


# Effects of immunosuppressive agents on the hemostatic system in normal dogs

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**Background:** In dogs, the effects of immunosuppressive medications on hemostasis are not well known.

**Hypothesis/Objectives:** The objective was to determine the effects of immunosuppressive medications on primary and secondary hemostasis. Our hypothesis was that cyclosporine and prednisone would increase markers of hypercoagulability and thromboxane synthesis, while azathioprine, mycophenolate mofetil, and leflunomide would have minimal effects on hemostasis.

**Animals:** Eight healthy dogs.

**Methods:** A randomized, cross-over study used aggregometry, the PFA-100 platelet function analyzer, viscoelastometry, platelet count, and prothrombin and activated partial thromboplastin times to evaluate hemostasis during the administration of prednisone, azathioprine, cyclosporine, mycophenolate mofetil, and leflunomide for 1 week each at standard oral doses. Urine 11-dehydro-thromboxane-B<sub>2</sub> (11-dTXB<sub>2</sub>) and 6-keto-prostaglandin-F<sub>1α</sub> (6-keto-PGF<sub>1α</sub>) concentrations, normalized to urine creatinine concentration, were measured.

**Results:** The aggregometry amplitude decreased from 51 ± 21 to 27 ± 14 (*P* = .002) during leflunomide treatment (ADP activation), but there were no differences in amplitude (*P* = .240) for any medications when platelets were activated with collagen. For all medications, there were no significant differences in viscoelastometry indices (ACT, *P* = .666; ClotRate, *P* = .340; and platelet function, *P* = .411) and platelet count (*P* = .552). Compared with pretreatment values, urinary 11-dTXB<sub>2</sub>-to-creatinine ratio increased (*P* = .001) after drug administration (from 3.7 ± 0.6 to 5.6 ± 1.1). Cyclosporine was associated with an increase (*P* < .001) in the 6-keto-PGF<sub>1α</sub>-to-creatinine ratio (from 10.3 ± 4.6 to 22.1 ± 5.3).

**Conclusions and Clinical Importance:** Most immunosuppressive drugs do not enhance platelet function or coagulation in healthy dogs, suggesting that these medications might not predispose hypercoagulable dogs to thromboembolism. The results of our study need to be correlated with the clinical outcomes of hypercoagulable dogs.

## KEYWORDS

cyclosporine, platelets, prostacyclin, thromboxane

**Abbreviations:** 6-keto-PGF<sub>1α</sub>, 6-keto-prostaglandin-F<sub>1α</sub>; 11-dTXB<sub>2</sub>, 11-dehydro-thromboxane B<sub>2</sub>; ACT, activated clotting time; ADP, adenosine diphosphate; aPTT, activated partial thromboplastin time; COX, cyclooxygenase; IMHA, immune-mediated hemolytic anemia; PFA, platelet function analyzer; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PT, prothrombin time

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## 1 | INTRODUCTION

Immune-mediated hemolytic anemia (IMHA) is a common cause of anemia in dogs.<sup>1</sup> The case fatality rate for canine IMHA approach 50%-70%,<sup>2</sup> and one of the most common cause of death is thromboembolic disease. The true incidence of thromboembolism in dogs with IMHA is unknown, but has been estimated to be as high as 50%-80%.<sup>3</sup> The exact mechanism of thrombus formation is unknown and probably multifactorial: dogs with IMHA have been shown to have an increase in hyperactive platelets,<sup>4</sup> procoagulant microparticles,<sup>5</sup> and intravascular tissue factor expression.<sup>6</sup> When activated, platelets release several vasoactive molecules that contribute to a hypercoagulable state and predisposition to thromboembolism.<sup>7</sup>

Treatment of IMHA in dogs is multimodal and is centered on immunosuppressive medications, drugs that inhibit hemostasis, and supportive care. Glucocorticoids are the most commonly used immunosuppressive medications for the treatment of IMHA in dogs but can be associated with undesirable adverse effects. Additional immunosuppressive agents such as cyclosporine, azathioprine, mycophenolate mofetil, and leflunomide are therefore often used concurrently with glucocorticoids, to reduce undesirable adverse effects by allowing reduced glucocorticoid doses, or to provide more rapid or potent immunosuppression.

