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Platelet aggregometry testing during aspirin or clopidogrel treatment and measurement of clopidogrel metabolite concentrations in dogs with protein-losing nephropathy

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Abstract

Background: Dogs with protein-losing nephropathy (PLN) are treated with antiplatelet drugs for thromboprophylaxis but no standardized method exists to measure drug response. It is also unknown if clopidogrel metabolite concentrations [CM] differ between healthy and PLN dogs.

Objectives: Assess response to aspirin or clopidogrel in PLN dogs using platelet aggregometry (PA) and compare [CM] between healthy and PLN dogs.

Animals: Six healthy and 14 PLN dogs.

Methods: Platelet aggregometry using adenosine diphosphate (ADP), arachidonic acid (AA), and saline was performed in healthy dogs at baseline and 1-week postclopidogrel administration to identify responders or nonresponders. A decrease of ≥60% for ADP or ≥30% for AA at 1 or 3 hours postpill was used to define a responder. At 1 and 3 hours postclopidogrel, [CM] and PA were measured in healthy and PLN dogs. Platelet aggregometry was performed in PLN dogs at baseline, 1, 6, and 12 weeks after clopidogrel or aspirin administration.

Results: In PLN dogs receiving clopidogrel, PA differed from baseline at all time points for ADP but not for AA at any time point. Most dogs responded at 1 or both time points except for 1 dog that showed no response. For PLN dogs receiving aspirin, no differences from baseline were observed at any time point for either ADP or AA. No differences in [CM] were found at either time point between healthy and PLN dogs.

Conclusions and Clinical Importance: Platelet aggregometry may represent an objective method to evaluate response to clopidogrel or aspirin treatment and PLN dogs appear to metabolize clopidogrel similarly to healthy dogs.

KEYWORDS
aspirin, renal disease, responder, thromboprophylaxis

1 | INTRODUCTION

Protein-losing nephropathy (PLN) in dogs is associated with a hyper-coagulable state and thromboembolic risk.[1-9] The true prevalence of thromboembolic complications in PLN dogs is difficult to ascertain but has been reported to be more than 40% in previous studies.[10] Medications for clot prevention or thromboprophylaxis therefore are recommended as part of standard management in PLN dogs.[10-12] Interestingly, this approach is not a standard recommendation in affected humans. In people, PLN refers to a diverse group of diseases including IgA nephropathy, post-infectious glomerulonephritis, lupus nephritis, and other glomerular diseases.[13] These diseases are considered major types of glomerulonephritis in people but thromboprophylaxis only is recommended for patients with membranous nephropathy (MN).[14-17] Specific medications and monitoring are not yet standardized in people with MN, but recently an algorithm has been developed.[16] Similarly, although thromboprophylaxis is advised in dogs with PLN, treatment recommendations and monitoring protocols have not been established.[10] Currently, no consensus exists on what medications are optimal for clot prevention and what monitoring if any should be performed in dogs once on treatment.

According to consensus guidelines for standard treatment in PLN dogs, aspirin or clopidogrel is recommended for thromboprophylaxis.[12] In humans, both aspirin and clopidogrel also are commonly utilized for clot prevention, but resistance is documented frequently, and patients are classified as responders or nonresponders (specifically referring to the antiplatelet effects of aspirin rather than anti-inflammatory effects). There are several possible explanations for resistance including non-compliance or drug interactions, but also genetic polymorphisms or alternative pathways that can circumvent inhibition resulting in platelet activation despite appropriate medication administration.[18] It is unknown if genetic polymorphisms or alternative pathways exist in dogs that could result in aspirin or clopidogrel resistance. It is estimated, however, that only about one third of dogs will respond consistently to aspirin treatment, and thus aspirin resistance is suspected to occur. It is unknown, however, if clopidogrel resistance occurs, but platelet response heterogeneity has already been observed in healthy dogs.[19-21] Because of resistance, it is generally agreed upon in human medicine that monitoring of treatment response using platelet function testing (PFT) is indicated, but specific protocols have not been determined.[18]

Additionally, people with kidney disease, particularly those with chronic kidney disease (CKD) can have a decreased response to anti-platelet treatment, but it is unknown if a similar effect occurs in dogs with renal disease such as those with PLN.[22] This is particularly relevant because proteinuria can lead to renal damage resulting in progression of disease and worsened azotemia, increased risk of uremic crisis, and a higher rate of mortality in dogs with CKD.[23,24] In addition to assessing treatment response using PFT, aspirin and clopidogrel administration also can be evaluated in dogs by measuring urinary or serum metabolites, but this approach has not been evaluated in dogs with renal disease.[25-27] Therefore, the purpose of our study was to assess response to aspirin or clopidogrel in dogs with PLN using whole blood impedance platelet aggregometry (PA) and to compare clopidogrel metabolite concentrations [CM] between healthy and PLN dogs.

