued revealing probable mitral valve endocarditis. The glucocorticoids were rapidly tapered and discontinued and additional antibiotic medications were added for treatment of probable bacterial endocarditis. The platelet count rebounded to normal levels after initiating this therapy and the valve lesion resolved. The patient

was euthanized approximately nine months later due to progressive renal disease (suspected to have suffered from an acute kidney injury initially) and never exhibited any signs of immune-mediated disease and did not require immunosuppressive medications. The second patient was referred for evaluation of urolithiasis, ureterolithiasis, unilateral severe renomegaly, and severe thrombocytopenia. During evaluation, the platelet count was checked several times and ranged from 11,000 to 27,000 platelets/µl with clumps and a mildly elevated MPV. This patient was a Cavalier King Charles Spaniel which is a breed known to have macrothrombocytopenia[28] however due to the lack of a significantly large MPV, there was a concern for idiopathic IMTP. Direct flow cytometry was performed and was negative for percent IgG. As a result, it was suspected that two major factors were contributing to the thrombocytopenia observed in this patient; breed-associated macrothrombocytopenia in addition to a consumptive process related to an inflammatory urinary process. The patient then underwent surgery to address the stones and renomegaly and upon recovery, the platelet count began to rebound and the MPV was significantly elevated consistent with the breed-associated macrothrombocytopenia. No immunosuppressive medications were required in this patient and recovery was uneventful. These clinical examples illustrate the benefit of documenting that a patient is negative for percent IgG which would infer an immune process is unlikely to be contributing to the observed thrombocytopenia. Documentation of a negative result can help guide diagnostic next steps, potentially decrease monetary burden and stress on clients and patients, and also decrease the use of unnecessary medications.

For this study, dogs were classified as either positive or negative for anti-platelet antibodies based on the percent IgG cut-off value. However, it was unknown if the magnitude of percentage binding for percent IgG or the magnitude of the fluorescent intensity (GMFI) would help differentiate etiologies in dogs that were classified as positive for percent IgG. If dogs with

idiopathic IMTP tended to have higher magnitudes of percent IgG compared to dogs with non-idiopathic IMTP such as neoplasia that would be of great diagnostic benefit. Similarly, if dogs with idiopathic IMTP tended to have much higher magnitudes of GMFI as compared to dogs with non-idiopathic IMTP such as an infectious disease, this would also be very helpful in determining the underlying etiology of thrombocytopenia. Based on our study, the magnitude of percentage binding and fluorescent intensity greatly varied in all dogs in each category with a substantial amount of overlap between etiologies. An important consideration is that only a very small number of dogs in the neoplasia and other category were positive for percent IgG so a comparison between a larger number of dogs is indicated. However, based on our initial findings, it is not recommended to use either of these markers for differentiating etiologies of thrombocytopenic dogs that are positive for anti-platelet antibodies.

All of the dogs that were diagnosed with idiopathic IMTP in this study were positive for percent IgG or anti-platelet antibodies. This is in contrast to the human literature where only 60-70% of people are documented to have antibodies potentially indicating that non-antibody dependent mechanisms are responsible for platelet destruction.[2,18] From our study, it is likely that platelet destruction in dogs with idiopathic IMTP occurs solely or in combination with an antibody-mediated mechanism. When considering platelet destruction in humans with ITP who lack antibodies, it has been shown that CD8+ T cells result in direct platelet destruction and are also involved in subsequent platelet desialylation.[2,18,25-28] Platelet desialylation involves the exposure of residues on the platelet surface following cleavage of terminal sialic acids from glycoproteins that result in targeting by hepatocytes and subsequent clearance of platelets.[28] As previously mentioned, it is unknown if these mechanisms occur in dogs however cell-mediated platelet destruction has not been historically observed when attempting to evaluate animal models

for studying human ITP.[18] Future studies could be performed in a larger number of dogs with idiopathic IMTP to determine if any dogs do lack anti-platelet antibodies, if cell-mediated mechanisms can be documented, and if lack of anti-platelet antibodies is related to clinically relevant topics such as response to therapy.

