Platelet count and plateletcrit in Cavalier King Charles Spaniels and Greyhounds using the Advia 120/2120

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Dedication

This thesis is dedicated to my supportive husband Rob for relentless encouragement and motivation.
Abstract

**Background:** Models for the regulation of thrombopoiesis predict that platelet mass is the biologically regulated parameter, yet clinical evaluations are often based on platelet number. When there is concurrent thrombocytopenia and variation in platelet size, platelet count may poorly estimate total platelet mass, confounding clinical decision making.

**Objective:** We hypothesize that plateletcrit provides clinically important information when platelet number is an incomplete representation of platelet mass, such as in genetic macrothrombocytopenia.

**Methods:** We retrospectively compared platelet count and plateletcrits with general reference intervals for four groups of dogs: sick Cavalier King Charles Spaniels and Greyhounds that presented for non-hematopoietic disease to the University of Minnesota (Advia 2120) and Auburn University Teaching Hospitals (Advia 120) over a 3 year period.

**Results:** A canine plateletcrit reference interval of 0.129 – 0.403 % was established. None of the four sample groups were found to have significantly more individuals below the reference interval for plateletcrit. For platelet count, only the Cavalier groups were found to have significantly more individuals below the reference interval than predicted.

**Conclusion:** Use of the plateletcrit as determined by the Advia 120/2120 appeared to avoid overestimation of low platelet mass in sick Cavalier Kind Charles Spaniels in a clinical setting. In contrast, the plateletcrit performed similarly to the platelet count in evaluation of platelet mass in sick Greyhounds. Evaluation of the plateletcrit should be considered in other conditions associated with increased mean platelet volume.
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Chapter 1: Introduction

Chapter one serves as an overview of the biology and diagnostic evaluation of platelets, and is an introduction to the plateletcrit, the main research focus of this thesis. The overview also consists of megakaryocyte and platelet biology, mechanisms of thrombocytopenia, lab assessment of platelet disorders, and other topics pertinent to the plateletcrit. A large section of chapter one is devoted to a review of current platelet research, and how models of disease can be used to enhance understanding of platelet disorders in clinical medicine. The introduction to the plateletcrit contains a review of a separate plateletcrit research paper that is important to the justification of why the plateletcrit is a useful research question.

Platelet Biology: Physiological Roles of Platelets and Platelet Function

The physiological roles platelets play in disease processes are numerous and diverse, and therefore are not described in depth. For example, activated platelets are involved in clot formation which, if excessive, can cause myocardial infarction or ischemic stroke in people. Each year 795,000 people experience a new or recurrent stroke. Platelets can also cause bleeding if they are dysfunctional or are too few in number. Acquired thrombocytopenia is very common while inherited thrombocytopenias are extremely rare. Balduini reports that 51% of people with inherited thrombocytopenias are actually undiagnosed as to actual etiology because their disease has not yet been described.

Platelets are a major component of the primary hemostasis system which is defined as the formation of the platelet plug. It can be described in three major steps that
occur simultaneously in vivo: Adhesion, activation, and aggregation. First, the normally quiescent platelets are exposed to the subendothelial matrix proteins collagen and membrane-expressed tissue factor. (Under ordinary conditions, endothelial cells constitutively release nitric oxide and generate prostacyclin to inhibit platelet activation.) Or the platelets are exposed to another powerful agonist called thrombin invoked by a pathological state such as an inflammatory process. Next, the platelet glycoprotein von Willebrand factor binds to the exposed collagen on the surface of damaged or activated endothelium. Platelets also bind directly to collagen via the membrane GPVI collagen receptor complex and the integrin α2β1 collagen receptor.4,5

In the second step, stored platelet compounds are secreted from their granules. Positive feedback signals from the α-granules and dense granules, thromboxane, ADP and other platelet agonists attract more platelets to the extending thrombus. Also, GPVI (collagen receptor) molecules, complexed with the Fc receptor gamma-chain, cluster and stimulate signaling that results in rapid shape change so that platelets spread to cover the damaged endothelium.4,5

In the third step, aggregation of platelets is accelerated by plasma von Willebrand factor and fibrinogen via the platelet integrin αIIbb3 (GPIIb–IIIa) complex. The platelets change shape and form filopodia and lamellipodia due to outside-in signaling of the actin/myosin complexes.4 Abnormalities in any parts of the steps of platelet plug formation can result in platelet dysfunction. Some abnormalities may be much more serious than others, based on the presence or absence of alternative pathways to complete the clot formation.5
Platelets also play a role in immunity. Viruses can be recognized and internalized by platelets resulting in new antigen expression. Some bacteria can bind to platelets, inducing granule secretion and platelet activation. Platelets can also interact with protozoa and fungi such as *Candida albicans* causing platelet aggregation.\(^6\)

**Platelet Biology: Individual, Species and Population Heterogeneity**

Platelet number and size are unique to each species, and can vary between genetic populations and within individuals in a population. For example, mice have extremely high numbers of small platelets, (an average platelet count of 1200 x 10\(^9\)/L and an MPV of 2.1 fL.) while porcupines have lower numbers of very large platelets (an average count of 30 x 10\(^9\)/L and an MPV of 105 fL.)\(^7\) Human platelets are small (3.0 – 0.5 µm), anucleate, discoid shaped cells with a mean volume of 7-11 fL. Human platelet counts range from 150 – 400 x 10\(^9\)/L with variance due to age, sex and ethnicity. On a population level, a Mediterranean population of people had a 95% platelet count range of 89,000-290,000/µL, and a Northern European population had a higher range of 148,000-323,000/µL. The MPV of the Mediterranean population was 17.8 while the Northern European population had a smaller MPV of 12.4.\(^8\) This and some animal data suggest that platelet number may decrease as size increases.\(^7\)

Circulating platelets within each individual can exhibit diversity in size, density, age and function.\(^9\) Larger platelets are more active in hemostasis than smaller ones.\(^10,11\) This is because larger platelets contain more proteins and granules that support thrombosis than do smaller platelets. Larger platelets are also reticulated and therefore are younger.\(^9\) Platelets that still contain RNA remnants are considered to be immature or
just released from the megakaryocyte, and are therefore the youngest platelets in circulation. These immature, or reticulated platelets are like reticulated red blood cells in that they contain increased levels of mRNA that stain with thiazole orange dye, are on average larger than other platelets, and they remain in circulation for an average of only 24 hours before maturing. Measurement of reticulated platelets has been utilized as a representation of platelet production and turnover. However, because different pools of mRNA exist, there are concerns about the dogma that platelet mRNA is unstable and can be used to measure turnover. 

Mangalpally supports the thought that larger platelets are younger and therefore more hemostatically active. Mangalpally isolated platelets based on size, and found that a larger proportion of large platelets had a greater proportion of reticulated platelets than the other smaller size groups. Larger platelets express more von Willebrand factor, fibrinogen, and P-selectin in response to antagonists such as aspirin as compared to smaller platelets. Also, alpha granule protein release, dense granule content, surface protein activation, and thromboxane synthesis were significantly greater in large platelets as compared to smaller ones.

While counts and size can differ between species, their hemostatic function remains the same with the exception of non-mammalian species. Avian and reptilian platelets are referred to as thrombocytes and are nucleated cells. In addition to coagulation properties, avian thrombocytes also possess phagocytic capabilities. Reptilian thrombocytes are unique in that they also play a role in wound healing.
Platelet Biology: Platelet Structure

Platelet structure is divided into 4 major zones: The peripheral zone, the structural zone, the organelle zone, and the membrane system. The peripheral zone is the outermost layer of the platelet. The peripheral zone has an exterior coat called the glycocalyx, an inner phospholipid bilayer, and a submembrane containing actin. The glycocalyx coats the surface of the phospholipid bilayer and also lines the inner channels of the platelet. The glycocalyx is rich in glycoprotein receptors, proteins, mucopolysaccharides, and adsorbed plasma proteins such as fibrinogen which are needed for clotting. Within the fluid matrix of the phospholipid bilayer are microdomain lipid rafts which are unique to platelets. The lipid rafts have lateral mobility important for cell signaling. Underneath the phospholipid bilayer is a submembranous region with a skeleton of microfilaments resembling actin that aid in the platelet’s shape-changing abilities and translocation of receptors.

The structural zone lies underneath the peripheral zone. The structural zone is responsible for maintaining the resting discoid shape of the platelet, and for changing the shape of the platelet during platelet activation through reorganization and polymerization of its microtubules and cytoskeletal network. The microtubules are composed of the protein tubulin and completely outline the periphery of the platelet. Tubulin is a heterodimer composed of α and β monomers that are encoded by separate genes creating different isoforms that can affect the microtubule’s function. Navarro-Nunez examined different tubulin RNA levels to look at different isotypes and found that Beta-1 tubulin is specifically expressed in platelets and megakaryocytes and accounts for up to
90% of total tubulin. Navarro- Nunez also suggests that high levels of beta-1 tubulin may not be critical to sustain platelet discoid shape.

The cytoskeletal network of the structural zone is composed of actin, myosin, spectrin and other contractile elements that can apply force to the circumferential tubulin microtubules and thereby change platelet shape. The cytoskeleton is also critical for granule movement, granule release, and clot retraction.\textsuperscript{14-16} Since it is the job of the cytoskeleton to change platelet conformation, if any of the cytoskeleton properties are modified or mutated, a congenital platelet disorder may arise.\textsuperscript{5}

The \textit{organelle zone} lies under the microtubule layer and is located throughout the platelet’s cytoplasm. The organelle zone consists of mitochondria, glycogen particles and four main types of granules: dense bodies, alpha granules, lysosomal granules and peroxisomes.\textsuperscript{14} The dense granules store ADP, ATP, polyphosphates, high concentrations of Ca\textsuperscript{2+}, and serotonin. The alpha granules are the large, basophilic granules that can be visualized using a light microscope.\textsuperscript{16} Many different types of proteins and membrane receptors are stored by the alpha granules. Fibrinogen, VWF, factor V, protein S, and tissue factor pathway inhibitor are all stored along with the chemokines SDF-1α, PF4, β-thromboglobulin, ENA-78 and RANTES. Also, platelet derived growth factor, endothelial growth factor, and trans-membrane proteins such as integrins α\textsubscript{IIb}β\textsubscript{3}, P-selectin, and CD40L are all found within the alpha granules.

The \textit{membrane system} is the fourth structural zone of the platelet and is composed of two systems: The canalicular system and dense tubular system. The dense tubular system is a remnant of the smooth endoplasmic reticulum from megakaryocytes. It
synthesizes prostaglandins, and it stores and releases intracellular Ca$^{2+}$. The canalicular system is actually connected to the surface of the platelet allowing for release of granules outside of the platelet and entry for external substances into the platelet. Since it is lined by the glycocalyx, it increases the surface area of the platelet available for spreading during activation. Some species such as ruminants and equine do not have a surface-connected open canalicular system as they fuse their granules directly to the outer membrane.\textsuperscript{16}

There are many platelet receptors important to platelet function. Two major platelet glycoprotein receptors involved in hemostasis are glycoproteins complexes Ib-IX-V and IIb-IIIa. (White) Glycoprotein Ib contains the binding sites for VonWillebrand’s Factor, thrombin, and ristocetin. Glycoproteins IIb and IIIa bind fibrinogen.