2 | MATERIALS AND METHODS

2.1 | Animals

Client and staff-owned dogs presented to the hospital between December 2015 and July 2017 were prospectively enrolled in this observational study. The study was approved by the Clinical Review Board and the Institutional Animal Care and Use Committee and all owners gave informed consent at study enrollment. For healthy dogs to be eligible for inclusion, a normal physical examination and minimum database were required (CBC, serum biochemistry profile, urinalysis with no proteinuria). Healthy dogs could not have any concurrent illnesses or be receiving medications or supplements other than heartworm and other parasite prevention treatments. A total of 10 healthy dogs were to be recruited for the study, but because of cost limitations, 6 dogs were enrolled. Based on previous studies, 6 dogs were deemed an acceptable number to determine overall representative response to clopidogrel when given to healthy dogs.[21,27] For PLN dogs, a diagnosis of PLN was required and was defined as persistent proteinuria with an inactive sediment, negative urine culture, and no known comorbidities that could result in proteinuria. Enrolled dogs were required to have a CBC, serum biochemical profile, urinalysis, and urine protein: creatinine ratio (UPC) performed before study enrollment. Diagnostic procedures such as abdominal ultrasound examination and tick-borne disease testing were encouraged but not required. Renal biopsy was not required for enrollment. Additionally, dogs receiving drugs that could cause proteinuria or affect platelet function such as glucocorticoids or nonsteroidal anti-inflammatory drugs were not eligible. The use of fish oil was not an exclusion criterion because antioxidant treatment is recommended for dogs with PLN.[12]

2.2 | Sample collection and medication administration

2.2.1 | Healthy dogs

At study enrollment, blood was collected from fasted dogs into heparin tubes (Sarstedt lithium heparin micro tube, Numbrecht, Germany) for baseline PA before starting clopidogrel. Clopidogrel (75 mg tablets, Aurobindo Pharma USA, East Windsor, New Jersy) was given once daily at approximately 2-3 mg/kg PO for 1 week. Then, at 1 week, PA was repeated at 1 and 3 hours postpill, but PA also was performed at 12 hours postpill in a subset of dogs. Aggregometry results were used to establish cutoff values to define a responder or nonresponder as described below in Section 3, and [CM] also were measured at 1 and 3 hours postpill and at 12 hours postpill in a small subset of dogs using heparinized blood samples (Covidien Monoject Sodium Heparin 3 mL tubes, Dublin, Ireland).

2.2.2 | PLN dogs

At study enrollment, blood was collected into heparin tubes for baseline PA before starting aspirin or clopidogrel. Clopidogrel was administered once daily at approximately 2-3 mg/kg PO or compounded aspirin in a gelatin capsule (Geri-Care Aspirin tablets 81 mg, Gulfport, Mississippi) was administered once daily at approximately 1 mg/kg PO at the discretion of the primary clinician. Aspirin was compounded based on body weight by the hospital's compounding pharmacy. Then, PA was repeated in addition to measurement of hematocrit and a manual platelet count was performed at 1, 6, and 12 weeks after starting the prescribed medication. For dogs receiving clopidogrel, at each evaluation, blood was collected within 1-3 hours postpill with the exception of 1 dog in which blood was collected 12 hours postpill. For dogs receiving aspirin alone, blood was collected 4-6 hours postpill. The [CM] were measured using heparinized blood samples (Covidien Monoject Sodium Heparin 3 mL tubes, Dublin, Ireland) at 1 of the evaluations (1 or 6 weeks) 1 and 3 hours postpill. All dogs were fasted for analyses. For each dog, any documented or suspected thrombotic complication was recorded. A thrombotic complication was defined as documentation of a thrombus in any location during the study time period or the development of acute neurologic, cardiovascular, pulmonary, or extremity (eg, lack of pulses, cold extremity) abnormalities during the study time period that could not be attributed to other causes.

2.2.3 | Whole blood impedance PA

For multiple channel electrical impedance PA (Multiplate 5.0 Analyzer, Diapharma Group Inc, West Chester, Ohio), heparinized blood samples were kept at room temperature and analyzed within 32 minutes of blood collection for 12 minutes. Before analysis, test cells were warmed to 37°C in the aggregometer and an automated pipette was utilized. Heparinized blood samples (300 µL) were diluted with 300 µL 0.9% NaCl and incubated for 3 minutes. Adenosine diphosphate (ADP) 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}) was recorded (PA for ADP and AA were performed in duplicate). The AUC value is an arbitrary unit. As recommended by the manufacturer (Diapharma Group Inc), final concentration of ADP was 6.5 µM and final concentration of AA was 0.5 mM. To serve as a control with no agonist added, an identical volume of saline was used in place of the agonists (AUC_{saline}). Reagents were reconstituted and stored in 60 µL aliquots according to the manufacturer's recommendations (Diapharma Group Inc). The test was repeated if any quality control flags were observed for ADP, AA, or saline.