When considering prognosis in dogs with idiopathic IMTP, it is a common anecdote that dogs with melena seem to have a poor prognosis which was shown in a previous study in addition to our study.[29] The reason for this could be related to the fact that many of these dogs require blood transfusions and longer hospitalizations so dogs may be euthanized due to financial constraints of the client. Additionally, dogs with melena can often appear much more clinically ill than other dogs affected by IMTP which can also lead to humane euthanasia if owners feel that the quality of life is not acceptable. However, a possible confounding factor in our study was that One dog was receiving appropriate doses of meloxicam for osteoarthritis whereas the other two dogs were given either ibuprofen (two doses of 13 mg/kg) or aspirin (12 mg/kg) by their owners. NSAIDs are known to not only affect platelet function but also can be associated with gastrointestinal bleeding in dogs.[33,34] Therefore, it is suspected that concurrent NSAID use may have contributed to the observed melena and overall poor prognosis and underlines the importance of thorough history taking when initially evaluating patients. With regards to prognosis as defined in this study, there was no association between prognosis and the persistence of antibodies. In humans, some studies have documented that the presence of antibodies is associated with more severe bleeding manifestations and chronic disease versus transient acute disease.[22] This type of analysis could not be performed in our study since all of the dogs were positive for antibodies. Based on our study, the persistence of antibodies for 4 weeks following initial diagnosis in dogs

with idiopathic IMTP should not be used to determine prognosis. However, the presence of melena may be associated with a worse outcome and decreased survival to discharge.

The response to therapy as defined in this study was not associated with the persistence of antibodies. In humans, there have been contradictory results but one study showed that the lack of antibodies was associated with a decreased response to rituximab which is considered a common second-line therapy in humans with ITP and works by depleting B cells.[24] Additionally, it has been shown that the persistence of these antibodies in people after treatment for ITP is associated with more severe disease.[20] In our study, antibodies persisted in multiple dogs for variable periods of time despite platelet counts returning to normal whereas other dogs quickly became negative and exhibited a rapid rebound in platelet count. This was particularly apparent in one dog that had a platelet count of 12,000 platelets/µl but had rapidly become antibody negative within 3 days and within 8 days demonstrated a rapid rebound in platelet count (425,000 platelets/µl). The rapid disappearance of antibodies is intriguing since the half-life of most of the immunoglobulin G (IgG) subclasses in dogs is estimated to be similar to that of humans, which is approximately 20-21 days.[35] One possible contributing factor could be that the antibody bound platelets are rapidly removed from circulation by the reticuloendothelial system[2] and are replaced by platelets not associated with antibody due to concurrent administration of immunomodulatory medications. In a previous study at our institution (see Chapter 5), dogs experimentally infected with *Ehrlichia canis* became negative for percent IgG in a short amount of time and it was theorized that this could potentially be related to the immunomodulatory non-anti-microbial effects of doxycycline.[36,37] In order to further investigate this finding in thrombocytopenic dogs not due to an *E. canis* infection, all of the idiopathic IMTP dogs enrolled in this study were evaluated for concurrent doxycycline administration. Eleven of the twenty-seven dogs (11/27, 41%) received

doxycycline but only nine of these dogs were able to be evaluated serially since one dog was euthanized in hospital and one dog died at home shortly after being discharged. With the exception of one dog who remained positive for percent IgG for eight weeks, all of these dogs were negative for percent IgG within 4 weeks of initial evaluation. However, eight of the sixteen dogs (8/16, 50%) that did not receive doxycycline also became negative within 4 weeks of initial evaluation. Therefore, the rapid decline in positivity occurs in some dogs regardless of whether they received doxycycline or not so this is unlikely to be due to doxycycline administration alone. It is suspected that doxycycline could contribute to the decrease in positivity since it has been shown to inhibit proliferating lymphocytes and to decrease antibody production.[36,37] However, since this rapid decline in positivity was observed in dogs that had not received doxycycline, this was suspected to be related to the effects of immunomodulatory medications such as glucocorticoids which are known to have rapid genomic and non-genomic effects on inflammation, cytokine expression, macrophage function, T-cell function, cellular apoptosis, and antibody production.[38,39] Therefore, it is possible that glucocorticoid therapy not only decreases antibody formation against platelets but also inhibits the targeting of newly emerged platelets. It has also been shown that glucocorticoid therapy in people with ITP results in changes in the platelet membrane involving platelet activation pathways so it is theoretically possible that this type of therapy results in multiple platelet membrane changes that could disrupt antigen-antibody binding.[40] These processes in combination with the expeditious removal of antibody coated platelets could explain why some dogs become negative for percent IgG so quickly. In certain circumstances, this rapid change to negativity could be clinically relevant if blood samples are not submitted at initial evaluation. For example, if a patient is diagnosed with presumptive idiopathic IMTP and placed on immunosuppressive therapy, antibodies could be absent within a week if there is a dramatic

