Platelet Function in Dogs with Chronic Liver Disease

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ABSTRACT (academic)

Background: Dogs with acquired chronic liver disease often have hemostatic derangements. It is currently unknown whether dogs with acquired chronic liver disease have decreased platelet function and alterations in von Willebrand factor (vWF) that may contribute to hemostatic abnormalities.

Hypothesis: Dogs with chronic liver disease have prolonged platelet closure time (CT), assessed with the PFA-100®, and buccal mucosal bleeding time (BMBT), and increased vWF concentration compared to healthy dogs.

Animals: Eighteen dogs with chronic acquired liver disease undergoing ultrasound-guided needle biopsy of the liver or laparoscopic liver biopsy and eighteen healthy age-matched control dogs.

Methods: Prospective study. BMBT, CT using the PFA-100®, and vWF antigen were measured in dogs with chronic liver enzyme elevation undergoing ultrasound-guided needle biopsy of the liver or laparoscopic liver biopsy. After undergoing ultrasound-guided needle biopsy, dogs were monitored for hemorrhage with serial packed cell volume measurements and focused assessment with sonography. An unpaired t-test was used for normally distributed data and the Mann-

Whitney test was used when non-Gaussian distribution was present. The level of significance was set at $P < 0.05$.

Results: The CT was not different between the two groups ($P = 0.27$). The BMBT was significantly longer in the liver disease group compared to the control group ($P = 0.019$). There was no difference in the mean vWF antigen of the two groups ($P = 0.077$).

Conclusions and clinical relevance: These results demonstrate mild impairment of primary hemostasis in dogs with chronic liver disease based on prolongation of BMBT.

Platelet function in dogs with chronic liver disease

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ABSTRACT (public)

Background: Dogs with chronic liver disease often have abnormal blood clotting activity. It is currently unknown whether dogs with chronic liver disease have decreased platelet function and alterations in von Willebrand factor (vWF) that may contribute to blood clotting abnormalities. Platelet function can be assessed using the PFA-100®, which measures platelet closure time (CT), and buccal mucosal bleeding time (BMBT). The PFA-100 simulates blood in circulation to assess platelet function. BMBT is a crude but readily available test to assess platelet function in practices without sophisticated methods of assessing platelet function.

Hypothesis: Dogs with chronic liver disease have prolonged CT and BMBT, which both suggest platelet dysfunction. Additionally, dogs with chronic liver disease have increased vWF concentration compared to healthy dogs.

Animals: Eighteen dogs with chronic acquired liver disease undergoing ultrasound-guided needle biopsy of the liver or laparoscopic liver biopsy and eighteen healthy age-matched control dogs.

Methods: Prospective study. BMBT, CT, and vWF antigen were measured in dogs with chronic liver disease undergoing ultrasound-guided needle biopsy of the liver or laparoscopic liver

biopsy. After undergoing ultrasound-guided needle biopsy, dogs were monitored for hemorrhage.

Results: The CT was not different between the two groups but the BMBT was significantly longer in the liver disease group compared to healthy dogs. There was no difference in the mean vWF antigen between the two groups.

Conclusions and clinical relevance: These results demonstrate mild impairment of blood clotting activity in dogs with chronic liver disease based on prolongation of BMBT compared to healthy dogs. Prolongation of BMBT compared to healthy dogs is suggestive of endothelial dysfunction and/or platelet dysfunction in dogs with chronic liver disease.

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A. Platelet physiology

a. Production

In adult mammals, platelets are formed from the cytoplasmic fragmentation of their precursor cells, megakaryocytes, primarily located within the bone marrow [1]. Mechanisms for platelet biogenesis have been an area of ongoing research but a widely accepted concept is the "proplatelet theory" [2]. The "proplatelet theory" states that megakaryocytes form multiple long cytoplasmic extensions toward the end of their maturation. These extensions, called proplatelets, originate as thick projections, and become thin, branch, and develop into platelet-sized segmental swellings as the megakaryocytes mature. Ultimately, the segmental swellings within the proplatelets fragment into individual platelets. The process of fragmentation is thought to occur primarily within circulation, or platelets would be trapped in the bone marrow stroma [3]. Once megakaryocytes are mature in the bone marrow, they extend their proplatelets into bone marrow sinusoidal vessels. Shear forces within the vasculature cause the extensions to break apart into individual proplatelets and are released into circulation as platelets [4].

Cytokines regulate megakaryocyte maturation and ultimately platelet production. The cytokine thrombopoietin plays the largest role in megakaryocyte differentiation and ultimately platelet production [3]. In adult mammals, thrombopoietin is primarily produced in the liver but also in bone marrow, kidney, and smooth muscle [5,6]. Hepatic synthesis of thrombopoietin is minimally affected by external signals, resulting in relatively constant production. Platelets and megakaryocytes have high affinity thrombopoietin receptors that continuously remove the cytokine from circulation. If there are fewer platelets in circulation, there is less thrombopoietin bound to platelets, which increases the level of thrombopoietin in circulation and ultimately

platelet production [6]. The average life span of platelets in circulation varies among species. The lifespan of circulating platelets in dogs is approximately 6 days [5]. Platelets are primarily eliminated from circulation through the splenic mononuclear phagocyte system (previously reticuloendothelial system) [7].

b. Platelet anatomy

Understanding platelet anatomy is necessary to understand platelet function. Despite their complexity and fundamental role in hemostasis, platelets lack a nucleus. They contain mitochondria that form adenosine triphosphate (ATP) and adenosine diphosphate (ADP) and residuals of an endoplasmic reticulum and Golgi apparatus. Platelets contain compounds important for wound healing, such as platelet-derived growth factor, which aids in the repair of damaged vessel walls, and fibrin-stabilizing factor. Platelets are coated with glycoproteins, which repel normal endothelium, but facilitate adherence to damaged endothelium. Their cell membranes contain phospholipids, which anchor activated clotting factors, and protect them from inhibition and neutralization [7].

Platelets carry several preformed substances within granules called alpha and delta (dense) granules. Substances stored within alpha granules include platelet-derived growth factor, fibronectin, transforming growth factor β, β-thromboglobulin, platelet factor 4, fibrinogen, clotting factors V and VIII, and von Willebrand factor (vWF). Delta (dense) granules contain ADP, ATP, epinephrine, serotonin, calcium ions, and histamine. Platelets also have contractile proteins, which include actin, myosin, and thrombosthenin, that allow platelets to undergo conformational changes upon activation [5]. Platelets at rest are discoid in shape and contain homogenously distributed granules. Once activated, they release the contents of their granules and their cytoskeleton is altered to form extensions called pseudopodia that originate from the

cell membrane. The pseudopodia ultimately help facilitate platelet aggregation and adherence to vascular endothelium, which leads to platelet plug formation [8].

c. Function

The ultimate product of primary hemostasis is platelet plug formation. Damage to a vessel wall leads to subendothelial matrix and collagen exposure. Platelets adhere to subendothelium along damaged endothelium and form a temporary platelet plug. A sequence of events, including platelet adhesion, activation, and aggregation, lead to platelet plug formation [5].

Platelet adhesion to damaged endothelium is a complex process facilitated by multiple adhesive ligands and platelet receptors. The adhesive properties are often dependent upon local blood flow conditions. Small and medium-sized arteries and arterioles create high shear conditions and veins and large arteries create low shear conditions [5]. Under high shear conditions, platelets interact with damaged endothelium using the adhesive glycoprotein Ibα (GPIbα) in the glycoprotein Ib-IX-V receptor (GPIb-IX-V) complex. Glycoprotein Ibα in the GPIb-IX-V complex is found on the surface of platelets and binds to vWF. Von Willebrand factor multimers bind to collagen along the exposed subendothelial matrix and serve as a binding site for platelets under high shear conditions [8]. The other major platelet receptor for vWF is the GPIIb-IIIa, an integrin that plays an important role in platelet aggregation [9].

The function of vWF multimers is largely dependent upon their size. Larger, high molecular weight multimers bind to collagen and platelets more avidly than low molecular weight multimers. Ultra-large vWF multimers are most capable of binding to platelets and inducing platelet aggregation [10]. When ultra-large vWF multimers and high molecular weight vWF multimers are secreted, they are broken down into smaller multimers by sheer stress forces within vasculature [10]. The size of vWF multimers is also regulated by metalloprotease

ADAMTS13. This metalloprotease is present in plasma and breaks ultra-large vWF multimers and high molecular weight multimers into smaller multimers [9]. Ultra-large vWF multimers are present in low concentration, if at all, in blood since they rapidly bind to platelet receptors and cause spontaneous platelet aggregation [9]. Because of this, ultra-large multimers are rapidly reduced to smaller molecules by the aforementioned mechanisms. In healthy individuals, there is a balance between large vWF multimer synthesis and degradation to prevent thromboembolic disease [9]. Von Willebrand factor is primarily removed from circulation by the liver, which accounts for 40-60% of its removal. Other routes of excretion of vWF are the kidneys and spleen [10].

Both megakaryocytes and endothelial cells synthesize vWF [5]. Once vWF is synthesized by the endothelium, it is released directly into circulation or stored in secretory vessels within the endothelium called Weibel-Palade bodies [9]. Histamine, estrogens, thrombin, and fibrin are all agonists that lead to secretion of vWF from Weibel-Palade bodies. Von Willebrand factor synthesized by megakaryocytes is stored within the alpha granules of megakaryocytes and platelets [5]. Approximately 20% of the total vWF present in blood is found within the alpha granules [9]. Alpha granules also store ultra-large vWF multimers. Agonists that lead to alpha granule degranulation include ADP, collagen, and thrombin. These agonists are often present at sites of vascular injury, which facilitates targeted delivery of ultra-large vWF and other vWF multimers to facilitate platelet adhesion [9]. The high molecular weight of vWF also causes platelets to slow down as they move through vasculature, which gives platelets the opportunity to bind to damaged endothelium [8].

Thrombospondin 1 (TSP-1), an adhesive glycoprotein, also helps facilitate platelet adhesion to exposed collagen. Thrombospondin 1 can serve as an alternative to vWF for

facilitating platelet adhesion under high shear conditions [8]. Thrombospondin 1 and vWF both bind to the platelet GPIb/V/IX membrane complex under these conditions. Although TSP-1 may encourage platelet recruitment with vWF to sites of vascular injury, TSP-1 weakly competes with platelet adhesion onto vWF. Thrombospondin 1 also regulates ADAMTS13's action on vWF multimers and regulates the degree of vWF multimer break down [11]. Similar to vWF, TSP-1 is released by endothelial cells and megakaryocytes [10].