**Platelet Biology: Thrombopoietin and Regulation of Total Platelet Biomass**

Primary hemostasis is facilitated by the constant maintenance of platelet mass in circulation. Total platelet biomass is controlled through the regulation of the thrombopoietin (TPO) feedback loop. Thrombopoietin is an acidic glycoprotein produced constitutively in the liver (and also produced to a lesser extent in the kidney and bone marrow.) Thrombopoietin binds to megakaryocytes and platelets via its specific high-affinity TPO receptors called c-MPL. When bound, the megakaryocytes are stimulated and thrombopoiesis is initiated. When bound to platelets, TPO is internalized and degraded and not available for stimulating thrombopoiesis. In the presence of normal circulating mass of platelets, most of the TPO is cleared by platelets and small basal
levels maintain the normal rate of platelet production from the bone marrow megakaryocytes. When there is a reduction in the ability of the circulating platelet mass to remove TPO from the circulation (due to decreased platelet number, decreased time of the platelet in circulation, or decreased platelet TPO receptor number or function), TPO levels rise, megakaryocytes are stimulated, and platelet production increases.\textsuperscript{7}

This amount of platelet functional mass can be thought of as the total amount of receptors that can be on many small platelets with few receptors, or few large platelets with many receptors when assuming receptor density is consistent. Therefore, it is not necessarily a specific number of platelets that the body regulates, but a total amount of TPO receptor present on platelets that must be maintained. In short, platelet mass, (not number) is the physiologically important hemostatic parameter to be monitored.\textsuperscript{7} Since mass is dependent not only on platelet number but also platelet size, few large platelets can have the same functionality as multiple smaller platelets by creating the same platelet mass. Therefore, platelet mass is a function of both platelet size and number.

The relationship of thrombopoietin to platelet mass was demonstrated by Kuter and Rosenburg \textsuperscript{19}when they created a model of nonimmune thrombocytopenia in rabbits and observed that the TPO levels increased. When platelets were transfused into the thrombocytopenic rabbits near the time of their platelet count nadir, the elevated levels of TPO decreased. Many other studies have identified TPO as the regulator of thrombopoiesis.\textsuperscript{20} For example, in a study of essential thrombocythaemia (ET), the TPO levels and platelet size in the patients with ET was the same as in controls. The difference between the patients with ET and the healthy subjects was that the patients
with ET had 5.6 ± 5.5 TPO binding sites per platelet, and the healthy subjects had 56 ± 17 TPO binding sites per platelet. Therefore, the number of TPO binding sites was maintained by increasing the number of platelets in patients with ET.\textsuperscript{21}

**Platelet Biology: Megakaryopoiesis**

Megakaryopoiesis precedes platelet and proplatelet formation. Megakaryocytes develop from hematopoietic stem cells: Embryonic hematopoietic stem cells (HSC) are found in the aorta-gonad-mesonephros region, and possibly the yolk sac and the placenta. They then migrate and expand in the fetal liver, and eventually reside in the endosteal niche of the bone marrow. Upon cellular division, HSC then migrate to the endothelial/vascular niche of the bone marrow. These bone marrow microenvironments support cellular proliferation and differentiation through local growth factor production, cell to cell interactions and distance signaling.\textsuperscript{22}

Thrombopoietin (TPO) is the key regulator of thrombopoiesis and affects all stages of megakaryocyte development. Colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) is a multipotent cell that develops from the pluripotent hematopoietic progenitor. It is identified by the expression of CD61 (integrin β3, GPIIIa) and elevated CD41 (integrin αIIb) levels. It has the potential to differentiate into bipotent megakaryocyte-erythroid progenitor (MEP) that can give rise to biclonal colonies composed of megakaryocytic and erythroid cells.\textsuperscript{23}

Stimulation of CFU-GEMM or MEP with TPO, Stem Cell Factor, IL-3, IL-6 and IL-11 causes differentiation into the first committed megakaryocyte precursor cell called burst-forming unit-megakaryocyte (BFU-Meg), and then further differentiates into
colony-forming unit-megakaryocyte (CFU-Meg). Both CFU-Meg and BFU-Meg express CD34, CD33, and CD41. (CD41 cell surface antigen is a megakaryocytic lineage marker.) The promegakaryoblast develops next and is the first recognizable megakaryocyte precursor in the bone marrow. Interleukin-3 by itself supports the early stages of megakaryocyte development up to the promegakaryoblast stage before polyploidization. It is followed by the megakaryoblast, then the promegakaryocyte, and finally the megakaryocyte. 

Megakaryocytes undergo endomitosis and become polyploid through repeated cycles of DNA replication without cell division. Megakaryocyte polyploidization results in a functional gene amplification which likely increases protein synthesis and subsequently enlarges the cell. Therefore, megakaryocytes are unlike most cells which have checkpoints and feedback controls to ensure that DNA replication and cell division are tightly coupled.

**Platelet Biology: Proplatelet Formation**

The mechanistic processes of platelet formation are currently not fully understood. The exact nature of platelet release from megakaryocytes and correlation to disease states needs to be fully described because knowing exactly how platelets are formed from megakaryocytes is important for the development of therapies for platelet disorders. Current therapies such as thrombopoietin mimetics are slow to stimulate megakaryocyte maturation from hematopoietic stem cells. (Five days are needed to increase platelet counts.) Platelet transfusions are a common therapy for thrombocytopenia. However, due to the short lifespan of a platelet and the difficulty in
finding platelet apheresis donors and whole blood donors, platelets are usually in short supply.24

Although little is known about platelet release, Junt25 has witnessed proplatelet formation in vivo. Platelets were released from megakaryocytes via the mechanism of proplatelet formation: Proplatelet formation begins after the process of endomitosis is completed, cytoplasm has been expanded, and the demarcation membrane system has formed. (The demarcation membrane system is a suborganization of the megakaryocyte cytoplasm that serves as an extensive network of flattened cisternae homogeneously distributed throughout the cytoplasm that subdivide the cytoplasm into “platelet fields.”)24

First the megakaryocyte’s centrosomes disassemble and its microtubules undergo a dramatic reorganization. The microtubules translocate to the cell cortex, the outside periphery of the megakaryocyte’s cytoplasm.”26 Next, thick pseudopodia form that erode one pole of the megakaryocyte. The large, thick pseudopodia structures are composed of thick bundles of microtubules that fill the shafts of the pseudopodia and extend outward from the megakaryocyte. The microtubule bundles are thickest in the portion of the proplatelet near the body of the megakaryocyte, and thin near proplatelet ends. As they elongate and thin, they branch to yield slender projections of uniform diameter. The microtubules loop around within the proplatelet to re-enter the shaft forming buds at the proplatelet tip, each measuring 3–5 μm in diameter.24 Coiling of microtubules, the signature of circulating platelets, occurs only at the proplatelet ends and not within the
platelet-sized swelling positioned along the proplatelet shaft. Therefore, the primary site of platelet assembly is at the end of each proplatelet.\textsuperscript{27}

Since the microtubules are hollow polymers assembled from $\alpha\beta$-tubulin dimers, they are the major structural component of the engine that powers the proplatelet elongation: Proplatelet extension is due to tubulin polymerization and dynein-powered sliding of overlapping microtubules. (Cytoplasmic dynein is a microtubular molecular motor protein.) Repeated actin-dependent bending and branching that bifurcates the proplatelet shaft is common, and serves to increase the number of free pro-platelet ends from which platelets are thought to be released. Elongation/sliding of these microtubules then generates new daughter proplatelet processes. Eventually the megakaryocyte is transformed into a residual naked nuclei surrounded by an anastomosing network of proplatelet processes. The proplatelets are chipped off into the bone marrow sinuses via sheer blood pressure forces and go into the bloodstream. The residual nuclear material is eliminated by macrophage-mediated phagocytosis.\textsuperscript{24}

There is a possibility that platelets may continue to divide while circulating in the periphery. Schwertz\textsuperscript{28} obtained peripheral blood platelets, and observed formation of new cell bodies by looking at microdrops of blood or maintaining the platelets in suspension culture. They also observed that platelet numbers increase during ex vivo storage. If anucleate platelets are able to generate their own progeny, the peripheral platelet number will be in a state of change while the platelet mass will always be maintained. Therefore, measuring platelet mass using the platelet count may be difficult to measure.
Mechanisms of Thrombocytopenia

There are many causes of true thrombocytopenia (which needs to be distinguished from pseudothrombocytopenia). The major mechanisms include: Increased platelet destruction, increased platelet consumption, decreased platelet production, and increased platelet sequestration.13

Increased platelet destruction can be caused by both immune and non-immune mediated pathways. Immune destruction is most commonly caused by primary immune thrombocytopenic purpura (ITP). In the case of ITP, it has been established that the presence young platelets that are larger than normal in size are a sign of healthy bone marrow regeneration and indicate that the thrombocytopenia is caused from peripheral destruction.29 Secondary ITP which can be associated with drugs, infections, neoplasia, and systemic autoimmune disease, is another source of immune destruction.30

Non-immune mediated mechanisms of thrombocytopenia can also be the result of increased consumption. Increased consumption is most commonly caused by disseminated intravascular coagulation in acutely ill patients.30 Other sources of platelet consumption are thrombotic microangiopathies such as thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. Rapid loss of platelets may occur with massive hemorrhage due to traumatic blood loss or anticoagulant rodenticides. Increased platelet destruction may result in the release platelets that are larger in size to compensate for the consumption.9, 12, 31

Abnormal platelet distribution can be caused by sequestration of platelets in the spleen. Normally 30-40% of the total circulating platelet pool may be stored in the
spleen. Splenomegaly is a condition in which up to 90% of platelets are sequestered in the spleen. Total platelet mass, survival and production are normal however, peripheral platelet counts are low. Hypersplenism is rare in animals, but has been reported in cases of canines with hypothermia. 13

Decreased or defective platelet production can be caused by bone marrow that is diseased or damaged by cancer, drugs, chemicals, toxins, irradiation, or infections. Primary bone marrow disorders such as leukemias, myelodysplasia, and myelofibrosis affect hematopoiesis and cause thrombocytopenia. Metastatic cancer and solid tumors can also affect hematopoiesis, but are rare in animals. Bone marrow defects that can cause pancytopenia may cause platelets to be smaller in size. 13 Examples of veterinary drugs that affect hematopoiesis and can display associated thrombocytopenia are: chemotherapy drugs, antibiotics such as sulfonamides and cephalosporins, phenobarbital, and antifungals such as griseofulvin in cats. Corticosteroid treatments are used to suppress the immune system causing platelet counts to rise in dogs with immune-mediated thrombocytopenia. In animals, many different types of viral, bacterial, fungal, and protozoan infections are common and can cause thrombocytopenias. In dogs, thrombocytopenia can occur with viral infections such as canine distemper and parvovirus. In cats, panleukopenia is observed in feline leukemia virus, parvovirus, and feline immunodeficiency virus. Equine infectious anemia and Rickettsial infections are further examples in horses. 31

A less common form of the decreased platelet production mechanism not frequently described because of its rarity, is congenital thrombocytopenia. 30 Affecting
only about 1 in 500,000 people, the inherited disorders manifest with varying degrees of morphological and functional platelet change, and other non-hematological symptoms.\textsuperscript{32}

Many congenital or inherited platelet disorders present with macrothrombocytopenia. Examples of syndromes that cause macrothrombocytopenia due to a mutation in the MYH9 gene are May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, and Epstein syndrome. \textsuperscript{33}The MYH9 gene is located on the long arm of chromosome 22 (22q13.1) and codes for the heavy chain of non-muscle myosin IIA (NMMHC-IIA), a protein involved in the contractile activity of the cytoskeleton.\textsuperscript{34}

Bernard-Soulier syndrome and its heterozygous forms, Mediterranean Macrothrombocytopenia and DiGeorge Syndrome, are caused by an absent von Willebrand factor receptor. (Nurden) Platelet β1-Tubulin Q43P Polymorphism may present with or without thrombocytopenia. However, enlarged spherocytic platelets with decreased functionality are caused by defects in the platelet cytoskeleton. \textsuperscript{35}

More of these uncommon disorders exist that are yet to be classified and fully characterized.