2.2.4 | Clopidogrel metabolite concentrations

For each dog, heparinized blood was centrifuged within 15 minutes of blood collection and plasma was stored at −80°C until analysis. The [CM] were determined using a previously validated and reported

assay in dogs using high-performance liquid chromatography.[21] Using this assay, the inactive metabolite SR 26334 was measured and the reported limit of quantification was 0.1 µg/mL.

2.2.5 | Statistical analysis

Statistical analysis was performed using R (R package, version 3.2.1 and 3.6.1). For each response variable, a repeated measures (RM) analysis (1-way analysis of variance [ANOVA]) was performed, and subsequent time points (1, 6, and 12 weeks) were compared to baseline results for ADP, AA, hematocrit, and platelet count. A RM analysis (2-way ANOVA) was performed separately to evaluate differences in either response variable (saline PA results and [CM]) between healthy and PLN dogs. Specifically for saline PA results, analysis was performed using the lme4 and emmeans packages. Log transformation of saline PA results was performed to satisfy model assumptions. Saline PA results of zero were assigned a value of 1 in order to perform the log transformation. Statistical significance was set at $P \leq .05$.

3 | RESULTS

3.1 | Animals

3.1.1 | Healthy dogs

Six staff-owned dogs were enrolled. Three were spayed females and 3 were castrated males. Median age was 4.25 years (range, 1-9 years). Median weight was 25.5 kg (range, 13.6-30.7 kg). Breeds included Standard Poodle (n = 2), Corgi, Corgi mix, Australian Shepherd mix, and Mastiff mix. No bleeding complications were observed in any dog during the study.

3.1.2 | PLN dogs

Fourteen client-owned dogs were enrolled. Ten were spayed females and 4 were castrated males. Median age was 9.5 years (range, 5-16 years). Median weight was 19.1 kg (range, 4.5-31.5 kg). Breeds included West Highland White terrier (n = 2), Pomeranian, Golden Retriever, Labrador Retriever, Corgi, Airdale, Standard Poodle, Weimaraner, Greyhound, French Bulldog, Shar Pei, mixed breed (n = 2). Abdominal ultrasonography was performed in 13/14 dogs but was not performed in 1 dog (Shar Pei) because of progression of renal disease and quality of life concerns. Ten dogs received clopidogrel alone (dose range 1.8-3.2 mg/kg PO q24), 3 dogs received aspirin alone (1.0-1.2 mg/kg PO q24), and 1 dog was started on aspirin (1 mg/kg PO q24) but was changed to clopidogrel (3 mg/kg PO q24) during the study. Six dogs (43%) were azotemic as determined by International Renal Interest Society (IRIS) staging (4/14 dogs were IRIS stage 2 with serum creatinine concentrations of 1.6-1.9 mg/dL, 1 dog was IRIS stage 3 with serum creatinine concentration of 3.0 mg/dL, and 1 dog was IRIS

stage 4 with serum creatinine concentration of 5.2 mg/dL). Six dogs (43%) were hypoalbuminemic (range, 1.8-2.6 g/dL). Of the 6 dogs that were azotemic at the time of enrollment, 2/6 dogs had proteinuria before the development of azotemia. The 4 remaining azotemic dogs all were hypoalbuminemic (range, 1.6-2.6 g/dL), had UPC values ranging from 3.75-15.32, 3/4 dogs had mild azotemia (IRIS stage 2), and 2/4 dogs had suspected or were documented to have thrombotic complications. Thrombotic complications were definitively identified in 3 dogs, suspected in 2 additional dogs during the study and suspected in 1 dog upon follow-up evaluation. In confirmed cases, all thrombi were visualized by ultrasound examination and included 1 left external iliac artery thrombus, a caudal aortic thrombus, and a portal vein thrombus with bilateral renal infarcts. Suspected cases included 2 dogs that presented for probable stroke or vascular event because of acute onset of neurologic signs that rapidly improved with no progression and 1 dog that died during sleep. One dog was euthanized during the course of the study and 4 dogs were euthanized upon follow-up evaluation after the study was concluded. Reasons for euthanasia included progressive CKD (n = 3), suspected nephrotic syndrome (n = 1), and suspected neoplasia. A necropsy was not performed in any of the cases. No bleeding complications were noted in any of the dogs receiving aspirin, but 2 dogs receiving clopidogrel had abnormal bleeding noted (eg, integument bruising after cystocentesis and mild gingival bleeding).