Platelets directly adhere to subendothelial collagen under low shear or static conditions. Collagen binds to the platelet membrane receptor glycoprotein Ia/IIa (GPIa/IIa), which ultimately leads to platelet activation and granule secretion. Other important platelet membrane receptors for collagen include CD36 and glycoprotein VI (GPVI). Glycoprotein VI is a member of the immunoglobulin family and its signaling pathway is similar to lymphocyte signaling [8].

Although effective platelet adhesion is vital to control hemorrhage, protective mechanisms are needed to prevent platelet adherence and aggregation to normal endothelium. Platelets and endothelial cells both have electronegative charges, which result in repulsive forces. Platelet inhibitors, such as nitric oxide, are also released from normal endothelium to prevent inappropriate platelet adhesion. Nitric oxide stimulates production of cyclic GMP (cGMP), which activates cGMP-dependent protein kinase (G kinase). Through unknown mechanisms, G kinase inhibits $G\alpha_q$-phospholipase C-inositol 1,4,5-triphosphate signaling, which prevents intracellular calcium mobilization that is needed for platelet agonists to exert an affect. The primary platelet agonist inhibited is thromboxane A_2 (TXA_2) [12]. Prostacyclin, which is a product of arachidonic acid metabolism, also inhibits platelet adhesion and aggregation. It is produced by vascular endothelium and is a potent vasodilator. Prostacyclin ultimately inhibits platelet aggregation by stimulating platelet adenyl cyclase, which leads to increased cyclic AMP

(cAMP). Cyclic AMP inhibits platelet activation and counteracts the increase in cytosolic calcium that thromboxane A2 initiates to cause granule release [13]. Substances are also present on the endothelial surface that inhibit or destroy platelet agonists, such as ADPases, heparin sulfate, and thrombomodulin [5].

Platelet activation can occur through different mechanisms, including platelet agonist and receptor binding, platelet receptor cross-linking, platelet plasma membrane alterations, and platelet adhesion [5,8]. The binding of vWF to GP Ib-IX-V complex is an example of receptor cross-linking that leads to platelet activation. Physiologic platelet agonists include ADP, serotonin, thrombin, thromboxane A2, platelet activation factor (PAF), epinephrine, and plasmin [8]. Thrombin is the most potent of physiological platelet agonists. Adenosine diphosphate promotes platelet activation under high shear conditions and thromboxane A2 promotes it under low shear conditions. Thrombin promotes platelet activation under all conditions [5]. Pathologic conditions can also create stimuli for platelet activation including antigen-antibody complexes and aggregated gamma globulin, which is produced by activated leukocytes [14].

A number of changes occur once platelets are activated. Platelets at rest have evenly distributed granules throughout their cytoplasm and are discoid in shape [8]. The discoid shape allows platelets to flow near the periphery of a vessel wall during circulation to promote exposure to damaged endothelium [15]. Once activated, platelets rearrange their cytoskeleton proteins to form pseudopodia and their granules move to the periphery of their cytoplasm. Platelets contract as well and release the contents of their granules [8]. Some of the substances secreted, such as ADP and serotonin, activate surrounding platelets and recruit other platelets to the site of injury, which ultimately leads to formation of platelet aggregates. ADP is the primary

amplifier of initial platelet activation. There are two primary ADP receptors on the platelet surface, including $P2Y_1$ -receptor and $P2Y_{12}$-receptor [8].

The binding of a platelet agonist to its specific receptor or receptor cross-linking leads to the production and release of intracellular messenger molecules. Agonists, including ADP, TxA2, epinephrine, serotonin, and thrombin, interact with seven different transmembrane receptors. All seven of the transmembrane receptors are coupled by GTP-binding heterotrimeric G-proteins, which initiate several signal pathways involving calcium, triacylglycerol, inositol-1,4,5-triphosphate (IP3), TxA_2, and products of the phospholipase C (PLC)-mediated phosphoinositol hydrolysis. A specific family of G-protein receptors, the Gq-family, ultimately results in activation of PLC. Phospholipase C catalyzes the hydrolysis of phosphatidyl inositolbisphosphate 2 (PIP2) to IP3. Inositol triphosphate mobilizes Ca^{2+} from the dense tubular system within the platelet. An increase in intracellular Ca^{2+} leads to changes in platelet shape and granule release and therefore platelet activation. Adenosine diphosphate is one of the substances secreted from the granules, which, as mentioned earlier, acts on the $P2Y_{12}$ receptor and amplifies platelet activation. Calcium mobilization also leads to TxA_2 synthesis and release. Thromboxane A_2 binds again to its Gq-coupled thromboxane-prostanoid (TP)-receptor and further amplifies platelet activation [8].

Platelet aggregation following platelet activation ultimately leads to the formation of a platelet plug. Platelets are primarily linked together with fibrinogen and the platelet receptor GP IIb-IIIa [8]. Von Willebrand factor also plays a role in platelet to platelet interaction and aggregation [16]. Resting platelets contain approximately 40,000-50,000 GP IIb-IIIa receptors but they cannot bind to fibrinogen until platelet activation results in conformational change to an

active GP IIb-IIIa receptor [8]. The platelet plug is only stable for a few hours unless secondary hemostatic forces reinforce the platelet plug with a cross-linked fibrin meshwork [5].

d. Pathophysiology

Primary hemostatic defects are divided into three major categories, including thrombocytopenia, thrombocytopathia, and endothelial disruption. Thrombocytopenia, or decreased platelet count, can be a result of platelet destruction, consumption, decreased production, or sequestration of platelets. Platelet destruction or immune-mediated occurs when immune system deregulation leads to abnormal antibody production directed against platelets. This abnormal antibody production results in early removal of platelets from circulation via the mononuclear phagocyte system. Platelet lifespan can be reduced from days to a few hours. Immune-mediated thrombocytopenia can be primary, where an underlying trigger for the immune system deregulation cannot be identified, or secondary to neoplasia, drug reaction, or infection [17]. Thrombocytopenia caused by platelet sequestration usually occurs due to splenomegaly or primary splenic disease. Decreased platelet production can be a result of decreased thrombopoietin levels, antimegakaryocyte antibody production, and bone marrow disease, which may include drug toxicity, infection, neoplasia, myelodysplasia, or an idiopathic process [5,17]. Increased platelet consumption is often a result of hemorrhage or disseminated intravascular coagulation (DIC) [17].

Thrombocytopathia, or abnormal platelet function, can occur due to inherited or acquired disease. Von Willebrand disease (vWD) is the most common inherited primary hemostatic defect in dogs. Von Willebrand disease is a deficiency or defect of vWF, resulting in impaired adhesion of platelets to damaged endothelium and normal platelet aggregation. Von Willebrand factor is also a carrier protein for factor VIII, which protects it from early proteolysis by

activated protein C [5]. Von Willebrand factor exists in multimers varying in size with larger multimers providing more hemostatic activity. There are three basic forms of vWD in dogs. All multimers of vWF are present in type I vWD but are reduced in number. Breeds predisposed include Doberman pinschers, Shetland sheepdogs, German shepherds, and standard poodles. Spontaneous hemorrhage is uncommon and usually occurs after surgery or trauma. Dogs with type II vWD do not have the larger and more effective vWF multimers. Bleeding can be severe in these cases. Type III is considered the most severe form of vWD, where all vWF multimers are absent and life-threating hemorrhage is more likely to occur [16].

Scott syndrome is a form of inherited thrombocytopathia resulting from impaired externalization of phosphatidylserine (PS) from the platelet membrane and reduced shedding of small membrane vesicles that contain PS. Reduced exposure and release of PS leads to reduced thrombin generation. All other aspects of platelet adhesion, aggregation, and secretion are normal in these patients [18]. The condition was first described in German shepherds from a single, inbred colony. These dogs had clinical signs of epistaxis, hyphema, intramuscular hematomas, and prolonged bleeding after surgery. Platelet count, platelet morphology under light microscopy, bleeding time, clot retraction, and platelet aggregation and secretion in response to thrombin, collagen, and ADP were all normal [19]. Canine Scott syndrome is usually diagnosed based on measures of platelet procoagulant activity, including serum prothrombin consumption and detection of platelet PS externalization after activation [18]. Glanzmann thrombasthenia (GT) is another inherited form of thrombocytopathia affecting dogs characterized by a deficiency in the platelet membrane glycoprotein (GP) IIb/IIIa complex. As discussed previously, GP IIb/IIIa is needed for normal platelet adhesion and aggregation. Glanzmann thrombasthenia is rare in dogs, but has been identified in great Pyrenees dogs and otterhounds. Dogs with GT often have

normal platelet counts but absent or reduced platelet aggregation and prolonged bleeding times. Mucosal bleeding and epistaxis are common clinical signs associated with GT in dogs [20].

Acquired thrombocytopathia can occur due to a number of conditions including *Ehrlichia spp.* infection, snakebite envenomation, neoplasia, uremia, DIC, anemia, endotoxemia, and pharmacologic manipulation with antiplatelet drugs [5,16,21]. Commonly used antiplatelet drugs include aspirin and clopidogrel. Aspirin alters platelet function by indirectly decreasing production of TXA_2, which results in decreased platelet activation and aggregation. The effect of aspirin on platelets is irreversible and inhibition lasts for the lifespan of the affected platelet. Clopidogrel is a $P2Y_{12}$ receptor antagonist. Blockade of the $P2Y_{12}$ receptor prevents binding of ADP to platelets, leading to decreased platelet activation and aggregation. These drugs are used to reduce the risk of thromboembolic disease [22].

