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Characteristics of hemostasis during experimental *Ehrlichia canis* infection

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

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

Animals: Four healthy laboratory beagles.

Methods: Dogs were given blood infected with *E. canis* IV. Platelet indices of activation, platelet aggregometry, antiplatelet antibodies (percent IgG), complete coagulation panel, and thromboelastography (TEG) were measured before inoculation and on weeks 1-8. Dogs were treated with doxycycline at approximately 5 mg/kg PO q12h between weeks 3 and 4 (day 24). For each variable, 1-way repeated measures analysis (1-way ANOVA) with post-hoc analysis was performed with statistical significance set at $P < .05$.

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

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

KEYWORDS
aggregometry, platelet, thrombocytopenia, thromboelastography

Abbreviations: α, rate of clot formation; AA, arachidonic acid; ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; AUC, area under the curve; AUC_{AA}, AUC for AA; AUC_{ADP}, AUC for ADP; AUC_{saline}, AUC for saline control; CL30, clot lysis 30 minutes after MA; CL60, clot lysis 60 minutes after MA; HCT, hematocrit; K, clotting time; L, total lysis; LY30, percent clot lysis 30 minutes after MA; LY60, percent clot lysis 60 minutes after the MA; MA, maximum amplitude; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MPC, mean platelet component concentration; MPM, mean platelet mass; MPV, mean platelet volume; MRTG, maximal rate of thrombus generation; OSPT, one-stage prothrombin time; PCDW, platelet component distribution width; PCT, plateletcrit; PDW, platelet volume distribution width; percent IgG, percent of immunoglobulin associated platelets; TEG, thromboelastography; TF, tissue factor; TF-TEG, TF-activated TEG; TG, total thrombus generated; TF + tPA-TEG, TF-activated TEG + tissue plasminogen activator; TMRTG, time to maximum rate of thrombus generation; MRL, maximum rate of lysis; TMRL, time to maximal rate of lysis; R, reaction time

1 | INTRODUCTION

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

2 | MATERIALS AND METHODS

2.1 | Experimental *E. canis* infection

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

A total of 8 mL of anticoagulated blood was collected from the client-owned *E. canis*-infected dog and all 4 of the *E. canis*-naïve dogs were each given 2 mL of this blood IV via the cephalic vein. After inoculation, the dogs were observed daily for clinical abnormalities and blood samples were scheduled to be collected on weeks 1–8. In addition to testing for infection, other assays performed included CBC (Siemens, ADVIA 120 Hematology System, Erlangen, Germany), plasma fibrinogen concentration (Tcoag AMAX Destiny Plus, Bray, Wicklow, Ireland), tissue factor (TF)-activated TEG (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation, Braintree, Massachusetts; TF-TEG), TF-activated TEG (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation) with tissue plasminogen activator (tPA) added (TF + tPA-TEG), whole blood impedance platelet aggregometry (Multiplate 5.0 Analyzer, Diapharma Group Inc, West Chester, Ohio), and direct flow cytometry detecting percent of immunoglobulin-associated platelets (percent IgG) and are described below. The dogs were fasted for 10 hours before each analysis and approximately 12 mL of blood from the jugular vein was drawn with a 20 gauge needle using a vacutainer (BD Vacutainer Single Use Needle Holder, Franklin Lakes, New Jersey). The blood for TEG and platelet aggregometry assays was drawn from the other jugular vein with a 20 gauge needle. For the TEG and aggregometry assays, each blood collection event had to have a single penetration of the vein with no redirection. The appropriate volumes of blood were placed into the following tubes: EDTA (BD Vacutainer K2 EDTA 3.6 mg 2.0 mL tubes, Franklin Lakes, New Jersey), red top (Covidien Monoject Blood Collection Tube No Additive 5.0 mL tubes, Minneapolis, Minnesota; serum), 3.2% sodium citrate buffered (BD Vacutainer 3.2% sodium citrate tubes, Franklin Lakes, New Jersey), and a heparin tube (Sarstedt lithium heparin micro tube, Numbrecht, Germany), respectively, and were gently inverted 5 times to allow proper mixing.

