Comparison of platelet function and viscoelastic test results between healthy dogs and dogs with naturally occurring chronic kidney disease

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OBJECTIVE
To compare platelet function and viscoelastic test results between healthy dogs and dogs with chronic kidney disease (CKD) to assess whether dogs with CKD have platelet dysfunction and altered blood coagulation.

ANIMALS
10 healthy control dogs and 11 dogs with naturally occurring CKD.

PROCEDURES
Blood and urine were collected once from each dog for a CBC, serum biochemical analysis, urinalysis, and determination of the urine protein-to-creatinine ratio, prothrombin time, activated partial thromboplastin time, plasma fibrinogen concentration, and antithrombin activity. Closure time was determined by use of a platelet function analyzer and a collagen-ADP platelet agonist. Thromboelastography (TEG) variables (reaction time, clotting time, α angle, maximum amplitude, and global clot strength [G value]) were determined by use of recalcified nonactivated TEG. Platelet expression of glycoprotein Ib (GPIb; receptor for von Willebrand factor), integrin αIIbβ3 (αIIbβ3; receptor for fibrinogen), and P-selectin (marker for platelet activation) was assessed by flow cytometry.

RESULTS
Compared with healthy control dogs, the median closure time was prolonged, the median maximum amplitude and G value were increased, and the median clotting time was decreased for dogs with CKD. Platelet expression of both αIIbβ3 and P-selectin was also significantly increased for dogs with CKD, compared with that for control dogs. Platelet expression of GPIb, αIIbβ3, and P-selectin was not correlated with closure time or any TEG variable.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that dogs with CKD frequently had evidence of platelet dysfunction and hypercoagulability that were not totally attributable to alterations in platelet surface expression of GPIb, αIIbβ3, and P-selectin. (*Am J Vet Res* 2017;78:589–600)

Chronic kidney disease is a common condition in many species including dogs and humans.

ABBREVIATIONS

αIIbβ3	Integrin αIIbβ3
aPTT	Activated partial thromboplastin time
AT	Antithrombin activity
BMBT	Buccal mucosal bleeding time
CKD	Chronic kidney disease
CT	Closure time
FACS	Fluoresence-activated cell sorting
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
GPIb	Glycoprotein Ib
IRIS	International Renal Interest Society
MA	Maximum amplitude
MFI	Mean fluorescence intensity
MPV	Mean platelet volume
PFA	Platelet function analyzer
PT	Prothrombin time
TEG	Thromboelastography
UPC	Urine protein-to-creatinine ratio
vWF	von Willebrand factor

The prevalence of CKD ranges from 0.37% to 3.74% in various populations of dogs[1–3] and up to 16.8% in various human populations.[4] Human patients and dogs with CKD frequently develop numerous complications such as anemia, renal secondary hyperparathyroidism, uremic gastritis, and hypertension.[5] Additionally, bleeding tendencies secondary to defects in platelet function or abnormalities in the coagulation cascade have been described in human patients with uremia.[6] Patients with uremia occasionally develop mild thrombocytopenia; however, abnormal bleeding can occur in patients with platelet counts, PT, and aPTT within the respective reference intervals, which suggests that the abnormal bleeding tendencies in those patients are secondary to inherent abnormalities in the interactions between platelets and between platelets and the endothelial lining of blood vessels rather than coagulation deficiences.[6] In human patients with CKD and uremia, abnormal bleeding is called ure-

mic thrombocytopathy. Compared with clinically normal individuals, patients with uremic thrombocytopathy are at an increased risk of thrombosis (likely owing to the loss of antithrombotic endothelial properties), hyperfibrinogenemia, hyperactivation of P-selectin, and the abnormal release of microparticles associated with inflammation.[6] All of those abnormalities can result in coagulation system imbalances that lead to bleeding or venous thrombosis.

Overt clinical bleeding is rarely described in dogs with naturally occurring CKD; however, a dramatic increase in BMBT has been reported in dogs with experimentally induced renal failure soon after the induction of azotemia.[7] Additionally, dogs with CKD often have a decrease in other markers of platelet function such as platelet glass bead retention.[7-9] Similar to human patients with renal failure, dogs with CKD and platelet dysfunction can have platelet counts and coagulation times within the respective reference intervals. This suggests that dogs can develop uremic thrombocytopathy, although information regarding the underlying mechanism of that condition in dogs with CKD is limited.

In human patients with CKD, uremic platelet dysfunction is the result of multiple factors such as inherent defects in platelet activation and abnormalities in the adhesion of platelets to the endothelial lining of blood vessels.[10] Additionally, concurrent diseases such as anemia or the administration of β-lactam antimicrobials can contribute to platelet defects.[11,12] Numerous intrinsic defects in platelets and platelet function have been identified in human patients with CKD, including α granule abnormalities and abnormally decreased production of intracellular platelet agonists such as ADP and serotonin, abnormally increased production of platelet inhibitors such as cAMP, abnormal calcium mobilization, and abnormally decreased synthesis of thromboxane A2.[6,13] Excessive bleeding in human patients with CKD has also been attributed to abnormalities in platelet adhesion to other platelets and the endothelial wall of blood vessels.[10] Those adhesion defects are associated with abnormalities in the platelet surface expression of receptors for GPIb and αIIbβ3, which are necessary for binding of vWF and fibrinogen, respectively.[10]

Platelet aggregation may be within, or increased or decreased from, the reference interval in dogs with naturally occurring or induced uremia.[7,8,14] Uremic dogs frequently have a prolonged BMBT and abnormal results for methods (eg, platelet glass bead assay) historically used to evaluate platelet activation and adhesion, which suggest that uremic thrombocytopathy in dogs is caused by platelet adhesion defects.[9] Azotemia does not alter the structure and function of plasma vWF, and the plasma concentration of that integral hemostatic protein is abnormally increased in uremic dogs.[15] Although uremic thrombocytopathy in dogs is suspected to be caused by multifaceted dysfunction of platelet activation and adhesion in a manner similar to that in human patients, elucidation of platelet function in dogs with CKD is lacking.

The aim of the study reported here was to compare platelet function and viscoelastic test results between healthy dogs and dogs with CKD to assess whether dogs with CKD have platelet dysfunction and altered blood coagulation. We also evaluated expression of platelet surface receptors for vWF (GPIb) and fibrinogen (αIIbβ3) and platelet surface expression of P-selectin (a marker of platelet activation) with flow cytometry. We hypothesized that, compared with healthy dogs, dogs with CKD would have impaired platelet function as determined by altered platelet glycoprotein receptor expression. We also hypothesized that dogs with CKD would have hypercoagulable TEG variables, similar to those observed for human patients with renal failure.