Cyclosporine has become a popular drug for the treatment of IMHA in dogs.<sup>8</sup> In dogs, as in humans,<sup>9,10</sup> administration of cyclosporine is associated with a significant increase in thromboxane A<sub>2</sub> synthesis, especially at the time of peak blood drug concentrations in dogs receiving immunosuppressive dosages of cyclosporine.<sup>11,12</sup> Thromboxane A<sub>2</sub>, which is primarily produced by activated platelets, triggers vasoconstriction, and enhances platelet activation and aggregation, which decreases blood flow, promotes coagulation, and could predispose patients to excessive thrombus formation.<sup>13</sup> In human renal transplant recipients, patients treated with cyclosporine have increased thromboxane A<sub>2</sub> synthesis, and an associated increased rate of thrombus formation within the renal parenchyma when compared with patients receiving azathioprine.<sup>10</sup>

Azathioprine, mycophenolate mofetil, and leflunomide are other potent immunosuppressive agents commonly used to treat dogs with IMHA.<sup>8,14</sup> In human transplant patients receiving azathioprine, thromboxane synthesis does not increase<sup>10</sup> and, in mice treated with mycophenolate mofetil, there is actually a reduction in thromboxane synthesis.<sup>15</sup> The effects of leflunomide on thromboxane synthesis has not been determined. Although previous studies have evaluated the effects of cyclosporine on the hemostatic system in dogs,<sup>11,12</sup> comparable studies evaluating the effects of other immunosuppressive drugs on hemostasis have either not been performed in dogs or have measured only a limited range of drugs or hemostatic indices.<sup>16-20</sup> In dogs, the effects of azathioprine, mycophenolate mofetil, and leflunomide on the hemostatic system are currently almost completely unknown.

Because cyclosporine induces thromboxane synthesis in dogs, with an associated concern about potentially predisposing hypercoagulable dogs to thromboembolic complications, alternative immunosuppressive agents such as azathioprine, mycophenolate mofetil, and leflunomide

have the potential to be safer therapeutic options. However, the effects of these medications on the canine hemostatic system are essentially unknown, and there is a possibility that, as with cyclosporine, these drugs could also be prothrombotic. The objective of our study was to determine the effects of five immunosuppressive agents (prednisone, cyclosporine, azathioprine, mycophenolate mofetil, and leflunomide) on a variety of indices that assess primary and secondary hemostasis in dogs.

## 2 | MATERIAL AND METHODS

### 2.1 | Study design, animals

Eight healthy Walker Hound dogs, 4 intact females and 4 neutered males, with a median age of 2 years (range, 1.3-7.3 years) were used in our study. A sample size calculation was performed using data from a previous study that measured urinary 11-dehydro-thromboxane B<sub>2</sub> (11-dTXB<sub>2</sub>) concentrations in dogs receiving cyclosporine.<sup>12</sup> Based on the differences in means and standard deviations, and using a power of 0.8 and alpha of 0.05, a total of at least 7 dogs per treatment group was determined to be needed to detect a significant difference of 50% among groups. Dogs were deemed healthy by detection of no abnormalities on physical examination, complete blood count (including manual platelet count), serum biochemistry, urinalysis, and heartworm testing. The dogs were not exposed to any medications or vaccines for at least 1 month before initiation of the study. The median body weight was 27.7 kg (range, 24.7-29.6 kg). Body weights obtained at the beginning of the study were used to calculate all drug doses.