**Clinical and Lab Assessment of Platelets**

When a patient presents with any signs of primary hemostatic defect such as ecchymosis, petechiae, mucosal bleeding, or prolonged bleeding after surgery or phlebotomy, thrombocytopenia will be suspected. But before any diagnostic testing is requested, a full clinical and family history is taken in order to screen for a potential platelet defect. The pattern of bleeding is established by knowing whether it is lifelong or recent, triggered by trauma, is present within other family members, or associated with any medication the patient may take.\textsuperscript{33} In the case of inherited macrothrombocytopenias,
if the thrombocytopenia is severe, the syndrome may be discovered at birth. Some congenital disorders go undetected until a bleeding episode associated with hemorrhage or surgery arises. Other congenital platelet disorders are apparent upon routine complete blood counts, but are disregarded due to no presentation of clinical symptoms.

There is a need for rapid and accurate classification of thrombocytopenia so that patients are not misdiagnosed and treated promptly. Because of the wide variation in practices between laboratories, and because disease classification systems are needed to make therapeutic decisions while providing a flexible framework for the integration of new data, a set of reliable platelet parameter are needed to aid in the diagnosis of thrombocytopenia. Routine laboratory requests for platelet parameter testing most commonly include the platelet count, mean platelet volume (MPV), and platelet distribution width (PDW). These indices, used together, have many potential diagnostic applications in hematology, cardiology, and other disciplines. However, the platelet parameters have technical limitations which must be considered while making each and every diagnosis. A universal standardization of platelet parameters is needed because there are variations in MPV measurements in optical versus impedance instruments. Both optical and impedance instruments may count the largest platelets as red blood cells which can result in a falsely low platelet count and MPV. These variations in measurement can be mitigated by standardization between different institutions and confirmation with microscopic techniques. After standardization and the creation of cut-off values using prospective cohorts, comparisons between different institutions can be possible. Without the verification by evaluation of platelet morphological appearance,
the hematology analyzer’s platelet volume distribution, and the platelet indices, diagnosis of platelet disorders may be inaccurate.\textsuperscript{29}

Platelet indices have potential to be useful in the diagnosis of thrombocytopenias, especially when evaluated as a panel. Leader\textsuperscript{11} stresses that there is great overlap between clinical syndromes and so the sensitivity and specificity of thrombocytopenia diagnosis can be increased by using the platelet count, platelet distribution width (PDW), MPV and microscopic examination all in combination. Leader demonstrated that patients with inherited platelet disorders have higher MPV and PDW than controls. An MPV above 12.4 fL in combination with an PDW greater than 3.3 µm had good diagnostic power for the diagnosis of inherited macrothrombocytopenias such as Bernard-Soulier Syndrome and MYH9-related diseases. This same study was able to distinguish inherited macrothrombocytopenias from ITP.

Overall, MPV and PDW are increased in destructive thrombocytopenia in comparison to diseases that result in decreased platelet production.\textsuperscript{11} In a study that attempted to distinguish different types of bone marrow disease apart from one another, MPV had limited sensitivity and specificity for many of the groups except for ITP.\textsuperscript{39} An MPV of less than 7.4fL was found to have significant predictive value when used to investigate the presence or absence of bone marrow metastasis. Usefulness of platelet parameters can be applied to more areas than just hematology. MPV has been reported to be increased in coronary artery disease, gestational diabetes, acute inflammatory disease, stroke, and more.\textsuperscript{11
Use of additional platelet indices may provide more important clinical data for use in disease classification. One such parameter not commonly reported by hematology laboratories is the plateletcrit. Analogous to the hematocrit, some hematology analyzers report the plateletcrit as the percentage of whole blood that is comprised of platelets.\textsuperscript{40} (It is also sometimes referred to as platelet mass.) Other analyzers calculate the plateletcrit as the product of the platelet count and platelet number.\textsuperscript{41} The plateletcrit encompasses both size and number to estimate total body platelet biomass.

**Animal Models in Platelet Research**

Animal models facilitate the search for cures for both animal and human diseases. Hyram Kitchen\textsuperscript{42} explains the importance of such research: “Comparisons of homologous disease processes between humans and animals may reveal the fundamental mechanisms common to all species affected by a given disease. The study of normal processes and spontaneous disease in animals mimic human aberrations and can be valuable to medical research.” Tumbleson\textsuperscript{43} suggests that animal models should strive to replicate human physical and environmental conditions as closely as possible. Therefore, a good model needs to be an animal that can most closely mimic the human process of platelet plug formation including the vasculature which is just one of the platelet’s environments. Ideally, models should also be able to provide multiple samples. Large animal species serving as platelet models can provide multiple samples because peripheral blood is easily attainable.\textsuperscript{43}

Since many platelet disorders are unique to individuals or individual families, creating a single model to emulate each individual disorder would be challenging due to
the great heterogeneity in the platelet disorders. There are many different animal models available for study of human platelet disease, disorders, and diseases that are related to platelets. Choosing the right model will depend on what question is being addressed by the research plan. Choosing carefully saves time, money and generates the solid scientific evidence needed to answer the research question.

Like many mammals, human platelets do not have the needed DNA required for many post-translational molecular studies because they do not have a nucleus. However, platelets do retain small amounts of residual megakaryocyte mRNA which makes a limited number of genetic studies possible. The studies based on the mRNA must then be confirmed via examination of protein expression due to possible contamination from nucleated cells. Analysis of the platelet proteome via mass spectrometry in conjunction with the molecular studies can reveal mechanistically how platelets function.44

Animal Models in Platelet Research: The Zebrafish

Danio rerio, or the zebrafish, has thrombocytes which are nucleated, and thus contain DNA which can be used for platelet molecular model studies. Other popular genetic model organisms such as Drosophila or Caenorhabditis elegans cannot be used because they do not have a cell equivalent to the human platelet. One major downfall of the zebrafish is that since the functional role of the nucleus in thrombocyte hasn’t been clearly defined, the full effect of the nucleus on the ability to extrapolate findings to human is unclear.44

The Zebrafish have highly conserved coagulation and hematological systems that are similar to humans. These biological similarities in combination with the opportunity
of the nucleus to provide genetic studies have made the Zebrafish a highly attractive model organism for studying hemostasis. Like humans, Zebrafish platelets have an open canalicular system, alpha-granules, dense granules, and they form pseudopod-like projections when activated. Also, their thrombocytes are able to function in a similar manner as humans. They aggregate in response to ristocetin, collagen, thrombin, ADP, and arachidonic acid. Biochemical studies suggest that all components of the coagulation network are present, and in vivo thrombus formation can be induced by chemical or laser injury of blood vessels.\textsuperscript{44} Thus, zebrafish can mimic the process of human platelet plug formation. They also have an adaptive immune system which helps to replicate similar environmental conditions as humans.\textsuperscript{44} Zebrafish also fulfill Tumbleson’s\textsuperscript{43} suggestions of an appropriate model by being able to provide many samples. They become sexually mature in only 3-4 months and can lay 200-300 eggs per week in a fish facility. Therefore, Zebrafish are also cost effective.\textsuperscript{44}

**Animal Models in Platelet Research: Mice**

Even though mice do not have nucleated platelets, they have served as powerful genetic models in the field of platelet research because mice models can be specifically genetically manipulated to mimic human conditions. The mouse is useful in genetic knock-out and transgenic studies. Targeting transcription factors is critical for normal thrombopoiesis (such as binding proteins GATA1 and GATA2, cofactor FOG1, core binding factor proteins RUNX family, and nuclear factor-erythroid 2), and inactivating genes relevant to platelet development, function, and survival has produced new ways to
define platelet protein function and is unraveling signal transduction pathways in platelets.\textsuperscript{45}

There are other structural and functional differences between mice and human platelets that do not replicate human physical conditions. Murine platelets are smaller, live only 3-4 days, and circulate in four-fold greater numbers than humans. The mouse genome lacks the gene for FcϒRIIA, the only Fc receptor found on human platelets. And mouse platelets do not express PAR-1, one of the major thrombin receptors on human platelets. Mice also fail to mimic human platelet plug formation when tail bleeds and vessel wall injury are induced by laser ablation.\textsuperscript{45} There are also important differences in participation of protein isotypes in signaling cascades.\textsuperscript{10} The total hemostatic impact of these differences must be compensated for in each mouse model. For example, although thrombopoietin is highly homologous across species, it is only 81\% homologous between mice and people.\textsuperscript{23} Nevertheless, the mouse was the model used to identify thrombopoietin as the key the humoral regulator of platelet production.\textsuperscript{46}

Mice are sometimes used to serve as models of thrombopoiesis after transplantation with human hematopoietic stem cells (HSC) because in vitro platelet and megakaryocyte culture of human platelets has low platelet yield. Because non-obese diabetic/severe combined immunodeficient mice present multiple defects in innate and adaptive immunity, transplantation of human HSC results in high levels of engraftment and differentiation of multiple blood cell lineages.\textsuperscript{44}

Thon used a mouse model to study pro-platelet production and platelet release.\textsuperscript{24} Mice are able to replicate the human expression of GPIIbIIIa, localization of alpha- and
dense-granules, morphological characteristics of proplatelet elongation, and transport of molecular constituents from the cell body to pro-platelet tips. Considering that these aspects are conserved across both human and murine species, it can indicate that the fundamental mechanisms of megakaryocyte differentiation and pro-platelet genesis in murine and human cells are also similar.\textsuperscript{24} Other methods of inducing experimental thrombocytopenia in mice and other models include the use of antiplatelet antibodies, ionizing irradiation, chemotherapy, deficiency of thrombopoietic effectors, high dose estrogen, cardiopulmonary bypass models, thrombotic thrombocytopenia purpura, and heparin-induced thrombocytopenia.\textsuperscript{10}