Materials and Methods

Animals

Dogs examined at The Ohio State University Veterinary Medical Center that were determined to have CKD on the basis of IRIS guidelines,[16] which included documentation of azotemia for > 2 months, were prospectively recruited for the study. For each dog prior to study enrollment, a CBC, serum biochemical analysis, determination of the UPC, and Doppler measurement of indirect systolic blood pressure were performed. The results were used to classify each dog into 1 of 4 IRIS stages on the basis of severity of CKD and ensure that subjects enrolled in the study did not have any concurrent diseases known to affect coagulation.

Clinically normal dogs owned by students, faculty, and staff of The Ohio State University Veterinary Medical Center were recruited as controls. All control dogs were determined to be healthy on the basis of owner-provided history and results of a physical examination, CBC, serum biochemical analysis, urinalysis, UPC, and indirect systolic blood pressure measurement.

Any dog with mild to moderate thrombocytopenia (platelet count < 90,000 platelets/μL) or moderate anemia (Hct < 25%) or that was receiving medications that might affect in vivo hemostasis (eg, glucocorticoids, NSAIDs, hetastarch, heparin, clopidogrel, or darbepoetin) was excluded from the study. Dogs with confirmed or suspected nonrenal systemic illness, including diseases associated with hypo- or hypercoagulability such as immune-mediated hemolytic anemia, hyperadrenocorticism, or neoplasia, were also excluded from the study.

All study procedures were reviewed and approved by The Ohio State University Institutional Animal Care and Use Committee and the College of Veterinary Medicine Clinical Research Advisory Committee. The owners of all dogs enrolled in the study provided written informed consent for the use of their pets in the study.

Sample collection

Blood and urine was collected once from each dog with CKD and control dog. Blood (15 mL) was collected by jugular venipuncture with a 21-gauge butterfly needle attached to a vacuum blood collection system[a] directly into 4 blood collection tubes in the following order: a 10-mL tube without any additives, two 4.5-mL tubes containing 3.2% sodium citrate, and a 2-mL tube containing 7.5% EDTA. Urine samples were collected by either midstream free catch or cystocentesis.

Minimum database

For all dogs, a CBC was performed with an automated hematologic analyzer,[b] and a manual differential cell count was performed by microscopic evaluation of a blood smear. A serum biochemical analysis was performed with an automated chemistry analyzer.[c] A standard urinalysis was performed, which included measurement of urine specific gravity by refractometry, dipstick analysis, and microscopic examination of urine sediment. Urine protein and creatinine concentrations were determined with an automated chemistry analyzer[c] by use of the turbidimetric and Jaffe methods, respectively.

Coagulation analysis

For all dogs, plasma harvested from the blood samples collected in the tubes with sodium citrate was used for coagulation testing. Prothrombin time, aPTT, and fibrinogen concentration were determined by use of an automated mechanical clot detection instrument.[d] Plasma AT was measured by use of a chromogenic test with the same automated instrument[d] and was reported as a percentage of a pooled normal canine plasma reference, which was assigned an AT value of 100%. Plasma D-dimer concentration was measured with a semiquantitative rapid latex slide agglutination test[e] in accordance with the manufacturer's instructions.

Evaluation of platelet function

PFA—A commercial point-of-care PFA[f] that has been previously evaluated for use in dogs[17] was used to assess in vitro platelet function. The analyzer records the number of seconds required for platelet plug formation (CT) after a whole blood sample is exposed to platelet agonists such as collagen and ADP. Briefly, 800 µL of citrated whole blood was aspirated through an aperture of a cartridge[g] containing a membrane coated with a collagen-ADP platelet agonist at a high shear rate. The time required for blood flow through the aperture to stop was recorded as the CT. All samples were analyzed in duplicate within 1 hour after collection. For each sample, the mean was calculated and used for analysis purposes. The CT reference interval established by our laboratory for the collagen-ADP platelet agonist cartridge was 52 to 86 seconds. The PFA analyzed blood flow through the cartridge for a maximum of 300 seconds, and 300 seconds was the CT recorded for all samples that failed to form a platelet plug.

TEG—For each dog, a recalcified nonactivated TEG analysis was performed as described.[18] Briefly, 20 µL of $CaCl_2$ was added to a prewarmed cup (37°C) of the TEG machine[h] followed by 340 µL of citrated whole blood to bring the volume in the cup to 360 µL. The run time for the analysis ranged from 120 to 180 minutes, after which a single TEG tracing was obtained and the reaction time (R), clotting time (K), α angle, MA, and global clot strength (G value) were determined and recorded. Because TEG variables vary with Hct,[18] our laboratory has established reference intervals for each variable at Hcts of 20% (Hct20) and 45% (Hct45). For this study, TEG variables for blood samples with an Hct > 32% were assessed with the Hct45 reference intervals, whereas those for blood samples with an Hct ≤ 32% were assessed with the Hct20 reference intervals.

Evaluation of platelet receptor expression and platelet activation

GPIb expression—Platelet surface expression of GPIb (CD42) was evaluated by use of flow cytometry[i] in a manner similar to that described for canine platelets in other studies.[19,20] Briefly, 2 mL of a 1:10 dilution of erythrocyte lysis buffer containing 0.5% paraformaldehyde[j] was added to 100 µL of citrated whole blood and incubated at 25°C for 15 minutes to obtain platelet-fixed, erythrocyte-depleted blood. The erythrocyte-depleted blood was washed with FACS buffer,[k] which had been filtered through a 0.45-µm pore filter immediately before use and then centrifuged at 435 × *g* at 8°C for 15 minutes. The resulting platelet pellet was resuspended in 100 µL of FACS buffer and incubated with 50 µL of a murine FITC-labeled, anti-human CD42 monoclonal antibody[l] at room temperature (approx 20°C) for 30 minutes in the dark. Then, the platelets were washed with FACS buffer, centrifuged at 435 × *g* at 8°C for 15 minutes, and resuspended in 500 µL of FACS buffer.

αIIbβ3 expression—Platelet surface expression of αIIbβ3 (CD61) was evaluated by use of flow cytometry as described for evaluation of GPIb expression and similar to methods described in other studies.[10,21] Briefly, 100 µL of washed, erythrocyte-depleted blood was incubated with 50 µL of murine FITC-labeled, anti-human CD61 monoclonal antibody[m] at room temperature for 30 minutes in the dark. Then, the platelets were washed with FACS buffer, centrifuged at 435 × *g* at 8°C for 15 minutes, and resuspended in 500 µL of FACS buffer.