3.2 | Whole blood impedance PA

3.2.1 | Healthy dogs

All dogs completed the study and received clopidogrel with a dose range of 2.0-2.8 mg/kg PO q24. All dogs had PA performed at 1 and 3 hours postclopidogrel administration but 4/6 dogs had PA performed at 12 hours postpill. Responder and nonresponder cutoff values were determined from ranges and average percentage decreases in AUC observed in healthy dogs receiving clopidogrel. When defining cutoff values, the approximate lowest percentage inhibition observed was chosen. For ADP and AA at 1 hour postpill, the average percentage decrease in AUC_{ADP} was 76.9% (range, 65.7%-91.9%) and in AUC_{AA} was 60.0% (range, 35.1%-88.5%). For ADP and AA at 3 hours postpill, the average percentage decrease in AUC_{ADP} was 81.1% (range, 60.2%-93.5%) and in AUC_{AA} was 55.1% (range, 29.2%-81.6%). For ADP and AA at 12 hours postpill, the average percentage decrease in AUC_{ADP} was 84.0% (range, 73.0%-95.5%) and in AUC_{AA} was 61.9% (range, 44.3%-80.3%). For ADP, a patient was considered a responder to clopidogrel if the AUC_{ADP} decreased by ≥60% from baseline at 1 or 3 hours. For AA, a patient was considered a responder to clopidogrel if the AUC_{AA} decreased by ≥30% from baseline at 1 or 3 hours.

3.2.2 | PLN dogs

Twelve dogs completed the entire study, but only baseline and 1-week samples were collected in 2 dogs (1 died acutely and 1 was euthanized).

For dogs receiving clopidogrel alone, 9/10 dogs had PA performed at 1 hour postpill and all dogs had PA performed at 3 hours postpill. Platelet aggregometry was performed 1 week after initiating clopidogrel in 8/9 dogs and was performed at the 6 week re-evaluation in 1 dog. Two of the dogs (2/9) had an increase in the AUC_{AA} and were not included in the data. For ADP and AA at 1 hour postpill, average percentage decrease in AUC_{ADP} was 61.5% (range, 7.2%-90.7%) and in AUC_{AA} was 48.9% (range, 13.6%-85.6%). For ADP and AA at 3 hours postpill, average percentage decrease in AUC_{ADP} was 68.0% (range, 12.4%-93.8%) and in AUC_{AA} was 39.5% (range, 0.8%-84.8%). The classifications of responder versus nonresponder are presented in supplemental information. Most dogs were classified as responders at 1 or both time points, except 1 dog classified as a nonresponder that showed no response to ADP or AA at any time point. All of the dogs classified as responders continued to be classified as such at subsequent re-evaluations during the study, and the 1 dog that was classified as a nonresponder never exhibited a response at any of the subsequent re-evaluations during the study. Average AUC_{ADP} and AUC_{AA} results at baseline, 1 hour, and 3 hours in healthy dogs and PLN dogs are shown in Figure 1. Statistical analysis was not performed for saline PA in PLN dogs because of the presence of 2 outliers. For the majority of PLN dogs, all saline results were <10, but 2 dogs had saline results of 101 and 122 at baseline, but at subsequent time points all saline results were <10. In PLN dogs receiving clopidogrel, PA results differed from baseline at all time points (1-week estimated difference, −141.1, standard error (SE) = 19.3, 95% confidence interval (CI) = −190.0 to −91.9, P < .001; 6-week estimated difference, −155.2, SE = 21.9, CI = −211.0 to −99.3, P < .001; and 12-week estimated difference, −141.0, SE = 23.2, CI = −200.0 to −82.0, P < .001) for ADP but no statistical differences were observed for AA (1-week estimated difference, −66.4, SE = 33.4, CI = −152.0 to 18.9, P = .15; 6-week estimated difference, −44.6, SE = 37.9, CI = −141.0 to 51.9, P = .51; and 12-week estimated difference, −45.8, SE = 40.1, CI = −148 to 56.1, P = .54), or platelet count (1-week estimated difference, −18.9, SE = 43.1, CI = −129.2 to 91.4, P = .92; 6-week estimated difference, 11.9, SE = 49.4, CI = −114.0 to −137.8, P = .98; and 12-week estimated difference, 40.4, SE = 52.2, CI = −92.7 to −173.5, P = .76) at any time point. The hematocrit was not different from baseline at 1 week (estimated difference, −0.8, SE = 1.84, CI = −5.50 to 3.90, P = .92) or 6 weeks (estimated difference, −2.8, SE = 2.1, CI = −8.16 to 2.59, P = .43) but was lower at 12 weeks (estimated difference, −5.9, SE = 2.22, CI = −11.55 to −0.18, P = .04). Median hematocrit was 51% (range, 34%-64%). In dogs receiving aspirin alone, no statistical differences were observed for saline (1-week estimated difference, 23, SE = 23.4, CI = −45.1 to −91.1, P = .65; 6-week estimated difference, 26.3, SE = 23.4, CI = −41.9 to 94.4, P = .57; and 12-week estimated difference, −19.3, SE = 25.9, CI = −94.6 to 56.0, P = .78), ADP (1-week estimated difference, 6.0, SE = 37.2, CI = −102.2 to 114.0, P = .99; 6-week estimated difference, 9.5, SE = 37.2, CI = −98.7 to 118, P = .97; and 12-week estimated difference, 20.5, SE = 41.2, CI = −99.1 to 140, P = .90), AA (1-week estimated difference, −12.0, SE = 20.0, CI = −70.2 to 46.2, P = .86; 6-week estimated difference, 14.5, SE = 20.0, CI = −43.7 to 72.7, P = .79; and 12-week estimated difference, 24.8, SE = 22.2, CI = −39.6 to 89.1, P = .57); hematocrit (1-week estimated difference, −0.25, SE = 2.0, CI = −6.18 to 5.68, P = .99; 6-week
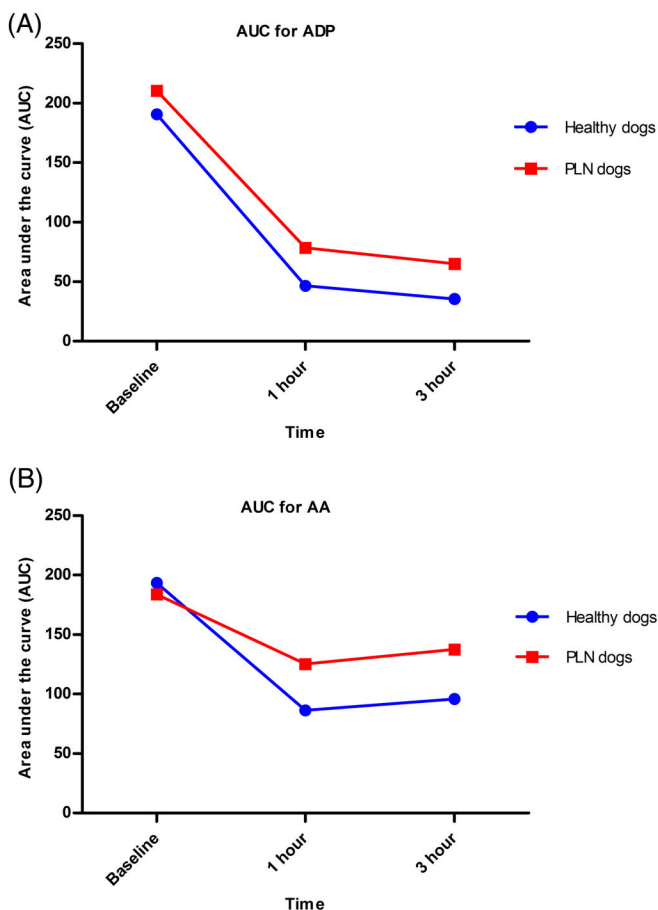
(A)