Endothelial damage can result in abnormal primary hemostasis as well. Endothelial damage may lead to an inadequate number of endothelial receptors or dysfunction of these receptors, which ultimately inhibits platelet adhesion. Potential causes of endothelial damage include infection, such as *Rickettsia rickettii*, heatstroke, drug toxicity, neoplasia, or immune-mediated illness [5].

e. Diagnostics to assess primary hemostasis

i. Platelet aggregometry

Many techniques have been used to assess platelet function and primary hemostasis. Platelet aggregometry has traditionally been used to assess *in vitro* platelet function under low shear flow conditions (<1,000 s^{-1}) [23]. There are two primary methods used to perform platelet aggregometry, including whole blood aggregometry and light transmission aggregometry [24]. Light transmission aggregometry is more sensitive than

whole blood aggregometry for subtle changes in platelet function [23]. Whole blood aggregometry uses electrical impedance to measure whole blood aggregation. An electric current is passed between two electrodes, which are inserted into a cuvette filled with whole blood. The electrodes initially become coated with a platelet monolayer. A platelet agonist is later added to the blood sample and platelets, red blood cells, and white blood cells aggregate along the electrodes and obstruct the current. The degree of current obstruction is used to determine the amount of platelet aggregation present and therefore platelet function. Light transmission aggregometry measures light diffusion through platelets in platelet rich plasma before and after a platelet agonist is added to the sample. The light signal is transferred electronically to a chart recorder or computer. An "aggregation curve" representing light transmission is later created. Minimal light transmission occurs when the platelets are in a resting state because they are evenly distributed. Once platelets are activated and aggregate, light transmission increases since it is transmitting around the clumped platelets. Both light transmission aggregometry and whole blood aggregometry are technically challenging, time consuming, and can only be performed in specialized laboratories [24]. Due to these limitations, platelet aggregometry is often impractical to use in a clinical setting.

The Multiplate® analyzer is an impedance aggregometer created for point-of-care patient assessment. Agonists used to induce platelet aggregation with the Multiplate® analyzer include ADP, collagen, and arachidonic acid [25]. The system is considered to be practical and simple to operate. The Multiplate® analyzer has been used to assess platelet function in healthy dogs treated with aspirin and clopidogrel, which revealed decreased platelet aggregation with these treatments [26].

The Plateletworks® system uses citrated whole blood and measures platelet count before and after aggregation is induced with a platelet agonist, such as ADP or arachidonic acid. A decrease in platelet count is indicative of platelet aggregation and therefore platelet function. Flow conditions that would occur within normal vasculature are not simulated. This test provides rapid results but can be impractical in some clinical settings since samples have to be run within minutes of collection [27]. The Plateletworks® system has been infrequently used in canine patients. One study did show it could effectively detect platelet inhibition caused by aspirin and clopidogrel in healthy dogs. The results had overall agreement with other methodologies, such as the PFA-100 [26].

ii. PFA-100®

The platelet function analyzer or PFA-100® is a whole blood platelet-function assessment tool [28]. It is considered to be one of the most practical, accurate, and efficient methods available to assess platelet function. The PFA-100® simulates primary hemostasis by creating high shear flow rates (>10,000 s^{-1}) and exposes platelets to biochemical stimuli that would be present within an arteriole. A whole blood sample is aspirated under constant pressure through an aperture in a collagen-coated membrane. The membrane is lined with either epinephrine or ADP, which are platelet agonists that lead to platelet activation. As the blood is aspirated through the cartridge, a platelet plug ultimately forms and occludes blood flow through the aperture. The time required for blood flow to cease is reported as the platelet closure time (CT). Closure time is prolonged if platelet function is decreased [28]. The PFA-100® is thought to better reflect *in vivo* platelet function than platelet aggregometry since flow conditions are created and platelets are exposed to a collagen-lined membrane.

Variables that can prolong CT include anemia, thrombocytopenia, *in vitro* platelet clumping, and vWF concentration and activity. In dogs, a packed cell volume (PCV) less than 30% and platelet count less than 100,000/uL can lead to prolongation of CT [29]. In humans, the PFA-100® is considered to be very sensitive for identifying severe platelet dysfunction and abnormalities in vWF level or function. The epinephrine-collagen cartridges are more sensitive for identifying mild platelet dysfunction than the ADP-collagen cartridges in humans. The PFA-100® is used routinely to screen for primary hemostatic defects in people prior to surgical or biopsy procedures [30]. In a study evaluating healthy dogs and dogs with primary hemostatic disorders, including vWD, thrombocytopenia, and intrinsic platelet defects (i.e. thrombopathia), the ADP cartridges had a clinical sensitivity and specificity of 95.7 and 100%, respectively. The epinephrine cartridges had a 95.7% and 82.8% sensitivity and specificity, respectively. In dogs, the cartridges lined with epinephrine tend to have high coefficients of variance compared to ADP lined cartridges [29]. The PFA-100® has been satisfactorily used to evaluate platelet function in healthy dogs, dogs administered aspirin, clopidogrel, and omeprazole, and dogs with cardiac disease, von Willebrand disease, and endotoxemia [21,29,31,32].

iii. Flow Cytometry

Flow cytometry measures specific attributes of platelets, such as receptor expression, conformational changes associated with platelet activation, platelet granule secretion, and platelet aggregation. The method detects fluorescent dyes that are conjugated to antibodies to specific epitopes on proteins of interest inside the platelets or within the platelet membrane. A light source is applied to platelets with the labeled antibody bound to them, which causes the molecules of the fluorescent dyes to be excited

to a higher energy state. Light is emitted at different wavelengths, termed emission spectra, when the molecules from the fluorescent dyes return to their resting state. Based on the emission spectra, it can be determined if specific epitopes are present within or on platelets and ultimately quantify platelet activation. Unlike many other methods to assess platelet function, results are not affected by thrombocytopenia. Flow cytometry has been used infrequently in canine patients. In dogs, it is feasible to use flow cytometry to quantify platelet microparticles, which are subcellular procoagulant vesicles released upon platelet activation [34,35]. Flow cytometry has also been used in dogs to demonstrate abnormal P-selectin expression in dogs with immune-meditated hemolytic anemia, which suggests that their platelets circulate in an activated state [33]. Although flow cytometry may be a useful method to assess platelet activation, the expense and need for specialized operators and devices makes it often impractical to perform [27].

iv. Buccal mucosal bleeding time

Buccal mucosal bleeding time (BMBT) is a crude but readily available and inexpensive test used to assess primary hemostasis. Platelet count, platelet function, and endothelial function all affect primary hemostasis and BMBT. Using a spring-loaded cassette, 1 or 2 incisions of precise length and depth are made into the buccal mucosa. The buccal mucosa is everted and maintained in place with a gauze tie while the test is performed. Absorbent filter paper is used to collect blood adjacent to the wound. Care is taken to not touch the filter paper directly to the wound to potentially disrupt the clot. The time it takes for bleeding to cease is recorded as the BMBT, which is a measure of platelet plug formation. Normal bleeding time in dogs is 2 to 3 minutes [23] but BMBT can be affected by the size of the spring loaded device used, which should be taken into

consideration [34]. Buccal mucosal bleeding time is not affected by secondary hemostatic defects [23,35]. This has been documented in dogs with primary hemostatic defects, such as VWD, in which all affected dogs had prolonged BMBT compared to controls. Dogs that had been diagnosed with secondary hemostasis defects, such as hemophilia A and B, did not have prolonged BMBT [35].

Buccal mucosal bleeding time has some limitations because measurements can be affected by operator skill, thrombocytopenia, anemia, and abnormal vascular tone [35,36]. There is also substantial interobserver and intraobserver variability when measuring BMBT. A study using two observers to measure BMBT on healthy greyhounds showed that for any two readings within the same dog, the BMBT could vary for up to two minutes. The reference interval was also found to be quite broad, ranging from 53-235 seconds. Interestingly, there was also no significant correlation between BMBT and von Willebrand factor concentration [37].

Buccal mucosal bleeding time in dogs has been assessed in several other studies. Buccal mucosal bleeding time has often been used to assess primary hemostasis defects associated with NSAID therapy. Most NSAIDs do not appear to have a dramatic effect on BMBT although BMBT prolongation does occur with high dose (10 mg/kg) aspirin administration [36]. Another study revealed BMBT prolongation by an average of 1.3 minutes after aspirin was administered at a dose of 12.5 mg/kg. This mild prolongation was not found to be statistically significant from baseline, but minimal change may have occurred since BMBT was measured only two hours after administration of aspirin [38]. Alternatively, platelet inhibition caused by low dose (1 mg/kg) of aspirin can be detected with the PFA-100 in dogs using collagen/epinephrine as an agonist[39]. Buccal mucosal

bleeding time in dogs is not significantly affected by robenacoxib, ketoprofen, carprofen, or meloxicam administration [40-42]. In contrast, platelet inhibition caused by carprofen and meloxicam can be detected by the PFA-100 when collagen/epinephrine lined cartridges are used [43]. In a study designed to assess safety of robenacoxib, BMBT prolongation was observed in a small proportion of dogs studied only after 178 days of treatment with doses far exceeding those recommended for therapy [44]. Buccal mucosal bleeding time has also been assessed after the administration of other pharmacologic agents, including yunnan baiyo and cephalothin. The effect of yunnan baiyo on BMBT was previously assessed in healthy Beagles, which showed no difference in BMBT after starting the medication. Thromboelastography was also assessed in these dogs, which showed no changes in coagulation status after receiving Yunnan Baiyo [45]. Alternatively, cephalothin, a cephalosporin utilized in surgical antibiotic protocols, prolongs BMBT when comparing values obtained 1 hour and 6 hours after cephalothin administration [46].

Buccal mucosal bleeding time may not be able to consistently predict clinical hemorrhage. Buccal mucosal bleeding time did not predict hemorrhage occurring during surgery in dogs infected with *Anaplasma platy*s and *Ehrlichia canis* [47]. Although BMBT has limitations, it has been useful for identifying dogs with von Willebrand disease. In a study evaluating BMBT in dogs with a variety of disease states, every dog with von Willebrand disease had prolonged BMBT compared to healthy dogs [36]. Buccal mucosal bleeding time is also useful in assessing response to treatment in dogs with von Willebrand disease. Administration of cryoprecipitate to Doberman Pinschers with type-I von Willebrand disease shortens BMBT. Fresh frozen plasma increases vWF concentration in Doberman Pinschers with type-I von Willebrand disease but does not

shorten BMBT [48]. Alternatively, desmopressin acetate significantly shortens BMBT in Doberman Pinschers with von Willebrand disease [49,50]. In addition to von Willebrand disease, BMBT has also been evaluated in dogs with other diseases including hypothyroidism and uremia. Dogs with uremia have significantly longer BMBTs compared to healthy dogs [36]. Dogs with experimentally induced hypothyroidism do not have significant changes in BMBT [51].