P-selectin expression—Platelet activation was assessed by evaluation of platelet surface expression of P-selectin as described.[19,22,23] Erythrocyte-depleted blood was incubated with 100 µL of a 1:100 dilution

of affinity-purified, monoclonal mouse anti-canine P-selectin antibody (provided by another researcher[n]) at room temperature for 30 minutes. The platelets were washed with FACS buffer, centrifuged at 435 X *g* at 8°C for 15 minutes, and resuspended in 100 µL of FACS buffer. Next, 100 µL of a 1:150 dilution of FITC-labeled, goat anti-mouse antibody[o] was added to the resuspended platelet solution. The solution was incubated at room temperature for 30 minutes in the dark, after which the platelets were washed and resuspended in 100 µL of FACS buffer.

All flow cytometry procedures were preceded by daily calibration of the machine with the manufacturer's recommended controls.[p] All samples were run in parallel with isotype controls and 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 versus log-side scatter plot. The platelet population was gated excluding platelet aggregates and nonlysed erythrocytes. A histogram was constructed from the gated platelet population with the logarithm of fluorescence intensity on the x-axis and platelet count on the y-axis. Platelet surface expression of GPIb, αIIbβ3, and P-selectin was quantified by the intensity of antibody staining, which was expressed as MFI.

Statistical analysis

Data were evaluated for normality with the Kolmogorov-Smirnov and Shapiro-Wilk tests. Comparisons between dogs with CKD and control dogs were performed by use of the Student *t* test for variables with normal distributions and the Mann-Whitney rank sum test for variables that were not normally distributed. The Spearman rank correlation coefficient (r_S) was used to evaluate the correlation among the various variables assessed (CT, TEG variables, expression of platelet surface receptors [GPIb, αIIbβ3, and P-selectin], and other coagulation and minimum database variables). All analyses were performed with a commercially available statistical software program,[q] and values of $P < 0.05$ were considered significant.

Results

Dogs with CKD

Eleven dogs with CKD were enrolled in the study. Those dogs had a median age of 9.7 years (range, 2.6 to 15.8 years) and median body weight of 8.7 kg (range, 2.7 to 45 kg). Breeds represented included mixed (n = 3), Labrador Retriever (2), and Boxer, Cocker Spaniel, Jack Russell Terrier, Puli, Shih Tzu, and Yorkshire Terrier (1 each). Of the 11 dogs with CKD, 10 were classified with IRIS stage III disease and 1 was classified with IRIS stage IV disease. No dogs with IRIS stage I or II disease were enrolled in the study.

Control dogs

Ten healthy control dogs were enrolled in the study. Those dogs had a median age of 4.4 years (range, 1.2 to 10.3 years) and median body weight of 26 kg (range, 17 to 41.3 kg). Breeds represented included mixed (n = 4), Golden Retriever (3), American Pit Bull Terrier (2), and Doberman Pinscher (1). Control dogs were significantly ($P = 0.01$ for both comparisons) younger and had a greater mean body weight than the dogs with CKD.

Minimum database variables

Select serum biochemical, CBC, and urinalysis variables and the UPC were summarized for the control dogs and dogs with CKD **(Table 1)**. The median BUN ($P < 0.001$), serum creatinine ($P < 0.001$), and total calcium ($P = 0.002$) concentrations and UPC ($P = 0.001$) were significantly greater and the median urine specific gravity ($P < 0.001$) and Hct ($P < 0.001$) were significantly less for dogs with CKD, compared with the correspond-

Table 1—Median (range) for minimum database variables for 10 healthy control dogs and 11 dogs with CKD.

Variable	Reference interval	Control dogs	Dogs with CKD	*P* value
BUN (mg/dL)	5–20	18 (12–34)	69 (28–200)	< 0.001
Creatinine (mg/dL)	0.6–1.6	0.9 (0.7–1.5)	4.1 (3.0–6.7)	< 0.001
Phosphorus (mg/dL)	3.2–8.1	3.8 (2.3–5.0)	4.0 (2.3–16.5)	0.3
Total calcium (mg/dL)	9.3–11.6	10.55 (10.1–11.2)	11.7 (10.1–13.2)	0.002
Albumin (g/dL)	2.9–4.2	3.65 (3.3–4.2)	3.5 (2.7–3.8)	0.08
Urine specific gravity	> 1.030	1.044 (1.014–1.048)	1.012 (1.007–1.014)	< 0.001
UPC	< 0.2	0.09 (0.06–0.12)	0.86 (0.06–4.99)	0.001
Hct (%)	37–56	47.5 (42–54)	36.4 (24.4–51)	< 0.001
Platelet count (X 10^9 platelets/L)	108–433	246.5 (154–313)	243 (93–482)	0.57
MPV (fL)	7.0–10.0	12 (9.1–14.6)	9 (7.4–14.4)	0.08
Systolic blood pressure (mm Hg)	< 150	122.5 (96–175)	130 (110–180)	0.13

Control dogs were owned by students, faculty, and staff of The Ohio State University Veterinary Medical Center and were determined to be healthy on the basis of owner-provided history and results of a physical examination, CBC, serum biochemical analysis, urinalysis, UPC, and indirect systolic blood pressure measurement. Dogs with CKD were prospectively recruited and consisted of client-owned dogs examined at The Ohio State University Veterinary Medical Center in which CKD was diagnosed on the basis of IRIS guidelines, which included documentation of azotemia for > 2 months. Values of $P < 0.05$ were considered significant.

ing variables for the control dogs. The platelet count for 1 dog with CKD was within the reference range 2 days prior to study enrollment, but a platelet count was not recorded on the day of study enrollment; therefore, that dog was not included in the statistical analyses for platelet count and MPV. The median platelet count ($P = 0.57$) and MPV ($P = 0.08$) did not differ significantly between the dogs with CKD and control dogs.

Coagulation function

The median PT (7.7 seconds; range, 5.3 to 8.7 seconds), aPTT (10.8 seconds; range, 9.7 to 16.0 seconds), and plasma AT (130%; range, 107% to 139%) for dogs with CKD did not differ significantly from the median PT (7.6 seconds; range, 6.8 to 8.3 seconds; $P = 0.49$), aPTT (10.45 seconds; range, 9.3 to 12.2 seconds; $P = 0.50$), and plasma AT (136%; range, 125% to 158%; $P = 0.30$) for control dogs. The median fibrinogen concentration (315.0 mg/dL; range, 183.0 to 821.0 mg/dL) for dogs with CKD was significantly ($P = 0.001$) greater than that for control dogs (179.5 mg/dL; range, 99.0 to 313.0 mg/dL; **Figure 1**). All control dogs tested negative for D-dimers. Of the 11 dogs with CKD, 2 had a semiquantitative D-dimer concentration between 100 and 200 ng/mL and 2 had a semiquantitative D-dimer concentration between 200 and 400 ng/mL; no dogs with CKD were strongly positive for D-dimer (concentration > 400 ng/mL).