response to therapy. Therefore, the lack of antibodies in this patient does not mean the initial diagnosis of idiopathic IMTP was erroneous. However, if a patient's platelet count is not responding well to standard therapy and antibodies are detected, this can be clinically helpful as it indicates there is still active disease present and additional diagnostics or therapeutic adjustments may need to be pursued. In contrast, if no antibodies are detected in a patient that is not responding to standard therapy, as demonstrated in previous examples, then that could indicate the initial diagnosis of idiopathic IMTP may need to be re-evaluated. However, based on the specific definitions in our study, persistence of antibodies did not appear to be related to the response to therapy in dogs with idiopathic IMTP.

In contrast to assessing prognosis or response to therapy, the dynamics of percent IgG did appear to be clinically relevant because the recurrence of antibodies was associated with the risk of relapse. As mentioned previously, both dogs that relapsed during the study had also relapsed prior to study enrollment. One of the dogs had remained persistently positive for percent IgG for months but had become negative and was doing clinically well. However, the patient started to become positive again three months later. During this time period, glucocorticoids had been discontinued and the patient was being transitioned from mycophenolate to cyclosporine. The percent IgG was evaluated weekly and remained positive for 4 weeks. During these four weeks, the platelet counts were 83,000 platelets/µl with numerous small clumps, 56,000 platelets/µl with moderate clumps, 65,000 platelets/µl with few clumps, and 41,000 platelets/µl with few clumps respectively. Additionally, the MPV was only slightly elevated in all of these samples. When evaluating the platelet count alone and in the presence of platelet clumping with a mild MPV elevation, it was difficult to determine if the patient was relapsing or not but the percent IgG had gone from negative to positive and was persistently positive. Due to concern for a relapse,

glucocorticoids were re-instituted and within one week, the platelet count had rebounded from 41,000 platelets/µl to 390,000 platelets/µl with few clumps and a normal MPV and the percent IgG was negative. In the second patient that experienced a relapse, the platelet count had historically ranged between 270-275 platelets/µl with no clumps and a normal MPV. This dog had been receiving only mycophenolate as glucocorticoids had been discontinued four months prior. At the time of relapse, the platelet count was 66,000 platelets/µl with many clumps and a mildly elevated MPV. However, again, due to the presence of many platelet clumps and only a mildly elevated MPV, it was unclear if the patient was truly experiencing a relapse or not so the patient came in 24 hours later for a recheck platelet count where a vacutainer would be used in addition to a fresh blood smear before EDTA contact to try to minimize platelet clumping. The second platelet count was 15,000 platelets/µl with few clumps and an elevated MPV. Both of these blood samples were positive for percent IgG whereas previous samples had been negative.

The documentation of recurrence of antibodies was very helpful in both of these cases because it helped to confirm that an active immune process was likely contributing to the thrombocytopenia and that the decreased platelet counts were not related to benign causes such as method of venipuncture or only exuberant platelet clumping. Additionally, documentation of antibodies was also helpful in a third patient that was enrolled into the study. As mentioned previously, this dog could not be classified as a relapse according to the definitions of the study because the dog was a historically diagnosed idiopathic IMTP dog that was enrolled at the time of initial relapse and did not experience a relapse during the course of the study. Specifically, this dog was receiving only mycophenolate as glucocorticoids had been discontinued eight months prior. The platelet count had been re-evaluated several times out of state with the primary veterinarian and had been steadily decreasing (101,000 – 49,000 platelets/µl) over three months