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

2.2 | Complete blood cell count and standard coagulation tests

A CBC was performed on each sample collection day and the following variables were recorded: hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV), plateletcrit (PCT), platelet volume distribution width (PDW), mean platelet mass (MPM), mean platelet component concentration (MPC), and platelet component distribution width (PCDW). Activated partial thromboplastin time (APTT; Tcoag AMAX Destiny Plus), one-stage prothrombin time (OSPT; Tcoag AMAX Destiny Plus), and antithrombin activity (Tcoag AMAX Destiny Plus) were measured at week 0 and weeks 2–6 in all dogs. D-dimer concentrations (Tcoag AMAX Destiny Plus) were measured at week 0 and weeks 2–8 in all dogs. Plasma fibrinogen concentration (Tcoag AMAX Destiny Plus) was measured at all time points (weeks 0–8).

2.3 | Thromboelastography

For TF-TEG, citrated blood samples were allowed to sit at room temperature for 30 minutes before analysis. Briefly, the cups were warmed

to 37°C and 20 μL of 0.2 M $CaCl_2$, 10 μL of TF (Tcoag TriniCLOT PT Excel, Bray, Wicklow, Ireland) at a final dilution of 1:1000, and 330 μL of citrated whole blood were added to the cup and analyzed. TF was prepared before each individual TEG analysis. The TEG tracings then were generated for at least 60 minutes and the variables reaction time (R), clotting time (K), rate of clot formation (α), and maximum amplitude (MA) were recorded.

For TF + tPA-TEG, citrated blood samples were allowed to sit at room temperature for 30 minutes before analysis. Briefly, the cups were warmed to 37°C and 20 μL of 0.2 M $CaCl_2$ and 10 μL of TF (Tcoag TriniCLOT PT Excel) at a final dilution of 1:1000 were added to the cup. To prepare tPA (Cathflo Activase [Alteplase], 2 mg Vial, Carroll, Ohio), the vial was reconstituted with sterile water resulting in 1.08 million units/mL. Then, 4.1 μL of the reconstituted tPA was added to 996 μL of a phosphate-buffered solution (PBS) to make the stock tPA solution. The stock solution (10 μL) was added to 400 μL of citrated whole blood, mixed gently, and 330 μL of this mixture was added to the cup and analyzed. The TF was prepared before each individual TEG analysis and the tPA solution was kept on ice between analyses but was discarded after each testing period. The TEG tracings then were generated for at least 60 minutes and the variables R, K, α, MA, percent of clot lysis 30 minutes after MA is reached (LY30), amount of clot lysis 30 minutes after MA is reached (CL30), percent of clot lysis 60 minutes after the MA is reached (LY60), and amount of clot lysis 60 minutes after MA is reached (CL60) were recorded. Additionally, velocity curve variables were recorded, denoted as the maximal rate of thrombus generation (MRTG), time to maximum rate of thrombus generation (TMRTG), total thrombus generated (TG), maximum rate of lysis (MRL), time to maximal rate of lysis (TMRL), and total lysis (L). Controls (Level I [Levels I and II controls; Haemonetics Corporation, Braintree, Massachusetts] and Level II [Levels I and II controls; Haemonetics Corporation]) were performed at each time point as recommended by the manufacturer (TEG 5000 Thromboelastograph Hemostasis Analyzer, Haemonetics Corporation) before analysis of the samples.

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

2.4 | Whole blood impedance platelet aggregometry

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

2.5 | Direct flow cytometry assay for platelet-associated immunoglobulin

The protocol used for our study was modified from other protocols previously described in the literature.[13,14] Blood (700 μL) anticoagulated with EDTA was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200*g* for 1 minute 30 seconds at 20°C to generate platelet-rich plasma (PRP). Platelet-rich plasma was removed from the erythrocyte layer and placed into an Eppendorf tube (Light Labs SNAPLOCK Microcentrifuge Tubes, Dallas, Texas). Each PRP sample was adjusted to 2×10^6 cells/mL using a manual hemocytometer to provide a standard volume of PRP that then was pelleted by centrifugation at 1000*g* for 5 minutes at 20°C. The platelets were resuspended and washed 3 times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature with 50 μL of a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled rabbit anti-dog IgG (FITC-conjugated AffiniPure rabbit anti-dog IgG (H + L) Jackson ImmunoResearch Labs, 304-095-003, West Grove, Pennsylvania) for 30 minutes. After incubation, the platelets were washed 3 times with PBS-EDTA-BSA solution and resuspended in 200 μL of PBS-EDTA-BSA solution for flow analysis. Gate settings used for our study were previously established with PE-labeled mouse anti-human CD61 (Anti-human CD61 [Integrin beta 3] PE, VI-PL2, 12–0619-42, eBioscience, San Diego, California) using healthy dog samples. Platelets from a healthy beagle served as a negative control at each time point. Samples were considered positive if the percent IgG was > 2 standard deviations above the reference range determined from the negative control and the 4 healthy beagles before inoculation with *E. canis*. All samples were analyzed using a Cyan ADP instrument (Cyan ADP instrument, Beckman Coulter, Miami, Florida) and the generated data was analyzed using FlowJo software (FlowJo software, Tree Star, Ashland, Oregon).