Platelet function

The median CT for dogs with CKD (83.0 seconds; range, 54.0 to 150.5 seconds) was significantly ($P < 0.01$) longer than that for control dogs (57.25 seconds; range, 51.0 to 90.5 seconds; Figure 1). Of the 11 dogs with CKD, the CT was > 86 seconds (ie, the upper limit of the reference interval) for 4 and within the upper third of the reference interval for 3; no dog with CKD had a CT < 52 seconds (ie, the lower limit of the reference interval). Two of the 10 control dogs had CTs outside the reference interval; the CT was 51 seconds for one of

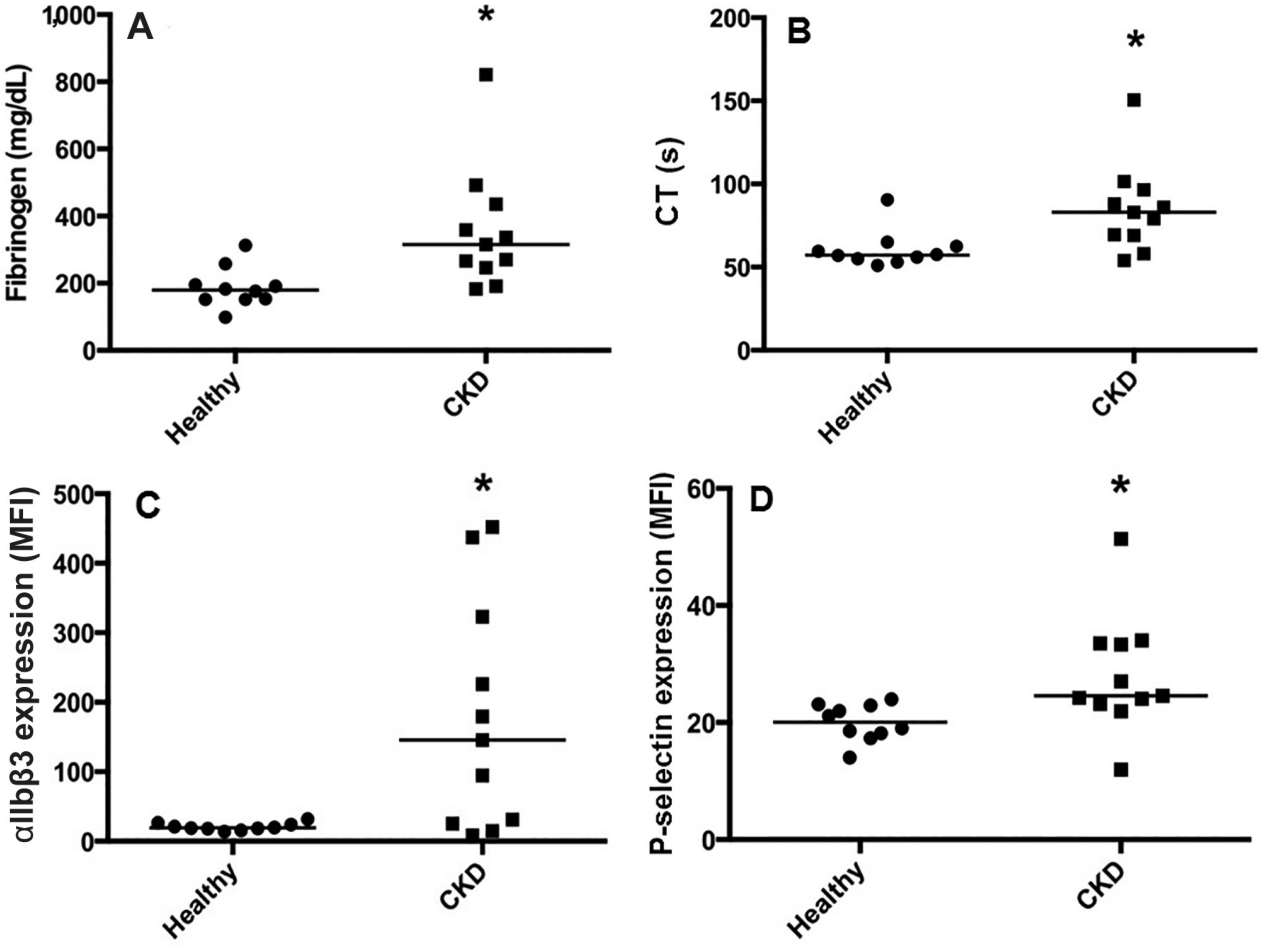

Figure 1—Scatterplot of plasma fibrinogen concentration (A), CT (B), and platelet membrane expression of αIIbβ3 (C) and P-selectin (D) for 10 healthy control dogs (circles) and 11 dogs with CKD (squares). Control dogs were owned by students, faculty, and staff of The Ohio State University Veterinary Medical Center and were determined to be healthy on the basis of owner-provided history and results of a physical examination, CBC, serum biochemical analysis, urinalysis, UPC, and indirect systolic blood pressure measurement. Dogs with CKD were prospectively recruited and consisted of client-owned dogs examined at The Ohio State University Veterinary Medical Center in which CKD was diagnosed on the basis of IRIS guidelines, which included documentation of azotemia for > 2 months. Horizontal lines represent the median for each group of dogs. *Median for dogs with CKD differs significantly ($P < 0.05$) from the median for control dogs.

those dogs and 90.5 seconds for the other. The CT was not significantly correlated with BUN or serum creatinine concentration, platelet count, or Hct.

TEG variables

Thromboelastography data for control dogs and dogs with CKD were summarized **(Table 2)**. The median MA (P = 0.008) and G value (P = 0.01) for dogs with CKD were significantly greater than the median MA and G value for control dogs. Conversely, the median clotting time for dogs with CKD was significantly (P = 0.035) less than that for control dogs. None of the other TEG variables differed significantly between dogs with CKD and control dogs.

The reaction time was within the appropriate reference interval (Hct45 or Hct20) established by our laboratory for all 10 control dogs and 10 of 11 dogs with CKD. One dog with IRIS stage III disease and an Hct ≥ 32% had a reaction time of 8.9 seconds, which was greater than the upper limit of the Hct45 reference interval and indicative of a hypocoagulable state. That dog also had a prolonged CT on the PFA. No dog had a reaction time below the lower limit of the appropriate reference interval. The clotting time was within the appropriate reference interval for all control dogs and 10 of the 11 dogs with CKD. One dog with CKD and an Hct < 32% had a clotting time of 0.8 seconds, which was less than the lower limit of Hct20 reference interval and indicative of a hypercoagulable state. No dog had a clotting time greater than the upper limit of the appropriate reference interval.

