An Investigation of the Multifaceted Platelet Dysfunction in Dogs with Naturally-Ocurring Chronic Kidney Disease

THESIS

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Abstract

Bleeding is a complication of chronic kidney disease (CKD) in humans and can occur despite normal coagulation times and platelet counts. These abnormalities are caused, in part, by platelet dysfunction and abnormal platelet-vessel wall interactions. Dogs with experimentally induced CKD have prolonged mucosal bleeding times, however this test is variable and error prone. In dogs with naturally occurring CKD, coagulation defects and platelet dysfunction have not been evaluated using new point-of-care tests of platelet function and global coagulation. Our objective was to compare platelet function between healthy dogs and dogs with CKD using the PFA-100 and thromboelastography (TEG) and to determine if changes in these tests can be explained by alterations in platelet GPIb and GPIIb-IIIa expression, membrane receptors for vWF and fibrinogen, respectively or in changes in platelet activation as determined by platelet P-selectin expression.

Blood samples from 11 dogs with naturally occurring CKD and 10 healthy control dogs were collected. Routine CBC with platelet count, serum biochemistry and urinalysis with urine protein to creatinine ratio were performed in both groups. Platelet function was assessed by measuring PFA-100 closure times (CT) using collagen and epinephrine (Col+EPI) or collagen and adenosine diphosphate (Col+ADP) agonists. Reaction time (R), clot formation time (K), α-angle (α), maximal amplitude (MA) and global clot strength (G) TEG variables were analyzed. Soluble coagulation factor function testing
was performed including PT, aPTT, fibrinogen concentration and AT activity. Platelet GPIb, GPIIb-IIIa and P-selectin expression was assessed by flow cytometry and expressed as mean fluorescence intensity (MFI).

Hematocrit was significantly decreased in CKD dogs (p < 0.0001). Platelet counts were not different between groups (p = 0.57). Dogs with CKD had significantly prolonged PFA-100 Col+ADP CT compared to healthy dogs (p = 0.01). No significant difference in Col+EPI CT was found between healthy and CKD dogs. There was a significant increase in TEG MA (p < 0.01) and G (p = 0.01) and a significant decrease in K-time (p = 0.035) in dogs with CKD compared to healthy controls. The remaining TEG variables (R, and angle) were not significantly different between CKD and healthy dogs. CKD dogs had increased platelet GPIIb-IIIa and P-selectin MFI compared with control dogs (p = 0.01 and 0.03 respectively). There was no difference in GPIb MFI between groups. No correlation was found between platelet surface receptor expression and any TEG value or PFA-100 closure times.

Dogs with CKD appear to have platelet dysfunction despite normal platelet counts. However, despite platelet dysfunction, these patients are hypercoagulable based on their significantly elevated MA and G TEG values. This discrepancy may be explained by the significantly decreased hematocrit and significantly increased fibrinogen concentrations in CKD dogs. However, platelet receptor expression cannot be used to explain changes in platelet function and changes in whole blood coagulation identified in these patients.
Additional studies are needed to identify the underlying platelet defect(s) in dogs with CKD and what role they play in whole body coagulation, as well as their clinical significance.
To my parents, without you I would be nothing.
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Chapter 1: Introduction

Chronic kidney disease (CKD) occurs commonly in many species including both dogs and humans. The prevalence of CKD in various canine populations ranges from 0.37-3.74%\textsuperscript{1,2,3} and affects as much as 16.8% of the human population.\textsuperscript{4} Numerous complications occur secondary to CKD in humans and dogs, including anemia, renal secondary hyperparathyroidism, uremic gastritis, and hypertension.\textsuperscript{5} Additionally, bleeding tendencies secondary to defects in primary hemostasis have been well described in uremic disease states in people.\textsuperscript{6,7,8}

Bleeding has long been described in humans with naturally occurring CKD dating back to the 1800’s.\textsuperscript{11} In 1907, Riesman described bleeding from multiple sites associated with kidney disease including a syndrome called hemorrhagic diathesis, a term used to describe bleeding into the skin and from mucus membranes. Riesman concluded that the cause of hemorrhagic diathesis in these patients is unknown but likely secondary to a toxic disease process “analogous to the hemorrhagins of snake venom.”\textsuperscript{12} Since this early work, uremic bleeding has been described in human patients to occur on mucosal surfaces as epistaxis, gingival bleeding, gastrointestinal bleeding,\textsuperscript{8} on the skin as petechiae and ecchymoses,\textsuperscript{7} as well as in body cavities including pleural and pericardical hemorrhagic effusions.\textsuperscript{9,10} While mild thrombocytopenia does occur in uremic states,
bleeding can occur even in patients with normal platelet numbers and with normal prothrombin time (PT) and partial thromboplastin time (PTT) suggesting that increased bleeding tendencies in these patients are secondary to an inherent abnormality of primary hemostasis due to platelet dysfunction rather than deficiencies in secondary hemostasis.\textsuperscript{6,7} In humans, this syndrome of increased risk of bleeding associated with chronic kidney disease and uremic states is termed uremic thrombocytopenia.

Overt clinical bleeding is often not described in dogs with naturally occurring CKD, however in experimentally induced renal failure, a dramatic increase in buccal mucosal bleeding times (BMBT) has been reported to occur soon after induction of azotemia.\textsuperscript{13} Additionally, despite the lack of clinical bleeding in these patients, BMBT is prolonged and there is a reduction in other markers of platelet function such as platelet glass bead retention (PR) in dogs with CKD.\textsuperscript{13-15} Similar to the findings in humans, dogs with CKD have normal platelet counts and coagulation times despite these platelet function defects.\textsuperscript{13} This suggests that dogs with CKD have a similar uremic thrombocytopenia, however research evaluating the cause underlying platelet dysfunction occurring in dogs is limited.

Previous studies in people have shown uremic platelet dysfunction to be multifaceted. Both inherent platelet defects as well as abnormalities in platelet adhesion to the endothelial wall have been implicated as causes of uremic bleeding in human patients
with CKD.\textsuperscript{16,17} Additionally, concurrent disease states, such as anemia, or the need for antibiotic therapy with $\beta$-lactam antibiotics, can contribute to platelet defects.\textsuperscript{18-20}

Platelets play a central role in hemostasis by forming a hemostatic plug at sites of endothelial damage and initiating the coagulation cascade and fibrin formation. Following a defect in the endothelial wall, functional platelets act by adhering to either collagen in the subendothelial matrix during low shear conditions or to von Willebrand factor (vWF) on the subendothelium during high shear conditions. Collagen and vWF bind to glycoprotein complex GPIa-IIa and GPIb-V-IX respectively, on the platelet surface.\textsuperscript{21,22} This platelet-vessel wall interaction induces a signaling cascade leading to platelet activation. The activation of platelets, as a result of adhesion, leads to a shape change of the platelet cytoskeleton,\textsuperscript{23} granule secretion\textsuperscript{24} and activation of another platelet receptor glycoprotein, GPIIb-IIIa.\textsuperscript{25} Platelet GPIIb-IIIa is a platelet glycoprotein membrane receptor, which is essential for fibrinogen binding and is a major source of platelet linking and aggregation secondary to fibrinogen bridging. Secretion of agonist-containing granules and activation of surface GPIIb-IIIa leads to the attraction and stimulation of additional platelets, amplifying primary aggregation.\textsuperscript{25}

Platelets contain dense and alpha-granules which are released upon platelet activation. Dense granules contain platelet agonists such as ADP, serotonin and calcium.\textsuperscript{24} ADP is an important agonist and is one of the primary amplifiers of initial platelet activation. ADP stimulates two receptors on the surface of the platelet (P2Y\textsubscript{1} and P2Y\textsubscript{12}), which
mediates activation of additional platelets, intracellular calcium mobilization and platelet aggregation. Alpha granules contain large proteins, which function as adhesins (such as vWF and fibronectin), growth factors (such as platelet-derived growth factor), and coagulation factors (Factor V, VII, XI, XIII). Glycoproteins, including P-selectin and CD40-ligand, are located on the membrane of the alpha-granules and are translocated to the platelet surface when the alpha-granule fuses with the platelet membrane during granule secretion. These glycoproteins mediate platelet binding to leukocytes and can be used as a marker of platelet activation.

After the initial platelet plug is formed at the site of endothelial injury, it is stabilized by fibrin formation and cross-linking during secondary hemostasis. The activated platelet membrane exposes phosphatidylserine (PS) and other phospholipids, which provide binding sites for clotting factors and calcium, leading to fibrin generation on the platelet surface. This leads to a stable platelet-fibrin clot.