2.6 | Statistical analysis

For each response variable, a repeated measures analysis (1-way ANOVA) was performed using the lme4 and lmerTest packages in R (R package, version 3.2.2). To account for repeated measures, dog was included as a random effect. Time points were compared with week 0

TABLE 1 Dunnett's test results for the TF-activated TEG, TF + tPA TEG, and whole blood impedance platelet aggregometry variables in beagles experimentally infected with *E. canis*

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

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

using Dunnett's method with the lsmeans package in R (R package, version 3.2.2). To investigate the effects of doxycycline on the measured variables, an additional analysis was performed where time points were compared with week 3 using Dunnett's method with the lsmeans package in R (R package, version 3.2.2). For all tests, a $P < .05$ was considered significant.

3 | RESULTS

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

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

Several statistically significant differences from baseline were identified in all dogs for TEG and platelet aggregometry at multiple time points (Table 1) with the exception of only 4 variables: TF + tPA-TEG variables R, TMRTG, and MRL and platelet aggregometry variable

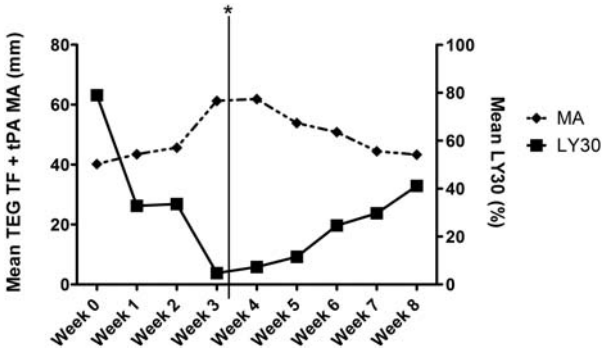

FIGURE 1 Mean MA values and mean percent clot lysis 30 minutes after MA (LY30) from TF + tPA TEG over time in beagles experimentally infected with *E. canis*. *The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline

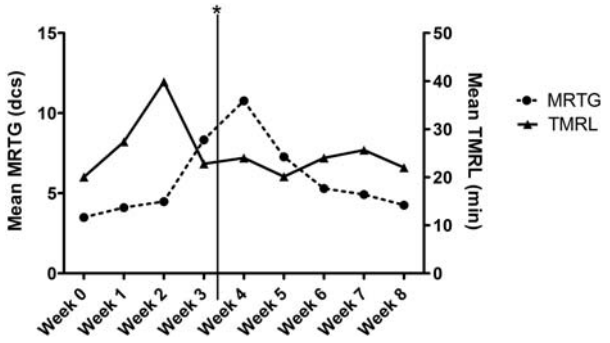

FIGURE 2 Mean MRTG and mean TMRL from TF + tPA TEG over time in beagles experimentally infected with *E. canis*. *The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline

AUC_{saline}. Similarly, Dunnett's method identified multiple statistically significant differences from week 3 in all dogs at multiple time points (Table 1) with the exception of 4 variables: TF + tPA-TEG variables K, TMRTG, and MRL and platelet aggregometry variable AUC_{saline}. For TF-TEG, dogs had shorter R times, smaller K values, larger α values, and higher MA values as compared with baseline at the respective time points (Table 1). For TF + tPA-TEG, dogs had smaller values for K, LY30, LY60, and higher values for α, MA, CL30, CL60, MRTG, TG, TMRL, and L as compared with baseline at the respective time points (Table 1). After doxycycline administration on day 24, several statistical differences were noted as compared to week 3 (Table 1). Overall, for TF-TEG, at week 3, dogs had shorter R times, smaller K values, larger α values, and larger MA values as compared to other time points. Overall, for TF + tPA-TEG, at week 3, dogs had smaller values for R, K, LY30, LY60, and TMRL and higher values for α, MA, CL30, CL60, MRTG, TG, and L as compared with the respective time points. The average MA values and average LY30 values over time in relationship to doxycycline administration are presented in Figure 1. The average MRTG and average TMRL values over time in relationship to doxycycline administration are presented in Figure 2.