**Animal Models in Platelet Research: Large Animal Models**

Primates also have not been commonly used in platelet studies probably because they are expensive, harbor lethal viruses that can be transmitted to humans, and they are of high political and ethical concern.\textsuperscript{43}

Pure-bred dogs can serve as special models of spontaneous disease. Due to many years of artificial selection and breeding of dogs with common founders, recessive diseases are common in purebreds. These diseases have been mapped in large canine pedigrees and have potential for use in genetic studies.\textsuperscript{47} The American Kennel Club registers at least 154 breeds of purebred dogs; and dog genetic resources exist such as National Center for Biotechnology Information’s dog resource page and the dog gene index at The Institute for Genomic Research.\textsuperscript{48}

Since both the human and canine genomes have been sequenced, great strides have been made in understanding how dog population genetics can be used to model
human disease. Canine genetic linkage disequilibrium studies can be used to compare haplotypes of breeds of affected dogs to breeds lacking the trait in order to pinpoint a locus for disease. The locus can hopefully be used to locate the human homolog and eventually identify the mechanism of the disease and find a cure for both species.\textsuperscript{48}

Obtaining DNA samples for analysis of these dogs requires a different approach than for lab animal species like mice and rats.\textsuperscript{48} Even though the dog genome has been completely sequenced, researchers must approach breed clubs and pet owners for phenotypes and DNA samples.\textsuperscript{49}

Because certain canine diseases can be more characteristic of specific breeds, and dogs can serve as spontaneous models of human disease, they are a good candidate for the study of inherited platelet disorders. Canines are more closely genetically related to humans than mice. Also, gross dog physiology is closer to humans than rats or mice. Likewise, canine thrombopoietin is 85\% homologous to human thrombopoietin at the amino acid level.\textsuperscript{23} Macrothrombocytopenia in Cavalier King Charles Spaniels, thrombasthenia in Great Pyrenees and Otterhound, cyclic hematopoiesis in the Grey Collie, and CalDAG-GEFI disorder in Basset Hounds and Eskimo Spitz are all examples of spontaneous canine inherited platelet disorders with potential to model human disease.\textsuperscript{34}

**Cavalier King Charles Spaniels as a Platelet Model for Inherited Macrothrombocytopenias**

In 2002 Pederson\textsuperscript{50} investigated the prevalence of an asymptomatic macrothrombocytopenia in Cavalier King Charles Spaniels (CKCS) and demonstrated
that it is an inherited autosomal recessive trait. The β-1 tubulin mutation discovered in this breed of canine can provide a spontaneous model of an inherited platelet disorder.\textsuperscript{51} In 2008, Davis\textsuperscript{17} described the molecular aspect of the macrothrombocytopenia in a paper called “Mutation in β-1 Tubulin Correlates with Macrothrombocytopenia in Cavalier King Charles Spaniels”. This inherited giant platelet disorder affects approximately 50% of Cavalier King Charles Spaniels. It is characterized by thrombocytopenia, macrothrombocytes, and decreased platelet aggregation in response to ADP. Platelet ultrastructure is normal and the animals do not have a bleeding diathesis.\textsuperscript{51}

Some Cavaliers have received inappropriate medical treatment because the asymptomatic inherited thrombocytopenia is not distinguished from acquired thrombocytopenia.\textsuperscript{17} Davis recognized the need to find a way to determine if a Cavalier has inherited or acquired macrothrombocytopenia and proposed that a mutation in the beta 1-tubulin gene could provide molecular evidence of the etiology of the giant platelets. PCR of 89 Cavalier King Charles Spaniels revealed the missense mutation substitution of an asparagine for an aspartic acid in the gene encoding beta-1 tubulin. 19% were clear of the mutation, 48% were heterozygous, and 33% were homozygous. Of the 52 non-CKCS tested for the mutation, only 1 was heterozygous.

CKCS were divided into groups based on presence or absence of the β1-tubulin mutation, and platelet numbers and mean platelet volume were reevaluated based on these groups. The means of the platelet number and mean platelet volume in each group were significantly different from one another (p<0.0002). Low platelet counts correlated with the homozygous state of the mutation, intermediate counts correlated with the
heterozygous state of the mutation, and normal to high platelet counts were correlated with animals there were clear of the mutation. These significant data support the idea that the point mutation affects platelet counts. (Also, the heterozygous state reveals half the phenotype.) The use of immunofluorescence for visualization of β1-tubulin was physical evidence that the β1-tubulin was present, but decreased in amount in the affected and heterozygous canines. In those same dogs, electron microscopy for visualization of the microtubules revealed an abnormal and most likely unstable structure.

In 2008 there was no β1-tubulin mutation reported in people that was similar to the CKCS mutation. (In the β1-tubulin Q43P mutation, the patients have giant platelets but they are not always thrombocytopenic.) But, now in 2012, Kunishima reports a human counterpart to the CKCS mutation: A β1-tubulin R318W mutation that replaces arginine with tryptophan and likely affects microtubule assembly in a similar manner as in the cavaliers. Structurally, both the CKCS mutation D249N and the human mutation R318W are located at the interface between tubulin beta and alpha subunits. Clinically, both mutations present with asymptomatic macrothrombocytopenia.

This thesis investigates the use of the plateletcrit as an assessment of total platelet biomass in Cavalier King Charles Spaniels with asymptomatic congenital macrothrombocytopenia. A better understanding of the laboratory evaluation of platelets with this disorder will aid in their medical care, however the data will have larger implications for application in other human and veterinary platelet disorders.
Chapter 2: Thesis Research Project

Genetic variation in dogs can represent a significant challenge to the interpretation of clinical pathology data in clinical situations but may also elucidate significant biological principles important to the understanding of disease. Two breeds of dogs, Cavalier King Charles Spaniels (CKCS) and Greyhounds, have been reported to have low platelet counts compared with outbred dogs and other breeds, yet neither seems predisposed to excessive bleeding associated with these low counts. The biological basis for the macrothrombocytopenia in CKCS has been associated with a missense mutation in the gene encoding β1-tubulin, resulting in abnormal proplatelet formation and platelet production by megakaryocytes due to impaired microtubule assembly. The etiology of the low platelet counts in Greyhounds has not been conclusively determined, although a recent review concludes that detailed clinical investigations of thrombocytopenia in otherwise healthy Greyhounds may not be warranted. Hypotheses for these low platelet counts include stem cell competition between megakaryocyte and erythroid precursors, splenic or pulmonary sequestration, and an inverse relationship with iron stores.

It has been proposed that platelet mass is a more biologically important hemostatic parameter than platelet count, and thus plateletcrit may be superior to platelet count as an indicator of primary hemostasis, particularly in CKCS, which have fewer but larger platelets when affected by the β1-tubulin mutation. Three recent studies of CKCS have demonstrated that estimates of platelet mass using the quantitative buffy coat method are higher than estimates derived from platelet counts generated by impedance or
laser-based analyzers in healthy dogs and in dogs in which disease was limited to mitral valve insufficiency. A validation study comparing the quantitative buffy coat method with the plateletcrit generated by the Advia 2120 showed good general agreement between the two methods that was considered satisfactory by the authors; however, the Advia plateletcrit demonstrated a negative bias and may have underestimated the platelet mass in CKCS with the lowest plateletcrits in the study. Since most laboratories are not equipped with analyzers using the quantitative buffy coat method, a better understanding of the diagnostic performance of the platelet parameters that are available from standard analyzers will support good clinical decision making. Additional studies evaluating the platelet status of sick CKCS presenting for medical care are needed to corroborate previous findings in more limited populations to avoid spectrum bias. Additionally, the plateletcrit of Greyhounds, another breed reported to have a tendency for low platelet numbers without macroplatelets, has not been investigated and provides an interesting contrast to CKCS. To evaluate how the plateletcrit and platelet count from a commonly utilized reference hematology analyzer compared in the assessment of platelet mass in clinical patients, we conducted retrospective data-base searches for CKCS and Greyhounds presenting as clinical patients with a variety of underlying illnesses to the University of Minnesota and Auburn University Veterinary Small Animal Teaching Hospitals. We compared the platelet status of these patients using the platelet count and plateletcrit determined by a laser-based hematology analyzer commonly utilized by reference laboratories.
Materials and Methods

Data retrieval

University of Minnesota (UMN): The electronic medical records database for the University of Minnesota Veterinary Medical Center was searched for complete blood counts performed on Cavalier King Charles Spaniels (CKCS) and Greyhounds from March 2008 through October 2010. The initial report included 187 results from CKCS and 134 from Greyhounds. When multiple results were reported from the same individual (57 CKCS and 55 Greyhound), only data from the first CBC was analyzed. Greyhounds were primarily retired racers, although information regarding the source of the dog was not available for all patients. Other exclusion criteria included evidence of platelet clumping; patients being less than six months of age; and a diagnosis of primary hematologic disease (i.e. immune-mediated hemolytic anemia or thrombocytopenia), neoplasia, or sepsis due to potential impact on platelet parameters. The medical records of each patient were reviewed for the diagnoses that coincided with the CBCs of interest. Diagnoses were categorized according to organ system; more than one diagnosis sometimes applied to an individual animal. The reference population consisted of 47 healthy dogs of various breeds and greater than 6 months of age owned by students, staff, and faculty at UMN. An a priori direct selection process was used with defined selection criteria, and samples were collected over a period of three weeks. Of the 47 dogs, 1 was excluded for the presence of anemia, and two were excluded for low platelet counts.
Auburn (AU): The electronic medical records data base for the Auburn University Veterinary Medical Center was searched for complete blood counts performed on Cavalier King Charles Spaniels (CKCS) and Greyhounds from January 2008 through February 2011. The initial report included 138 results from CKCS and 80 from Greyhounds. When multiple results were reported from the same individual (66 CKCS and 53 Greyhound), only data from the first CBC was analyzed. Other exclusion criteria were identical to those for UMN dogs; however, patients with platelet clumping were excluded as part of the initial data base search. Disease processes were characterized as described above. The UMN reference population was used for comparison for both the UMN and Auburn CKCS and Greyhound cohorts because the original reference interval data for Auburn could not be retrieved.

Sample collection and processing

Due to the retrospective nature of the study, sample collection and processing could not be controlled or evaluated beyond the exclusion of samples with microscopic evidence of platelet clumping. At both institutions, samples were collected and submitted in EDTA, with an average collection to processing time of less than two hours during normal operating hours with the potential for up to a 12 hour delay in processing over nights or on weekends. No samples were more than 24 hours old when analysis was performed. All CBCs included blood smear evaluation by a licensed medical technologist, which included scanning for platelet clumps. Samples were analyzed using either an Advia 2120 (UMN) or 120 (AU). The technology for platelet assessment is
identical for these two analyzers, with an international multicenter trial revealing excellent correlation between the two analyzers, even for platelet counts less than 50,000/ul. Parameters evaluated included the automated platelet count, mean platelet volume and the plateletcrit (PCT). PCT is calculated by the Advia 2120 by multiplying platelet number in thousands by mean platelet volume in femtoliters and dividing that product by 10,000. PCT was not programmed to report out by the Advia 120 at AU, and was calculated in Microsoft Excel using the same equation as the Advia 2120: PCT = (PLT x MPV) / 10,000.