In a 5-way, randomized, cross-over study, the dogs were given either prednisone (2.0 ± 0.1 mg/kg, [mean ± SD], PO, q24h; Prednisone, West-Ward Pharmaceutical Corp, Eatontown, New Jersey), azathioprine (1.8 ± 0.1 mg/kg, PO, q24h; Azathioprine, Mylan Pharmaceuticals Inc, Morgantown, West Virginia), cyclosporine (10.3 ± 0.2 mg/kg, PO, q12h; Atopica, Novartis Animal Health, Greensboro, North Carolina), mycophenolate mofetil (9.9 ± 0.4 mg/kg, PO, q12h; Mycophenolate mofetil, West-Ward Pharmaceutical Corp), or leflunomide (4.0 ± 0.2 mg/kg, PO, q24h; Leflunomide, Trigen Laboratories, LLC, Sayreville, New Jersey). All drugs were administered for 7 days, followed by a recovery period of at least 21 days between dosing. In previous cyclosporine studies, changes in thromboxane production were seen within one week of commencing the drug, and had returned to pretreatment values within 2 weeks of discontinuing the medications.<sup>11,12</sup> After the recovery period, the dogs switched groups, and the study was continued until all dogs had received all medications.

For all medication groups, blood and urine samples were collected on day 0 (before drug administration) and on day 7 of drug administration, ~2 hours after drug administration. Blood was collected by jugular venipuncture with a 20-gauge needle directly into 4 separate 4.5 mL Vacutainer tubes containing 3.2% sodium citrate anticoagulant (aggregometry, Platelet Function Analyzer [PFA-100], SonoClot, one-stage prothrombin time [PT] and activated partial thromboplastin time [aPTT]; 3.2% sodium citrate, Vacutainer tube, Becton Dickinson, Franklin Lakes, New Jersey) and 1 tube containing EDTA (manual

platelet count). Two of the citrate tubes were used for aggregometry, 1 tube was used for PFA-100 and SonoClot analysis, and the final tube was used for the PT and aPTT. For the PFA-100 and SonoClot, the tube of blood was held at room temperature without disturbance. Immediately before analysis, the tube was inverted 3 times and the blood was inserted into the instruments via pipetting. Urine was collected by an ultrasound-guided cystocentesis using a 22-gauge 1.5 inch needle with a 6 mL syringe. The volume of urine collected varied between 2 and 5 mL, and urine was stored at  $-80^{\circ}\text{C}$  for later analysis. There were no notable technical problems encountered with venipunctures in individual animals that would be expected to adversely influence the results, and the collection of additional blood samples was not required.

## 2.2 | Optical platelet aggregometry

To create platelet-rich plasma (PRP), whole blood collected into 3.2% sodium citrate was centrifuged for 3 minutes at 1200g at room temperature. The PRP supernatant was collected and combined to create a single PRP sample. The remaining blood sample was centrifuged for 8 minutes at 1800g at room temperature to create platelet-poor plasma (PPP). A 2-channel platelet aggregometer (Chronolog 700 Whole Blood/Optical Lumi-Aggregometer, Chronolog Corporation, Haverton, Pennsylvania) was used to analyze platelet aggregation at a temperature of  $37^{\circ}\text{C}$  and a stir speed of 1200 rpm. Based on the manufacturer's standard guidelines (Chronolog 700 Manual, Chronolog Corporation), 247  $\mu\text{L}$  of PRP was placed into a glass cuvette containing a siliconized magnetic stir bar, and 250  $\mu\text{L}$  of PPP was placed into a cuvette without a stir bar. Samples were incubated for 1 minute at  $37^{\circ}\text{C}$ , inserted into the aggregometer, and stable baseline values for minimal (PRP) and maximal (PPP) aggregation were obtained. ADP (40  $\mu\text{M}$ ) or collagen (10  $\mu\text{g}/\text{mL}$ ) was added to the PRP, and aggregation was monitored for 6 and 8 minutes, respectively. The percentage aggregation at maximum amplitude was calculated by computer software (AGGRO/LINK 8, Chronolog Corporation). At each time point and drug group, 3 samples per dog were analyzed, and the results were averaged. All samples were analyzed within 5 hours of collection. Based on recommendations published by the International Society of Thrombosis and Hemostasis Platelet Physiology and Scientific and Standardization Committee, the platelet count in the PRP was not adjusted to a standardized number by dilution with PPP before analysis.<sup>21-24</sup>