Numerous intrinsic defects of platelet function have been identified in humans with CKD, including abnormalities of alpha-granules, decreased intracellular platelet agonists such as ADP and serotonin, increased platelet inhibitors such as cAMP, abnormal calcium mobilization and a decrease in TxA2 synthesis. ADP is typically a potent agonist of platelet aggregation. However, in human patients with CKD, ADP-induced platelet aggregation is decreased, with aggregation being more severely affected with increasing uremia. Moreover, total ADP concentration is decreased in platelets of
uremic individuals. A defect in platelet ATP secretion\textsuperscript{33,34} and a reduction in membrane ATPase concentration, which is responsible for secretion of many intracellular molecules in an energy-dependent manner, have also been identified.\textsuperscript{32} Similar to ADP, there is also a decreased platelet concentration of other agonists such as serotonin.\textsuperscript{32,34,35} While platelet ADP and serotonin concentrations are lower in uremic patients, this phenomenon is corrected in individuals undergoing dialysis or in renal transplant patients likely contributing to the normalization in platelet aggregation seen after resolution of uremia.\textsuperscript{32}

Both ADP and serotonin are present in dense granules in the platelet storage pool. Gawatz, et al. found a decreased number of dense granules in end-stage renal failure patients.\textsuperscript{36} Storage pool deficiencies in CKD, either related to reduced ADP or serotonin within dense granules, have correlated with prolonged bleeding times in these patients.\textsuperscript{34} This is similar to inherited storage pool deficiencies or other acquired storage pool deficiencies such as myelodysplastic syndromes, which are also correlated with increased skin bleeding times.\textsuperscript{37}

Normally, intracellular calcium rises upon platelet stimulation. Numerous normal platelet functions are dependent on cytoplasmic calcium, including shape changes, granule secretion and activation. Platelets from uremic patients have been shown to have a reduced intracellular calcium concentration after stimulation with various platelet agonists including ADP, arachidonate and epinephrine. This decrease in agonist-induced calcium burst was associated with decreased aggregation. Unlike other platelet defects,
which are corrected with resolution of uremia, alterations in platelet intracellular calcium flux are not reversed when uremic platelets are suspended in non-uremic plasma.\textsuperscript{38} In opposition to this study showing a defect in calcium mobilization within platelets, other work has shown an increase in total resting platelet calcium concentrations, which resolved after treatment with active vitamin D.\textsuperscript{39} The exact defect in platelet calcium in CKD and the underlying cause is unknown, however alterations in PTH are suspected to play a role.\textsuperscript{40}

Another platelet agonist that is decreased in kidney disease is thromboxane. Normally thromboxane is formed from the release of arachidonic acid from membrane phospholipids by phospholipase A\textsubscript{2} and the actions of cyclooxygenase and thromboxane synthase.\textsuperscript{41} Platelet thromboxane is then secreted and binds to its receptor, propagating platelet activation and aggregation. The stimulatory effects of the thromboxane pathway, however, are not essential to platelet function since other agonists, including ADP, can lead to activation and aggregation despite inhibition of cyclooxygenase.\textsuperscript{42} Several studies have shown a reduction in thromboxane synthesis in human platelets in uremia and decreased aggregation response to arachidonic acid in these patients.\textsuperscript{43,44} This inhibition can be induced in normal platelets incubated in uremic plasma\textsuperscript{44} and, in uremic patients, this thromboxane defect can be reversed with dialysis.\textsuperscript{43} These results are controversial, as other work describes a normal to increased amount of thromboxane in platelets of uremic patients, although reduced aggregation to arachidonic acid and other agonists was still documented.\textsuperscript{33,45}
The decrease in platelet agonists does not appear to be the sole factor in platelet dysfunction in CKD. Platelets in uremic patients have been shown to have increased inhibitors of platelet function such as cAMP, and it is this imbalance between agonists and antagonists within the platelet that contribute to a reduction in platelet function and aggregation. cAMP alone inhibits platelet aggregation \textit{in vitro} and many platelet inhibitors exert their effects through upregulation of cAMP within the platelet.\textsuperscript{46} In health, endothelial-derived prostacyclin increases platelet cAMP, by the action of adenylate cyclase, to inhibit platelet aggregation and keep thrombosis in check.\textsuperscript{47} In uremia, both adenylate cyclase and cAMP levels are increased in platelets,\textsuperscript{48,49} contributing to the decreased aggregation in these patients. Similar to many of the derangements of platelet function found in CKD, both cAMP and adenylate cyclase return to normal levels after dialysis.\textsuperscript{48}

In addition to defects in inherent platelet function, abnormalities in platelet adhesion to both the endothelial wall and to other platelets have been implicated as a cause of bleeding in human patients with CKD. Platelet-vessel wall and platelet-platelet adhesion defects include abnormalities in binding of von Willebrand factor (vWF) and fibrinogen to their receptors as well as abnormalities in surface expression of GPIb and GPIIb-IIIa receptors, which are necessary for vWF and fibrinogen binding, respectively.\textsuperscript{16,17}
Glycoprotein Ib (GPIb) is a receptor for vWF on the surface of platelets and is critical for platelet adhesion to the subendothelium. There are also large intracellular reserve stores of GPIb, which can be more than three times as large as the surface pool, and can be mobilized to the platelet surface. Many studies have shown a reduction in surface expression of GPIb using flow cytometry. This decrease in GPIb expression correlates with severely decreased platelet aggregation and an increase in soluble glycocalicin, a fragment of GPIb, due to proteolytic damage to GPIb on the platelet surface. GPIb expression is inversely correlated with serum creatinine concentration and the reduction in GPIb expression is not improved with dialysis, which may be due to whole platelet depletion of the molecule. Sloand, et al showed that uremia causes a reduction in total platelet GPIb, however surface GPIb levels were only mildly decreased, suggesting that membrane expression of GPIb can be maintained at a constant level by mobilization of intracellular stores until depletion.

Glycoprotein IIb-IIIa (GPIIb-IIIa) is a platelet fibrinogen receptor and is integral for platelet-platelet adhesion and aggregation. When a platelet is activated, GPIIb-IIIa undergoes a conformational change to its active state, where it can bind fibrinogen and soluble vWF. In uremia, the total number of surface GPIIb-IIIa is normal to increased. However, the activation and function of GPIIb-IIIa, and its ability to bind fibrinogen and vWF, are decreased. This reduced binding ability may be secondary to a dialyzable uremic toxin since these effects are reversed with dialysis, or due to receptor occupation by fibrinogen fragments.
von Willebrand factor is an important glycoprotein which binds to collagen in the subendothelial matrix and plays a large role in platelet-vessel wall adhesion during endothelial wall defects by binding to the GPIb receptor on the platelet membrane. Plasma vWF also binds to platelet GPIIb-IIIa to mediate platelet-platelet interactions, which can occur weakly, even in the absence of fibrinogen. Plasma vWF has been shown to be normal to increased in uremic patients. While plasma vWF can be increased, platelet vWF, which is stored and released from alpha-granules upon activation, has been shown to be decreased in uremia. Despite normal serum vWF concentrations, adhesion defects between vWF and GPIIb-IIIa have been shown. However, adhesion defects between vWF and GPIb are rarely found. It has been hypothesized that this functional defect between vWF and GPIIb-IIIa is compensated for by the higher than normal plasma vWF found in uremic patients, since platelet adhesion is worsened when the elevated vWF is normalized. A compensatory effect of increased vWF is also shown by the improvement of bleeding times and normalization of platelet aggregation with the addition of cryoprecipitate and desmopressin.

Concurrent disease states, such as anemia, can also contribute to platelet defects in CKD patients. During normal blood flow, erythrocytes displace platelets toward the vessel wall increasing platelet contact with the subendothelium at sites of injury. Erythrocytes also enhance platelet activation by releasing ADP. A negative correlation between hematocrit and bleeding time has been shown in patients with CKD.
and platelet aggregation are significantly improved with increasing the hematocrit to greater than 30% in uremic individuals.\textsuperscript{64} Besides the positive impact on hemostasis by improving hematocrit, treatment with recombinant human erythropoietin (rHuEPO) also has a direct effect on thrombosis by increasing platelet aggregation independently of hematocrit.\textsuperscript{65,66}

It has long been hypothesized that the abnormalities of platelet function and adhesion in patients with CKD was due to small dialyzable compounds found in the serum of uremic individuals. Evans, \textit{et al.} found decreased platelet aggregation of platelets from healthy individuals when uremic plasma was added \textit{in vitro}.\textsuperscript{6} Additionally, bleeding times and many of the underlying platelet abnormalities observed in uremia are reversed by dialysis.\textsuperscript{16,32,33,67} Initially blood urea nitrogen (BUN) was theorized to be the major uremic toxin implicated in platelet defects. In 1969, Eknoyan, \textit{et al.} described an inverse relationship between platelet adhesiveness and BUN.\textsuperscript{68} Additionally, ingestion of urea prolonged bleeding time and reduced platelet adhesiveness in otherwise healthy individuals.\textsuperscript{68} These results have not been repeatable, however, with many studies finding no correlation between platelet defects or aggregation and urea\textsuperscript{6,32} even when oral urea was used to experimentally induce a uremic state.\textsuperscript{32} Furthermore, in a study of a rare inherited condition of familial azotemia where individuals have unexplained defects in urea excretion with otherwise normal renal function, platelet function and bleeding times were normal.\textsuperscript{69} Based on these reports it appears that another compound other than urea is involved with platelet defects described in CKD.
PTH has also been theorized as a uremic toxin inhibiting platelet function in CKD. PTH has been shown to inhibit platelet aggregation and platelet secretion of serotonin \textit{in vitro} in humans.\textsuperscript{40,70} However, no correlation was found between bleeding time and PTH or PTH fragments \textit{in vivo}, suggesting that PTH does not play a role in platelet dysfunction in uremia.\textsuperscript{71}