Bleeding time is sometimes assessed in humans to detect defects of primary hemostasis. When bleeding time is assessed in humans, a skin wound is made instead of a gingival wound. Similar to dogs, the test is practical and efficient to perform but test results can be influenced by several variables. Factors include skin thickness, body temperature, and operator skill. In humans, bleeding time is usually used as a screening tool to identify congenital and acquired defects of primary hemostasis when other platelet function tests are not readily available. Bleeding time does not appear to be a test that can reliably predict risk of hemorrhage in humans [27]. It also is less sensitive at detecting platelet dysfunction related to drug therapy compared to other methodologies, such as platelet aggregation [52].

v. Thromboelastography

Thromboelastography (TEG) provides a global overview of hemostasis, assessing primary hemostasis, secondary hemostasis, and fibrinolysis. To perform TEG, a whole blood sample is placed into a rotating system, which is composed of a pin that is suspended by a torsion wire in a cup. The pin moves up and down at an ultrasonic rate. After reagents are added to the sample and rotating forces are applied, a blood clot forms that entraps the pin. Once the pin is entrapped, it promotes motion that increases as the

clot strengthens and decreases as the clot breaks down. The motion is recorded throughout the whole process and different parameters are computed by specialized software, which represent different aspects of hemostasis. The K index indicates platelet function and angle alpha represents rate of fibrin formation. Maximal amplitude (MA) reveals the platelet and fibrin contribution to clot formation [27].

Thromboelastography is often used in humans as a tool to predict risk of bleeding in post-operative and emergency settings and predicts the need for blood product administration [27]. In dogs, TEG is usually employed to assess for potential coagulopathies and hypercoagulable states. Thromboelastography has been evaluated in dogs with a variety of diseases, such as chronic hepatopathies [53], chronic kidney disease [54], immune-mediated hemolytic anemia [55], and dogs infected with *Ehrlichia canis* [56]. It has also been used to assess coagulation changes in response to treatment with medications, such as rivaroxaban [57] and Yunnan Baiyo [45]. Although TEG can be used to assess platelet function, it is rarely used in dogs to solely assess primary hemostasis.

B. Hemostatic derangements with chronic liver disease

a. Chronic liver disease in dogs

Liver disease is common in middle age and older dogs, and is reported to be the cause of death in approximately 5% of adult dogs [58]. Dogs can be afflicted with a variety of acquired conditions including chronic hepatitis, cirrhosis, cholangiohepatitis, and copper storage disease. One study found that 12% of dogs undergoing necropsy had evidence of chronic hepatitis [59]. Chronic liver disease usually causes an increase in liver enzyme activity with routine serum biochemistries. In a study of 661 dogs where serum biochemistries were evaluated for unspecified reasons, alanine aminotransferase (ALT)

activity was elevated in 13% and 23% and alkaline phosphatase (ALP) activity was elevated in 28% and 51% of dogs 1-8 and >8 years of age, respectively [60]. Persistent elevation of ALT and ALP frequently results in additional diagnostic investigation.

Abdominal ultrasound is useful in investigating the cause of liver enzyme elevations. While it is effective in identifying focal hepatic disease, it is insufficient for identifying the cause of diffuse hepatic disease. In fact, 64% of dogs with sonographically unremarkable livers had histopathologic abnormalities identified on biopsy [61]. Sampling of the liver is usually necessary to elucidate the cause of laboratory test abnormalities. Fine-needle aspiration of the liver is generally safe, but cytologic assessment of these samples agrees with histopathology in only 30% of cases in one hospital [62]. Therefore, the clinical importance of persistent liver enzyme elevation can be best determined by liver biopsy. Biopsy from multiple liver lobes is recommended in most cases to obtain a sample that is reflective of the overall disease process within the liver [63].

Liver biopsy carries the serious risk of hemorrhage. In a study evaluating complications associated with ultrasound guided Tru-cut biopsies, major complications were encountered in 4.2% of dogs and minor complications occurred in 18.5% of dogs. Of the dogs experiencing major complications, which included death and the necessity for blood transfusion or IV fluid therapy, three of the 13 dogs (23%) had received a liver biopsy [64]. In humans, excessive hemorrhage is not always predicted by standard methods of assessing coagulation, including prothrombin time (PT), partial thromboplastin time (PTT), and platelet count [65]. Pet owners and veterinarians are often reluctant to pursue liver biopsies due to the risk of potentially life-threatening hemorrhage that cannot be

adequately predicted with routine testing. A study evaluating complications associated with ultrasound guided liver biopsy in dogs found no statistically significant correlation between PT and PTT prolongation and ultrasonographic evidence of hemorrhage after biopsy [66]. A study evaluating the safety of laparoscopic hepatic biopsy in dogs showed that preoperative thrombocytopenia was associated with the need for blood transfusion or conversion to a laparotomy. Preoperative coagulopathies were not associated with the development of a complication [67]. Platelet function was not routinely assessed in these studies.

b. Primary hemostasis abnormalities

Through a variety of mechanisms, up to 70% of humans with cirrhosis develop thrombocytopenia, which is potentially due to decreased platelet production and platelet sequestration [68,69]. The liver, in conjunction with the kidneys, is responsible for producing thrombopoietin (TPO), a cytokine that regulates megakaryocyte formation and platelet production [70]. Thrombopoietin mRNA levels in the liver are decreased in humans with cirrhosis [71]. Interestingly, both thrombopoietin levels and platelet count are increased after liver transplantation in humans with cirrhosis [69]. Although decreased platelet production is thought to contribute to thrombocytopenia, it is unlikely to be the sole cause [70]. Platelet sequestration contributes to thrombocytopenia as well. Humans with portal hypertension often have splenomegaly and subsequent platelet sequestration [72]. Partial splenic embolization has been shown to increase platelet count [70]. Immune-mediated platelet destruction is thought to play a role in the thrombocytopenia as well, because patients with chronic liver disease have substantially elevated platelet-associated IgG [73] and increased numbers of B cells that produce antibodies to GPIIb/IIIa [74].

Dogs with liver disease have significantly lower platelet counts compared to healthy individuals. A study comparing platelet count among dogs with normal livers and dogs with a variety of liver diseases, including chronic hepatitis, chronic hepatitis with cirrhosis, non-specific reactive hepatitis, congenital portosystemic shunt, and steroid-induced hepatopathy, showed that mean platelet concentration was significantly lower in dogs with chronic hepatitis plus cirrhosis compared to other dogs in the study. Although the mean platelet concentration was significantly lower, the mean platelet count was within reference interval [75]. Another study showed that dogs with chronic hepatopathies had significantly lower platelet counts compared to reference intervals. In this group of dogs, 10/21 had a platelet count below reference interval [53]. Thrombocytopenia has been documented in dogs with acute liver failure as well with thrombocytopenia documented in 51% of dogs and 29% of 49 dogs with acute liver failure and evidence of hemorrhage [76]. The platelet count of dogs with congenital portosystemic shunts has been found to be lower than healthy dogs in at least 1 study [77].

Thrombocytopathia is present in some humans with chronic liver disease and is more likely to be present in patients with severe liver disease and cirrhosis than those with milder disease [70]. Forty percent of humans with cirrhosis have bleeding times that exceed the reference interval (<10 minutes). Within this population, 43% of patients were thrombocytopenic, which may have contributed to the prolonged bleeding time. However, 11.5% of patients had a prolonged bleeding time despite a platelet count greater than >100,000/uL, which suggests thrombocytopathia. Interestingly, 14.2% of patients with a platelet count <100,000/uL had a bleeding time less than 10 minutes. Thrombocytopenia and bleeding time prolongation appeared to be directly related to the degree of liver failure in these patients [78].

Platelet function has been investigated further in a study utilizing the PFA-100® in humans with cirrhosis that revealed prolongation of CT in 44.4% of patients. While concurrent thrombocytopenia and anemia may have contributed, 21% of the patients with prolonged CT were not anemic (HCT ≤27%) or thrombocytopenic (platelet count ≤150,000/uL). Interestingly, some of the patients had normal CT but were thrombocytopenic [79]. Platelet counts less than 100,000 /uL and hematocrit <20% typically result in prolonged closure time even when thrombocytopathia is not present [80].

Humans with cirrhosis and thrombocytopenia, but normal CT, have significantly higher vWF-antigen levels compared to patients with prolonged platelet closure times. In thrombocytopenic patients with cirrhosis, hematocrit and vWF-antigen are independent predictors of CT. A study evaluating the addition of vWF and anti-vWF-antibody to blood samples of patients with cirrhosis and thrombocytopenia found that there was a reduction or prolongation in CT when recombinant vWF or anti-vWF-antibody was added, respectively. Based on these results, increased vWF levels may reduce hemorrhage due to thrombocytopenia and that platelet count may not reflect risk of hemorrhage. [79]. Currently, it is unknown how well CT and platelet count predict bleeding in humans with cirrhosis [81].

Humans with cirrhosis have higher levels of circulating vWF compared to normal individuals. The degree of vWF elevation increases with the severity of cirrhosis and hepatic dysfunction. Humans with cirrhosis have progressive increases in vWF as their albumin decreases and PT becomes prolonged [82]. The increase in vWF may be a result of endothelial alterations due to recurrent bacterial infection and endotoxemia secondary to decreased hepatic clearance of bacteria. Release of vWF from endothelial cells injured secondary to endotoxemia has been documented *in vitro*. Supporting this association, vWF and bacterial endotoxin

concentrations increase with severity of cirrhosis. Once patients with cirrhosis and endotoxemia are treated with antimicrobials, there is a subsequent decrease in vWF [83]. Although it is currently unknown if vWF increases in dogs with cirrhosis, dogs with hepatic disease and multiple portosystemic shunts are more likely to have Gram-positive bacteria, compared to control dogs, in blood cultures collected from portal and systemic circulation [84].