(B)

FIGURE 1 Whole blood impedance platelet aggregometry was performed in 6 healthy dogs after receiving clopidogrel for 1 week and in 14 protein-losing nephropathy (PLN) dogs after receiving clopidogrel for 1 or 6 weeks. A, The area under the curve (AUC) for platelet agonist adenosine diphosphate (ADP) was recorded at baseline, 1 hour, and 3 hours postclopidogrel administration. B, The AUC for platelet agonist arachidonic acid (AA) was recorded at baseline, 1 hour, and 3 hours postclopidogrel administration

estimated difference, −1.25, SE = 2.0, CI = −7.18 to 4.68, P = .85; and 12-week estimated difference, 3.3, SE = 2.3, CI = −3.28 to 9.83, P = .39) or platelet count (1-week estimated difference, 58.3, SE = 44.6, CI = −71.4 to 187.9, P = .47; 6-week estimated difference, −42.3, SE = 44.6, CI = −171.9 to 87.4, P = .67; and 12-week estimated difference, −20.4, SE = 49.5, CI = −163.3 to 122.5, P = .93) at any time point. Median hematocrit was 47% (range, 38%-55%). Saline platelet aggregometry results were statistically different between healthy and PLN dogs at baseline (estimated difference, −1.71, SE = 0.61, CI = −2.95 to −0.47, P = .01) but not at 1 (estimated difference, 0.32, SE = 0.61, CI = −0.91 to 1.56, P = .60) or 3 hours (estimated difference, −0.32, SE = 0.61, CI = −1.56 to 0.92, P = .60) postclopidogrel administration.