Many other uremic toxins have been identified in human patients with kidney disease.\textsuperscript{72} Several of these toxins have also been implicated in dysfunction of primary hemostasis. Increases of phenolic acids and guanidinosuccinic acid (GSA) have been shown to inhibit \textit{in vitro} platelet aggregation.\textsuperscript{73,74} Dietary phenolic acids, such as hippuric acid, have been shown to inhibit \textit{in vitro} platelet aggregation at supra-physiologic concentrations. Additionally, some of these phenolic acids also inhibited platelet P-selectin expression. Ostertag, et al. concluded that it is unlikely that the concentrations of phenolic compounds required to inhibit platelet function in this \textit{in vitro} study could be achievable through dietary ingestion, however it is unclear if these concentrations could be reached in patients with significant renal dysfunction.\textsuperscript{75} Dialysis has been shown to reduce the serum levels of GSA and phenolic acids in uremic patients with improvement in platelet function.\textsuperscript{73,74} Although while GSA has been shown to prolong bleeding times when injected into mice,\textsuperscript{76} bleeding time or increased incidence of clinical bleeding has not been directly correlated with GSA or phenolic acids in uremic individuals.\textsuperscript{77}
Platelet function can be evaluated with both *in vivo* and *in vitro* testing. One of the earliest used tests of platelet function is *in vivo* bleeding time. Ivy and Duke methods of bleeding time exist in humans where a small cut is made on the forearm and the time until bleeding stops is recorded. A similar method exists in dogs called the buccal mucosal bleeding time (BMBT). Abnormalities in bleeding time can be seen with thrombocytopenia, anemia, platelet or endothelial cell dysfunction and von Willebrand disease (vWD). Assessment of bleeding time is a simple and cost effective method to assess platelet function; however there is poor reproducibility and is prone to error in both humans and dogs. In humans with CKD, bleeding time is often prolonged and has been useful to assess which patients are likely to develop clinical bleeding, however bleeding times have had poor correlation with *in vitro* tests of platelet function such as aggregation. Additionally, there is poor correlation with bleeding times and BUN, creatinine or glomerular filtration rate (GFR). While there is no correlation between bleeding time and markers of azotemia including BUN and creatinine, bleeding time normalizes after dialysis.

Platelet aggregometry is used extensively as an *in vitro* test of platelet function and response to specific agonist stimulation. Platelet aggregation is assessed by either the amount of light transmitted in platelet-rich plasma in turbidimetric aggregometry or electrical impedance in whole blood in impedance aggregometry. While turbidimetric aggregometry is considered the gold standard for evaluation of platelet function, electrical impedance whole blood aggregometry correlates well with this test.
Aggregometry can be used to detect a wide range of platelet defects including inherited platelet function defects,\textsuperscript{89} von Willebrand disease,\textsuperscript{90} acquired platelet function defects\textsuperscript{91} and drug monitoring.\textsuperscript{92} Aggregation studies in uremia have variable results. Platelet aggregation has been shown to be decreased\textsuperscript{6,32,33} or normal\textsuperscript{38,84} in response to various agonists. When aggregometry is decreased, it has been shown to improve with dialysis.\textsuperscript{33} There is poor correlation of platelet aggregometry in uremia with bleeding times,\textsuperscript{84} which correspond with clinical bleeding,\textsuperscript{86} so aggregometry may be an insensitive marker of platelet dysfunction in CKD.

With bleeding times and aggregometry being unreliable markers of platelet dysfunction, invasive and technically difficult to perform in clinical patients, respectively, newer point-of-care tests of platelet function have been developed. The Platelet-Function Analyzer-100 (PFA-100) is a bench-top analyzer of primary hemostasis under high shear rate, mimicking \textit{in vivo} conditions of platelet plug formation. Whole blood is aspirated through an aperture in a collagen membrane, which is coated with either ADP or epinephrine agonists to stimulate platelet aggregation. The time until full closure of the aperture is recorded and reported as the closure time (CT).\textsuperscript{80} The PFA-100 has been used in humans as a screening test to identify defects in primary hemostasis\textsuperscript{78} and has been validated for use in dogs\textsuperscript{93} and cats.\textsuperscript{94} In people, the PFA-100 has been shown to be more sensitive than aggregometry\textsuperscript{95,96} and skin bleeding time\textsuperscript{97} for detecting von Willebrand disease and similar to aggregometry and skin bleeding time for detecting platelet dysfunction.\textsuperscript{96,97} A linear correlation between the PFA-100 closure times and bleeding
times has been reported in individuals with von Willebrand disease and platelet dysfunction. However, other studies have shown poor correlation between PFA-100 closure times, bleeding times and aggregometry.

PFA-100 closure times are prolonged in up to 75% of humans with end-stage renal disease and can be used to detect platelet dysfunction in CKD which has been documented with other tests of primary hemostasis. Additionally, more patients were identified with platelet dysfunction using the PFA-100 than with aggregometry or skin bleeding time. The prevalence of abnormal PFA-100 closure times increases with the stage of CKD, and PFA-100 closure times are correlated with serum concentrations of BUN and creatinine. Similar to what has been documented in uremic patients with prolonged skin bleeding times, abnormal PFA-100 closure times normalize after dialysis.

Despite the high frequency of prolonged PFA-100 closure times in patients with renal disease, PFA-100 closure times in general are poor at predicting bleeding complications in patients with CKD after renal biopsy or renal transplant. In fact, the degree of azotemia was better correlated with clinically significant bleeding and the need for a transfusion after renal biopsy. However, a prolonged PFA-100 was more likely to correlate with severe bleeding when compared with skin bleeding times. Despite this, the PFA-100 appears to have a poor ability to detect clinically significant defects in hemostasis.
In recent years, viscoelastic point-of-care instruments, such as thromboelastography (TEG), have become widely used to evaluate global coagulation to detect hyper- and hypocoagulability. These instruments detect changes in the viscosity of blood during clot formation. These viscosity changes are translated to a pin connected to a torsion wire. This transmits the torque placed on the pin as clot formation occurs and records several measurements including R, K, $\alpha$, maximum amplitude (MA), G and clot lysis at 30 and 60 minutes (LY30/LY60).

The R, or reaction time, is the time it takes for initial fibrin formation and corresponds with coagulation factor function but has been shown to have variable correlation with standard methods of coagulation factor testing such as prothrombin time (PT) and activated partial thromboplastin time (aPTT). The K time and alpha ($\alpha$) angle measure the speed of clot formation and are affected by fibrinogen, factor XIII, which facilitates fibrin cross-linking, and to a lesser degree platelets. While the angle corresponds with fibrinogen function, it does not always correlate with fibrinogen concentration.

The maximum amplitude (MA) represents clot strength and assesses fibrin and platelet binding and the G, which is calculated from the MA, is another indicator of clot strength. The MA correlates well with platelet count, and, to a lesser degree, fibrinogen concentration. However conflicting reports exist on the use of MA in detecting platelet dysfunction with some studies showing a decreased MA consistent with platelet
dysfunction\textsuperscript{110} with others showing no correlation between MA and platelet function.\textsuperscript{111} In general, the TEG does not appear to be a sensitive indicator of impaired platelet function.\textsuperscript{112} However a new application of TEG, called TEG platelet mapping, is a method where four TEG tracings are performed using different platelet agonists in heparinized blood to eliminate the influence of thrombin.\textsuperscript{107} Platelet mapping has been used in monitoring antiplatelet therapies in dogs and correlates well with aggregometry\textsuperscript{113} and therefore may be a better indicator of platelet function than traditional TEG.