Nine of 11 dogs with CKD had an α angle that was within the appropriate reference interval. One of the 2 remaining dogs with CKD had an Hct < 32% and an α angle of 78.3°, which was greater than the upper limit of the Hct20 reference interval and indicative of a hypercoagulable state, whereas the other had an Hct ≥ 32% and an α angle of 46.6°, which was less than the lower limit of the Hct45 reference interval and indicative of a hypocoagulable state. Nine of the 10 control dogs had an α angle within the appropriate reference interval; the remaining control dog had an Hct ≥ 32% and an α angle of 47.8°, which was indicative of a hypocoagulable state.

Six of 11 dogs with CKD had an MA that was increased from the appropriate reference interval, which was consistent with a hypercoagulable state. None of the dogs with CKD had an MA below the reference interval. Nine of the 10 control dogs had an MA within the appropriate reference interval, and the remaining control dog had an MA of 70.7 mm, which was increased from the reference interval.

Six of 11 dogs with CKD had a G value that was increased from the appropriate reference interval, which was consistent with a hypercoagulable state, and 3 of the remaining 5 dogs had a G value in the upper third of the appropriate reference interval. Nine of 10 control dogs had a G value within the appropriate reference interval, and the remaining control dog had a G value that was increased from the upper limit of the reference interval.

A significant strong negative correlation was identified between clotting time and α angle (r_S = –0.80; P = 0.002) and between clotting time and platelet count (r_S = –0.81; P = 0.003); however, platelet count was not correlated with any other TEG variable. A significant (P = 0.049) negative correlation was identified between Hct and α angle (r_S = –0.61), but Hct was not correlated with any other TEG variable. A significant (P = 0.04) moderate positive correlation was identified between reaction time and CT; however, CT was not significantly correlated with any other TEG variable.

The G value is a logarithmic derivation of the MA; therefore, variables correlated with MA are also correlated with the G value, and the magnitude of those correlations is the same. A moderate positive correlation was identified between MA (and the G value) and serum creatinine concentration (r_S = 0.63; P = 0.04), and a strong positive correlation was identified between MA and plasma fibrinogen correlation (r_S = 0.77; P = 0.007). The degree of azotemia was not correlated with any other TEG variable. The MA (and G value) was not correlated with Hct, platelet count, or CT.

Platelet surface expression of GPIb

Median platelet surface expression of GPIb did not differ significantly (P = 0.09) between dogs with CKD (6.16 MFI; range, 3.26 to 12.69 MFI) and con-

Table 2—Median (range) for TEG variables for the dogs of Table 1.

Variable	Hct20 reference interval	Hct45 reference interval	Control dogs	Dogs with CKD	*P* value
R (s)	2.9–5.9	1.0–6.1	3.5 (1.9–5.1)	3.4 (2.0–8.9)	0.49
K (s)	0.9–1.3	0.9–3.6	1.85 (1.2–2.9)	1.2 (0.8–3.2)	0.035
α Angle (°)	70.2–76.6	51.8–73.4	64.0 (47.8–71.3)	71.4 (46.6–78.3)	0.43
MA (mm)	63.8–71.8	43.9–67.9	59.95 (54.1–70.7)	71.4 (57.9–82.2)	0.008
G value (kdyn/cm^2)	8.66–12.86	3.10–10.02	7.5 (5.9–12.0)	12.5 (6.9–23.1)	0.01

Samples were analyzed by use of a nonactivated TEG method (ie, no platelet activator was used). Because TEG variables vary with Hct, our laboratory has established reference intervals for each variable at Hcts of 20% (Hct20) and 45% (Hct45). For this study, TEG variables for blood samples with an Hct > 32% were assessed with the Hct45 reference interval, whereas those for blood samples with an Hct ≤ 32% were assessed with the Hct20 reference interval.

K = Clotting time. R = Reaction time.

See Table 1 for remainder of key.

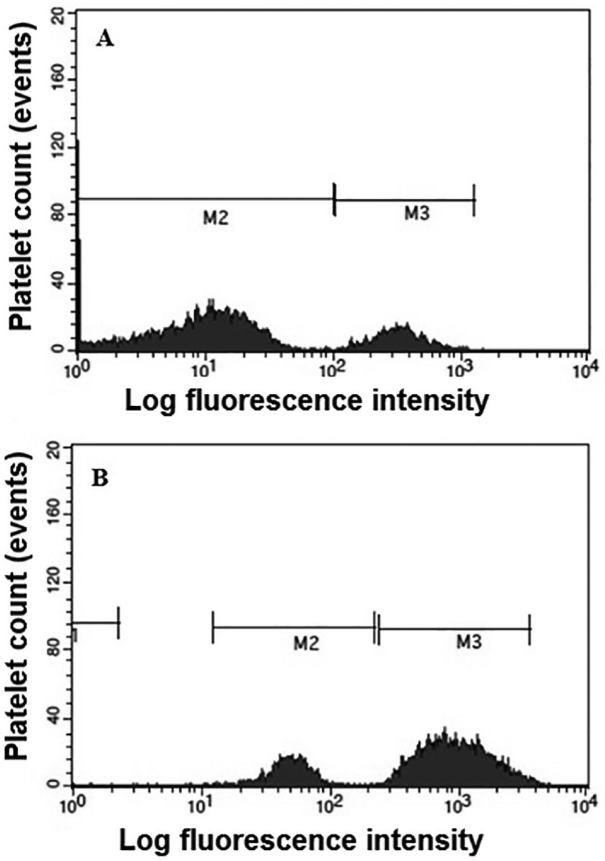

Figure 2—Representative fluorescence histogram of platelet surface expression of αIIbβ3 for a healthy control dog (A) and dog with CKD (B). Two subpopulations (M2 and M3) of platelets were identified in all dogs. The percentage of platelets in the high-intensity subpopulation (M3) for dogs with CKD was significantly (P = 0.007) greater than that for control dogs.

trol dogs (5.12 MFI; range, 3.93 to 7.47 MFI). Platelet surface expression of GPIb was not significantly correlated with CT, any TEG variable, platelet surface expression of αIIbβ3, or BUN or serum creatinine concentration. A moderate positive correlation was identified between platelet surface expression of GPIb and P-selectin (r_S = 0.67; P = 0.03), and a strong positive correlation was identified between platelet surface expression of GPIb and MPV (r_S = 0.78; P = 0.01).