## 2.3 | Platelet function analyzer

A PFA-100 (PFA-100, Siemens Healthcare Diagnostics, Deerfield, Illinois) that has been previously evaluated for use in dogs was used to analyze platelet function in whole blood.<sup>25,26</sup> The PFA-100 assesses platelet function under high shear forces after activation by platelet agonists, and measures the closure time, in seconds, needed to form a platelet plug and inhibit blood flow. The cut-off time for the instrument is  $>300$  seconds. The instrument was used according to manufacturer's instructions. For analysis, 800  $\mu\text{L}$  of citrated whole blood was placed into PFA-100 cartridges and analyzed. The collagen/ADP cartridge

(PFA Collagen/ADP Test Cartridge, Siemens Healthcare Diagnostics, Duluth, Georgia) was used initially to ensure normal platelet function in each dog before enrolment in the study, and the collagen/epinephrine cartridge (PFA Collagen/EPI Test Cartridge, Siemens Healthcare Diagnostics) was used during the study to assess drug-associated platelet dysfunction. Two collagen/epinephrine cartridges were analyzed concurrently, and the closure times were averaged. All samples were analyzed within 2 hours of collection.

## 2.4 | Viscoelastometry (SonoClot)

The SonoClot (SonoClot, Sienco Inc, Arvada, Colorado) uses a viscoelastometric system to detect mechanical changes that occur within a whole blood sample as it clots, and provides information on coagulation, fibrin gel formation, and clot retraction. The SonoClot contains a tubular probe that oscillates within a blood sample and detects the resistance to motion that the probe encounters as the sample clots, generating a clot signal. Clotting was triggered using the gbACT Kit (gbACT Kit, Sienco Inc), and the results were calculated by the Signature Viewer Program (Signature Viewer Program, Sienco Inc). The clot signal includes the activated clotting time (ACT; the time until the 1st detectable clot) in seconds, the clot rate (the slope of the SonoClot signature during the fibrin gel formation) in clot signal units per minute, and platelet function (an index which reflects the role of platelets in clot formation). The instrument was used according to the manufacturer's instructions. Samples were analyzed in duplicate and averaged. All samples were analyzed within 3 hours of collection.

## 2.5 | Urine 11-dehydro-thromboxane B<sub>2</sub>

Urinary 11-dTXB<sub>2</sub> (a stable thromboxane A<sub>2</sub> metabolite) concentration was measured using an ELISA kit (11-dehydro-thromboxane B2 EIA kit-Monoclonal, Cayman Chemical Co, Ann Arbor, Michigan) that has been previously validated in dogs.<sup>27,28</sup> For analysis, samples were thawed to room temperature, diluted with the assay buffer, and handled according to the manufacturer's instructions. Samples were analyzed in triplicate, analyzed at a wavelength of 412 nm using a plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, California), and reported in picograms per milliliter of urine. Urine creatinine concentration was measured using a biochemistry analyzer (ACE Alera, Clinical Chemistry System, Alfa Wasserman, Inc, West Caldwell, New Jersey) and used to calculate a urinary 11-dTXB<sub>2</sub>-to-creatinine ratio.

## 2.6 | Urine 6-keto-prostaglandin F<sub>1 $\alpha$</sub>

Urinary 6-keto-prostaglandin F<sub>1 $\alpha$</sub>  (6-keto-PGF<sub>1 $\alpha$</sub> , a stable prostacyclin metabolite) concentration was analyzed using an ELISA kit (6-keto-prostaglandin-F1 $\alpha$  EIA, Cayman Chemical Co) that has undergone analytic validation by the manufacturer and has been used previously in dogs.<sup>29</sup> Using a similar technique as described with urinary 11-dTXB<sub>2</sub>, urinary 6-keto-PGF<sub>1 $\alpha$</sub>  was analyzed according to the manufacturer's instructions. Samples were analyzed in triplicate, averaged, and reported in picograms per milliliter of urine. Urine creatinine

concentration was measured to calculate a urinary 6-keto-PGF<sub>1α</sub>-to-creatinine ratio.