When compared to the PT and aPTT, the TEG variables are a more sensitive indicator of hypercoagulable states in people.\textsuperscript{114} Similarly, TEG has been used to detect hypercoagulability in many disease states in dogs.\textsuperscript{115-118} The sensitivity for the TEG to detect hypocoagulability is also superior to PT and aPTT with a better ability to detect clinical bleeding in various hypocoaguable states including hemophilia A.\textsuperscript{119,120} However, TEG tracings are normal in patients with vWD unless severe Factor VIII deficiency is present, likely due to the high shear rate needed for vWF-platelet binding which is not present in a static assay such as TEG.\textsuperscript{107}

In patients with CKD, TEG tracings often appear hypercoagulable with an increased MA, G and alpha angle and a shortened K.\textsuperscript{85,121,122} However, an increased fibrinogen and reduced hematocrit are also found in uremic patients and these findings correlate with the elevated MA in these individuals.\textsuperscript{121} Hypocoagulability in uremic patients has also been documented using TEG with resolution of the hypocoagulable tracing after
administration of DDAVP similar to previously reported normalization of bleeding times with DDAVP administration. Darlington, et al. highlighted the variable TEG tracings observed in CKD, describing some patients as having a hypocoagulable state as indicated by an increased R time, others showing an increase in platelet and fibrin binding and increased aggregation with an increased MA, and others having decreased aggregation as evidenced by a decreased MA indicating that defects in both primary and secondary hemostasis occur in CKD.

While the TEG is a poor indicator of platelet function, the TEG has been found to be a better predictor for clinical bleeding than other tests of platelet function including bleeding time and platelet counts. TEG tracings indicating hypocoagulability, primarily a prolonged K time and decreased angle, are associated with an increased risk of bleeding after renal biopsy. And while the sensitivity of the TEG to detect patients with an increased risk of bleeding is similar to bleeding times, the specificity is greater indicating that patients with abnormal TEG values are at an increased risk of hemorrhage compared to those with normal TEG tracings. These findings have lead to the use of the TEG in clinical settings to guide transfusion requirements. Similar findings have been demonstrated in dogs using tissue factor activated TEG where the G value was more accurate than PT and aPTT for detecting clinical bleeding.

Few studies have been conducted to evaluate platelet function in dogs with CKD. Similar to humans, platelet aggregometry appears variably affected in natural or induced
uremic states with normal, increased and decreased aggregation all being reported in uremic dogs.\textsuperscript{13,15,128} However, BMBT and older methods of evaluating platelet function and adhesion, such as the platelet glass bead retention assay, are abnormal in uremic dogs suggesting defects in platelet adhesion rather than platelet dysfunction as a cause of uremic thrombocytopenia in dogs.\textsuperscript{13,14} Also similar to human studies, plasma vWF structure and function is unaltered in azotemic states and the concentration of this integral hemostatic protein is elevated in uremic dogs.\textsuperscript{129} While the cause of uremic bleeding in dogs is suspected to be a multifaceted dysfunction of platelets and platelet-vessel wall interaction similar to humans, the literature on platelet function in dogs with CKD is lacking.

The aim of this study is to evaluate platelet function in dogs with CKD using two point-of-care hemostatic analyzers, the PFA-100 and thromboelastography (TEG). We also aim to evaluate platelet receptors of vWF and fibrinogen, GPIb and GPIIb-IIIa respectively, and surface platelet P-selectin expression, as a marker of platelet activation, with flow cytometry in dogs with CKD to elucidate the cause of the underlying primary hemostatic defect in this species. We hypothesize that platelet function will be decreased in dogs with CKD compared to clinically healthy dogs and can be explained by platelet receptor glycoprotein expression.
Chapter 2: Methods

Patient enrollment

Dogs being presented to the Ohio State University Veterinary Medical Center (OSU-VMC) and diagnosed with CKD based on IRIS guidelines, including documented azotemia for greater than 2 months duration, were recruited for enrollment. Dogs were classified into respective IRIS Stage (I-IV). Baseline complete blood count (CBC) with platelet count, serum chemistry, urinalysis, urine protein:creatinine ratio and systolic blood pressure via Doppler were performed to classify patients based on their IRIS stage and ensure no other concurrent disease known to affect coagulation status was present prior to entry into the study.

Clinically healthy control dogs were recruited from students, faculty and staff of the OSU-VMC. Control dogs were deemed healthy based on history, physical examination and minimum database of CBC, serum chemistry, urinalysis, urine protein:creatinine ratio and systolic blood pressure.

Dogs were excluded from enrollment in either group if any of the following were identified: mild to moderate thrombocytopenia (platelet count < 100,000/µL), moderate anemia (classified as a hematocrit of less than 25%) or if receiving medications that may
impact their coagulation status (including glucocorticoids, non-steroidal anti-inflammatory medications, hetastarch, heparin, clopidogrel or darbepoetin). Dogs with either documented or suspected non-renal systemic illness, including diseases that have been associated with hypo- or hypercoagulability, such as immune-mediated hemolytic anemia, hyperadrenocorticism or neoplasia, were also excluded from enrollment.

Platelet function and global coagulation status was evaluated on CKD and healthy control dogs with the PFA-100 and thromboelastography (TEG), respectively. Additional coagulation testing, including prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, antithrombin and d-dimers, were performed on all CKD and healthy control dogs. Platelet receptor expression and platelet activation were evaluated on all CKD and control dogs using flow cytometry. Blood was collected for all testing via a single jugular venipuncture using a 21-gauge butterfly needle attached to a vacutainer directly into blood collection tubes. Blood was initially collected into a 10 mL additive-free blood tube and then into 4.5 mL tubes containing 3.2% sodium citrate followed by a 2 mL tube containing 7.5% EDTA. All urine samples were obtained either by midstream free catch or cystocentesis.

Minimum Database

All dogs had a hemogram performed by an automated hematology analyzer and manual blood smear review. Serum biochemistry was performed using an automated chemistry analyzer. Standard urinalyses were performed and included urine specific gravity by
refractometer measurement, urine dipstrip analysis and microscopic sediment examination. Urine protein and urine creatinine concentrations were determined by the turbidimetric and Jaffé methods, respectively, by an automated chemistry analyzer.

Coagulation Analysis

Coagulation testing, including PT, aPTT, fibrinogen and antithrombin (AT) was performed on citrated plasma from all CKD and control dogs. Prothrombin time, aPTT and fibrinogen values were determined by use of an automated mechanical clot detection instrument. Plasma AT activity was measured with a chromogenic test using the same automated instrument. Activities of plasma AT were reported as a percentage of a standard, which was assigned a value of 100% activity. Plasma d-dimer concentration was measured using a semi-quantitative rapid latex slide agglutination test in accordance with manufacturer’s instructions.

Evaluation of Platelet Function

PFA-100

The PFA-100 has previously been evaluated for use in dogs as a commercial, point-of-care, platelet function analyzer. The PFA-100 is an in vitro platelet function analyzer that records the closure time, in seconds, needed to form a platelet plug after activation by either collagen and epinephrine (Col+EPI) or collagen and ADP (Col+ADP) platelet agonists. Briefly, 800 µl of citrated whole blood is added to a cartridge containing a membrane coated in either Col+ADP or Col+EPI platelet agonists. The citrated blood is
aspirated at a high shear rate through an aperture in the membrane and the time taken to stop blood flow through the aperture is recorded as the closure time (CT).

Both Col+ADP\(^b\) and Col+EPI\(^i\) cartridges were used to evaluate platelet function in CKD and control dogs. Samples were run in duplicate and averaged to obtain the mean closure time for the individual dog. The CT reference range used by our laboratory is 52-86 seconds for the Col+ADP agonist and 97-225 seconds for the Col+EPI agonist. The analyzer can detect a maximum closure time of up to 300 seconds, and samples exceeding the maximum closure time were recorded as 300 seconds. All samples were analyzed within 1 hour of collection.

*Thromboelastography (TEG)*

Re-calcified, non-activated TEG analysis was performed in each dog as described in a previous study.\(^1\)\(^3\)\(^0\) Briefly, 20 µL of CaCl\(_2\) was placed in a pre-warmed cup (37 °C) of the TEG-5000\(^j\) and 340 µL of citrated whole blood added to make a total volume of 360 µL. A single TEG tracing per dog was obtained after 120-180 min of running time. In each dog the following TEG parameters were recorded: reaction time [R], clotting time [K], \(\alpha\) angle, maximum amplitude [MA] and calculated G value. Reference ranges for each of these have been previously established by our hospital using this methodology of re-calcified, non-activated citrated whole blood TEG.
Evaluation of platelet receptor expression and platelet activation

**GPIb expression**

Expression of platelet membrane GPIb was evaluated using flow cytometry with a FACSCalibur flow cytometer, similar to previously described methods using canine platelets. Briefly, platelet-fixed, erythrocyte depleted blood was obtained by the addition of 2mL of a 1:10 dilution of erythrocyte lysis buffer containing 0.5% paraformaldehyde to 100 µl of citrated whole blood and incubating at 25°C for 15 minutes. Erythrocyte depleted blood was washed with FACS buffer which was filtered through a 0.45 µm pore filter directly before use. The erythrocyte-depleted blood was centrifuged at 435g at 8°C for 15 minutes and the platelet pellet was resuspended in 100 µl of FACS buffer. The platelet suspension was incubated with 50 µl of a murine, fluorescein isothiocyanate (FITC)-labeled, anti-human CD42 monoclonal antibody for 30 minutes in the dark at room temperature. Platelets were washed with FACS buffer, centrifuged at 435g at 8°C for 15 minutes and resuspended in 500 µl of FACS buffer.