despite doubling the mycophenolate dose. The MPV or presence of platelet clumping was unknown or inconsistently reported during this time period so it was again difficult to know if the patient was truly experiencing a relapse. The patient was then referred for further investigation and the platelet count was 60,000 platelets/µl with a mildly elevated MPV and few clumps and was positive for percent IgG. This raised further concern for a relapse so glucocorticoids were re-instituted and 2 weeks later, the platelet count was 202,000 platelets/µl with a normal MPV and few clumps. The patient was still positive for percent IgG at that time point but was negative within 4 weeks of relapse. In clinical practice, dogs with idiopathic IMTP are typically monitored by evaluating trends in platelet counts but it can be difficult to discern if a relapse is occurring or not in some scenarios as demonstrated in the three dogs detailed above. In addition to the platelet count, another component that can be evaluated during monitoring of IMTP dogs includes the mean platelet volume. This can be evaluated to try to determine if younger more immature platelets are being released into the periphery due to suspected platelet destruction however multiple mechanisms other than immune processes can result in an elevated MPV so it is unlikely that MPV can be used for determining risk of relapse in dogs with IMTP.[41-44] Additionally, previous studies have shown variable results as to whether dogs with idiopathic IMTP consistently have an elevated MPV.[16,45] As a result, it can be challenging to determine if a relapse is truly occurring in some patients. It is very important to determine if a relapse is occurring because not only can life-threatening bleeding events occur but it is important for appropriate patient management and client communication. In the three cases mentioned above, it was not particularly clear whether the patient was relapsing or not due to the presence of platelet clumping and relatively gradual drops in platelet count. The documentation of recurrence of antibodies helped give additional information about each patient so that appropriate recommendations could be

made. It is possible that these patients could have become positive due to a concurrent vector-borne disease or neoplastic condition rather than a relapse of IMTP but based on the clinical assessment and diagnostic results, this was considered unlikely. However, repeat infectious disease testing or imaging could have been pursued if clinically indicated. Therefore, when monitoring dogs diagnosed with idiopathic IMTP over time, the dynamics of antibodies can be clinically useful when determining risk of relapse of disease.

There were several limitations to this study. It would have been interesting to know if the dynamics of the antibodies were related to specific treatment protocols but the study design did not dictate treatment protocols. This was not pursued because the aim of the study was to evaluate these antibodies over time in dogs diagnosed with idiopathic IMTP regardless of treatment protocol to determine if they were related to prognosis, response to therapy, or risk of relapse. Our study was designed to reflect what commonly occurs in clinical practice where dogs are placed on various treatment protocols. Future studies could be performed in dogs utilizing specific treatment protocols to further investigate this however it is unknown if this would be of benefit clinically. A second limitation includes that the dogs were not monitored at specific follow-up times. Therefore, it is unknown exactly when certain dogs became positive or negative for percent IgG during the course of therapy. However, unless the dogs were sampled daily for months, this would also have been true even if weekly evaluations had been performed in all dogs. Although the exact characteristics of how the antibodies changed over time would have been interesting to standardize, this was not the aim of this study. Our study evaluated patients for persistence or recurrence of antibodies over time at each recheck appointment and was not designed to determine the specific time it takes to shift from positive to negative or vice versa. Another limitation of the study could include the smaller number of dogs enrolled that were diagnosed with idiopathic

IMTP. However, most of the dogs were able to evaluated for prolonged periods of time which garnered additional data for evaluation. In our study, the presence of melena at initial evaluation was associated with a decreased probability of survival to discharge. However, a possible limitation of this analysis was that some of these dogs had received concurrent NSAIDs which could have contributed to a worse clinical outcome. It is still possible that melena at initial evaluation is truly associated with a worse clinical outcome but this may be exacerbated by NSAID administration and therefore it is very important to obtain a thorough drug history from clients.

Overall, anti-platelet antibodies were detected in dogs with thrombocytopenia from infectious, neoplastic, toxic and immune-mediated etiologies. From our study, the absence of antibodies in thrombocytopenic dogs is therefore unlikely to be related to an immune process; primary or secondary. All dogs diagnosed with idiopathic IMTP were positive for percent IgG indicating that an antibody-mediated process is involved in platelet destruction rather than a non-antibody-mediated process. The presence of melena at initial presentation in dogs diagnosed with idiopathic IMTP was associated with a decreased survival to discharge. The persistence of antibodies was not associated with prognosis or response to therapy however the recurrence of antibodies was associated with risk of relapse in dogs diagnosed with idiopathic IMTP. Therefore, determining if anti-platelet antibodies are present can help determine if the observed thrombocytopenia is related to an immune process and can also be of clinical benefit when serially monitoring idiopathic IMTP dogs for risk of relapse. Therefore, the combination of platelet count and direct flow cytometry for detection of anti-platelet antibodies can improve understanding of a patient's evolving disease and enhance patient care.