Platelet function is altered in humans with cirrhosis through a variety of mechanisms. First, their platelets appear to have transmembrane signaling defects. The normal release of intracellular messenger molecules after a platelet agonist binds to its transmembrane receptor does not occur. Intracellular IP3 production that usually occurs after stimulation with thrombin is significantly decreased in humans with cirrhosis [85]. Decreased IP3 production appears to be due to a defect in receptor-dependent phospholipase C activation [85]. As discussed previously, phospholipase C is an intracellular messenger molecule that catalyzes the hydrolysis of phosphatidyl inositol bisphosphate (PIP2) to IP3 [8]. Inositol triphosphate is needed to mobilize Ca^{2+} from the dense tubular system within the platelet. A decrease in intracellular Ca^{2+} leads to decreased platelet granule release and decreased activation [8]. Increases in intracellular calcium are also needed to cause conformational change of the GP IIb/IIIa receptor, which facilitates interactions with fibrin to reinforce the platelet plug [85]. In addition, calcium mobilization is needed for TxA_2 synthesis and release, which further amplifies platelet activation [8]. Finding reduced metabolites of cyclooxygenase and thromboxane synthase, which are activated during thromboxane formation, is evidence for decreased intracellular calcium in patients with cirrhosis [85]. While it is not completely understood why patients with cirrhosis have transmembrane platelet signaling defects, the lipid content in the platelet plasma membrane may be altered and lead to inhibition of phospholipase C activation [86]. As discussed previously, phospholipase C

catalyzes the hydrolysis of phosphatidyl inositol bisphosphate (PIP2) to IP3. Inositol triphosphate mobilizes Ca2+ from the dense tubular system within the platelet, leading to an increase in intracellular calcium and ultimately platelet activation [8].

Impaired platelet aggregation in humans with cirrhosis is associated with reduced platelet thromboxane and prostaglandin E2 as a result of decreased arachidonic acid availability [87]. Dietary arachidonic acid supplementation improves collagen-induced platelet aggregation in patients with cirrhosis [89]. This study was complicated by the fact that participants had concurrent renal dysfunction cirrhosis [88].

Platelet storage pool (granule) defects have been demonstrated in some humans with cirrhosis. Decreased amounts of ATP and serotonin (5HT) in delta granules and decreased amounts of platelet factor 4, b-thromboglobulin (BTG), and P-selectin in alpha granules has been found. ATP and serotonin are both powerful platelet agonists needed for normal platelet aggregation [85,89,90]. P-selectin is a glycoprotein that helps facilitate platelet adhesion but also promotes platelet activation and aggregation [91]. Although the content of the platelet granules is altered, the actual platelet granule morphology remains unchanged [90]. Finding that humans with cirrhosis have abnormal platelet granule content supports the "platelet exhaustion" theory. The "platelet exhaustion" theory states that the portal hyperdynamic circulation in cirrhosis applies shear forces to the platelets that result in an inappropriate intravascular activation. This early activation leads to granule release and ultimately depletion of the platelet storage pools, ultimately leading to platelet dysfunction [85].

In addition to intrinsic platelet defects, plasma factors may play a role in abnormal platelet function. High-density lipoprotein apolipoprotein E levels are altered in humans with cirrhosis. High-density lipoprotein apolipoprotein E inhibits agonist-induced platelet

aggregation at normal plasma concentration by occupying saturable platelet cell-surface receptors. High-density lipoprotein apolipoprotein E likely promotes an anti-atherogenic effect in healthy individuals. Humans with cirrhosis have significantly higher apolipoprotein E content in their high-density lipoprotein compared to healthy individuals and impairment of ADP-induced platelet aggregation correlates with high-density lipoprotein E concentration [92]. Fibrinogen degradation products, which may be increased in patients with liver disease, can also inhibit platelet function by adhering to the platelet surface and interfering with transmembrane signaling [85].

Although thrombocytopathia has been the primary focus of research in humans with cirrhosis, patients may be at increased risk of thrombosis due to primary hemostasis abnormalities as well. Humans with chronic hepatitis, with and without cirrhosis, have significantly decreased ADAMTS13 levels compared to healthy individuals, most likely because the enzyme is primarily produced in the liver. The degree of ADAMTS13 deficiency correlates with the severity of liver disease [93]. As discussed previously, ADAMTS13's main function is to cleave ultra-large or large vWF multimers into smaller multimers that have lower thrombogenic activity. ADAMTS13 deficiency leads to an excess of ultra-large vWF in circulation and spontaneous platelet aggregation that results in abnormal thrombus formation. Humans with cirrhosis and portal vein thrombosis have significantly lower ADAMTS13 levels than humans with cirrhosis without portal vein thrombosis [94].

Platelet function has been well studied in humans with chronic liver disease and cirrhosis but has received little attention in dogs with acquired chronic liver diseases. Dogs with congenital portosystemic shunts have normal capillary bleeding time, platelet closure time, platelet count, and ADP-induced platelet aggregation. However, platelet aggregation in response

to collagen and arachidonic acid is moderately impaired in these dogs [95]. In a study of 11 dogs with a congenital portosystemic shunt and six with chronic active hepatitis, decreased aggregation was identified using arachidonic acid and collagen but not ADP using a whole blood platelet aggregation assay. Six of the dogs in the study also had evidence of hemorrhage, including epistaxis, gingival bleeding, melena, and hematuria, despite having a normal PT, PTT, and platelet count [96]. Platelet aggregation in response to ADP and collagen has been shown to be reduced in dogs following common bile duct ligation [97]. Platelet dysfunction in dogs with chronic hepatopathies is also supported by increased K and decreased MA using thromboelastography (TEG) when portal hypertension exists [53]. Further information is needed to determine whether dogs with chronic acquired liver disease have thrombocytopathia, as this may be an important component to predicting the risk of hemorrhage in these patients.

c. Secondary hemostasis abnormalities

Secondary hemostasis is the series of events through which coagulation factors become activated and ultimately lead to the generation of thrombin. Thrombin catalyzes the creation of a cross-linked fibrin mesh that stabilizes the initial platelet plug [98]. Chronic liver disease can result in secondary hemostasis defects through decreased coagulation factor production (II, V, VII, IX, X, XI, and XIII), decreased fibrinogen production, decreased vitamin K mediated carboxylation of coagulation factors (II, VII, IX, and X), and changes in production of anticoagulants [98]. A study evaluating dogs with chronic hepatitis with cirrhosis showed lower activities for factors II, V, VII, VIII, IX, X, XI, and XII but only factor IX activity was significantly lower when compared to control dogs [75]. Using TEG analysis, 24% of dogs with chronic hepatitis were shown to have an overall hypocoagulable state, while 33% had a hypercoagulable state. The maximal amplitude (MA), which is the platelet contribution to clot formation, was not found to

be significantly different between dogs with chronic hepatopathies and control dogs. The K value, which is indicative of platelet function, was significantly prolonged in dogs with chronic hepatopathies [53]. Interestingly, there was no difference in PT and PTT in dogs with chronic hepatitis compared to reference intervals [53]. Thromboelastography abnormalities occur with acute liver disease in dogs as well. A study evaluating TEG in dogs with acute liver disease revealed 52% of dogs to be hypocoagulable, 38% to be normocoagulalbe, and 10% to be hypercoagulable [99]. Ninety-three percent of dogs with a variety of liver diseases, including degeneration, inflammation, cirrhosis, or neoplasia, had at least 1 abnormal coagulation test value. The PT and PTT were abnormal in 50% and 75% of these dogs respectively [100].

CHAPTER 2: PLATELET FUNCTION IN DOGS WITH CHRONIC LIVER DISEASE

A. Introduction

Liver disease is common in middle aged and older dogs and is reported to be the cause of death in approximately 5% of dogs [58]. Additionally, chronic hepatitis has been reported to have a prevalence of 12% in dogs undergoing necropsy [59]. Elevation of plasma alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities has been reported in 23% and 51%, respectively, of dogs over 8 years of age [60]. However, determination of the clinical importance and etiology of the elevated enzyme activity necessitates further investigation. Since up to 64% of dogs with sonographically normal livers may have histopathologic abnormalities and cytology of the liver only agrees with histopathology in about 30% of cases, liver biopsy is often required to obtain a diagnosis for diffuse liver disease [61,62]. Liver biopsy carries the risk of serious hemorrhage, a risk made greater by hemostatic derangements caused by liver disease.

Unfortunately, standard tests of hemostasis do not consistently predict hemorrhage associated with biopsy. Two separate studies evaluating laparoscopic and ultrasound guided Tru-cut liver biopsy did not show a relationship between a coagulopathy and clinically relevant hemorrhage after liver biopsy [66,67]. To better predict risk of hemorrhage, the hemostatic derangements associated with chronic acquired liver disease need to be better characterized.

Secondary hemostatic abnormalities are well documented in dogs with chronic liver disease but minimal information is available about potential primary hemostasis defects [98]. Humans with cirrhosis may experience thrombocytopenia, endothelial dysfunction, increased von Willebrand factor (vWF) concentration, and thrombocytopathia [68,82,85,101]. Thrombocytopathia has been documented in 11.5% of humans with cirrhosis as determined by prolonged bleeding time and in 21% by finding prolonged platelet plug formation when assessed by closure time (CT) using the Platelet Function Analyzer-100 (PFA-100®, Siemens Healthcare, Deerfield, IL) [79,102]. Proposed mechanisms for thrombocytopathia in cirrhosis include platelet transmembrane signaling defects, reduced thromboxane and prostaglandin E2 due to reduced arachidonic acid availability, and platelet storage pool (granule) defects [85,87,90]. In addition to intrinsic platelet defects and changes in vWF, a variety of plasma factors may play a role in abnormal platelet function. High-density lipoprotein apolipoprotein E is increased in humans with cirrhosis and inhibits platelet aggregation [92]. Fibrinogen degradation products, which are increased in some patients with liver disease, can inhibit platelet function by adhering to the platelet surface and interfering with transmembrane signaling [85]. The authors are aware of only one small study evaluating platelet function in dogs with chronic hepatitis using whole blood platelet aggregation, which identified thrombocytopathia [96]. To our knowledge, CT, buccal

mucosal bleeding time (BMBT), and vWF antigen have not been evaluated in dogs with acquired chronic liver disease.

Both BMBT and CT have been used in several studies in dogs with a variety of disorders. Buccal mucosal bleeding time is a crude but practical and readily available test. Platelet count, platelet function, and endothelial function are assessed by BMBT [23]. Closure time is a practical and efficient way to assess platelet function. The PFA-100® simulates *in vivo* primary hemostasis under high shear conditions and exposes platelets to biochemical stimuli that would be present within an arteriole. The amount of time it takes for a platelet plug to form and occlude blood flow through the analyzer is recorded as the CT [28]. In a study evaluating healthy dogs and dogs with primary hemostatic disorders, including von Willebrand disease (vWD), thrombocytopenia, and intrinsic platelet function defects, CT using ADP as an agonist had a sensitivity and specificity of 95.7 and 100%, respectively, for identifying thrombocytopathia [29]. Unlike BMBT, the CT should not be affected by endothelial function.