**GPIIb-IIIa expression**

Platelet membrane GPIIb-IIIa expression was evaluated using flow cytometry as described above and similar to previously described methods. 100 µl of washed, erythrocyte-depleted blood was incubated with 50 µl of a murine FITC-labeled anti-human CD61 monoclonal antibody for 30 minutes in the dark at room temperature. Platelets were washed with FACS buffer, centrifuged at 435g at 8°C for 15 minutes and resuspended in 500 µl of FACS buffer.
Platelet activation was assessed by surface platelet P-selectin expression as previously described.\textsuperscript{28,131,133} Erythrocyte-depleted blood was incubated with 100 µl of a 1:100 dilution of affinity-purified, monoclonal mouse anti-canine P-selectin antibody\textsuperscript{p} for 30 minutes at room temperature. Platelets were washed, centrifuged at 435g at 8°C for 15 minutes and resuspended in 100 µl of FACS buffer. 100 µl of a 1:150 dilution of FITC-labeled goat anti-mouse antibody\textsuperscript{q} was added and incubated for 30 minutes in the dark at room temperature. After incubation, platelets were washed and resuspended in FACS buffer.

All flow cytometry samples were stored in the dark at 4°C prior to analysis. A total of 5,000 gated events were recorded for each surface marker and displayed on a log forward scatter vs. log side scatter plot. The platelet population was gated, excluding platelet aggregates and non-lysed erythrocytes (Figure 1). A histogram plot was constructed from the gated platelet population with log fluorescence intensity on the x-axis and platelet number on the y-axis. Surface expression of GPIb, GPIIb-IIIa and P-selectin were quantified by the intensity of antibody staining and expressed as mean fluorescence intensity (MFI).
Statistical Analysis

Data was evaluated for normality with the Kolmogorov-Smirnov and Shapiro-Wilk tests. PFA-100 CT, TEG variables, and platelet surface receptor expression were compared between control and CKD dogs using the Student’s t-test for normally distributed data and the Mann-Whitney Ranked Sum test for nonparametric data. Spearman’s rank correlation coefficient was performed to evaluate the correlation between PFA-100 CT, TEG variables and platelet surface receptor expression in CKD dogs. A Spearman’s rank correlation coefficient was also performed to evaluate the correlation between PFA-100 CT, TEG variables and other coagulation and minimum data base variables. All data was analyzed using a commercially available statistical software package\textsuperscript{5}. A p-value of 0.05 was used for statistical significance.
Chapter 3: Results

Patient Demographics

There were 11 CKD dogs enrolled. There were eight different breeds represented with Mixed Breed (n=3) and Labrador Retrievers (n=2) being the most commonly represented breeds. There was one each of the following breeds: Yorkshire Terrier, Puli, American Cocker Spaniel, Jack Russell Terrier, Boxer and Shih Tzu. The median age of CKD dogs was 9.7 years (range 2.6-15.8 years) and the median body weight was 8.7 kg (range 2.7-45 kg). 10 out of 11 (91%) CKD dogs were classified as IRIS Stage III with 1 dog classified as IRIS Stage IV. No dogs with CKD IRIS Stage I or II were enrolled.

There were 10 healthy control dogs with four different breeds represented. There were four Mixed Breed dogs, three Golden Retrievers, two American Pit Bull Terriers and one Doberman Pinscher. The median age of control dogs was 4.4 years (range 1.2-10.3 years). The median body weight was 26 kg (range 17-41.3 kg). Control dogs were significantly younger (p = 0.01) and weighed significantly more (p=0.01) than CKD dogs.
Minimum database

Select biochemistry, CBC, urinalysis and UPC values in CKD and healthy dogs are summarized in Table 1. Dogs with CKD had significantly elevated BUN (p <0.0001) and creatinine (p<0.0001) and significantly decreased urine specific gravity (p<0.0001) when compared with controls. The UPC was significantly elevated in CKD dogs compared with control dogs (p =0.001), and the hematocrit in CKD dogs was significantly decreased compared to healthy dogs (p<0.0001). One CKD dog had a normal platelet count two days prior to study inclusion, but a platelet count was not recorded on the day of enrollment, therefore this dog was not included in statistical analysis of platelet count or mean platelet volume (MPV). There was no difference in platelet count or MPV between healthy and CKD dogs (p=0.57 and p=0.08, respectively).

Coagulation Analysis

Median prothrombin time was 7.6 seconds (range 6.8-8.3s) in control dogs and was 7.7 seconds (range 5.3-8.7s) in CKD dogs. Median activated partial thromboplastin time (aPTT) in control dogs was 10.45 seconds (range 9.3-12.2s) and was 10.8 seconds (range 9.7-16.0s) in CKD dogs. No difference between PT or aPTT was found between the two groups (p = 0.49 and 0.5, respectively) (Figure 2). Median fibrinogen concentration for control dogs was 179.5 mg/dL (range 99.0-313.0 mg/dL) and 315.0 mg/dL (range 183.0-821.0 mg/dL) in CKD dogs. Fibrinogen concentration was significantly elevated in CKD dogs compared to healthy controls (p = 0.001) (Figure 3). No difference in plasma antithrombin activity was found between control (median 136%; range 125-158) and
CKD dogs (median 130%; range 107-139) (p = 0.3). All of the control dogs had negative d-dimers. Four of 11 (36%) dogs with CKD had weakly positive d-dimers. Two of these four CKD dogs had positive d-dimers between 100-200 ng/mL and 2/4 had positive d-dimers between 200-400 ng/mL. No dogs were strongly positive for the presence of d-dimers.

**PFA-100**

The median PFA-100 closure time with collagen and ADP agonists (Col+ADP) was 57.25 seconds (range 51.0 – 90.5) in healthy controls (n = 10) and 83.0 seconds (range 54.0-150.5) in CKD dogs (n=11). The PFA-100 Col+ADP CT was significantly prolonged in CKD dogs when compared to control dogs (p < 0.01) (Figure 4). The median PFA-100 closure time with collagen and epinephrine agonists (Col+EPI) was 136.3 seconds (range 88.0-300.0) in healthy controls (n=10) and 106.0 seconds (range 86.5 – 300.0) in CKD dogs (n=9). Two CKD dogs did not have PFA-100 Col+EPI CT recorded due to analyzer malfunction and were not included in statistical analysis. There was no difference in PFA-100 Col+EPI CT between control and CKD dogs (p= 0.41) (Figure 5).

PFA-100 Col+ADP CT were outside the reference range in 4/11 CKD dogs, all of these dogs had prolonged CT. Three of the remaining seven dogs with Col+ADP CT within the reference range, had values in the upper third of the reference range. No dog had a Col+ADP CT below the reference range. Two of the control dogs had PFA-100
Col+ADP CT outside the reference range. One of these dogs had a shortened Col+ADP CT at 51 seconds and one dog had a prolonged Col+ADP CT at 90.5 seconds.

PFA-100 Col+EPI CT were outside the reference range in 2/9 CKD dogs; one dog had a shortened CT at 86.5 seconds and one had a prolonged CT at 300 seconds. Two control dogs had Col+EPI CT outside the reference range with one control dog having a prolonged CT at 300 seconds and one having a shortened closure time at 88 seconds.

There was a strong positive correlation between PFA-100 Col+ADP CT and PFA-100 Col+EPI CT (r = 0.8; p = 0.01). No correlation exists between PFA-100 Col+ADP or Col+ERI CT and BUN, creatinine, platelet count or HCT.

Thromboelastography (TEG)

TEG data for CKD and control dogs is summarized in Table 2. CKD dogs had significantly increased MA and G values compared to healthy control dogs (p = 0.008 and p = 0.01, respectively). There was a significant decrease in the K-time in CKD dogs compared with healthy controls (p = 0.035). There was no difference between CKD and control dogs for any other TEG variable.

All of the control dogs and 10/11 of the CKD dogs had an R-time within the reference range established by our laboratory. One CKD dog had an R-time outside the reference range, which was prolonged at 8.9 seconds indicative of hypocoagulability. This dog also
had prolonged PFA-100 CT for both Col+ADP and Col+EPI. No dog had an R time below the reference range. All of the control dogs and 10/11 of the CKD dogs had a K-time within the reference range. One CKD dog had a decreased K-time of 0.8 seconds, consistent with hypercoagulability. No dogs had an increased K-time.

Two of the CKD dogs had angle values outside of the reference range. In one CKD dog, the angle was prolonged at 78.3 degrees suggestive of hypercoagulability and in one dog the angle was decreased at 46.6 degrees suggestive of hypocoagulability. Six of the remaining nine dogs that had angle values within the reference range were within the upper third of the reference range. One control dog had an angle below the reference range at 47.8 degrees. The remaining nine control dogs had angle values within the reference range.