For platelet aggregometry, no significant differences were identified in the values for AUC_{saline} as compared with baseline or week 3. However, the AUC_{ADP} and AUC_{AA} were significantly lower than baseline at the respective time points (Table 1). To further evaluate the effect of doxycycline administration, for AUC_{ADP}, at week 3, dogs had significantly lower values as compared to other time points (Table 1). Specifically, the AUC_{ADP} was statistically higher at weeks 5 (difference, 106.50; *P*-value, .042), 6 (104; 0.049), 7 (166.75; 0.001), and 8 (149.00; 0.002) as compared with week 3. Similarly, for AUC_{AA}, at week 3, dogs also had significantly lower values as compared to other time points (Table 1). Specifically, the AUC_{AA} was statistically higher at weeks 0 (difference, 98.00; *P*-value, .009), 4 (86.00; 0.026), 5 (100.25; 0.007), 6 (115.25; 0.002), 7 (150.00; $<$0.001), and 8 (149.25; $<$0.001) as compared with week 3.

Results from the repeated measures 1-way ANOVA for the hematology and flow cytometry were significantly different for all variables with the exception of PDW. Multiple statistically significant differences from baseline in all dogs for the hematology and flow cytometry variables at multiple time points (Table 2), with the exception of only 4 variables: OSPT, PDW, MPC, and PCDW, were observed. Similarly, Dunnett's method identified multiple statistically significant differences from week 3 in all dogs at multiple time points (Table 2) with the exception of PDW. Overall, dogs had lower antithrombin concentrations, platelet counts, PCT, HCT, and MCHC as compared to baseline at the respective time points (Table 2). In contrast, dogs had higher D-dimers, OSPT, plasma fibrinogen concentration, MPV, MPM, MCV, and percent IgG as compared to baseline at the respective time points (Table 2). The average plasma fibrinogen concentrations and average MPM values over time in relationship to doxycycline administration are presented in Figure 3. After doxycycline administration on day 24, several statistical differences were noted as compared with week 3. Overall, at week 3, dogs had lower antithrombin concentrations, platelet counts, PCT, MPC, HCT, and MCHC as compared with other time points (Table 2). In contrast, at week 3, dogs had higher D-dimers, OSPT, APTT, plasma fibrinogen concentrations, MPV, MPM, PCDW, MCV, and percent IgG as compared with the respective time points (Table 2). The average platelet counts and average percent IgG over time in relationship to doxycycline administration are presented in Figure 4.

4 | DISCUSSION

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

The results from TF-TEG and TF + tPA-TEG showed that experimentally inoculated dogs appeared hypercoagulable as compared with baseline as defined by a higher MA value and hypofibrinolytic as defined by a lower LY30 and LY60 and a higher CL30 and CL60.[16]

TABLE 2 Dunnett's test results for hematology and flow cytometry variables in beagles experimentally infected with *E. canis*

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

Abbreviations: APTT, activated partial thromboplastin time; HCT, hematocrit; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPC, mean platelet component concentration; MPM, mean platelet mass; MPV, mean platelet volume; OSPT, one-stage prothrombin time; PCT, plateletcrit; PCDW, platelet component distribution width; PDW, platelet volume distribution width; percent IgG, percent of immunoglobulin associated platelets.

Additional analyses were performed to compare week 3 results to those from subsequent time points, because it was unknown if doxycycline administration would affect TEG parameters. The time point week 3 was chosen for comparison because the dogs were most clinically affected at this time and because doxycycline administration was started on day 24 of the study. After doxycycline administration, TF-TEG MA and TF + tPA-TEG MA did not statistically differ from week 3 until week 6 or week 7, respectively. After doxycycline administration, it took 2 weeks (week 6) for TF-TEG MA and 3 weeks (week 7) for TF + tPA-TEG MA to return to baseline. Before doxycycline administration, the fibrinolysis variables LY30, CL30, LY30, and CL60 all were statistically different from week 3 but did not return to baseline until week 8 with the exception of CL60. Thus, the results suggest that the dogs were hypofibrinolytic until week 8 (Figure 1) approximately 5 weeks after doxycycline administration. The velocity curve variables are generated from the TEG tracing and represent the first derivative of the associated waveform. These variables provide information regarding clot formation and clot breakdown.[16] The MRTG reflects the maximal velocity of increase in clot strength and observed clot growth whereas TMRTG reflects the time interval required to reach maximal rate of clot formation.[17,18] Total thrombus generated reflects amount of clot strength produced and represents total area under the velocity