The primary aim of this study was to determine if dogs with acquired chronic liver disease experience thrombocytopathia and alteration of plasma vWF concentration. A secondary aim was to determine whether hemorrhage, assessed by change in PCV and abdominal fluid accumulation after an ultrasound-guided needle biopsy of the liver, is associated with BMBT, CT, and plasma vWF concentration. We hypothesized that dogs with acquired chronic liver disease have prolonged BMBT and CT as markers of thrombocytopathia. A secondary hypothesis was that dogs with chronic liver disease would have an increased plasma vWF concentration compared to healthy control dogs.

B. Materials and Methods

This prospective study evaluated dogs presented for assessment of acquired chronic liver disease at the Virginia-Maryland College of Veterinary Medicine (VMCVM) Veterinary Teaching Hospital (VTH). The study was approved by the Institutional Animal Care and Use Committee at Virginia Tech and the VTH Hospital Board. Informed consent was obtained from the owner prior to enrollment in the study.

Dogs were included if they were older than one year of age and had increased serum activity of ALT (>2.5 times upper end of reference interval) for more than 2 weeks duration. In addition, dogs with biochemical evidence of hepatic dysfunction (hypocholesterolemia, decreased BUN, hypoglycemia, hypoalbuminemia, hyperbilirubinemia, or elevated fasting or postprandial bile acids) for more than 2 weeks duration were included, regardless of the ALT activity. Dogs with normal liver or vacuolar hepatopathy diagnosed on liver histopathology were excluded. Dogs were excluded from the study if they were a sighthound breed or had been diagnosed with a condition known to affect platelet function, including a heart murmur, diabetes mellitus, hyperadrenocorticism, renal failure, dirofilariasis, malignant neoplasia, or other major concurrent illness. Additional exclusion criteria included the presence of an extrahepatic biliary obstruction, acute pancreatitis, congenital portosystemic shunt, or liver disease confined to one liver lobe (focal mass) on abdominal ultrasound. Dogs receiving drugs that could alter platelet function, including non-steroidal anti-inflammatory drugs, calcium channel blockers, beta blockers, fish oil supplementation, or corticosteroids within 7 days or a beta-lactam antibiotic within 2 days prior of evaluation were excluded.

Dogs with a PCV less than 35% or platelet count less than 150,000/uL were also excluded, as these changes may affect CT.

Dogs with suspected acquired chronic liver disease were assessed using plasma biochemistry, CBC (including platelet count), prothrombin time (PT), activated partial thromboplastin time (aPTT), and abdominal ultrasound. In order to identify infection with *D. immitis*, a SNAP 4Dx Plus test (Idexx Laboratories, Westbrook, ME) was performed. Liver biopsy was obtained via ultrasound-guided needle biopsy or laparoscopy in dogs with chronic liver disease.

Healthy dogs that were age matched to the study population were recruited from VMCVM faculty, staff, and students to act as controls. Dogs were determined to be healthy based on results of history, physical examination, CBC, and plasma chemistry (including normal plasma ALT activity), and negative for *D. immitis* on SNAP 4Dx Plus test. The above tests in control and affected dogs were performed VMCVM ViTALS laboratory. Control dogs had not received any of the aforementioned drugs that could alter platelet function within the time listed above.

Closure time was measured using the PFA-100® and collagen/ADP cartridges in both groups of dogs. To evaluate CT, 5.4 ml of venous blood was collected using a 20g or 22 g needle into a syringe and then immediately placed into 3.2% buffered sodium citrate (1 part anticoagulant to 9 parts blood). The tube was inverted 3-4 times after collection. The samples were stored at room temperature for 30 -120 minutes before analysis. Each assay was performed in duplicate simultaneously using both channels of the instrument. The assay was repeated if the coefficient of variation between the 2 CTs was greater than 16%[103]. Immediately before analysis, the blood sample was inverted 2-3

times and 0.8 ml was placed into a disposable test cartridge that consists of a membrane containing collagen and ADP. After incubation of the blood sample in the cartridge at 37 °C for 3 minutes, the analyzer automatically pulled blood through a central aperture within the membrane under a steady vacuum. This creates an environment similar to high shear conditions within an arteriole, which along with the agonists in the membrane stimulate platelets to adhere and aggregate within the aperture. This results in formation of a platelet plug that occludes the aperture. Closure time was measured as the time from initial flow to occlusion of the aperture with a platelet plug and is representative of platelet function, with prolongation indicating reduced function.

The BMBT was performed by a single investigator (AW) in all dogs as previously described [34]. Briefly, the upper lip was lifted and held in place by placing a lightly tightened muzzle of gauze so the buccal mucosal surface was exposed. A bleeding template (Surgicutt; Jorgensen Labs Loveland, Colorado) was used to make a linear buccal mucosal incision in an area devoid of large vessels parallel to the lip margin. Blood from the incision was blotted every 5 seconds using filter paper held approximately 2-3 mm below the incision without contacting it. The time from incision to cessation of bleeding was recorded as BMBT.

Plasma vWF:Ag concentration was measured in blood collected at admission in all dogs. Blood was collected (2.7 mL) into a syringe and immediately placed into 3.2% buffered sodium citrate tubes and centrifuged for 10 minutes to collect plasma. The plasma was frozen at -70 °C until of vWF:Ag concentration was measured by the Comparative Coagulation Laboratory, Cornell University College of Veterinary Medicine.

Ultrasound-guided or laparoscopically-assisted liver biopsy was performed on dogs with chronic liver disease, but not in the control group. A histopathologic diagnosis was determined by one of several board-certified pathologists in the VMCVM ViTALS laboratory. Patients received sedation or general anesthesia for needle biopsy and general anesthesia for laparoscopic biopsy. Drugs administered for sedation were at the discretion of the primary clinician based on patient characteristics, and included the use of an opioid and either propofol or dexmedetomidine. Ultrasound guided needle biopsy was performed by a board certified veterinary radiologist or veterinary radiology resident supervised by a board certified veterinary radiologist. Aseptic technique included clipping the hair from the abdomen and scrubbing it with povidone iodine scrub and isopropyl alcohol. A 3-5 mm skin incision using a #11 blade was made in the cranial abdomen to allow advancement of the biopsy instrument into the liver. Using ultrasound guidance, 2-5 liver biopsies were obtained using a 14 ga Vet-Core® (Surgivet, Dublin, OH) biopsy needle. Multiple liver lobes were sampled when possible.

In order to estimate the severity of hemorrhage associated with needle biopsy, dogs were monitored in a standardized manner after the biopsy. A PCV and total solids was performed within 24 hours prior to and 12 hours after the liver biopsy. When possible, IV fluids were not administered, and patients that received IV fluid therapy were excluded from analysis of post-biopsy hemorrhage. Post-biopsy hemorrhage was also assessed using serial ultrasound examinations 2-3 hours after biopsy and the morning after the biopsy was obtained (17-24 hours). During each examination, the following sites were evaluated for free fluid: Biopsy site, the diaphragmatico-hepatic (DH), spleno-

renal (SR), cysto-colic (CC), and hepato-renal (HR). A point was allocated for each site when fluid was present and was considered to the "fluid score" [104].

Power analysis with an alpha of 0.05 and power of 80% determined 25 cases were needed in both groups. When data was normally distributed, an unpaired t-test was used. When non-Gaussian distribution was present, a Mann-Whitney tested was used. The level of significance was set at $P < 0.05$. Correlations were determined using Spearman correlation with Fisher's Z transformation. Data will be listed as mean; standard deviation (SD) throughout the manuscript.

C. Results

Twenty-one dogs were initially enrolled into the liver disease group, with 3 dogs excluded due to normal liver histopathology (n = 2) or diagnosis of vacuolar hepatopathy (n = 1). The mean age of the 18 dogs liver disease group remaining in the study was 6.5 (SD 3.5) years and sex distribution included 8 spayed females, 7 castrated males, 2 intact females, and 1 intact male. The mean age of the control group was 6.2 (SD 2.8) years and sex distribution included 8 spayed females and 10 castrated males. Age between the two groups was not different (P = 0.77). Breeds of dogs in the liver disease group included mixed (n = 5), Labrador retriever (n = 2), standard poodle (n = 2), Yorkshire terrier (n = 2), Bichon Frise (n = 1), bloodhound (n =1), Bouvier Des Flandres (n = 1), cairn terrier (n = 1), Canaan dog (n = 1), miniature schnauzer (n = 1), and vizsla (n = 1). Chronic hepatitis was diagnosed in 12 dogs, copper-associated hepatitis in 5 dogs, and nodular hepatopathy in 1 dog. Breeds of dogs in the control group included mixed (n = 10), chihuahua (n = 1), golden retriever (n = 2), doberman pinscher (n = 1), Australian cattle dog (n = 1), Walker hound (n = 1), coonhound (n = 1), and Boston terrier (n =1). Nine of the 18 dogs presenting for evaluation had no clinical signs associated with their liver disease. Clinical signs observed in the remaining dogs included anorexia or hyporexia (n =7), lethargy (n = 5),

weight loss (n = 2), and vomiting (n = 2). Alanine aminotransferase elevation was initially detected in 2 dogs 2 weeks prior to presentation, 5 dogs 2 weeks to 2 months prior to presentation, and 11 dogs more than 2 months prior to presentation.

The mean (SD) CT (Figure 1) in the liver disease group (76.1; 15.8 seconds) was not different ($P = 0.27$) than the control group (70.5; 10.0 seconds). The mean (SD) BMBT (Figure 2) was longer ($P = 0.019$) in the liver disease group (145; 40.9 seconds) than the control group (112.3; 39.4 seconds U). The mean (SD) vWF antigen (Figure 3) in the liver disease group (195.3; 13.2 %) was not different ($P = 0.0769$) than the control group (161.7; 12.8 %).

Indirect markers of hepatic function (Table 1), including plasma total bilirubin ($P = 0.1010$), cholesterol ($P = 0.0638$), albumin ($P = 0.1931$), BUN ($P = 0.3323$), and glucose ($P = 0.0616$) were not different between the liver disease group and control group. Four dogs in the liver disease group had total bilirubin above the reference interval but only 2 of these dogs had a bilirubin concentration above 0.6 mg/dL. Within the liver disease group, 7 dogs had hypoalbuminemia, 3 dogs had hypoglycemia, and 1 dog had a BUN concentration below the reference interval. The mean (SD) plasma ALT activity was higher ($P < 0.001$) in the liver disease group (628.6; 476.9 U/L) than the control group (36.7; 10.5 U/L). The mean (SD) plasma ALKP activity was higher ($P < 0.001$) in the liver disease group (376.6; 351.3 U/L) than the control group (40.1; 27.8 U/L). Mean platelet count ($P = 0.0558$) and hematocrit ($P = 0.1338$) were not different between the groups. Three dogs with liver disease had PT above reference interval and 2 dogs with liver disease had PTT above reference interval but none had prolongation greater than 25% of the reference interval in either test.