MA values were increased consistent with hypercoagulability in 6/11 CKD dogs and were in the upper third in 4/5 CKD dogs that had MA values within the established reference range. None of the CKD dogs had MA values below the reference range. One control dog had an MA value increased from the reference range at 70.7 mm. The remaining nine control dogs had an MA value within the reference range. Similar to MA values, 6/11 CKD dogs had an increased G value consistent with hypercoagulability and 3/5 CKD dogs had G values in the upper third of the reference range. One control dog had an increased G value and 9/10 control dogs had a G value within the reference range.
A strong negative correlation exists between K-time and $\alpha$-angle ($r = -0.8; \ p = 0.002$) and between K-time and platelet count ($r = -0.81; \ p = 0.003$) but no correlation exists between $\alpha$-angle and platelet count. Platelet count was not correlated with any other TEG variable. A negative correlation was identified between HCT and $\alpha$-angle ($r = -0.61; \ p = 0.049$). HCT was not correlated between any other TEG variable. A strong negative correlation exists between the $\alpha$-angle and PFA-100 Col+EPI CT ($r = -0.75; \ p = 0.02$) but not with PFA-100 Col+ADP CT. R-time was strongly correlated with PFA-100 Col+EPI CT ($r = 0.77; \ p = 0.02$) and moderately correlated with PFA-100 Col+ADP CT ($r = 0.62; \ p = 0.04$). No other correlations were identified between either PFA-100 CT and any other TEG variable.

TEG MA and G values were significantly positively correlated with each other ($r = 1.0; \ p < 0.0001$) but not with any other TEG variable. A moderate positive correlation exists between MA and G values and serum creatinine concentration ($r = 0.63; \ p = 0.04$) and a strong positive correlation exists between MA and G values and plasma fibrinogen concentration ($r = 0.77; \ p = 0.007$). Degree of azotemia was not correlated with any other TEG variable. No correlation was found between MA or G values and HCT, platelet count or PFA-100 Col+ADP and Col+EPI CT.
Platelet receptor expression

**GPIb expression**

There was no difference in platelet GPIb MFI in dogs with CKD (median 6.16 MFI; range 3.26-12.69) and platelet GPIb MFI in healthy control dogs (median 5.12 MFI; range 3.93-7.47) (p = 0.09) (Figure 6).

No correlation exists between GPIb MFI and Col+ADP CT, Col+ EPI CT, any TEG variable or platelet GPIIb-IIIa MFI. Additionally, no correlation exists between serum BUN and creatinine concentration and GPIb MFI. A moderate positive correlation was found between platelet GPIb MFI and P-selectin MFI (r = 0.67; p = 0.03). A strong positive correlation was also found between GPIb MFI and mean platelet volume (MPV) (r = 0.78; p = 0.01).

**GPIIb-IIIa expression**

Platelet GPIIb-IIIa MFI was significantly higher in dogs with CKD (median 145.5 MFI; range 8.74-452.4) than in healthy control dogs (median 19.35 MFI; range 13.76-31.91) (p = 0.01) (Figure 7). The GPIIb-IIIa fluorescence histogram showed two populations of platelets in all dogs, both with and without CKD (Figure 8). Dogs with CKD had a significantly higher percentage of platelets in the higher fluorescence intensity subpopulation (median 54.9%; range 2.38-96.08) than healthy dogs (median 14.21%; range 11.32-21.32) (p = 0.007).
A moderate positive correlation exists between platelet GPIIb-IIIa MFI and serum creatinine in CKD dogs ($r = 0.66$; $p = 0.03$) but not between GPIIb-IIIa MFI and serum BUN concentration. No correlation exists between GPIIb-IIIa MFI and Col+ADP CT, Col+ EPI CT, any TEG variable or between platelet GPIIb-IIIa MFI and either platelet P-selectin MFI or platelet GPIb MFI.

**P-selectin expression**

Platelet P-selectin MFI in dogs with CKD (median 24.56 MFI; range 11.97-51.38) was significantly higher than platelet P-selectin MFI in healthy control dogs (median 20.08; range 14.0-23.96) ($p = 0.03$) (Figure 9).

No correlation was found between PFA-100 Col+ADP CT, Col+EPI CT or any TEG variable and platelet P-selectin expression in CKD dogs. A moderate positive correlation was found between platelet P-selectin MFI and GPIb MFI ($r = 0.67$; $p = 0.03$) but not between P-selectin MFI and GPIIb-IIIa MFI. A strong negative correlation was found between platelet P-selectin MFI and PT values ($r = -0.71$; $p = 0.02$). No correlation exists between serum BUN and creatinine concentration and P-selectin MFI.
Chapter 4: Discussion

This study provides evidence of platelet dysfunction as well as global changes in coagulation in dogs with CKD. Dogs with CKD have defects in platelet function as indicated by the significantly prolonged PFA-100 Col+ADP CT in these patients. People with end-stage renal disease have been shown to have platelet dysfunction with between 14-60% of patients reported to have prolonged closure times.\(^9^9,1^0^3\) This is similar to what was found in the present study where 36% of dogs with CKD had a PFA-100 Col+ADP CT outside the reference interval.

Similar to what has been documented in people with renal disease,\(^9^9\) there was a strong correlation between the Col+ADP and Col+EPI closure times in CKD dogs in our study. However, we found no difference in closure times between healthy dogs and dogs with CKD when using Col+EPI as the platelet agonist. The reference range for Col+EPI CT in dogs is wide and the use of this activator with the PFA-100 has been previously shown to have a large degree of variability in dogs.\(^9^3\) Epinephrine is known to be a weak platelet agonist in dogs\(^1^3^4,1^3^5\) which may contribute to the variability seen in closure times when using this in dogs. Because of the variability of epinephrine to stimulate platelet activation, the PFA-100 Col+EPI activator may not be useful for monitoring platelet function in dogs.
Anemia has been shown to cause prolongation of the PFA-100 CT in humans and dogs. In humans, the PFA-100 CT will become prolonged once the hematocrit falls below 25% and the CT will become unreadable at a hematocrit below 10%. A previous study in dogs identified a statistically significant prolongation in PFA-100 Col+ADP CT as the hematocrit was diluted in vitro from 35% to 27%. However, although a statistically significant prolongation was found, the closure times were likely not clinically significant. In human patients with end-stage renal disease on hemodialysis, PFA-100 CTs have been found to be negatively associated with HCT with the affect of hematocrit on CT in these patients lost above 35%. However, other studies in humans with CKD have shown no correlation between hematocrit and closure time. The effect of hematocrit on primary hemostasis has been previously highlighted in dogs with experimentally induced renal failure whose prolonged BMBT improved with red blood cell transfusion alone and this phenomenon has been well established in people with naturally-occurring CKD. In the present study, dogs with CKD had a significantly lower HCT than control dogs, however only 2/11 CKD dogs had a hematocrit below 30%. We found no correlation between HCT and PFA-100 CT in our patients with CKD so hematocrit does not appear to be playing a role in the prolonged closure times identified in these patients.

A previous study by Kim, et al. showed a correlation between BUN and creatinine in people with kidney disease and both Col+ADP and Col+EPI closure times with the
percentage of patients with abnormal closure times increasing with the severity of azotemia.\textsuperscript{102} However, a different study in people found poor correlation with GFR and creatinine and no correlation with BUN and other tests of primary hemostasis including skin bleeding time and aggregometry.\textsuperscript{85} We found no correlation between BUN and creatinine and Col+ADP or Col+EPI closure times in the study presented here. To the authors’ knowledge, this is the first time that PFA-100 closure times have been evaluated in dogs with CKD, and all of the dogs presented here were in IRIS stage 3 or 4 CKD. Further studies in dogs across all IRIS stages of CKD are needed to better correlate the degree of azotemia with PFA-100 closure times and to evaluate if the percentage of CKD dogs with prolonged closure times increases with increasing IRIS stage.

Despite finding a significant increase in PFA-100 Col+ADP CT in dogs with CKD, 64% of the dogs in this study still had closure times within the normal reference range. In humans with CKD, there is poor correlation between PFA-100 closure times and skin bleeding times\textsuperscript{85} and the PFA-100 analyzer is unable to predict which individuals will have bleeding complications after renal biopsy.\textsuperscript{103} We did not evaluate for clinical bleeding complications in dogs with CKD in our study so a correlation between PFA-100 closure times and clinically relevant bleeding in dogs with CKD remains unknown. However, with such a large percentage of dogs in our study having closure times within the normal reference range, this suggests that while statistically significant, it may be of little clinical relevance. Instead, monitoring PFA-100 Col+ADP CT trends in an
individual CKD patient may be of more utility than a single point-in-time measurement to detect platelet dysfunction in these patients.