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Chapter 8: Concluding Remarks

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8.1 Significance of work

Vector-borne diseases such as *Ehrlichia canis* in dogs are quite common, have a substantial effect on canine morbidity and mortality, and have been studied for many years. Despite this, the underlying mechanisms for different disease manifestations is not completely understood. Gaining additional insight into the pathophysiology of this disease could translate to other vector-borne diseases not only in dogs and cats but also in humans. Of particular interest includes evaluating the coagulation system in ehrlichiosis beyond traditional assays and evaluating specialized assays such as thromboelastography (TEG) and platelet function. However, before these assays should be assessed in animals with disease, it is of paramount important to determine the variability and stability of test results in healthy animals. In Chapter 3, we determined that the reagents required for platelet function testing via whole blood impedance platelet aggregometry are stable when stored for prolonged periods thus substantially decreasing the costs associated with this assay and increasing the potential use for this test in veterinary medicine. In Chapter 4, we evaluated the inter- and intra-individual variability in healthy dogs for both TEG and platelet aggregometry determining that subject-based intervals are more appropriate for platelet aggregometry whereas

population-based intervals are reasonable for most variables of TEG analysis. Then, in Chapter 5, traditional and specialized components of the coagulation system were evaluated in dogs experimentally infected with *E. canis* allowing better characterization for why some dogs do not bleed despite significant levels of thrombocytopenia. Thus, one of the most common abnormalities noted in dogs infected with *E. canis* is thrombocytopenia. Thrombocytopenia can occur due to a variety of processes in the body and is one of the most common blood work abnormalities appreciated in canine blood samples. Therefore, determining the underlying etiology or mechanism that is contributing to the thrombocytopenia can represent a diagnostic challenge. The formation of anti-platelet antibodies can occur in ehrlichiosis as well as in several other disease states but the underlying mechanism is thought to be related to an immune process. Determining if an immune process is contributing to thrombocytopenia can help direct next diagnostic steps and case management. Additionally, in humans, the characteristics of anti-platelet antibodies can be related to important parameters such as response to therapy, risk of relapse, and prognosis. The development of a consistent and readily available assay for detection of these antibodies would be of benefit to veterinary practitioners when evaluating thrombocytopenic dogs. In Chapter 6, a flow cytometric assay was validated for detection of anti-platelet antibodies in dogs where blood samples were shown to be stable for up to 72 hours of storage at 4°C which allows for more veterinarians to be able to access this test for use in clinical practice. In Chapter 7, we utilized this assay to evaluate anti-platelet antibodies in dogs with presumptive idiopathic immune-mediated thrombocytopenia (IMTP) and thrombocytopenia due to infectious, neoplastic, or other etiologies. For dogs with IMTP, we determined that melena is associated with a decreased survival to discharge and that the recurrence of anti-platelet antibodies is associated with relapse of disease in dogs with IMTP.

Therefore, this research helped enhance our understanding of specialized assays that are becoming more commonly used in veterinary medicine not only in dogs with ehrlichiosis but also in healthy dogs and also better characterized the dynamics of anti-platelet antibodies in dogs with IMTP and how they relate to important parameters such as risk of relapse.

8.2 Specific Aim 1 (Chapter 3: Reagent stability for platelet aggregometry)

Specialized assays such as whole blood impedance platelet aggregometry are becoming more commonly utilized in veterinary research and clinical practice.[1-9] However, the costs associated with this assay can be substantial which could preclude the use of this technology in certain institutions. One obvious contribution to the increased cost of this assay are the reagents that are used to stimulate platelet aggregation. The manufacturer recommends to only store the reagents for a short time resulting in the frequent disposal of potentially useable reagent. Therefore, the aim of this chapter was to determine the stability of certain platelet agonist reagents after prolonged storage in two different conditions for up to 6 months. We determined that the reagents are stable in both conditions for up to 6 months which would significantly decrease the cost associated with performing this assay. Future directions could include evaluating these agonists for storage beyond 6 months and repeating this study utilizing other platelet agonists such as collagen.