3.3 | Clopidogrel metabolite concentrations

The [CM] were determined in all healthy dogs and 8/10 PLN dogs receiving clopidogrel alone at 1 and 3 hours postpill. Four healthy
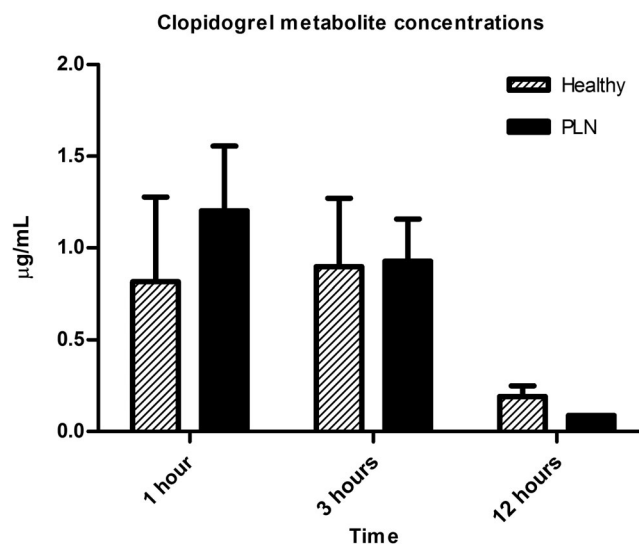

FIGURE 2 Clopidogrel metabolite concentrations (μg/mL) were measured in 6 healthy dogs and 8 protein-losing nephropathy (PLN) dogs at 1, 3, and 12 hours postclopidogrel administration

dogs (67%) and 1 PLN dog also had [CM] measured at 12 hours post-pill. No statistical differences were found in [CM] at 1 (estimated difference, −0.39, SE = 0.48, CI = −1.38 to 0.60, P = .43) or 3 hours (estimated difference, −0.03, SE = 0.48, CI = −1.02 to 0.96, P = .95) between healthy and PLN dogs (Figure 2). Statistical analysis was not performed at the 12-hour time point because too few samples were available.

4 | DISCUSSION

In dogs, PLN is associated with thromboembolic complications and thromboprophylaxis is recommended. However, no established protocols exist for determining which medications are optimal and what type of monitoring should be followed.[1-11] In veterinary medicine, thromboprophylaxis, using clopidogrel or aspirin, is recommended for all dogs with PLN. It is unknown if prophylaxis is indicated for all underlying causes of PLN.[12-16] Resistance to both of these drugs is a well-known phenomenon in people, which has resulted in genetic screening and also a need for PFT.[22,28-31] To complicate matters, CKD in people also is associated with resistance to antiplatelet treatment and is a consideration for cardiologists and nephrologists when treating patients with a variety of diseases, but in particular cardiovascular disease.[22,32-39] Interestingly, a recent study of human patients showed that presence of proteinuria independent of estimated glomerular filtration rate was associated with treatment resistance for clopidogrel, but not aspirin.[40] As mentioned previously, dogs with PLN can progress to CKD,[23,24] which raises concern for potential lack of treatment efficacy in veterinary patients. Objective ways to evaluate for treatment response in humans include PFT and measurement of various drug metabolites, but doing so is not common in veterinary practice. It is unknown if PFT is necessary in dogs with PLN, but aspirin resistance is known to occur

and clopidogrel resistance is possible.[19,20] These are important considerations for veterinarians because prescribed medications may not be efficacious in all patients, and individualized treatment may be necessary. Therefore, the purpose of our study was to evaluate the response to aspirin or clopidogrel in PLN dogs using PA in addition to comparing [CM] between healthy and PLN dogs.

The results of our study showed that a small sample of healthy dogs demonstrated a response (ie, decrease in AUC) after receiving a standard dose of clopidogrel. This phase of the study was completed so that cutoff values could be determined to classify if a PLN patient was a responder or nonresponder. In human medicine, the cut-off values for what determines an appropriate response vary depending on the study, but classifications also are considered with clinical outcome (ie, whether a thromboembolic complication occurred).[18] These studies have not yet been performed in veterinary medicine. The data from our small population of healthy dogs showed that an expected response to clopidogrel was at least a 60% decrease in AUC_{ADP} and at least 30% decrease in AUC_{AA} at 1 and 3 hours postpill, respectively. The reason why 1 and 3 hours were chosen was because it has been shown previously that the maximum decrease in AUC occurs at these time points postclopidogrel in healthy dogs.[21] Our study findings were similar to a recent study that evaluated different PFT in healthy dogs receiving aspirin, clopidogrel, or combination treatment, but the timing of blood collection was not described in that study.[27] The investigators of the previous study showed that, for PA, all dogs (6/6) had >50% decrease in AUC_{ADP} and 5/6 dogs had between 25% and 50% decrease in AUC_{AA} when receiving clopidogrel.