## 2.7 | Additional tests of hemostasis

A manual platelet estimate was performed by calculating the average platelet number from 10 high power (oil immersion 1000 × magnification) microscopic fields representing a typical red blood cell monolayer, and multiplying this value by 16 to obtain a final platelet count (×10<sup>9</sup>/L). The PT and aPTT were determined by using the STA Compact (STA Compact, Diagnostica Stago, Parsippany, New Jersey), which was used according to the manufacturer's instructions to measure the clotting time of citrated plasma. Briefly, after recalcification, the PT was determined after activation with a freeze-dried thromboplastin prepared from human recombinant tissue factor (STA-Neoplastine, Diagnostica Stago), and the aPTT was determined by activation with kaolin (factor XII activator) and cephalin (platelet substitute; STA-C.K. Prest, Diagnostica Stago), using a viscosity-based mechanical clot detection system.

## 2.8 | Statistical analysis

The effect of immunosuppressive drugs on hemostatic indices were analyzed by mixed model analysis using PROC MIXED in a statistical computer program (SAS for Windows version 9.4, SAS Institute, Cary, North Carolina). Separate models were made for each assessment of hemostasis. Models included sampling day, drug, and sampling day-drug interaction as fixed effects. Run and dog within run were included as random effects. If the interaction term was not significant, it was removed and the model refit with just main effects. For significant interaction effects, differences in least square means between drugs on sample day 0 were calculated as well as between sampling days for each drug by LSMESTIMATE statement with a SIMULATE adjustment for multiple comparisons. Conditional residual plots were assessed to ensure that the assumptions of normal distribution of the residuals and equal variance had been met for the statistical methods. Although the initial analysis of 11-dTXB<sub>2</sub> did not meet these assumptions, they were met after log-transformation of 11-dTXB<sub>2</sub> values. In the interpretation of results, *P*-values ≤ .05 were considered significant.