The present study also identified whole blood coagulation defects in dogs with CKD based on TEG tracings. The most common abnormalities were increased MA and G values suggestive of hypercoagulability. Previous studies in dogs with glomerular disease and non-protein-losing CKD have also shown similar hypercoagulable TEG tracings.\textsuperscript{117,138} Likewise, hypercoagulable TEG tracings have been identified in people with CKD.\textsuperscript{85,121,122,124} Some of these patients have also simultaneously been documented to have platelet dysfunction based on prolonged skin bleeding times, PFA-100 closure times and aggregometry.\textsuperscript{85} In people with CKD, increased MA and G values are similarly one of the most consistently reported abnormal hypercoagulable finding in these patients.\textsuperscript{85,121}

Only one dog in the present study had a hypocoagulable TEG tracing with the remaining dogs having normal or hypercoagulable results. This dog also had prolongation of both PFA-100 Col+ADP and PFA-100 Col+EPI closure times, however other CKD dogs had prolongation of PFA-100 closure times with concurrent hypercoagulable TEG tracings. It is unclear why the \textit{in vitro} coagulation testing in our CKD patients show conflicting results with the prolonged PFA-100 Col+ADP closure times indicating decreased platelet function and the shortened K-time and increased MA and G TEG values suggesting hypercoagulability in these patients. Hematocrit has been shown to impact TEG
Interestingly, anemia creates a hypercoagulable TEG tracing, with a decreasing hematocrit strongly correlating with increased MA and alpha angles and decreased K-time.\textsuperscript{139} This is despite anemia contributing to a hypocoagulable state characterized by prolonged skin or buccal mucosal bleeding times in clinical patients.\textsuperscript{13,140} In the present study, CKD dogs had a significantly lower HCT than healthy controls. However, in our study, HCT was only inversely correlated the alpha angle but was not correlated with any other TEG variable including MA or G making the role of HCT on the hypercoagulable tracings found in CKD dogs unclear.

Fibrinogen has also been correlated with TEG variables with an elevated plasma fibrinogen being positively correlated with MA values.\textsuperscript{115,121,138} The CKD dogs evaluated in this study had significantly increased fibrinogen concentrations, which were strongly correlated with increased MA and G values. Dogs with both CKD and glomerular disease have been previously documented to have increased plasma fibrinogen concentrations\textsuperscript{138,141} which may be secondary to a pro-inflammatory disease state\textsuperscript{142,143} or, in CKD, may be due to decreased catabolism of fibrinogen by the kidneys.\textsuperscript{141} The elevated fibrinogen concentrations found in our study may have contributed to an \textit{in vitro} effect of hypercoagulability in TEG analysis, however if the presence of a true \textit{in vivo} hypercoagulable state in these CKD patients truly exists is unknown.

Although the PFA-100 has been shown, in previous studies, to be a poor indicator of clinical bleeding, thromboestography has been shown to be the best assay for detecting
mild hemostatic deficits associated with an increased risk of post-kidney biopsy bleeding in people, with a decreased angle and a prolonged K-time being the best TEG variables to predict bleeding risk.\textsuperscript{125} Thromboelastography also correctly identifies dogs at risk for clinical bleeding with a high positive and negative predictive value.\textsuperscript{119} One CKD dog in the present study had a hypocoagulable TEG tracing including a prolonged R-time and decreased angle. Occurrence and risks of bleeding was not evaluated in this study and only one out of eleven CKD dogs had a hypocoagulable TEG tracing so we are unable to make any conclusions on the accuracy of the TEG to predict clinical bleeding in dogs with CKD.

TEG has also been a useful tool to predict thrombus formation in people following simultaneous pancreas-kidney transplantation by combining K, R, angle and MA TEG variables into one coagulation index.\textsuperscript{144} However, to the authors’ knowledge, there is currently no data available on the use of the TEG to predict thrombosis formation in dogs with CKD. Hypercoagulable TEG tracings have been identified in dogs with parvoviral enteritis associated thrombosis, however a direct correlation between specific TEG variables and increased thrombotic risk was not identified.\textsuperscript{145} While 54\% of the CKD dogs in the present study had an increased MA or G value, the development of thrombotic complications was not recorded. While, four CKD dogs did have an increased d-dimer level, all of these were weakly positive and below 400 ng/mL which is below the previously established cut-off of 500 ng/mL, shown to have a 100\% sensitivity
for predicting thromboembolic disease in dogs. Additional studies are needed to evaluate the ability of TEG to predict thrombembolic complications in dogs with CKD.

Flow cytometric evaluation of platelet GPIb and GPIIb-IIIa, the surface receptors for vWF and fibrinogen respectively, was performed on CKD dogs to evaluate the underlying cause of platelet dysfunction and viscoelastic coagulation abnormalities found in these patients. No difference was found in surface GPIb expression between CKD and healthy dogs. Many studies in humans with uremia have shown decreased expression of platelet GPIb, which is negatively correlated with the degree of azotemia and is associated with decreased platelet aggregation in these patients. A large percentage of platelet GPIb is stored in the intracellular storage pool and redistributes to the platelet surface. In humans with CKD, total platelet pool of GPIb is severely reduced with less reduction of surface-bound GPIb consistent with translocation of intracellular GPIb to replace cleaved surface GPIb with eventual depletion of total platelet GPIb stores. To the author’s knowledge, the present study is the first time platelet GPIb expression has been evaluated in dogs with CKD. Although no difference in GPIb expression was found between CKD and healthy dogs in the current study, we only evaluated surface GPIb expression and further studies are needed in dogs to determine if intracellular pools of GPIb are reduced.

Platelet GPIIb-IIIa is a receptor for fibrinogen and mediates platelet-platelet binding and aggregation. GPIIb-IIIa can also bind vWF and can weakly contribute to platelet-
platelet interactions in the absence of fibrinogen.\textsuperscript{56} In people with uremia, platelet surface expression of GPIIb-IIIa has been shown to be normal to increased.\textsuperscript{17,51,53,70} We similarly found increased expression of GPIIb-IIIa on the surface of canine platelets in dogs with CKD and GPIIb-IIIa expression was positively associated with serum creatinine concentration. This is similar to what was shown in humans with CKD where GPIIb-IIIa expression increases with increasing creatinine.\textsuperscript{53} The cause of the increased expression of GPIIb-IIIa in CKD dogs is unknown but may be compensatory for other defects in platelet function and the increased GPIIb-IIIa expression may contribute to the normal PFA-100 closure times and increased MA and G TEG values seen in some dogs with CKD.

The current study found normal platelet GPIb expression and increased expression of platelet GPIIb-IIIa in dogs with CKD. Neither GPIb nor GPIIb-IIIa expression was correlated with PFA-100 closure times or any TEG variable suggesting that alterations in platelet glycoprotein receptor expression cannot explain the changes in platelet function and whole blood coagulation seen in these patients. However, we did not assess the function or binding ability of these receptors, so while their expression may be normal or increased, their ability to bind ligands and contribute to platelet aggregation may be reduced. In humans with CKD, vWF and fibrinogen binding to GPIIb-IIIa has been shown to be decreased despite normal receptor expression.\textsuperscript{54,70} Normal binding of fibrinogen and vWF to GPIIb-IIIa may be inhibited due to the receptor being occupied by fibrinogen fragments\textsuperscript{55} or other uremic compounds as this defect in ligand binding is
reversed with dialysis. Further studies would be needed in dogs with CKD to determine if similar functional defects are present in platelet glycoprotein receptors unrelated to their surface expression.

Platelet P-selectin is expressed on the membrane of alpha granules and is transported to the platelet cell membrane after degranulation. P-selectin functions in the recruitment of inflammatory cells and generation of thrombin during thrombus formation\textsuperscript{147} and its expression on the platelet surface has been used experimentally as a marker of platelet activation in humans and dogs.\textsuperscript{131,148} Flow cytometric studies in humans with CKD have shown an increase in unstimulated expression of markers of platelet activation, including P-selectin, suggesting an increase in circulating activated platelets, which is inversely correlated with glomerular filtration rate in these patients.\textsuperscript{51,149} These findings are consistent with what was found in the present study where platelet P-selectin expression was increased in dogs with CKD. However, no correlation between platelet P-selectin expression and serum BUN or creatinine was identified in dogs with CKD. Additional evaluation of P-selectin expression and more sensitive assessments of GFR in dogs with CKD, such as iohexol clearance, should be performed to verify any relationship, or lack thereof, between P-selectin expression and alterations in renal function. Increased platelet activation and platelet hyperresponsiveness may contribute to the hypercoagulable TEG tracings seen in CKD dogs in this study. However, no correlation between any TEG variable and platelet P-selectin expression was identified. Additional
research is needed to evaluate the clinical relevance of increased platelet activation in dogs with CKD.