Statistical methods

For the plateletcrit and platelet count variables, a 95% reference interval was calculated from the reference sample by taking the 2.5% percentile of the normal distribution with mean and standard deviation equal to the sample mean and standard deviation; normality was checked using quantile-quantile plots and the Shapiro-Wilk test. A 95% bootstrap confidence interval for each end of the reference interval was calculated by taking 1000 bootstrap samples, computing the reference interval for each, and finding the appropriate quantiles. The percent of each non-reference sample that fell below the lower reference limit was then calculated; to test if this percent was significantly different than the expected 2.5%, a binomial test was performed using the lower limit from each bootstrap reference interval, and the results combined across all bootstrap samples. This procedure was used to properly account for both the uncertainty in the reference interval and the variability in each sample. These p-values were adjusted for multiple
comparisons using the Bonferroni-Holm procedure. Additionally, 95% confidence intervals for the percent falling below the reference limit was computed by finding the range of percents for which the p-value was greater than 0.05.

To test if there was a difference between the percent with plateletcrit values below the lower reference limit and the percent with platelet count values below the lower reference limit, McNemar's test was used to compare two measurements from each individual. Results were computed for each non-reference sample separately, and again combined across all bootstrap reference intervals. Differences between the non-reference groups were also of interest. However, because of uncertainty in the reference interval and the unpaired nature of this comparison, there was very little power to test if the percent falling below the reference limit differed between groups. Instead, it was tested if the 10% percentile was different between the groups; this was chosen instead of 2.5% because of the relatively small sample size of some of the groups. A bootstrap test was again used for this test; 1000 bootstrap samples were taken from each group and the 10% percentile calculated for each; a two-sided p-value for each pair was then computed and adjusted for multiple comparisons using the Bonferroni-Holm procedure. Results were considered significant at >0.05.
Results

Patient population

UMN: Of the 130 initial CBCs from CKCS, excluded samples included 34 due to microscopic evidence of platelet clumping, 7 for dogs not being purebred, 6 due to patient age, 4 due to the diagnosis of neoplasia and 1 for immune-mediated hemolytic anemia. Of the 78 remaining dogs with CBCs, there were 4 intact females, 28 spayed females, 6 intact males, and 40 neutered males. Median age was 4 years (range 8 months-11 years). Of the 79 Greyhounds from UMN, 1 was not purebred, 21 were excluded due to platelet clumping, 9 for neoplasia, and 1 for immune-mediated thrombocytopenia. Of the 47 remaining dogs, 7 were intact females, 10 were spayed females, 14 were intact males, and 16 were neutered males. Median age was 4 years (range 1-14 years). The distribution of diagnoses at presentation for both breeds is presented in Table 1.

Auburn: Of the 72 initial CBCs from CKCS, 11 were excluded due to the diagnosis of neoplasia or primary hematologic disease. Of the 61 remaining dogs, 4 were intact females, 32 were spayed females, 5 were intact males, and 20 were neutered males. Median age was 3 years (range 6 months to 10 years). Of the 27 CBCs from Greyhounds, 9 were excluded due to neoplasia. Of the 18 remaining dogs, 4 were spayed females, 5 were intact males, and 9 were neutered males. Median age was 6 years (range 2-12 years). The distribution of diagnoses at presentation for both breeds is presented in Table 1.
Figure 1. The distribution of individual plateletcrit values determined by the Advia 2120 (MN) or 120 (Auburn) for Cavalier King Charles Spaniels (Cavalier) and Greyhounds at the University of Minnesota (MN) and Auburn University compared with a reference population of healthy dogs of mixed genetic background. The solid vertical grey line is the lower limit of the 95% reference interval, with the dashed vertical lines indicating a 95% bootstrap confidence interval. After Bonferroni-Holm correction for multiple comparisons, none of the groups were found to have significantly more individuals below the reference interval than expected.
Figure 2. The distribution of individual platelet counts determined by the Advia 2120 (MN) or 120 (Auburn) for Cavalier King Charles Spaniels (Cavalier) and Greyhounds at the University of Minnesota (MN) and Auburn University compared with a reference population of healthy dogs of mixed genetic background. The solid vertical grey line is the lower limit of the 95% reference interval, with the dashed vertical lines indicating a 95% bootstrap confidence interval.

Platelet parameters

For plateletcrit, the lower limit of the 95% reference interval was 0.129 (95% bootstrap confidence interval 0.101- 0.163), and the upper limit of the reference interval was 0.403 (95% bootstrap confidence interval 0.368- 0.437). After the Bonferroni-Holm
correction for multiple comparisons, none of the four sample groups was found to have significantly more individuals below than the reference limit for plateletcrit than expected (Figure 1 and Table 2). For platelet count, the lower limit of the 95% reference interval was 116.2 (95% bootstrap confidence interval 88.4-149.9), and the upper limit of the reference interval was 398.2 (95% bootstrap confidence interval 364.5-429.0). In contrast to the plateletcrit, both Cavalier cohorts were found to have significantly more individuals below the reference limit than predicted (p<0.0001 for both (Figure 2 and Table 2). Neither of the Greyhound cohorts had significantly more individuals below the reference limit than expected (UMN: p=0.32, AU: p=0.13).

CKCS from both Auburn and UMN had significant differences between the percentage of the population having low plateletcrits and the percentage having low platelet counts (UMN, p<0.0001, AU, p=0.023), with the platelet count underestimating platelet mass compared with the plateletcrit (Table 2). In contrast, there were not significant differences between the two tests in reflecting platelet status for either of the Greyhound groups (for UMN, p=0.27, AU, p=0.42), suggesting that these different parameters function similarly in estimating platelet mass for this breed in our populations. The numbers of dogs falling below the reference interval are greater from the Auburn cohort given that out of necessity, UMN reference intervals were used.

The reliability of the plateletcrit as an estimate of platelet mass was further supported by the similarity of 10th percentile values between the breeds. Only Auburn Greyhounds had significantly lower 10th percentile plateletcrits than UMN CKCS, which is of uncertain significance and may be related to extrapolation of reference intervals or
breed differences. In contrast, UMN CKCS had significantly lower 10\textsuperscript{th} percentile platelet counts than either population of Greyhounds, and Auburn CKCS was lower than UMN Greyhounds, however the two Auburn populations were not different. There were no regional differences between breed cohorts, suggesting that plateletcrit may be a more consistent indicator of platelet mass than platelet count for CKCS, even when the plateletcrit is measured indirectly by the Advia 120/2120.

<table>
<thead>
<tr>
<th>Group</th>
<th>CV</th>
<th>Eye &amp; ear</th>
<th>Urogenital</th>
<th>MS</th>
<th>Nervous</th>
<th>GI/liver</th>
<th>Behavior</th>
<th>Healthy</th>
<th>Endo</th>
<th>Resp</th>
<th>Renal</th>
<th>Derm</th>
<th>Other</th>
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<tbody>
<tr>
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<td>13</td>
<td>2</td>
<td>18</td>
<td>20</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Minnesota Greyhound</td>
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<td>3</td>
<td>6</td>
<td>3</td>
<td>17</td>
<td>2</td>
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<td>0</td>
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<td>0</td>
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<td>56</td>
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<tr>
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<td>10</td>
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<td>1</td>
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<td>2</td>
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<td>81</td>
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<tr>
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<td>8</td>
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<td>5</td>
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</tr>
</tbody>
</table>

Table 1. Distribution of organ system pathology at presentation based on medical record review. Note that some dogs have more than one pathologic process. The large number of GI/liver cases at the University of Minnesota is related to many dogs presenting for evaluation of periodontal disease. CV=cardiovascular, MS=musculoskeletal, Endo=endocrine, Resp=respiratory, Derm=dermatologic.
Table 2. Percentage of dogs with plateletcrits and platelet counts below the reference interval. *Percentage below the reference interval that is significantly greater than the expected 2.5%, (binomial test, Bonferroni-Holm correction for multiple comparisons, p<0.05). Significant differences between the percent with low plateletcrit and the percent with low platelet count within a population are denoted by lower case letters (McNemar’s test, Bonferroni-Holm correction for multiple comparisons, p<0.05)

<table>
<thead>
<tr>
<th>Location and Breed</th>
<th>% Low Platelet Count</th>
<th>Confidence Interval</th>
<th>% Low Plateletcrit</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota Cavaliers (n=78)</td>
<td>30.8*a</td>
<td>(18.0 – 45.7)</td>
<td>5.1*a</td>
<td>(0.5 – 24.6)</td>
</tr>
<tr>
<td>Auburn Cavaliers (n=61)</td>
<td>23.0*b</td>
<td>(10.8 – 45.4)</td>
<td>11.5*b</td>
<td>(1.3 – 27.0)</td>
</tr>
<tr>
<td>Minnesota Greyhounds (n=47)</td>
<td>8.5</td>
<td>(0.0 – 44.2)</td>
<td>8.5</td>
<td>(0.0 – 63.0)</td>
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<tr>
<td>Auburn Greyhounds (n=18)</td>
<td>33.3</td>
<td>(0.0 – 71.4)</td>
<td>38.9</td>
<td>(0.0 – 77.9)</td>
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</table>

Table 3. Tenth percentile values for platelet count and plateletcrit by cohort. A bootstrap test was used; 1000 bootstrap samples were taken from each group and the 10%
percentile calculated for each; a two-sided p-value for each pair was then computed and adjusted for multiple comparisons using the Bonferroni-Holm procedure. Results were considered significant at the 0.05 significance level and are indicated by a shared superscript lower case letter denoting the groups that are different.