Platelet surface expression of αIIbβ3

Median platelet surface expression of αIIbβ3 for dogs with CKD (145.5 MFI; range, 8.74 to 452.4 MFI) was significantly (P = 0.01) greater than that for control dogs (19.35 MFI; range, 13.76 to 31.91 MFI; Figure 1). The fluorescence histograms for αIIbβ3 expression revealed that all study dogs had 2 distinct subpopulations (M2 and M3) of platelets **(Figure 2)**. The median percentage of platelets in the high-intensity subpopulation (M3) for dogs with CKD (54.9%; range, 2.38% to 96.08%) was significantly (P = 0.007) greater than that for control dogs (14.21%; range, 11.32% to 21.32%). A moderate positive correlation was identified between platelet surface expression of αIIbβ3 and serum creatinine concentration (r_S = 0.66; P = 0.03), but platelet surface expression of αIIbβ3 was not significantly correlated with BUN concentration. Platelet surface expression of αIIbβ3 was not correlated with CT, any TEG variable, or platelet surface expression of GPIb or P-selectin.

Platelet surface expression of P-selectin

Median platelet surface expression of P-selectin for dogs with CKD (24.56 MFI; range, 11.97 to 51.38 MFI) was significantly (P = 0.03) greater than that for control dogs (20.08 MFI; range, 14.0 to 23.96 MFI; Figure 1). A moderate positive correlation was identified between platelet surface expression of P-selectin and GPIb (r_S = 0.67; P = 0.03). A strong negative correlation was identified between platelet surface expression of P-selectin and PT (r_S = –0.71; P = 0.02). Platelet surface expression of P-selectin was not correlated with CT, any TEG variable, platelet surface expression of αIIbβ3, or BUN or serum creatinine concentration.

Discussion

Results of the present study indicated that dogs with CKD frequently had platelet dysfunction and global alterations in coagulation. Platelet dysfunction and prolonged CTs have been reported in 14% to 60% of human patients with end-stage renal disease,[24,25] which is similar to the proportion (4/11 [36%]) of dogs with CKD in the present study that had an abnormally prolonged CT.

Anemia causes prolonged CT in both humans and dogs.[26,27] In human patients, the CT becomes prolonged when Hct decreases to < 25% and unreadable when Hct is < 10%.[26] In an in vitro study[27] involving canine blood, the CT (as determined by PFA and use of a collagen-ADP platelet agonist) became prolonged as the Hct was diluted from 35% to 27%; however, although there was a significant negative association between CT and Hct in that study,[27] the magnitude of that association is unlikely to be clinically relevant. In a study[25] of human patients with end-stage renal disease who were undergoing hemodialysis, there was a significant negative association between CT and Hct, although that association became nonsignificant when the Hct exceeded 35%. Conversely, in another study[28] of human patients with CKD, Hct and CT were not correlated. In a study[7] of dogs with experimentally induced renal failure, BMBT improved with RBC transfusion alone, and a similar phenomenon has been described in human patients with CKD.[29] In the present study, the median Hct for dogs with CKD was significantly less than that for control dogs; however, only 3 of 11 dogs with CKD had an Hct < 32%. Unlike TEG variables, our laboratory has not established reference ranges for CT at various Hcts. In fact, for the dogs with CKD in the present study, Hct was not significantly correlated with CT, which suggested that Hct did not contribute to the prolonged CT identified in some of those dogs.

Results of a study[28] involving human patients with CKD indicate that BUN and serum creatinine concentrations are both positively correlated with CT (as determined by PFA and use of a collagen-ADP platelet agonist), and the percentage of patients with an abnormally prolonged CT increased as the severity of azotemia increased. Conversely, results of a study[30] involving uremic human patients indicate a poor correlation between CT and GFR and between CT and serum creatinine concentration, and CT was not correlated with BUN concentration and other indicators of platelet function such as skin bleeding time and aggregometry variables. In the present study, CT was not correlated with BUN or serum creatinine concentrations. To our knowledge, the present study was the first to evaluate PFA CT in dogs with CKD. All dogs with CKD evaluated in the present study were classified with either IRIS stage III or IV disease. If CT is truly correlated with the severity of azotemia in dogs, that correlation should be more readily detected in dogs with advanced CKD than in dogs in the earlier stages of the disease. Further studies that involve dogs at all IRIS stages of CKD are necessary to better elucidate the relationship between azotemia and CT.

Although the median CT for dogs with CKD was significantly greater than that for control dogs in the present study, 7 of the 11 dogs with CKD had a CT that was within the reference interval. In human patients with CKD, there is poor correlation between CT and skin bleeding time,[30] and PFA results are unable to reliably predict which patients will have bleeding complications after renal biopsy.[24] In the present study, we did not evaluate dogs with CKD for clinical bleeding complications, so the correlation between CT and clinically relevant bleeding remains unknown. Nevertheless, the majority (7/11 [64%]) of dogs with CKD had a CT within the reference interval, which suggested that, although the CT differed significantly between dogs with CKD and healthy control dogs, the magnitude of that difference may not be clinically relevant. Instead, monitoring CT over time in an individual dog with CKD might be more useful than measurement of CT at 1 point in time to detect platelet dysfunction.

In the present study, many dogs with CKD had TEG tracings that were indicative of blood coagulation defects. The most common defects identified were an abnormally increased MA and G value, which were suggestive of hypercoagulability. The G value is derived from the MA and is an index of clot strength; thus, it was not surprising that those 2 variables had parallel trends. Dogs with glomerular disease and nonprotein-losing CKD also have TEG tracings that are suggestive of hypercoagulability (hypercoagulable TEG tracings).[31,32] Similarly, human patients with CKD often have hypercoagulable TEG tracings, with abnormally increased MA and G values being the most consistently reported abnormalities.[30,33]

Only 1 of the 11 dogs with CKD in the present study had an abnormally decreased α angle and prolonged reaction time, which were indicative of hypocoagulability (hypocoagulable TEG tracing), whereas the TEG tracings for the remaining 10 dogs were either within reference limits or indicative of hypercoagulability. For the dog with the hypocoagulable TEG tracing, a platelet count was not performed on the day that the blood for TEG was collected but was within the reference interval 2 days earlier. Although an abnormally decreased platelet count would result in an altered G value, it should not alter the α angle and reaction time. The dog with the hypocoagulable TEG tracing also had a prolonged CT; however, the other dogs with CKD and a prolonged CT had hypercoagulable TEG tracings. Hematocrit can affect the TEG tracings of dogs.[18,34] Anemia is associated with a hypercoagulable TEG tracing, and decreasing Hct is strongly correlated with an abnormally increased MA and α angle and an abnormally decreased clotting time.[18,34] The dog with the hypocoagulable TEG tracing had an Hct of 37.7%, which was slightly less than the lower limit of the reference interval (40% to 59%) and would be expected to result in a clinically normal or hypercoagulable TEG tracing. Thus, the origin of the hypocoagulable TEG tracing for that dog was unknown.