Based on our established cutoff values, PLN dogs were classified as responders or nonresponders. Overall, most (8/9) PLN dogs were considered responders when evaluating AUC_{ADP} at 3 hours postpill whereas this was less consistently appreciated at 1 hour postpill. Interestingly, unlike the healthy dogs, the majority of PLN dogs were considered non-responders for AUC_{AA} at 1 and 3 hours postpill. It is possible that dogs with PLN have more excitable platelets as a consequence of hypercoagulability and therefore have the ability to stimulate platelets by other mechanisms and overcome inhibition via the ADP pathway, as has been suggested in human medicine.[22] To evaluate for evidence of potential increased inherent platelet activity when no agonist was added, the AUC_{saline} was compared between healthy and PLN dogs and showed that PLN dogs had higher AUC_{saline} values at baseline compared to healthy dogs. Whether PLN dogs have platelet hyper-excitability or platelet dysfunction has not been evaluated previously. It is suspected, however, that this depends on many factors and is likely a dynamic process, similar to what is observed in human medicine.[41,42] A recent study evaluating platelet function in healthy dogs and dogs with CKD using a different platelet function assay concluded that CKD dogs exhibited platelet dysfunction. Some of the reported dogs had proteinuria. In that study, although CKD dogs had higher expressions of receptors associated with platelet activation (fibrinogen, P-selectin), this did not translate to overall platelet performance and function.[43] The use of fish oils was allowed in our study and fish oil previously has been documented to enhance platelet inhibition when given with aspirin.[44] Only 5 of the study dogs were

given fish oil; 1 of these dogs was receiving aspirin and 4 were receiving clopidogrel. None of the dogs receiving aspirin showed a response when evaluating PA and thus fish oil use did not seem to enhance platelet inhibition in this patient. Additionally, in the remaining 4 dogs that received fish oil, 1 of the dogs was the dog that was classified as a nonresponder to both agonists at both time points. Additionally, the dosage of fish oil that was used in all of our study patients was lower than the dosage used in the previous study.[44] Taken together, our classifications were unlikely to have been influenced by the use of fish oil. To determine if our classifications of the PLN dogs is clinically useful, correlating these classifications with clinical outcome will be necessary for future studies. Although our study was not designed to correlate responders or nonresponders with frequency of thromboembolic complications, we could more closely evaluate our classification scheme in the patients with suspected or documented vascular or thrombotic events.

In the 3 dogs with documented thrombus formation, thrombi were present at diagnosis of PLN. Two of 3 dogs were treated with clopidogrel and 1 dog was treated with aspirin at study enrollment. In the 2 dogs receiving clopidogrel, 1 of the dogs could not be classified because it had PA performed at 12 hours postpill rather than 1 and 3 hours postpill because of subsequent changes made to the study protocol. The other dog was classified as a responder only at 3 hours postpill for ADP but was classified as a nonresponder at 1 hour for ADP and at both time points for AA. This classification did not change throughout the study and the documented portal vein thrombus remained static but the associated peritoneal effusion resolved upon starting clopidogrel. In the dog receiving aspirin, no inhibition in ADP or AA was observed after initiating treatment and the caudal aortic thrombus persisted throughout the study without progression. In dogs with suspected thrombus formation, 2 were treated with clopidogrel and 1 was treated with aspirin at study enrollment. The dog that died acutely during sleep was receiving clopidogrel for 1 week. The second dog receiving clopidogrel was classified as a responder during the study period but, upon follow-up (33 weeks after starting clopidogrel), presented with acute neurologic cranial nerve deficits and vestibular signs. These signs were suspected to be the result of a vascular event. At that time, PA was performed 20 hours postpill during an emergency visit and, in contrast to the original PA findings (performed within 3 hours postpill), showed no platelet inhibition. Platelet aggregometry was repeated the next day 10 hours postpill and also showed no inhibition. Upon further questioning, the only change that had been made was that the patient was receiving clopidogrel in the evening with a larger amount of food. The owner was advised to give the clopidogrel in the morning with less food and repeat PA was performed at 1, 3, and 12 hours postpill. Based on these results, the patient would have been classified as a responder to AUC_{ADP} at 3 hours but as a nonresponder to AUC_{AA}. Additionally, PA also was markedly decreased at 12 hours postpill for ADP after making the change in medication administration. Consequently, it was hypothesized that the amount of food may have interfered with clopidogrel absorption.[30,45] The patient did not exhibit any additional signs consistent with thromboembolic complications but was euthanized for severe progressive azotemia and proteinuria

approximately 4 months later. In the dog receiving aspirin, no response was noted by PA at any time point and at the time of acute neurologic signs (week 12), the primary care clinician switched the dog to clopidogrel. Platelet aggregometry was performed 10 and 34 days (3 hours postpill) after starting clopidogrel and this patient would have been classified as a responder for AUC_{ADP} and a nonresponder for AUC_{AA} on both days. The neurologic signs markedly improved but the dog was euthanized because of progressive CKD approximately 3 months later but had no additional signs of thrombotic complications.