**Discussion**

The use of the plateletcrit determined by the Advia 120/2120 rather than the platelet count to evaluate primary hemostasis in CKCS avoids overestimation of low platelet mass and risk of bleeding in sick CKCS, especially when general canine reference intervals are utilized. Since CKCS are predisposed to a number of medical conditions such as mitral valve disease and syringomyelia, these dogs frequently present to referral centers, where extensive testing is often performed. We propose that the use of plateletcrit in addition to the platelet count to evaluate primary hemostasis in sick CKCS may help prevent unnecessary additional testing and treatment by veterinarians and mitigate concern on the part of owners. Our data agree with that of previous investigations that plateletcrit is superior to platelet count for assessing platelet count in CKCS but suggest that quantitative buffy coat analysis may not always be required for the determination of plateletcrit in a clinical setting. This finding is supported in a recent study by Tvedten et al validating the Advia plateletcrit against quantitative buffy coat analysis. Tvedten’s data suggested that the Advia may underestimate platelet mass in CKCS with the lowest plateletcrits, so the diagnostic application of the Advia plateletcrit likely has limitations in some patients. In contrast, and as expected for a breed with a characteristically low platelet count and normal platelet size, platelet count and plateletcrit seem to function similarly as estimates of platelet mass in our populations of
Greyhounds. While excessive bleeding has been described in Greyhounds, coagulopathic dogs usually have normal platelet counts and the cause of bleeding is not directly attributed to thrombocytopenia.58

The use of plateletcrit may augment the evaluation of primary hemostasis in other situations. Although the diagnostic value of plateletcrit has not been extensively evaluated in people, one study demonstrated that the replacement of platelet count-based transfusion guidelines in the neonatal intensive care unit with plateletcrit-based guidelines resulted in lower transfusion rates, especially ones given prophylactically, with no detectable increase in hemorrhagic events in the patient population.59 More attention in human medicine has been focused on the use of platelet volume indices such as the MPV, which is the basis for the calculation of the Advia plateletcrit. There is preliminary evidence that consideration of platelet size in addition to platelet count may have diagnostic utility in evaluating human patients with thrombocytopenia11, especially for distinguishing inherited macrothrombocytopenia from immune-thrombocytopenia.38 However, technical issues such as variation between analyzers and lack of commercially available quality assurance materials for MPV need to be addressed for further progress to be made.29

There are several potential limitations to our study methodology. Because the original reference interval data from Auburn University was not available and the statistical methodology could not be verified, we applied UMN reference intervals to the AU CKCS and Greyhound populations. This likely impacted the proportion of dogs falling outside the reference intervals; however, the similarity between UMN and Auburn
breed data for the 10th percentile of platelet count and crit suggests that analysis was not significantly impacted. The relatively small number of dogs used to generate the UMN reference intervals resulted in wide confidence intervals, which may have reduced the statistical power for the multiple comparisons. The UMN reference interval for Advia plateletcrit was lower than that reported by Tvedten’s value of 0.18-0.44, and the lower limit of the reference interval for platelets was lower than some published values as well (ref). Although the reference interval study for this project was completed prior to the availability of the ASVCP Reference Interval Guidelines (http://www.asvcp.org/pubs/qas/index.cfm), a brief review of the documentation for our process confirmed that we conformed to recommendations. Because our study compares the relative performance of platelet count and plateletcrit using the same reference population, unusually low limits might have reduced the statistical power for the detection of differences between groups; however, this does not appear to have been a problem. To further evaluate our reference intervals, we queried the American Society for Veterinary Clinical Pathology listserv regarding reference intervals for the Advia 2120/120. Results revealed wide variation in the reference interval lower limit (see supplemental data) between facilities and institutions, illustrating limitations to our current approaches for reference interval determination. The study is retrospective, so we were unable to absolutely verify sample collection and handling methods. Overexposure to EDTA and refrigeration may artifactually increase MPV, which could have impacted our results. However, samples collected in large veterinary teaching hospitals are usually processed and analyzed rapidly, and both institutions do not routinely refrigerate
samples prior to analysis during regular business hours. Additionally, although all samples and smears were evaluated by medical technologists for evidence of clots and platelet clumping, clumping may not have been evenly distributed among or within some tubes and on smears,\textsuperscript{61} making it possible that some samples may have had undetected platelet clumping. Finally, there may have been gaps or inaccuracies in the patient medical records that could have impacted our results.

Regardless of these limitations, our results generated using data from clinical patients presenting for medical care agree with previous studies suggesting that plateletcrit is superior to platelet count for evaluation of dogs with genetic macrothrombocytopenia,\textsuperscript{40,56} but minimal additional information is gained in dogs with more normal platelet size such as greyhounds. The calculated plateletcrit using the Advia 120/2120 appears to be adequate for use in sick dogs that do not have primary hematologic disease or neoplasia but may still underestimate platelet mass at very low plateletcrits based on other studies.\textsuperscript{41} Data from human studies and the apparent utility of the plateletcrit in dogs with congenital macrothrombocytopenia suggest that the evaluation of the plateletcrit for additional clinical applications may be warranted.
Chapter 3: Detailed Description of Methods and Results

Platelet Determination by Advia 2120 & 120

Samples were analyzed on the Bayer Advia 2120 (Minnesota) or 120 (Auburn.) Platelets and red blood cells are sphered by the Advia RBC/PLT reagent which contains sodium dodecyl sulfate and glutaraldehyde. When the cells are isovolumetrically sphered, they become similar in shape while maintaining cell volume. This allows for measurement via laser light scatter using the Mie theory of light scatter for homogeneous spheres (per the Advia’s 2120 Systems User’s Manual located onboard the instrument.)

Volume/size data are obtained from the 2-dimensional integrated analysis measurement of both platelets and red blood cells on the platelet scatter cytogram. (See figure 3 below.) The platelet scatter cytogram is formed by pairing light scatter signals acquired at low-angle, high gain and at high-angle, high gain. Low angle (2 to 3 degrees) is plotted on the y-axis of the PLT scatter cytogram and measures volume. High angle light scatter (5 to 15 degrees) is plotted on the x axis and measures refractive index values. The refractive indexes of platelets and red blood cells are different due to the internal cellular contents. This integrated analysis of size and refractive index is used to distinguish platelets, large platelets, red blood cells, red blood cell fragments, and red blood cell ghosts from each other. The platelet volume cytogram is a graphical representation of cells with volumes of 0-30fL. Cells with volumes greater than 30 fL are displayed on the red blood cell cytogram. Thus, not all large platelets are visualized on the platelet scatter cytograms.
The Advia 120/2120 MPV is the mean of the platelet volume histogram. (Canine examples are in Figures 4 and 5.) The volume histogram shows distribution of cells by volume up to 60 fL only. Cells larger than 60 fL are counted as red blood cells. Therefore, not all platelets are included in the mean platelet volume measurement.

However, considering that the MPV range of all the Cavalier platelets in this study was 7.3-35.4 fL, which is not near 60 fL, the number of giant platelets not counted is minimal.

The plateletcrit is a calculated value: \[ PCT = \frac{PLT\# \times MPV}{10,000} \]

where PLT# is platelet count in thousands/μL and MPV is the mean of the platelet volume histogram in femtoliters.
Figure 3: Advia 120/2120 Human Platelet Scatter Cytogram

1 Platelets
2 Large platelets
3 Red blood cells
4 RBC fragments
5 RBC ghosts

A: Cell Density: High angle light scatter is plotted on the X-axis
B: Cell Volume: Low angle light scatter is plotted on the Y-axis
Collection of Genotyped Data

The genotype status for the missense mutation in the gene encoding beta-1 tubulin correlating with macrothrombocytopenia in 44 Cavalier King Charles Spaniels used in a previous study\textsuperscript{17} were kindly provided by Auburn University (Davis.)

Macrothrombocytopenia in Cavalier King Charles Spaniels is caused by a single nucleotide change (G to A) in the gene encoding beta-1 tubulin. This change results in the substitution of an asparagine for an aspartic acid in a highly conserved area of the protein.\textsuperscript{17} The platelet numbers and mean platelet volumes of these samples were determined at the various sample collection sites of North Carolina State University, Texas A&M University, Dublin Ireland’s University College Veterinary Hospital, and the University of Florida as well as by private practitioners. The exact methodologies used for the platelet determinations were unknown. The plateletcrits were calculated from the platelet numbers and MPV by using the equation: $PCT = \frac{PLT\# \times MPV}{10,000}$. 
Figure 4: Example of Advia 120 results for complete blood count, platelet scatter cytogram, and platelet volume histogram for an affected Cavalier King Charles Spaniel.

<table>
<thead>
<tr>
<th>Routine CBC</th>
<th>PLT Scatter</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC: 23.54 x10^11 cells/µL</td>
<td></td>
</tr>
<tr>
<td>RBC: 5.73 x10^6 cells/µL</td>
<td></td>
</tr>
<tr>
<td>HGB: 14.2 g/dL</td>
<td></td>
</tr>
<tr>
<td>HCT: 39.1 %</td>
<td></td>
</tr>
<tr>
<td>MCV: 68.2 fl</td>
<td></td>
</tr>
<tr>
<td>MCH: 24.8 pg</td>
<td></td>
</tr>
<tr>
<td>MCHC: 36.3 g/dL</td>
<td></td>
</tr>
<tr>
<td>CHCM: 13.3 %</td>
<td></td>
</tr>
<tr>
<td>CH: 26.4 pg</td>
<td></td>
</tr>
<tr>
<td>RDW: 1.73 g/dL</td>
<td></td>
</tr>
<tr>
<td>MPV: 103 x10^11 cells/µL</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5: Example of Advia 120 results for complete blood count, platelet scatter cytogram, and platelet volume histogram for a normal Cavalier King Charles Spaniel.

<table>
<thead>
<tr>
<th>Routine CBC</th>
<th>PLT Scatter</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC: 18.46 x10^11 cells/µL</td>
<td></td>
</tr>
<tr>
<td>RBC: 6.23 x10^6 cells/µL</td>
<td></td>
</tr>
<tr>
<td>HGB: 14.5 g/dL</td>
<td></td>
</tr>
<tr>
<td>HCT: 43.4 %</td>
<td></td>
</tr>
<tr>
<td>MCV: 69.6 fl</td>
<td></td>
</tr>
<tr>
<td>MCH: 23.3 pg</td>
<td></td>
</tr>
<tr>
<td>MCHC: 33.4 g/dL</td>
<td></td>
</tr>
<tr>
<td>CHCM: 34.6 g/dL</td>
<td></td>
</tr>
<tr>
<td>CH: 24.0 pg</td>
<td></td>
</tr>
<tr>
<td>RDW: 13.1 %</td>
<td></td>
</tr>
<tr>
<td>HDW: 2.00 g/dL</td>
<td></td>
</tr>
<tr>
<td>PLT: 494 x10^11 cells/µL</td>
<td></td>
</tr>
<tr>
<td>MPV: 22.0 fl</td>
<td></td>
</tr>
</tbody>
</table>
Creation of Reference Ranges and Statistical Analysis

A non-parametric central 95% normal reference interval for platelet number and plateletcrit was created using 43 random, normal outbred dogs from the vicinity of the University of Minnesota. Slides were reviewed for microscopic platelet clumping, and plateletlet number was confirmed by four medical technologists. (Complete blood counts with a manual differential were also performed.) Since only 43 normal canines were available, normality of the reference interval was checked using quantile-quantile plots and the Shapiro-Wilk test. 95% bootstrap confidence intervals were also generated by taking 1000 bootstrap samples, computing the reference interval for each, and finding the appropriate quantiles.62

To test if the Cavalier and Greyhound groups had more than 2.5% of individuals with platelet counts or plateletcrits below the reference interval, a binomial test was performed. The binomial test used the lower limit from each bootstrap reference interval, and the results combined across all bootstrap samples because of the small number of 43 canines used to generate the reference interval. This procedure was used to properly account for both the uncertainty in the reference interval and the variability in each sample. Also, 95% confidence intervals for the percent of canines that fell below the plateletcrit and platelet count reference intervals was computed by finding the range of percents for which the p-value was greater than 0.05.