None of the dogs undergoing laparoscopic (n = 7) or needle biopsy (n = 11) experienced serious hemorrhage that resulted in death or necessitated blood transfusion after biopsy. One

dog that received a needle biopsy through an intercostal approach developed a pneumothorax that resolved with supportive care. In dogs undergoing needle biopsy of the liver (n = 10), change in PCV prior to liver biopsy (mean 47.3%) and 12 hours post liver biopsy (mean 47.5%) was not significantly different ($P = 0.7480$). One dog was excluded from the PCV analysis due to an inaccurate PCV measurement prior to biopsy.

Mean fluid scores immediately, 2-3 hours, and 17-24 hours after biopsy were 1.27, 1.36, and 0.82, respectively. There was no correlation of fluid scores at any time with CT, BMBT, vWF:Ag concentration, or change in PCV. A correlation between BMBT and CT was found (Figure 4) when all dogs were evaluated as a group ($r = 0.435$; $P = 0.0084$).

D. Discussion

Platelet dysfunction has been well documented in humans with cirrhosis [79,102]. Results of the present study provide conflicting evidence of the effects on primary hemostasis of chronic acquired liver disease in dogs since BMBT was prolonged but CT was not different between groups. Because BMBT is dependent on normal endothelial function while CT is not, these results may indicate that dogs with acquired chronic liver disease experience endothelial dysfunction. In humans, cirrhosis is associated with increased vascular nitric oxide, a powerful vasodilator and platelet inhibitor, and hyperactive endothelial cells within systemic and splanchnic circulation. These changes lead to vasodilation within systemic vasculature and a hyperdynamic circulatory state [105]. Humans with cirrhosis also experience pathologic bacterial and bacterial product translocation from the gastrointestinal tract [106]. Increased circulating endotoxin likely causes endothelial damage since plasma vWF, which is a marker of endothelial disruption and is released from endothelial cells in response to endotoxin *in vitro*, increases as circulating endotoxin increases in cirrhosis [83]. A similar phenomenon may occur in dogs since

Gram-positive bacteremia and endotoxemia have been documented in dogs with induced hepatic disease and multiple acquired portosystemic shunts [84,107].

Since BMBT and CT were correlated in the present study, thrombocytopathia in our population of dogs with liver disease may be present, but not detected by CT. The PFA-100® simulates *in vivo* primary hemostasis under high shear conditions that would likely be present within an arteriole [28]. Because BMBT is a more global test of primary hemostasis, its ability to detect abnormal platelet function might be less restricted than CT. When platelet function was measured by whole blood platelet aggregation in dogs with chronic liver disease, a technique performed under low shear stress, thrombocytopathia was documented [18]. Platelet dysfunction in dogs with chronic hepatopathies and portal hypertension is also supported by increased K and decreased MA using thromboelastography [53]. While direct comparisons cannot be made between these studies for a variety of reasons, including differing severity and etiology of liver disease and use of different platelet agonists, limitations of CT may account for some of the difference. The platelet agonist used to measure CT in the present study may have affected our ability to identify thrombocytopathia as well. For instance, 13.5% of people with cirrhosis will have prolonged CT with epinephrine/collagen cartridges but will have normal CT with ADP/collagen cartridges [79]. Platelet activation in response to certain agonists may be more impacted by liver disease compared to other agonists. Previous studies in dogs have shown a discrepancy in CT when using the ADP/collagen versus epinephrine/collage cartridges when identifying platelet inhibition caused by NSAID therapy [43]. Future studies could evaluate platelet function using different aggregometry techniques or flow cytometry to allow use of different platelet agonists.

Humans with cirrhosis have increased levels of circulating vWF compared to healthy individuals [82]. The increase in vWF may may result from recurrent endotoxemia [83] or increased

hepatic synthesis of vWF as liver tissue from patients with cirrhosis has increased levels of vWF mRNA and vWF protein distribution [108]. While the mean vWF plasma concentration was higher in dogs with chronic liver disease, the difference was not significant. A contributing factor to our inability to detect a difference may be a result of the relatively mild hepatic disease in the population of dogs studied. In humans, the degree of vWF elevation correlates with the severity of cirrhosis and hepatic dysfunction since vWF increases as albumin decreases and PT becomes prolonged in these individuals [82]. Von Willebrand factor concentration correlates well with severity of liver disease in humans and can be used to predict the onset of ascites, variceal bleeding, and mortality [109]. Future studies may be aimed at evaluating plasma vWF concentration in dogs with more advanced liver disease and hepatic failure.

None of the dogs in our study experienced serious hemorrhage after liver biopsy and no change in PCV after liver biopsy in dogs undergoing Tru-cut liver biopsy was found. These results indicate that clinically relevant hemorrhage is unlikely to occur as a result of ultrasound guided liver biopsy in dogs with chronic hepatitis that have few overt signs of hepatic dysfunction. Buccal mucosal bleeding time, CT, and plasma vWF concentration did not correlate with change in PCV or abdominal fluid accumulation post biopsy. Our small case numbers and lack of procedure-related complications may have prevented our ability to identify a correlation between BMBT, CT, and vWF concentration and post biopsy hemorrhage. In addition, no dog in our study had serious hemorrhage, which would have been necessary to identify a correlation between hemorrhage post biopsy and BMBT, CT, and vWF.

Weaknesses in the current study include that the subject number determined in the initial power analysis was not met. Inclusion of more patients or selection of dogs with overt hepatic dysfunction might have led to different results. Inadequate subject numbers may have led to a

type II statistical error. The severity of liver disease in the dogs in the present study was relatively mild in general compared with the marked functional impairment and portal hypertension in humans with cirrhosis where platelet dysfunction has been documented [13, 14]. Additional studies of dogs with more severe liver disease and impaired hepatocellular function may detect abnormalities not noted in our population. Buccal mucosal bleeding time is an imprecise test with variability affected by operator technique, platelet number, anemia, vascular tone and other factors. Closure time also has limitations and is affected by thrombocytopenia and anemia. By having a single investigator perform all BMBT and excluding dogs with thrombocytopenia and anemia, the present study attempted to minimize the imprecision.

CHAPTER 3: CONCLUSIONS AND FURTHER RESEARCH

The results of the present study demonstrate mild impairment of primary hemostasis in dogs with chronic liver disease based on prolongation of BMBT. Normal CT with BMBT prolongation could indicate endothelial dysfunction in dogs with chronic liver disease. Because BMBT and CT correlated in the study, thrombocytopathia in our population of dogs with liver disease may exist as well but may not be sufficiently detected by CT. Potential primary hemostasis defects should be considered in dogs with chronic liver disease prior to pursuing liver biopsy. Von Willebrand factor concentration was not found to be significantly different between populations. Bleeding associated with Tru-cut liver biopsy, which was assessed with serial ultrasound examination and PCV measurements, did not correlate with BMBT, CT, and vWF concentration. Future studies with increased case numbers may be aimed at assessing BMBT, CT, and vWF concentration in dogs with hepatic dysfunction and cirrhosis rather than primarily chronic liver enzyme elevation.

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APPEXDEX A: TABLES

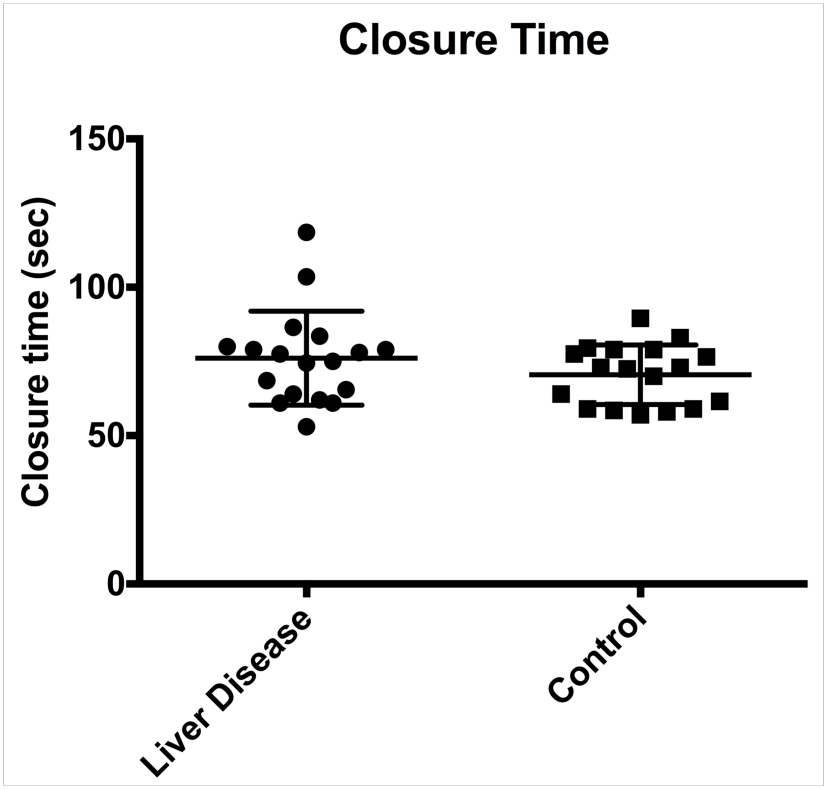
Table 1. Biochemical changes

	Liver group (n = 18)	Control group (n =18)	*P* - value
ALT (U/L)	628.6 (476.9)	36.7 (10.5)	<0.001
ALKP (U/L)	376.6 (351.3)	40.1 (27.8)	<0.001
BUN (mg/dL)	15.7 (7.1)	16.1 (3.9)	0.3323
Glucose (mg/dL)	97.1 (12.9)	104.7 (11.9)	0.01616
Cholesterol (mg/dL)	282.4 (141.7)	211.5 (65.9)	0.0638
Albumin (g/dL)	2.9 (0.5)	3.2 (0.2)	0.1931
Total bilirubin (mg/dL)	0.9 (1.75)	0.2 (0.08)	0.1010

The mean (SD) is listed for each value within each group.