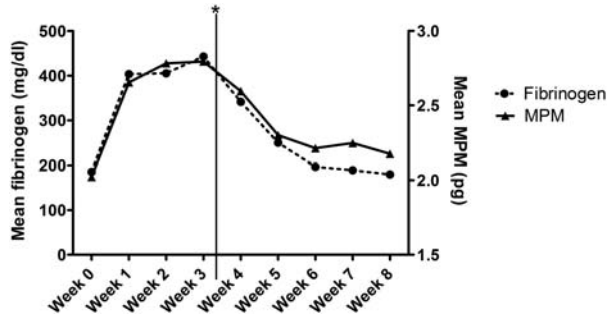

FIGURE 3 Mean fibrinogen and MPM over time in beagles experimentally infected with *E. canis*. *The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline

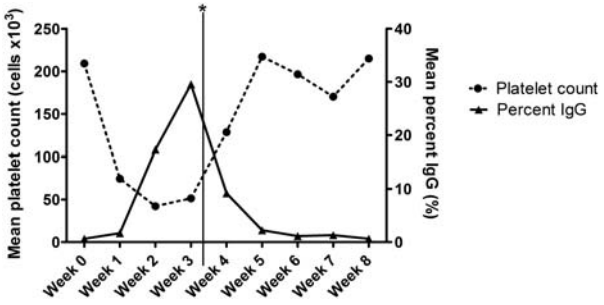

FIGURE 4 Mean platelet counts and mean percent IgG over time in beagles experimentally infected with *E. canis*. *The vertical solid line represents when all dogs were administered 5 mg/kg PO, twice daily of doxycycline

curve during clot formation.[18] As a result, an increased MRTG, decreased TMRTG, and increased TG could represent a hypercoagulable state.[17,19] With regards to clot breakdown, MRL, TMRL, and L can be evaluated. The MRL reflects maximal velocity of clot breakdown, TMRL reflects the time interval required to reach maximal rate of clot lysis, and L represents total amount of clot lysis. Therefore, an increased MRL, decreased TMRL, and increased L could represent a hyperfibrinolytic state and vice versa for a hypofibrinolytic state.[20] Overall, velocity curve results aligned with traditional TEG results in that the dogs appeared hypercoagulable and hypofibrinolytic. When evaluating the results from the dogs as compared with baseline, the dogs appeared hypercoagulable based on significantly larger MRTG and TG values (at weeks 3, 4, and 5) and also demonstrated hypofibrinolysis based on a longer TMRL value (at week 2). It also was observed that the dogs appeared hypercoagulable by week 3 (increased MRTG) and week 5 (increased TG) and these changes persisted and did not return to baseline until week 8. The cumulative findings from both TEG analyses and from velocity curve results indicated that dogs were hypercoagulable and hypofibrinolytic at certain time points over the course of infection and that these changes persisted for variable amounts of time after doxycycline administration.[16] A hypercoagulable and hypofibrinolytic state could result in a hemostatic phenotype rather than a bleeding phenotype, which could help explain why bleeding may not be observed in dogs infected with *E. canis* despite severe thrombocytopenia.

The overall function of platelets also could explain why bleeding is or is not observed in *E. canis* infection. If platelets are in an activated state, they will function in primary hemostasis to prevent bleeding.[21] However, if a thrombocytopathy is present, bleeding may be observed regardless of the platelet count, but particularly if the platelet count is low. We investigated platelet function using both whole blood impedance aggregometry and parameters measured on the Advia 120 hematology analyzer. The platelet aggregometry was normal except for time periods when the dogs were thrombocytopenic. Significant decreases in platelet count were observed by week 1 and remained statistically lower than baseline until week 4 and similarly, AUC_{ADP} and AUC_{AA} were statistically lower than baseline at weeks 2 and 3. Additionally, the initiation of doxycycline administration between weeks 3 and 4 likely affected the platelet count and resultant AUC readings.[22] The results show that platelet count returned to baseline by week 5 (Figure 2) and AUC returned to baseline by weeks 4 and 5, approximately 1–2 weeks after doxycycline administration. Therefore, no evidence of platelet dysfunction was detected before thrombocytopenia was documented or once the platelet count started to rebound and normalize. Additionally, platelet activation parameters were increased. Therefore, another reason why dogs infected with *E. canis* may not show evidence of bleeding despite severe thrombocytopenia is if platelets are in an activated state. Variables indicating increased platelet activity include increased MPV, MPM, and PCDW and decreased MPC.[11] These changes occur in the platelet because of release of intracellular components such as dense granules in addition to morphology changes in their integral shape.[11] Based on our results and aforementioned indices, there was evidence of platelet activation at weeks 2, 3, and 4 (increased MPV) and at weeks 1, 2, 3, and 4 (increased MPM) as compared with baseline. After doxycycline administration, MPV and MPM returned to baseline and were statistically different when results from week 3 and week 5 were compared. The dogs were noted to have significant decreases from their baseline platelet counts during weeks 1, 2, and 3 and had platelet counts that ranged from 20 to 100 $\times$ $10^3/\mu L$. Therefore, there was evidence of platelet activation during the periods of most severe thrombocytopenia, but it is also plausible that these changes could have been related to increased platelet release from the bone marrow as a response to thrombocytopenia. The differentiation between these 2 possibilities was not possible in our study. Because there was evidence of potential platelet activation during these time periods, such a situation could have contributed to why no bleeding events were observed despite the dogs having significant thrombocytopenia.