McNemar’s test62 was used to compare the plateletcrit to platelet count for each individual by assuming that if an individual’s platelet count is below the reference interval, then so should its count. 10th percentile differences were used to compare all
four groups to one another. 10th percentile test comparison was used due to the low power and uncertainty in the reference interval. Results were considered significant at the 0.05 level.62

Results

For healthy, normal dogs of mixed breed, age and gender, the central 95% reference interval for platletcrit was 0.129 – 0.403%. The central 95% platelet number reference interval was 116.2 – 398.2 x 10³/µL.

The 24 Cavalier King Charles Spaniels genotyped as heterozygous for the β1-Tubulin mutation at Auburn University had plateletcrits ranging from 0.11 – 0.50%. The 16 cavaliers genotyped as homozygous affected had plateletcrits ranging from 0.06 – 0.34%. The 4 cavaliers genotyped as wild type had plateletcrits ranging from 0.33 – 0.68%. However, these results are not included in the manuscript or discussed in the context of this study because the plateletcrits were determined via unknown methodologies. That is, not all data were collected from an Advia 2120/120. The plateletcrits of the genotyped Cavaliers were not included also because of the very small number of wild-type Cavaliers that made statistical evaluation underpowered.

Binomial testing revealed that the plateletcrit values of both cavalier and both greyhound groups followed a normal distribution pattern with no groups having significantly more individuals below the reference range than expected. (Table 4) The platelet counts of both the greyhound groups also followed a normal distribution pattern. However, 30.8% of the Minnesota cavaliers and 23% of the Auburn cavalier platelet
counts were below the reference interval which was significantly more than the expected 2.5% (p<0.0001 for both.) See Table 5.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Crit Below RI</th>
<th>Crit Low CI</th>
<th>Crit High CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota</td>
<td>5.1282</td>
<td>0.013181</td>
<td>0.2086</td>
<td>0.15645</td>
</tr>
<tr>
<td>Cavalier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auburn</td>
<td>11.4754</td>
<td>0.024288</td>
<td>0.2357</td>
<td>0.14632</td>
</tr>
<tr>
<td>Cavalier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minnesota</td>
<td>8.5106</td>
<td>0.019849</td>
<td>0.5409</td>
<td>0.14632</td>
</tr>
<tr>
<td>Greyhound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auburn</td>
<td>38.8889</td>
<td>0.176472</td>
<td>0.7183</td>
<td>0.06647</td>
</tr>
<tr>
<td>Greyhound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4**: Plateletcrit Percent Less than Reference Interval, with 95% Confidence Intervals (CI) and p-value compared to 0.025.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Count Below RI</th>
<th>Count Low CI</th>
<th>Count High CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota</td>
<td>30.7692</td>
<td>0.204168</td>
<td>0.4240</td>
<td>3.516×10^{14}***</td>
</tr>
<tr>
<td>Cavalier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auburn</td>
<td>22.9508</td>
<td>0.130334</td>
<td>0.4092</td>
<td>9.691×10^{-8}***</td>
</tr>
<tr>
<td>Cavalier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minnesota</td>
<td>8.5106</td>
<td>0.000000</td>
<td>0.2609</td>
<td>0.3184</td>
</tr>
<tr>
<td>Greyhound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auburn</td>
<td>33.3333</td>
<td>0.018038</td>
<td>0.6368</td>
<td>0.1340</td>
</tr>
<tr>
<td>Greyhound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5**: Platelet Count Percent Less than Reference Interval (RI), with 95% Confidence Intervals (CI) and p-value compared to 0.025
McNemar’s test showed that both the Cavalier populations had a significant number of individuals whose platelet counts were below the reference range while their plateletcrits were not below the reference range. Neither of the Greyhound populations had evidence of a significant number of individuals whose platelet counts were low while their plateletcrits were normal. (See Table 6.)

<table>
<thead>
<tr>
<th>Group</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota Cavalier</td>
<td>$4.818 \times 10^{-5}$ ***</td>
</tr>
<tr>
<td>Auburn Cavalier</td>
<td>0.02249 *</td>
</tr>
<tr>
<td>Minnesota Greyhound</td>
<td>0.27419</td>
</tr>
<tr>
<td>Auburn Greyhound</td>
<td>0.42293</td>
</tr>
</tbody>
</table>

**Table 6:** McNemar’s Test. Each individual’s plateletcrit compared to its own platelet count.

Pair-wise differences between the two locations revealed that there were no significant differences between the plateletcrit or platelet count distributions for the same breed at the 10th percentile. That is, the Minnesota Cavaliers and the Auburn Cavaliers had similar 10th percentile distributions, and so did the Greyhounds. (See tables 7 and 8 for 10th percentile values, and see table 9 for the p-values.) There were multiple differences when comparing between the two different breeds at the different locations: At the 10th percentile plateletcrit distribution, the Minnesota Cavaliers and the Auburn Greyhounds had a significantly different 10% percentile value. At the 10th percentile platelet count distribution the Minnesota Cavaliers were different from both Greyhound groups, and the Auburn Cavaliers were different from the Minnesota Greyhounds. (See table 9.)
Table 7: Plateletcrit Pairwise differences at the 10th Percentile.

<table>
<thead>
<tr>
<th>Group</th>
<th>Crit Estimate</th>
<th>Bootstrap Crit Low</th>
<th>Bootstrap Crit High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota Cavalier</td>
<td>0.139000</td>
<td>0.11900</td>
<td>0.16900</td>
</tr>
<tr>
<td>Auburn Cavalier</td>
<td>0.121368</td>
<td>0.10249</td>
<td>0.17456</td>
</tr>
<tr>
<td>Minnesota Greyhound</td>
<td>0.128000</td>
<td>0.10000</td>
<td>0.13000</td>
</tr>
<tr>
<td>Auburn Greyhound</td>
<td>0.099821</td>
<td>0.09776</td>
<td>0.10560</td>
</tr>
</tbody>
</table>

Table 8: Platelet Count Pairwise differences at the 10th Percentile.

<table>
<thead>
<tr>
<th>Group</th>
<th>Count Estimate</th>
<th>Count Low</th>
<th>Count High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota Cavalier</td>
<td>46.8</td>
<td>34.9</td>
<td>60.6</td>
</tr>
<tr>
<td>Auburn Cavalier</td>
<td>51.2</td>
<td>37.0</td>
<td>77.4</td>
</tr>
<tr>
<td>Minnesota Greyhound</td>
<td>122.0</td>
<td>113.0</td>
<td>139.8</td>
</tr>
<tr>
<td>Auburn Greyhound</td>
<td>100.2</td>
<td>66.0</td>
<td>113.0</td>
</tr>
</tbody>
</table>

Table 9: P-values for pairwise differences. Astericks denote significant differences at the 0.05 significance level.

<table>
<thead>
<tr>
<th></th>
<th>Crit</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN Cavalier-Auburn Cavalier</td>
<td>0.9443</td>
<td>0.59443</td>
</tr>
<tr>
<td>MN Cavalier-MN Greyhound</td>
<td>0.4122</td>
<td>1.2×10⁻⁵ *</td>
</tr>
<tr>
<td>MN Cavalier-Auburn Greyhound</td>
<td>0.0110 *</td>
<td>0.00829 *</td>
</tr>
<tr>
<td>Auburn Cavalier-MN Greyhound</td>
<td>0.9713</td>
<td>0.02925 *</td>
</tr>
<tr>
<td>Auburn Cavalier-Auburn Greyhound</td>
<td>0.1786</td>
<td>0.29744</td>
</tr>
<tr>
<td>MN Greyhound-Auburn Greyhound</td>
<td>0.2546</td>
<td>0.06432</td>
</tr>
</tbody>
</table>
All analyses are presented as separate groups based on location using reference intervals created at the University of Minnesota. There were two reasons why not to combine the Minnesota and Auburn Cavaliers, and the Minnesota and Auburn Greyhounds in order to analyze just one large group of each breed. First, the raw data from the reference intervals in use at Auburn University were unavailable, so it was not possible to generate a combined reference interval using Minnesota and Auburn raw data. This could have skewed the analysis of the combined groups. The other reason is that the Auburn had only 18 Greyhounds while Minnesota had 47.
Chapter 4: Case Presentations

Case Presentation #1: Unexplained Macrothrombocytopenia in a Chow Chow

A 7-month-old intact male Chow Chow was presented to the University of Minnesota Veterinary Medical Center for thrombocytopenia. The referring veterinarian discovered a platelet count of 69,000/µL on pre-operative blood work for a neuter surgery. Six days later the platelet count was reported as 79,000/µL and clumped, with a minimally elevated PT of 9.3 seconds and a normal PTT. A 4DX test for Dirofilaria immitus, Anaplasma phagocytophilum, Ehrlichia canis and Borrelia burgdorferi was negative. No post-venipuncture hematoma was observed with blood draw, nor was there any blood noticed in his stools. Also, no excessive bleeding or bruising was seen when the dog’s owner was trimming the puppy’s nails and accidentally clipped him. The owner declined corticosteroid treatment offered by the referring veterinarian for presumptive idiopathic (immune) thrombocytopenic purpura (ITP).

Three days later at the University of Minnesota Veterinary Medical Center, the PT and PTT were within normal reference range, there was a moderate macrothrombocytopenia, and the plateletcrit was within reference intervals. The Chow Chow appeared otherwise healthy. He had a normal appetite. The vaccination record was current, and no known drugs or toxins were known to be ingested.
Table 10: Summary of the Chow Chow’s Lab Results

<table>
<thead>
<tr>
<th>Normal Range</th>
<th>Platelet Count (10^3/µL)</th>
<th>MPV (fL)</th>
<th>PCT %</th>
<th>Microscopic Exam of Platelet Size</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>Referral Results</td>
</tr>
<tr>
<td>Day 3</td>
<td>79 and clumped</td>
<td></td>
<td></td>
<td></td>
<td>Referral Results</td>
</tr>
<tr>
<td>Day 11</td>
<td>49</td>
<td>28.8</td>
<td>0.14</td>
<td>3+ Large</td>
<td>Automated</td>
</tr>
<tr>
<td>1 Month</td>
<td>56</td>
<td>27.5</td>
<td>0.15</td>
<td>3+ Large</td>
<td>Automated</td>
</tr>
<tr>
<td>2 Months</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td>Manual Unopette</td>
</tr>
<tr>
<td>2 Months, 3 weeks</td>
<td>52</td>
<td>27.7</td>
<td>0.15</td>
<td>2+ Large</td>
<td>Automated</td>
</tr>
</tbody>
</table>

The platelet indices were checked again about two weeks later with very similar results. Because of the slight risk of the ITP, the owner was told to continue to monitor for signs of bleeding. One month later the platelet count was about the same at 61,000/µL as determined by the manual unopette counting method.