We did not find any differences in [CM] between healthy dogs and PLN dogs. In people with renal disease, certain enzymes and a transporter necessary for clopidogrel metabolism may be decreased.[32,46] In human patients in whom clopidogrel and associated metabolites were measured, moderate-to-severe CKD was associated with much lower [CM] than reported in people without renal disease, suggesting that clopidogrel biotransformation is decreased in individuals with renal disease.[39] The pathways and receptors for clopidogrel metabolism have been much more extensively studied in humans, and very little currently is known in dogs. To our knowledge, no studies have compared [CM] in people with proteinuric renal disease and healthy individuals. It was hypothesized that PLN dogs, particularly azotemic PLN dogs, might have different [CM] as compared to healthy dogs based on studies performed in humans with renal disease. Unfortunately, [CM] could not be measured in the dog with IRIS stage 4 CKD because it died 1 week into the study, and the dog that had IRIS stage 3 CKD was receiving aspirin. Future studies evaluating [CM] in a larger number of azotemic PLN dogs as compared to healthy dogs would be useful to determine if differences in drug metabolism associated with renal disease could have implications for the success of thromboprophylaxis. Interestingly, the dog that was defined as a nonresponder for both ADP and AA had similar [CM] as compared to the healthy dogs and other PLN dogs. This patient had no evidence of platelet inhibition in response to clopidogrel at any re-evaluation. Thus, despite [CM] that would have been expected to result in platelet inhibition, this dog showed little to no inhibition. This observation could indicate clopidogrel resistance and also was similar to findings in a recent study in healthy dogs receiving clopidogrel in which [CM] did not always correlate with differences in platelet inhibition.[27] This dog has not experienced any known thromboembolic events and has remained non-azotemic with a UPC < 2.5 and normal serum albumin concentration for at least 2 years.

Limitations of our study included the small number of dogs enrolled, particularly for dogs receiving aspirin at our institution, where clopidogrel is more commonly used. The dogs receiving aspirin were included in our study for several reasons. First, it is possible that the dogs in our study receiving aspirin would have shown a response to treatment based on PA if a higher dosage had been utilized.[25] Aspirin is still a reasonable drug for thromboprophylaxis, but PFT and future studies are indicated. Additionally, dogs receiving aspirin could have had further assessment of response to treatment in addition to PFT such as measurement of serum salicylate or urinary 11-dehydro-thromboxane B_2 concentrations.[25-27] Another reason dogs with aspirin were included despite small numbers was because PA appeared clinically useful when assessing a patient with a suspected severe

vascular event. If PA is available, this patient demonstrated how PFT potentially could be used in clinical practice when assessing response to treatment. Future studies could be pursued in patients with thromboembolic complications that have been treated with drugs for thromboprophylaxis to determine if PFT corroborates clinical signs and outcomes and helps guide future treatment. Other limitations include that renal biopsies were not performed in any of the cases. Ideally, studies comparing different types of glomerular diseases could be pursued in the future, and important factors such as response to treatment and outcomes (eg, frequency of thrombosis) could be assessed. Another limitation was that a single assay was chosen for PFT in our study. This assay was the only available assay at our institution. A potential reason why none of the dogs receiving aspirin showed a response or the single dog receiving clopidogrel showed no response was because the assay used in our study was too insensitive and that a more sensitive assay could have been utilized.[27,47] However, the optimal PFT in dogs has not been determined and the assay used in our study has been shown to detect platelet inhibition to both drugs in healthy dogs.[27,48] Therefore, it was suspected that these patients were not responding to their respective medication. For dogs receiving aspirin, this finding also could have been a consequence of the dosage of aspirin used because some evidence suggests that higher dosages of aspirin may more reliably inhibit platelet function.[25]

Our results showed that most dogs receiving clopidogrel at standard dosages were classified as responders at most time points for AUC_{ADP}, but 1 dog showed no platelet inhibition throughout the study despite having [CM] similar to those of other PLN dogs and healthy dogs. In dogs receiving aspirin at standard dosages, no platelet inhibition was appreciated at any time point. The [CM] were similar between healthy dogs and PLN dogs, indicating that metabolism and absorption after PO administration were likely similar between the groups. Clopidogrel and aspirin resistance may occur in some dogs, and PFT may aid in determining if such resistance is present. Hypothesized mechanisms for suspected resistance to aspirin or clopidogrel in our study that have been documented in people with renal disease include increased platelet turnover, increased platelet activity (increased fibrinogen, increased tissue factor), changes in the release of ADP and serotonin from platelet granules, and changes in the cytoskeletal arrangement of platelets (eg, glycoprotein IIb/IIIa, thrombin).[22,32,34,38-40] For aspirin resistance in people with renal disease, increased inflammation and oxidative stress have been shown to lead to increased concentrations of thromboxane A2 via pathways not inhibited by aspirin.[36] Future studies to evaluate the efficacy of these medications in various clinical settings as well as associations with clinical outcome are needed in veterinary medicine.

helping with interpretation of results, and all of the pet owners who allowed their dogs to participate in this study.

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

This study was approved by the IACUC; 16-6953A in addition to the Clinical Review Board; VCS #2015-38 at CSU.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.