There are several limitations with the present study. There was a small number of study dogs and increasing the sample size may allow for detection of other subtle or less common hemostatic derangements in CKD dogs. Additionally, the majority of dogs with CKD in this study were in IRIS Stage 3 with only one of eleven being in IRIS Stage 4. Further research in dogs in all IRIS Stages is needed to evaluate if the platelet defects identified in our study are present throughout the disease course and if they change with progression of CKD. The only abnormality correlated with degree of azotemia in the present study was a positive correlation between creatinine and GPIIb-IIIa expression, which suggests that the presence of other soluble uremic compounds may be responsible for the changes identified in CKD.

A significant weakness of the current study is the use of recalcified, unactivated TEG analysis, as poor reproducibility and high variability has been shown when no activator is used.\textsuperscript{150} When compared with kaolin-activated TEG, significant differences were found between TEG variables with only moderate correlation between unactivated and kaolin activated samples. Values obtained with an activator cannot be directly compared with unactivated samples or between the different activators, with stronger activators having less variability.\textsuperscript{150-152} Unactivated TEG was used in this study since our institution reference ranges have been established with this methodology. When kaolin activated
TEG was directly compared with unactivated TEG samples, the unactivated samples show longer R- and K-times and decreased angle, MA and G values consistent with relative hypocoagulable tracings when compared to kaolin activation. So the hypercoagulable tracings identified in CKD dogs in the present study using unactivated TEG are likely to also be identified if this study were to be repeated using kaolin activated samples. Additionally, previous work in dogs with PLN has been performed using unactivated TEG, allowing comparison of our results to the findings of that study.

In conclusion, platelet dysfunction, as evidenced by prolonged PFA-100 Col+ADP closure times, and changes in whole blood viscoelastic coagulation consistent with hypercoagulability were identified in dogs with CKD. Changes in expression of platelet GPIIb-IIIa and P-selectin were also identified, but do not entirely explain the changes seen in PFA-100 and TEG testing in these dogs, and their correlation with declining GFR are still unclear. Additional testing is needed to fully elucidate the causes of platelet defects in uremia and how these defects relate to clinical signs of bleeding or thrombosis.
References


Appendix A: Endnotes

b. BD Vacutainer® Safety-Lok blood collection set; Becton, Dickinson and Company; Franklin Lakes, NJ.
c. ADVIA 2120i, Siemens Healthcare Diagnostics, Deerfield, IL
d. COBAS 6000 c501, Roche, Indianapolis, IN
e. STA compact, Diagnostica Stago, Parsippany, NJ
f. D-dimer Slide agglutination kit, Remmel, Lenexa, KS
g. PFA-100®, Siemens Healthcare Diagnostics, Deerfield, IL
h. PFA-100® Collagen/ADP Test Cartridge, Siemens Healthcare Diagnostics
i. PFA-100® Collagen/Epinephrine Test Cartridge, Siemens Healthcare Diagnostics
j. TEG® 5000 Thrombelastograph Hemostasis Analyzer System, Haemonetics Corporation, Niles, IL
k. FACSCalibur, BD Biosciences, San Jose, CA
l. Erythrolyse Red Cell Lysing Buffer, AbD Serotec, Raleigh, NC
m. Cell Staining Buffer, BioLegend Inc, San Diego, CA
n. Clone SZ2 mouse IgG1 FITC-conjugated, Beckman Coulter Inc, Brea, CA
o. Clone SZ21 mouse IgG1 FITC-conjugated, Beckman Coulter Inc, Brea, CA
p. Clone MD6 IgG1, generously provided by Dr. C. Wayne Smith, Baylor College of Medicine, Houston, TX
q. Clone STAT70, Serotec USA, Washington, DC
r. GraphPad Prism 6, La Jolla, CA
Appendix B: Tables
Table 1. Minimum data base findings for CKD and healthy control dogs.
Results reported as median (range). **Indicates statistical significance. BUN = blood urea nitrogen; USG = urine specific gravity; UPC = urine protein:creatinine ratio; HCT = hematocrit; MPV = mean platelet volume; bp = blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Control Dogs</th>
<th>CKD Dogs</th>
<th>Reference Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dL) **</td>
<td>18 (12-34)</td>
<td>69 (28-200)</td>
<td>5-20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine (mg/dL) **</td>
<td>0.9 (0.7-1.5)</td>
<td>4.1 (3.0-6.7)</td>
<td>0.6-1.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>3.8 (2.3-5.0)</td>
<td>4.0 (2.3-16.5)</td>
<td>3.2-8.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Total calcium (mg/dL) **</td>
<td>10.55 (10.1-11.2)</td>
<td>11.7 (10.1-13.2)</td>
<td>9.3-11.6</td>
<td>0.002</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.65 (3.3-4.2)</td>
<td>3.5 (2.7-3.8)</td>
<td>2.9-4.2</td>
<td>0.08</td>
</tr>
<tr>
<td>USG **</td>
<td>1.044 (1.014-1.048)</td>
<td>1.012 (1.007-1.014)</td>
<td>&gt;1.030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UPC **</td>
<td>0.09 (0.06-0.12)</td>
<td>0.86 (0.06-4.99)</td>
<td>&lt;0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>HCT (%) **</td>
<td>47.5 (42-54)</td>
<td>36.4 (24.4-51)</td>
<td>37-56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>246.5 (154-313)</td>
<td>243 (93-482)</td>
<td>108-433</td>
<td>0.57</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>12 (9.1-14.6)</td>
<td>9 (7.4-14.4)</td>
<td>7.0-10.0</td>
<td>0.08</td>
</tr>
<tr>
<td>Systolic bp (mmHg)</td>
<td>122.5 (96-175)</td>
<td>130 (110-180)</td>
<td>&lt;150 mmHg</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Table 2. TEG values for CKD and healthy control dogs. Results reported as median (range). **Indicates statistical significance.

<table>
<thead>
<tr>
<th></th>
<th>Control Dogs</th>
<th>CKD Dogs</th>
<th>Reference Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>R time (sec)</td>
<td>3.5 (1.9-5.1)</td>
<td>3.4 (2.0-8.9)</td>
<td>1.0-6.1</td>
<td>0.49</td>
</tr>
<tr>
<td>K (sec) **</td>
<td>1.85 (1.2-2.9)</td>
<td>1.2 (0.8-3.2)</td>
<td>0.9-3.6</td>
<td>0.035</td>
</tr>
<tr>
<td>α angle (degrees)</td>
<td>64.0 (47.8-71.3)</td>
<td>71.4 (46.6-78.3)</td>
<td>51.8-73.4</td>
<td>0.43</td>
</tr>
<tr>
<td>MA (mm) **</td>
<td>59.95 (54.1-70.7)</td>
<td>71.4 (57.9-82.2)</td>
<td>43.9-67.9</td>
<td>0.008</td>
</tr>
<tr>
<td>G (kdyn/cm²) **</td>
<td>7.5 (5.9-12.0)</td>
<td>12.5 (6.9-23.1)</td>
<td>3.10-10.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Appendix C: Figures
Figure 1. Flow cytometric scattergram of canine platelets. This figure depicts platelet gating (R1) excluding microparticles, platelet aggregates and other cells.
Figure 2. Prothrombin (PT) and activated partial thromboplastin time (aPTT) in healthy control and CKD dogs. The horizontal line indicates median value. No significant difference found between controls and CKD dogs for either PT or aPTT (p = 0.49 and p = 0.5, respectively).

Figure 3. Plasma fibrinogen concentration in healthy and CKD dogs. The horizontal line indicates median value. ** Indicates a significant difference. Plasma fibrinogen concentration is significantly increased in CKD dogs compared to healthy controls (p = 0.001).
Figure 4. PFA-100 Col+ADP CT in healthy and CKD dogs. The horizontal line indicates median value. ** Indicates a significant difference. PFA-100 Col+ADP CT are significantly increased in CKD dogs compared to healthy controls (p < 0.01).

Figure 5. PFA-100 Col+EPI CT in healthy and CKD dogs. The horizontal line indicates median value. No significant difference found between groups (p = 0.41).
Figure 6. Platelet GPIb mean fluorescence intensity (MFI) in healthy and CKD dogs. The horizontal line indicates median value. There was no significant difference in platelet GPIb MFI in dogs with CKD compared to healthy control dogs (p = 0.09).

Figure 7. Platelet GPIIb-IIIa mean fluorescence intensity (MFI) in healthy and CKD dogs. The horizontal line indicates median value. **Indicates a significant difference. Platelet GPIIb-IIIa MFI is significantly higher in dogs with CKD than healthy controls (p = 0.01).
Figure 8. Platelet GPIIb-IIIa fluorescence histogram from a healthy control dog (A) and a dog with CKD (B).
Two subpopulations of platelets were identified in all dogs. CKD dogs had significantly higher percentage of platelets in the high intensity subpopulation (p = 0.007).
Figure 9. Platelet P-selectin mean fluorescence intensity (MFI) in healthy and CKD dogs. The horizontal line indicates median value. **Indicates a significant difference. Platelet P-selectin MFI is significantly higher in dogs with CKD than healthy controls (p = 0.03).