Possible causes for thrombocytopenia and subsequent bleeding in *E. canis* infected dogs include destruction of platelets by platelet-directed antibodies, increased clearance of platelets from circulation by the spleen and sequestration as a result of vasculitis.[4–7,9,23] The percent IgG was statistically higher than baseline at week 3 but statistically significant thrombocytopenia also was identified at weeks 1, 2, and 3. This finding is in agreement with other studies in which the thrombocytopenia observed in *E. canis* infections likely was caused by several mechanisms and not solely platelet-directed antibodies. Interestingly, the percent IgG decreased after administration of doxycycline in all dogs and they all were considered negative by week 5 (Figure 2). This finding was surprising because the half-life of most of the immunoglobulin G (IgG) subclasses in dogs is estimated to be similar to that of humans, which is approximately 20–21 days. Four subclasses of IgG occur in both humans and dogs, but they are categorized differently.[24] A previous study reported that natural and experimental *E. canis* infections in dogs are associated with a predominance of IgG2 rather than IgG1.[25] Immunoglobulin G2 is the functional human analog to the canine IgG subclass A which is not thought to be involved in antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, but research is ongoing to better characterize the canine IgG subclasses.[24] The rapid decrease in percent IgG after doxycycline may be related to other nonantimicrobial effects of this drug. For example, it has been previously documented that dogs infected with *E. canis* and healthy dogs had an increase in platelet counts suggesting that doxycycline may result in platelet proliferation.[22] Additionally, doxycycline has been shown to decrease antibody production and inhibit proliferating lymphocytes.[22,26] It also has been shown to decrease the expression of IgG on granulocytes, but it is unknown if this occurs with platelets.[27] Therefore, if the platelets have decreased survival in circulation because of antibody binding but doxycycline causes proliferation of platelets and decreased antibody production, this could explain why the percent IgG decreased so rapidly. It is currently unknown if doxycycline decreases IgG expression on platelets similar to granulocytes or if it interferes with antibody-antigen binding on the platelet surface. Further studies are needed to determine the relationship between doxycycline and the percent IgG response in thrombocytopenic dogs.

Another notable finding observed during our study was that all of the dogs were strongly positive on serology (SNAP 4Dx Plus, IDEXX

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

Dogs infected with *E. canis* have been shown previously to have decreased platelet function based on several platelet aggregation studies,[3,10] but to our knowledge, no studies have been performed using multiple channel electrical impedance platelet aggregometry. However, when using impedance platelet aggregometry, decreased platelet counts and a high HCT can cause in vitro effects on platelet aggregometry resulting in lower AUC readings.[28–30] As a result, AUC readings may appear erroneously low because of thrombocytopenia rather than reflecting true platelet dysfunction. To address this, an alternative technique is to standardize the platelet count in all samples before performing aggregometry. Doing so requires manipulation of the platelets (centrifugation) and the test is not performed in whole blood which may not reflect the true changes occurring in vivo (in whole blood).[3,10] Our testing was performed using whole blood, and the platelet count was not adjusted. As a result, the observed AUC readings likely were affected by the significant changes in platelet count and HCT observed at certain time points, which is a limitation of the study.

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

Although the mechanisms of thrombocytopenia and bleeding are not completely understood in dogs infected with *E. canis*, our results showed that activated platelets and a hypercoagulable, hypofibrinolytic state may explain the lack of a bleeding phenotype in some dogs despite substantial thrombocytopenia. Additionally, further studies are needed to investigate the relationship between doxycycline administration and platelet dynamics in thrombocytopenic dogs.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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