The relationship between anemia and hypocoagulability is characterized clinically by abnormally prolonged skin bleeding times or BMBTs.[7,35] In the present study, the median Hct for dogs with CKD was significantly less than that for healthy control dogs. However, when the TEG variables for the dogs with CKD were evaluated with the reference interval adjusted for the lower Hct (Hct20), they were still indicative of hypercoagulability. This suggested the dogs with CKD that had hypercoagulable TEG tracings in the present study were truly in a hypercoagulable state, and the abnormal TEG variables were not simply a reflection of assay artifact.

Hyperfibrinogenemia and hypercoagulable TEG tracings have been reported for dogs with protein-losing nephropathy and renal interstitial disease.[32] In the present study, the median plasma fibrinogen concentration for the dogs with CKD was significantly greater than that for the control dogs, and there was a strong positive correlation between plasma fibrinogen concentration and both the MA and G value. In patients with kidney disease, hyperfibrinogenemia is believed to be a consequence of a proinflammatory disease state[32,36] or the result of abnormally decreased catabolism of fibrinogen by the kidneys.[37] The abnormally increased plasma fibrinogen concentrations for the dogs with CKD in this study might have contributed to the hypercoagulable TEG tracings in vitro; however, whether those dogs were in a true (in vivo) hypercoagulable state remains unknown.

In human patients, PFA is a poor indicator of clinical bleeding, and TEG is the best assay for the detection of mild hemostatic deficits that may increase the risk of bleeding following kidney biopsy, with an abnormally decreased α angle and prolonged clotting time being the best predictive indicators of bleeding

risk.[38] Thromboelastography also has high positive and negative predictive values for correctly identifying dogs at risk of clinical bleeding.[39] We did not evaluate bleeding times for the dogs of the present study; therefore, we cannot draw any conclusions regarding the ability of TEG variables to predict clinical bleeding in dogs with CKD. Serial evaluation of TEG variables for a larger number of dogs with CKD than that evaluated in this study is warranted to further elucidate the use of TEG variables for prediction of bleeding in dogs.

Thromboelastography is also a useful tool to predict thrombus formation in human patients following simultaneous pancreas and kidney transplantations.[40] To our knowledge, use of TEG to predict thrombosis in dogs with CKD has not been evaluated. Hypercoagulable TEG tracings have been described for dogs with thrombosis associated with parvoviral enteritis[41]; however, a correlation between specific TEG variables and risk of thrombosis has not been identified. Although 6 of 11 dogs with CKD in the present study had an abnormally increased MA or G value, the development of thrombosis-associated complications was not recorded. Only 4 dogs with CKD were weakly positive for D-dimers, and the D-dimer concentration for all 4 dogs (≤ 400 ng/mL) was well below the cutoff (500 ng/mL) that was previously determined to have a clinical sensitivity of 100% for the prediction of thromboembolic disease in dogs.[42] In human patients, CKD is associated with abnormally increased fibrinolysis and the generation of fibrinogen fragments and D-dimers.[6] Additional studies are necessary to evaluate the ability of TEG to predict thromboembolic complications in dogs with CKD.

The contribution of platelets to clot strength (ie, G value) is controversial. Recent developments in TEG technology have led to new platelet analyses, which specifically evaluate the effect of platelets on clot strength by comparing TEG variables with and without platelet inhibition. Unfortunately, that technology was not available at the time the present study was conducted; therefore, we had to rely on the direct comparison of TEG variables for 2 groups of dogs (dogs with CKD and healthy control dogs), which were obtained from samples that were processed in an identical manner. Future studies to determine the effect of platelet function on viscoelastic measurements should use the recently developed specific test protocols.

Flow cytometric evaluation of platelet surface expression of GPIb and αIIbβ3, the platelet surface receptors for vWF and fibrinogen, respectively, was performed to evaluate the underlying cause of platelet dysfunction and viscoelastic coagulation abnormalities in dogs with CKD. Platelet surface expression of GPIb did not differ between dogs with CKD and the healthy control dogs. Human patients with uremia have abnormally decreased platelet surface expression of GPIb, which is negatively correlated with the extent of azotemia and associated with abnormally decreased platelet aggregation and adhesion between platelets and the endothelial lining of blood vessels.[43] In healthy individuals, a large percentage of GPIb is stored in the intracellular storage pool of platelets and is redistributed to the platelet surface when necessary (ie, platelets are activated).[44] In humans with CKD, the total platelet pool of GPIb becomes severely depleted, but there is only a mild decrease in the expression of surface-bound GPIb, which is consistent with translocation of intracellular GPIb to replace cleaved surface GPIb.[45] To our knowledge, the present study was the first in which platelet surface expression of GPIb was evaluated in dogs with CKD. However, we did not evaluate the intracellular pool of GPIb, and further investigation is necessary to determine whether the total platelet pool of GPIb is depleted in dogs with CKD.

The platelet receptor for fibrinogen, αIIbβ3, mediates platelet-platelet binding and aggregation. It can also bind vWF and weakly contributes to platelet-platelet interactions in the absence of fibrinogen.[6,46] Integrin αIIbβ3 is converted to an active state by an inside-out mechanism, which occurs secondary to the binding of extracellular agonists such as ADP and thrombin, resulting in G-protein–coupled release of intracellular calcium ions.[47] In human patients and mice with uremia, release of ADP and thrombin from α granules is impaired, which limits the downstream aggregation of platelets.[6] In the present study, the platelet surface expression of αIIbβ3 was significantly greater for dogs with CKD than for control dogs and was positively associated with serum creatinine concentration, findings that were similar to results for human patients with CKD.[6] The cause of the increase in platelet surface expression of αIIbβ3 in patients with impaired kidney function is unknown but may be a compensatory response for other defects in platelet function. That compensatory response may explain why some dogs with CKD had a CT within the reference interval but abnormally increased TEG variables. However, in patients with CKD, normal binding of fibrinogen is frequently impaired, even in the face of an increase in platelet surface expression of αIIbβ3, because of receptor occupation by fibrinogen fragments.[6] The presence of fibrinogen fragments was not assessed in the present study; thus, the effect of fibrinogen fragments on αIIbβ3 binding was not determined. The risk of bleeding can increase subsequent to a lack of adequate conversion of platelets to an active state by ADP or thrombin or the lack of appropriate fibrinogen binding, even in the presence of clinically normal or abnormally increased expression of αIIbβ.