After three more weeks, on the day of possible neuter surgery, there still was no significant change in the platelet indices. The chemistry profile and buccal mucosal bleeding time were normal. Since the buccal mucosal bleeding time indicates that platelet number and function are sufficient for platelet plug formation, and because no clinical evidence of bleeding was ever observed, the neuter surgery was performed. A bone marrow biopsy was also performed during the surgery in order to bone marrow disorder.

Only a minimal amount of bleeding was observed during the surgery which was considered normal. The bone marrow aspirate and core were normal and so the cause of
the persistent thrombocytopenia was not apparent on the bone marrow aspirates.
Molecular testing for the characterized congenital macrothrombocytopenia β1-tubulin mutation found in Cavalier King Charles Spaniels was negative. However, other inherited platelet disorders of unknown etiology exist that are not fully characterized or classified, which was strongly suspected in the patient based on the prevalence of large platelets. Since the canine seems hematologically normal despite the low platelet counts, the cause of the macrothrombocytopenia remains idiopathic. Use of the plateletcrit is recommended to estimate the patient’s total platelet mass during periodic wellness visits.

**Case Presentation #2: Waxing and Waning Platelet Counts in a Cairn Terrier**

A 4-year-old female spayed Cairn Terrier was presented to the University of Minnesota Veterinary Medical Center for thrombocytopenia. Upon finding the low platelet count, the referring veterinarian also reported that all blood chemistry testing, thyroid levels, coagulation tests, and tick-borne disease titers were within normal reference ranges. More complete blood counts performed by the referring veterinarian revealed waxing and waning low platelet counts. (The counts were estimated at 50,000-120,000 platelets/µL.) Platelet size was not reported by the referring veterinarian.

A complete physical examination at the University of Minnesota Veterinary Medical Center 5 months later revealed that the dog was obese but appeared healthy, and that no petechiae or ecchymosis were observed. The initial university platelet count was 105,000/µL via a manual unopette count which does not report platelet size. The BUN, albumin, and total protein were very mildly elevated which was mostly likely consistent with mild dehydration. Further work-up revealed an unremarkable abdominal ultrasound,
and a mildly enlarged spleen. Aspirates indicated mild lymphoid reactivity, a common finding in the spleen. The patient was negative for Bartonella (determined by National Veterinary Laboratory, New Jersey), and also negative for antiplatelet antibodies performed at Kansas State University, indicating no immune destruction of platelets.

Initial medications included preventative flea/tick and heartworm. After a two week trial of doxycycline, the platelet count was 95,000 platelets/µL with 3+ large platelets noted. Prednisone therapy was introduced one month later and counts remained below the normal reference interval. (See Table 11.) After 2 months of being seen at the University, Cyclosporine therapy was given, and the prednisone therapy was discontinued due to lack of response. The platelets counts were stable but slightly below normal while on the cyclosporine.

A bone marrow biopsy revealed possible mild megakaryocytic hyperplasia and dysplasia, and possible mild erythroid hyperplasia. Molecular testing for the β1-tubulin mutation for congenital macrothrombocytopenia was negative. After an extensive work-up and no significant response to therapy, the patient was diagnosed with immune mediated thrombocytopenia.

This case of waxing and waning platelet counts may have been better described as asymptomatic macrothrombocytopenia since the diagnosis of immune mediated thrombocytopenia was not supported by a response to therapy. The platelet counts ranged from 61,000 – 112,000 which were never near critical levels at the low end and never recovered to within normal range. The mean platelet volume ranged from 29.0-33.0 fL which is more than twice the normal range of 8.14-13.7fL. The mild megakaryocytic
hyperplasia and dysplasia, and possible mild erythroid hyperplasia could indicate decreased or abnormal platelet production by the bone marrow. Although the molecular testing for the congenital macrothrombocytopenia β1-tubulin mutation found in Cavalier King Charles Spaniels was negative, the potential for another inherited platelet disorder of unknown etiology could not be excluded because not all inherited platelet disorders have been fully characterized. Considering the Cairn Terrier’s large platelet size, utilization of the plateletcrit is the recommended measure of total platelet mass. Subsequently, a mutation similar to Cavalier King Charles Spaniels has been described in Cairn Terriers which is further discussed in the thesis conclusion.

**Table 11: Summary of the Cairn Terrier’s Lab Results**

<table>
<thead>
<tr>
<th>Date</th>
<th>Platelet Count (10^3/µL)</th>
<th>MPV (fL)</th>
<th>PCT %</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td>116.2-398.2</td>
<td>8.14-13.7</td>
<td>0.129-0.403</td>
<td></td>
</tr>
<tr>
<td>Day 1 at University</td>
<td>105</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>2 Weeks</td>
<td>95</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>3 Weeks</td>
<td>79</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>3 Weeks, 5 Days</td>
<td>87</td>
<td>29.0</td>
<td>0.25%</td>
<td>Automated</td>
</tr>
<tr>
<td>3 Weeks, 5 Days</td>
<td>86</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>4 Weeks, 2 Days</td>
<td>61</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>7 Weeks</td>
<td>87</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>71</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>69</td>
<td>33.0</td>
<td>0.23%</td>
<td>Automated</td>
</tr>
<tr>
<td>9 Weeks, 4 Days</td>
<td>Clumped</td>
<td></td>
<td></td>
<td>Automated</td>
</tr>
<tr>
<td>11 Weeks</td>
<td>112</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>13 Weeks</td>
<td>106</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>15 Weeks</td>
<td>Clumped</td>
<td></td>
<td></td>
<td>Manual unopette</td>
</tr>
<tr>
<td>15 Weeks</td>
<td>108</td>
<td>30.2</td>
<td>0.33%</td>
<td>Automated</td>
</tr>
</tbody>
</table>
Case Presentation Discussion/Summary

In the case of both the Chow Chow and the Carin Terrier the etiology of the macrothrombocytopenia is not fully elucidated. Neither dog presented with signs of bleeding, and both patients were put through extensive laboratory work-ups. The Carin Terrier was further subjected to ultrasound, spleen aspirates, x-rays, and corticosteroid treatments. All these treatments led to unnecessary interventions for the patient and increased costs to the owner. Many of the additional diagnostic tests and treatments could have been avoided if the plateletcrit had been reported and accepted as the more appropriate clinical measure of the platelet functional status. An interpretation of the patient’s plateletcrits would have revealed that both canines had a normal platelet mass. (A canine plateletcrit reference range of 0.129 – 0.403 % has been established at the University of Minnesota.) Also, the Chow Chow’s surgical procedure could have been expedited if the plateletcrit had been utilized.

It is recommended that in similar cases the plateletcrit may augment traditional lab assessment of platelet function. Total platelet mass depends on not only platelet number but also platelet size. Platelet number alone can underestimate total platelet mass. Plateletcrit is the product of both platelet number and size, and therefore should be used to routinely evaluate similar cases. Utilization of the plateletcrit may also aid in distinguishing acquired case of thrombocytopenia from thrombocytopenias secondary to infectious agents, consumption, medications, immune-mediated causes, and other genetic congenital abnormalities.
In both cases of macrothrombocytopenia an “interpretive comment” that could be used in a clinical setting to describe a recommendation or good policy about the use of the plateletcrit would be: “Use of the plateletcrit may provide important information for disease classification, and may be warranted especially in cases of abnormal or varying platelet volume.”
Chapter 5: Conclusion

Thrombocytopenia is a medical condition that requires immediate diagnosis and treatment due to the risk of hemorrhage. Since total platelet biomass is the physiologically important hemostatic parameter to be monitored in order to avoid bleeding, it is imperative to accurately assess total platelet biomass. The plateletcrit is a representation of total platelet mass, and more accurately represents mass than platelet number in some circumstances because it is a function of both platelet size and number combined. This thesis compared the use of the plateletcrit and the platelet number as an assessment of total platelet biomass in a spontaneous model of congenital macrothrombocytopenia.

In this particular model of an inherited asymptomatic macrothrombocytopenic platelet disorder, Cavalier King Charles Spaniels represent a group of individuals in which the bleeding risk is better evaluated using plateletcrit than platelet number. The platelet mass appears significantly lower in affected dogs when measured by count, but when measured by plateletcrit, more of the patients are appropriately within the reference range. Therefore, for the Cavalier King Charles Spaniels affected by this mutation, plateletcrit better represents the true total platelet biomass in peripheral circulation. For Greyhounds, platelet number and plateletcrit similarly represent total platelet biomass, as we anticipated based on their normal and consistent platelet size.
In the case of both the macrothrombocytopenic Chow Chow and Cairn Terrier presenting to the University of Minnesota, the total platelet mass was likely underestimated by platelet count alone since neither canine presented with signs of bleeding, even during surgery in the case of the Chow Chow. An interpretation of the patients’ plateletcrits eventually revealed that both dogs had normal platelet mass, suggesting the risk of clinical bleeding to be minimal. Based on the data presented in this thesis, we have added reference intervals for the plateletcrit to our routine laboratory data reporting system so that clinicians can have a more robust data set for platelet evaluation. This should help avoid over utilization of invasive testing and unnecessary treatment in spontaneous or unrecognized cases of genetic macrothrombocytopenia. Recently, a single nucleotide polymorphism was detected in the beta-1 tubulin gene in asymptomatic macrothrombocytopenic Norfolk and Cairn Terriers. All twelve out of 20 terriers with the genetic defect were macrothrombocytopenic. The 4 heterozygous terriers and the 4 normal terriers all had normal platelet counts and size. As in the case of the Cavalier King Charles Spaniels, Norfolk and Cairn Terriers may carry an inherited form of asymptomatic macrothrombocytopenia. This new finding further supports the use of the plateletcrit for the Cairn Terrier presenting for thrombocytopenia at the University of Minnesota.

Not only may the plateletcrit be useful for other uncharacterized genetic macrothrombocytopenias as described in the Cairn Terrier, it may also be useful in regenerative thrombocytopenias such as ITP. Regenerative thrombocytopenia is characterized by the release of macroplatelets, therefore there is a similar rationale for the
diagnostic application of the platelet crit to augment evaluation of primary hemostasis in these patients. Based on the data generated by this thesis, additional studies of MPV and plateletcrit in dogs with ITP have been initiated. Unfortunately, adequate quality assurance materials to support standardization of platelet parameters like the MPV are lacking and will be required for optimal clinical application of these indices.  

There is a paucity of studies that have investigated the use of the plateletcrit. In order to enhance understanding of congenital platelet disorders, and in order to study, classify, and provide treatment for platelet diseases in general, appropriate tools of measure are needed to accurately assess the total platelet biomass. The plateletcrit should be further investigated as the best tool for utilization as a routine platelet parameter. Encompassing both size and number, plateletcrit may be a supportive measure of platelet mass in hematological disease or considered an alternative measure of mass in some cases of genetic platelet size predisposition.
Images

Figure 6: Microscopic image of a human blood smear with normal platelets

Figure 7: Microscopic image of a canine blood smear with normal platelets and a single macroplatelet
Figure 8: Microscopic image of a Cavalier King Charles Spaniel blood smear with a single giant platelet

Figure 9: Microscopic image of a Cavalier King Charles Spaniel blood smear with giant platelets
References