8.3 Specific Aim 2 (Chapter 4: Thromboelastography and platelet aggregometry validation)

It is good practice to know how a test or analyte of interest varies in healthy individuals before applying them to ill individuals. This degree of variability is related to several factors such as analytical and biological variation. Some of the components that make up biological variation include intra-individual variability (CV_I) and inter-individual variability (CV_G) which help to

determine if population- or subject-based intervals should be used. Additionally, another factor utilized to determine what type of intervals should be used include the index of individuality which is a calculation that includes biological variation.[10] Knowing the components of variability for a specific test can lead to more appropriate interpretation of results which can have a strong clinical impact. The specific aim of this chapter was therefore to assess the CV_G, CV_I, and index of individuality over time for tissue factor (TF)-activated TEG and heparinized whole blood impedance platelet aggregometry variables in healthy dogs. The results of this chapter showed that subject-based intervals are more appropriate for platelet aggregometry and one TEG variable (maximum amplitude; MA) whereas population-based intervals are reasonable when considering the remaining TEG variables. The optimal timing of when to assess the variability in healthy dogs is unknown so future studies could include different timing strategies in dogs such as multiple times during the same day, once daily for multiple days (e.g. 7 days), once a week for a month, etc. Our study was performed in all male dogs of similar age so another future study could include preforming this study to evaluate variability in groups of dogs of different sexes, evaluating variability in different age groupings of dogs (young dogs versus geriatric dogs), or different breeds (Greyhounds versus Labrador Retrievers).

8.4 Specific Aim 3 (Chapter 5: Coagulation evaluation in experimental E. canis *infection)*

Ehrlichiosis in dogs can manifest in many different ways including bleeding presentations. However, some dogs never show signs of bleeding despite having significantly low platelet counts.[11] The reason for this clinical discrepancy had not been previously evaluated by combining objective measurements of platelet activation, specialized coagulation testing, and dynamics of anti-platelet antibodies. Therefore, the specific aim of this chapter was to assess platelet indices of activation, platelet function as assessed by whole blood impedance platelet aggregometry,

percentage of anti-platelet antibodies, and TEG measurements including velocity curve (Vcurve) variables in dogs experimentally infected with *E. canis*. The findings from this chapter showed that possible reasons for the lack of bleeding in some dogs could include activated platelets and a hypofibrinolytic state. There are multiple future studies that could be pursued following this study. Examples include studying these factors in experimentally infected dogs using a competent vector such as *Rhiphicephalus sanguineus*. It is unknown if the vector would change the characteristics of the coagulation system but in order to try to model a natural infection, that could be investigated. Studying these changes in naturally *E. canis* infected dogs is also an option but the timing would be difficult since when an animal became infected would be unknown and dogs could present at different time points in the disease. An intriguing finding from our study also included how rapidly antibodies disappeared following doxycycline administration. It is unknown if this is a direct or indirect effect of the antimicrobial drug however future directions could include studying how the platelet membrane changes once doxycycline has been administered. For example, if doxycycline changes the surface characteristics of the platelet membrane, this could explain the decreased binding or presence of antibodies (i.e. less antigen to bind). Flow cytometry evaluating CD61+ platelets which is glycoprotein IIIa or integrin β-3 could be evaluated in dogs before and after doxycycline administration. If doxycycline resulted in an increased expression of CD61+ platelets, then it could be inferred that this antimicrobial resulted in changes along the platelet membrane.[12] Additionally, platelet samples could be radiolabeled to determine if doxycycline results in more rapid clearance of abnormal platelets or the ultrastructure of platelets before and after doxycycline administration via scanning electron microscope could be evaluated to determine integrity of platelet membranes.[13,14] Future studies that could further evaluate whether a hypofibrinolytic state was present could include measurement of additional factors associated with

fibrinolysis such as plasminogen, urokinase plasminogen activator, tissue plasminogen activator, alpha2-antiplasmin, plasminogen activator inhibitor 1 (PAI-1), protein C activity, etc. Finally, in order to determine if these changes are consistently seen in dogs infected with ehrlichiosis, a larger group of dogs could be evaluated. For example, if a large group of dogs was studied and some dogs exhibited bleeding, their testing may show completely different findings such as a hypocoagulable or hyperfibrinolytic state. As with infections with *Angiostrongylus vasorum* where specific derangements of the coagulations system are related to treatment options and prognosis, this could also be the case in canine ehrlichiosis and warrants additional investigation.[15,16]