In the present study, although platelet surface expression of GPIb was within the reference interval and expression of αIIbβ3 was abnormally increased for dogs with CKD, neither GPIb nor αIIbβ3 expression was correlated with PFA CT or any TEG variable, which suggested that alterations in platelet surface expression of glycoprotein receptors are not respon-

sible for the changes in platelet function and blood coagulation observed in those patients. We did not assess the function or binding ability of the GPIb and αIIbβ3 receptors, so even though their expression may be clinically normal or abnormally increased, their ability to bind ligands and contribute to platelet adhesion and aggregation may be impaired. In human patients with CKD, vWF and fibrinogen binding to αIIbβ3 is abnormally decreased despite clinically normal receptor expression because those receptors are occupied by fibrinogen fragments or other uremic compounds, and that defect in ligand binding is reversed with dialysis.[6] Further investigation is necessary to determine whether similar disruptions in ligand binding are present in dogs with CKD.

P-selectin is expressed on the membrane of α granules and is transported to the platelet surface after activation.[48] P-selectin aids in the recruitment of inflammatory cells and generation of thrombin during thrombus formation,[48] and its expression on the platelet surface has been used experimentally as a marker of platelet activation in humans and dogs.[6,19] In human patients with CKD, flow cytometric evaluation of platelets indicates an increase in unstimulated expression of markers of platelet activation, including P-selectin, suggestive of an increase in circulating activated platelets, which is inversely correlated with the GFR for those patients.[6,49] Those findings are consistent with the results of the present study in which platelet surface expression of P-selectin was abnormally increased in dogs with CKD. However, platelet surface expression of P-selectin was not significantly correlated with BUN and serum creatinine concentrations in the dogs with CKD. Additional evaluation of P-selectin expression and more sensitive assessments of GFR, such as iohexol clearance, in dogs with CKD should be performed to elucidate the relationship between P-selectin expression and alterations in renal function. An abnormal increase in platelet activation may contribute to the risk of thrombosis in dogs with CKD, as has been suggested in human patients with CKD.[6] Additional research is needed to evaluate the clinical relevance of abnormally increased platelet activation in dogs with CKD.

The present study had several limitations. The study population was small, and a larger study population might have enabled us to detect other subtle or less common hemostatic derangements in dogs with CKD. The small study population also might have caused type II error in regard to platelet surface expression of GPIb. Additionally, 10 of the 11 dogs with CKD in this study were classified with IRIS stage III disease, and the remaining dog was classified with IRIS stage IV disease. We expect that alterations in platelet function or coagulation are more likely to be detected in patients with advanced disease. Further research that involves dogs in all IRIS stages is necessary to determine whether the platelet deficits identified in the present study change as CKD progresses. In the present study, the only significant correlation identified for the platelet surface markers evaluated was a positive correlation between platelet surface expression of αIIbβ3 and serum creatinine concentration, which suggested that other soluble uremic compounds are responsible for the coagulation changes observed in dogs with CKD. Additional studies should also include a control group that is matched to dogs with CKD on the basis of age and breed.

We expected the platelet surface expression of P-selectin in healthy dogs to be much lower than that observed in the control dogs of the present study. The reason for that unexpected finding may have been an artifact of the erythrolysis protocol used, which has been reported to increase the expression of P-selectin on unstimulated platelets.[50] Additionally, although we followed protocols used by other investigators, we used a different anti-canine P-selectin monoclonal antibody, which may have been more sensitive than the anti-mouse or anti-human antibody used in that other study.[51] For the present study, use of the same protocol and reagents to evaluate samples for both the dogs with CKD and control dogs allowed us to make comparisons between the 2 groups.

An important weakness of the present study was the use of recalcified nonactivated TEG analysis because when no platelet activator is used, the resulting TEG variables have high variability and poor reproducibility.[52] In fact, there is only moderate, albeit significant, correlation between TEG variables for split samples that are analyzed by nonactivated TEG and kaolin-activated TEG.[53] Thromboelastography variables obtained for activated samples cannot be directly compared with those obtained for nonactivated samples or among variables obtained by use of various activators, and TEG variables obtained for samples that are activated by strong platelet activators are less variable than those obtained for samples that are activated by weak platelet activators.[52–54,r] Nonactivated TEG was used for the present study because the laboratory at our institution has established reference intervals for that method.[18] Results of 1 study[53] involving healthy dogs indicate that, compared with kaolin-activated samples, nonactivated samples yield reaction and clotting times that are longer and α angles, MAs, and G values that are decreased (ie, hypocoagulable tracings). Therefore, given that nonactivated TEG resulted in hypercoagulable tracings for many of the dogs with CKD in the present study, it is likely kaolin-activated TEG would have also resulted in hypercoagulable tracings for those dogs.

Many dogs with CKD evaluated in the present study had prolonged CTs (as determined by PFA and a collagen-ADP platelet agonist) and alterations in blood viscoelastic coagulation that were consistent with hypercoagulability. Many of those dogs also had alterations in the platelet surface expression of αIIbβ3 and P-selectin, but those changes did not entirely explain the changes observed for CT and the TEG variables, and their correlation with a declining GFR remains

unclear. Additional studies are warranted to elucidate the causes of platelet defects and coagulopathies in patients with uremia and how those causes affect the risk of clinical bleeding or thrombosis in dogs with CKD.

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Footnotes

a. BD Vacutainer Safety-Lok blood collection set, Becton, Dickinson and Co, Franklin Lakes, NJ.
b. ADVIA 2120i, Siemens Healthcare Diagnostics, Deerfield, Ill.
c. COBAS 6000 c501, Roche, Indianapolis, Ind.
d. STA compact, Diagnostica Stago, Parsippany, NJ.
e. D-dimer Slide agglutination kit, Remmel, Lenexa, Kan.
f. PFA-100, Siemens Healthcare Diagnostics, Deerfield, Ill.
g. PFA-100 Collagen/ADP Test Cartridge, Siemens Healthcare Diagnostics, Deerfield, Ill.
h. TEG 5000 Thrombelastograph Hemostasis Analyzer System, Haemonetics Corp, Niles, Ill.
i. FACSCalibur, BD Biosciences, San Jose, Calif.
j. Erythrolyse Red Cell Lysing Buffer, AbD Serotec, Raleigh, NC.
k. Cell Staining Buffer, BioLegend Inc, San Diego, Calif.
l. Clone SZ2 mouse IgG1 FITC-conjugated, Beckman Coulter Inc, Brea, Calif.
m. Clone SZ21 mouse IgG1 FITC-conjugated, Beckman Coulter Inc, Brea, Calif.
n. Smith CW, Baylor College of Medicine, Houston, Tex.
o. Clone STAT70, Serotec USA, Washington, DC.
p. BD Calibrite, BD Biosciences, San Jose, Calif.
q. GraphPad Prism 6, La Jolla, Calif.
r. Shelton M, Griffith E, Spencer J, et al. Mode of activation significantly impacts thromboelastographic results and assay variability (abstr). *J Vet Intern Med* 2014;38:1092.

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