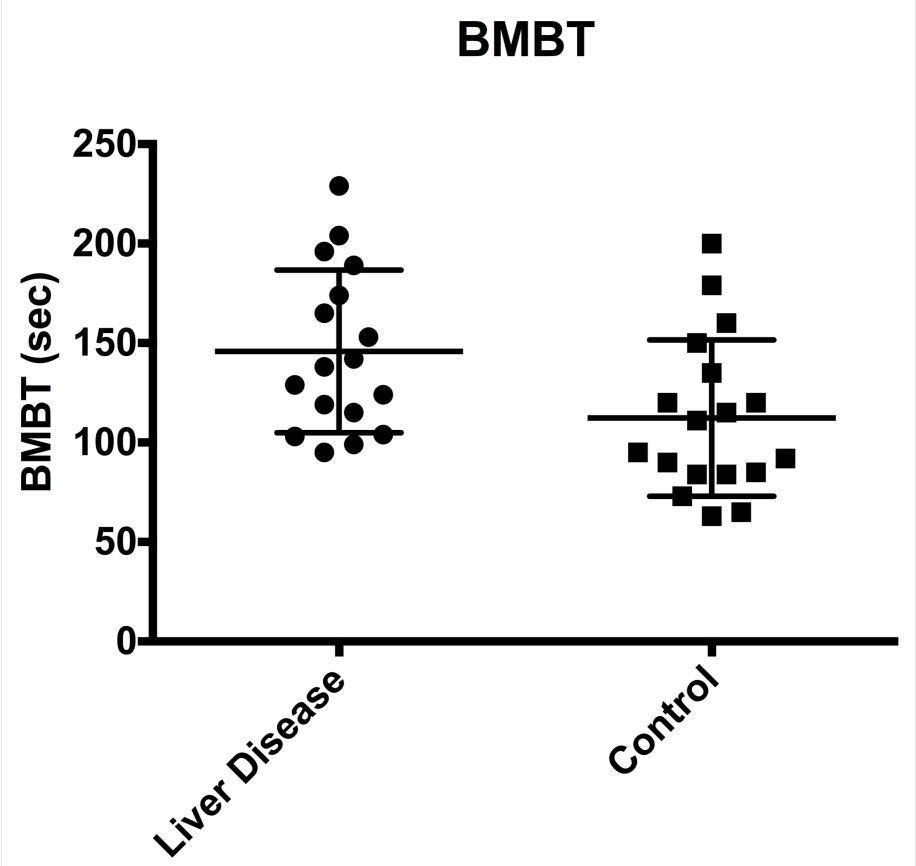
APPENDEX B: FIGURES

Figure 1. Closure time



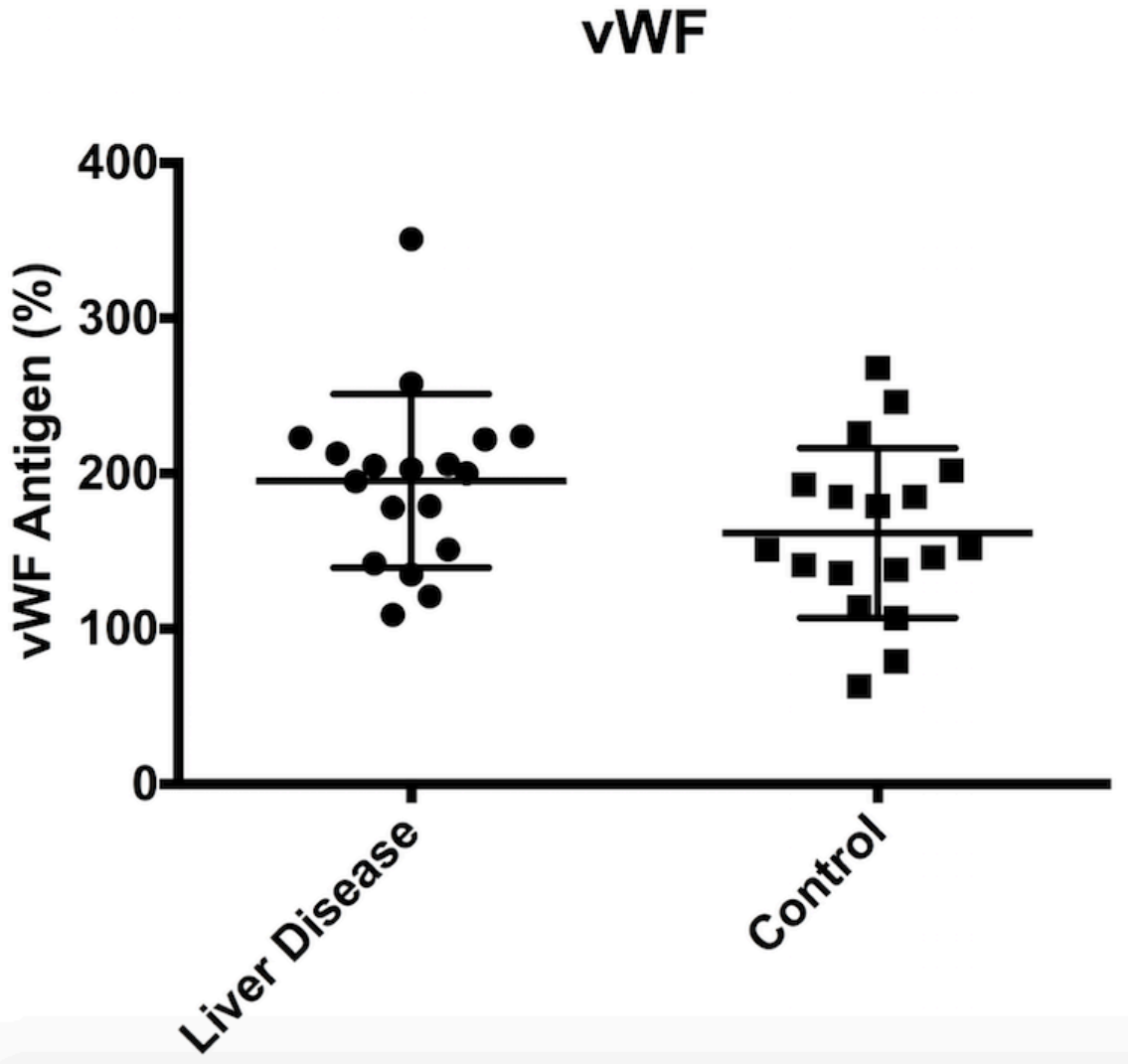
The middle bar represents the mean and the top and bottom bars represent standard deviation within each group. Individual values are represented by the circles and squares.

Figure 2. Buccal mucosal bleeding time



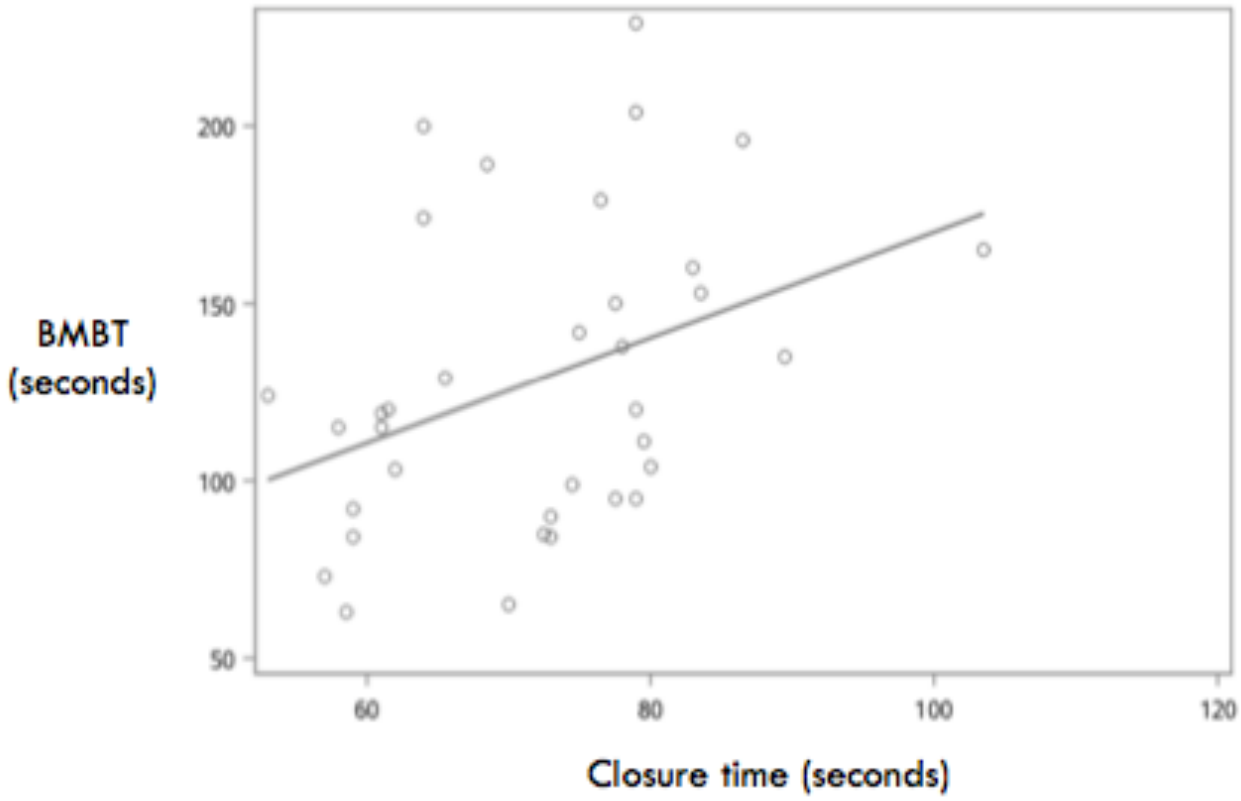
The middle bar represents the mean and the top and bottom bars represent the standard deviation within each group. Individual values are represented by the circles and squares.

Figure 3. von Willebrand Factor antigen concentration



The middle bar represents the mean and the top and bottom bars represent the standard deviation within each group. Individual values are represented by the circles and squares.

Figure 4. Correlation between closure time and buccal mucosal bleeding time in all dogs



Individual values are represented by the circles. The line represents the linear regression line. For this set of data, r = 0.435 and *P* = 0.0084.