8.5 Specific Aim 4 (Chapter 6: Flow cytometry validation)

Thrombocytopenia is one of the most common blood abnormalities to see in dogs and can occur due to a variety of immune and non-immune processes.[17,18] Therefore, determining the underlying etiology can represent a diagnostic challenge. However, if it is determined that an immune process could be contributing to the observed thrombocytopenia, this can help guide diagnostic steps and possible treatment options. This can be accomplished by evaluating for the presence of anti-platelet antibodies which can be pursued by several different methods including flow cytometry. Therefore, the specific aim of this chapter was to develop a clinically useful flow cytometric assay for the detection of anti-platelet antibodies in dogs. The results of this study showed that when using this particular assay, blood samples can be evaluated for analysis after up to 72 hours of storage at 4°C. The ability to evaluate blood for anti-platelet antibodies beyond 24 hours of storage allows veterinarians increased access to this test for use in clinical practice. Future studies could include validating this assay in dogs that are thrombocytopenic due to etiologies other than canine IMTP. However, our laboratory has evaluated fresh samples compared to

samples collected within 72 hours and have found no statistical difference. It is suspected that these findings will be the same as the findings from our initial study but a prospective study could be performed in this subset of dogs.

8.6 Specific Aim 5 (Chapter 7: Flow cytometry in thrombocytopenic dogs)

Anti-platelet antibodies can be detected in a variety of disease syndromes in dogs including infectious, neoplastic, inflammatory, and immune-mediated.[19-26] Previous studies in veterinary medicine have focused on detecting these antibodies instead of how they might change over time in diseases such as canine IMTP. In humans, the dynamics of these antibodies has been shown to be related to factors such as prognosis, risk of recurrence/relapse, and response to different treatments.[27-30] This has also never been previously investigated in dogs so the specific aim of this chapter was to evaluate thrombocytopenic dogs (due to IMTP, infectious, neoplasia, and other causes) for the presence of anti-platelet antibodies, to characterize the dynamics of these antibodies over time in dogs diagnosed with idiopathic IMTP, and how anti-platelet antibodies relate to prognosis, risk of recurrence/relapse, and response to therapy. This study showed that anti-platelet antibodies were detected in a subset of dogs with non-IMTP while all dogs with presumptive IMTP had detectable antibodies. Additionally, it was shown that melena was associated with a decreased survival to discharge and that the persistence of antibodies was not associated with prognosis or response to therapy but recurrence of antibodies was associated with risk of relapse in dogs with IMTP. Similar to the findings noted with experimentally *E. canis* infected dogs, antibodies resolved very quickly in some dogs with various treatments for IMTP. Future studies could be pursued to investigate if glucocorticoids in particular result in platelet membrane changes or increased clearance of antibodies as mentioned in section 8.4. Although all of the dogs in our study had detectable antibodies, a study could be pursued in a much larger number of dogs to see

if any dogs diagnosed with idiopathic IMTP lack antibodies. If it was documented that dogs did lack antibodies, future studies could be pursued in this subset of dogs. Examples of potential future directions could include evaluating this subset of dogs for T cell mediated platelet destruction via flow cytometry utilizing markers of cell-mediated toxicity such as granzyme B or a platelet lysis assay.[31,32] To determine if other processes such as platelet desialylation are contributing in this potential subset of dogs, flow cytometry evaluating for residues indicating this process such as fluorescein-conjugated lectins Ricinus communis agglutinin I (RCA-1) could be pursued as well.[33] Other interesting future studies could include investigating different anti-platelet antibodies such as glycoprotein (GP) IIb/IIIa and/or glycoprotein Ib/IX (most common targets in people) in dogs with refractory IMTP to determine if the different types of antibodies influence response to therapy.[34] There are many very interesting avenues to investigate in canine IMTP and this research helped to show that it is not as simple of a disease process as originally thought and that idiopathic IMTP behaves quite differently in some dogs bolstering the argument for individualized tailored patient management.

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