## 3 | RESULTS

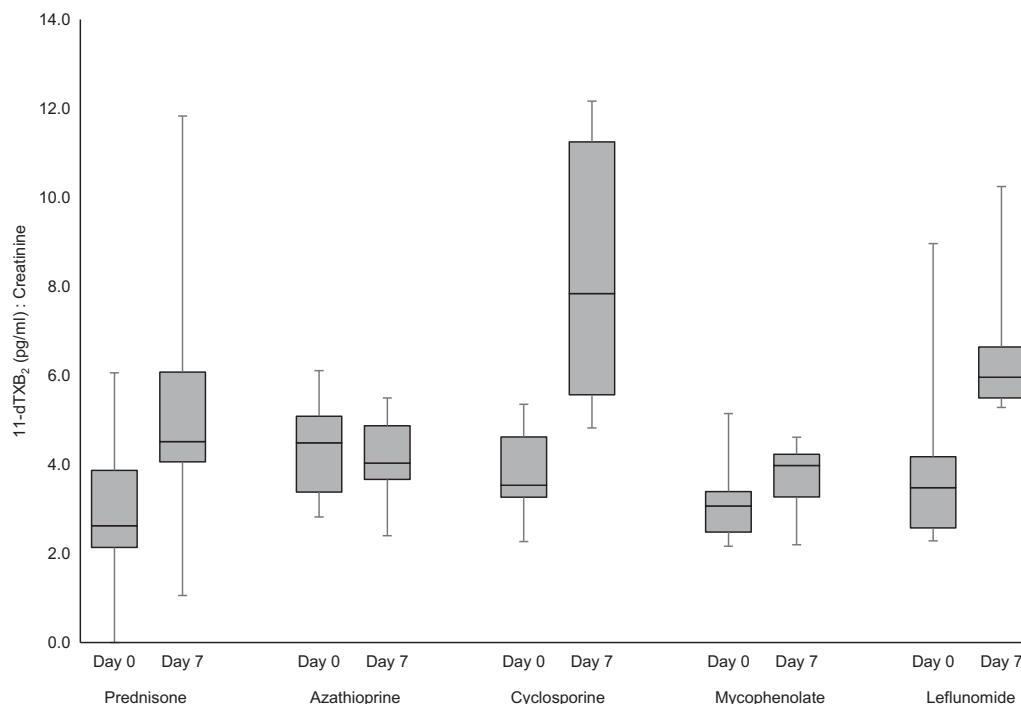
### 3.1 | Optical aggregometry

Mean optical aggregometry maximum amplitudes, activated with ADP and collagen, are presented in Table 1. When ADP was used as an agonist, the maximum amplitude was significantly (*P* = .002) lower than pretreatment values after leflunomide administration. There were no other differences in amplitude after treatment with any remaining immunosuppressive agent (*P* = .114). When collagen was used as an agonist, there were no differences from pretreatment values after treatment with any of the immunosuppressive medications (*P* = .173).

**TABLE 1** Mean and standard deviation of indices of hemostasis in 8 healthy dogs administered 5 immunosuppressive medications for 7 days

	Prednisone		Azathioprine		Cyclosporine		Mycophenolate mofetil		Leflunomide	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Aggregation: ADP (%)	52.5 ± 17	42.7 ± 27	48.2 ± 22	44.4 ± 23	44.2 ± 21	58.9 ± 11	43.6 ± 18	50.5 ± 21	51 ± 21	27 ± 14*
Aggregation: Collagen (%)	60.1 ± 12	55.7 ± 19	59.7 ± 15	53.9 ± 13	55.8 ± 17	59.5 ± 7	55 ± 9	57.1 ± 13	59.3 ± 14	44.3 ± 18
PFA-100 Closure Time (s)	142.5 ± 36	178.2 ± 36	152.1 ± 35	140.6 ± 38	158 ± 36	161 ± 52	178.9 ± 67	176.8 ± 43	159.1 ± 41	213.1 ± 65*
Platelet Count (K/μL)	232 ± 58	246.3 ± 53	230.3 ± 76	223.4 ± 44	248 ± 51	223 ± 57	229 ± 61	250.9 ± 51	247.5 ± 54	208 ± 55
SonoClot ACT (S)	158.3 ± 24	142.3 ± 32	141.9 ± 30	136.6 ± 20	140.4 ± 32	163.5 ± 27	155.3 ± 36	139.9 ± 22	139.6 ± 23	141.1 ± 20
SonoClot ClotRate (Units/minute)	17.3 ± 2	20.9 ± 3	19.3 ± 4	20.5 ± 2	19.5 ± 5	19.1 ± 7	17.9 ± 5	18.8 ± 4	18.6 ± 3	17.3 ± 5
SonoClot Platelet Function	3.3 ± 0.4	3.6 ± 0.8	3.5 ± 0.8	3.4 ± 0.6	3.1 ± 0.9	2.9 ± 0.8	3.2 ± 1	3.1 ± 0.6	3.9 ± 0.6	3.5 ± 0.5
PT (Seconds, Normal 5–12)	8.6 ± 0.7	7.9 ± 0.6*	8.5 ± 0.5	8.5 ± 0.5	8.5 ± 0.5	8.4 ± 0.5	8.6 ± 0.5	8.6 ± 0.6	8.5 ± 0.5	8.4 ± 0.4
aPTT (Seconds, Normal 10–20)	10.0 ± 0.5	9.4 ± 0.7	10.1 ± 0.4	10.0 ± 0.9	9.7 ± 0.8	10.4 ± 0.6	10.1 ± 0.7	10.1 ± 0.5	9.9 ± 0.4	10.4 ± 0.5

**Abbreviations:** ADP, adenosine diphosphate; ACT, activated clotting time; PT, prothrombin time; aPTT, activated partial thromboplastin time. Results labeled "\*" illustrate significant (*P* ≤ .05) differences from the respective day 0 sample.



**FIGURE 1** Urine 11-dehydro-TXB<sub>2</sub>:creatinine ratios in 8 dogs treated with 5 immunosuppressive medications for 7 days. There was no individual drug effect on ratios, but day 7 values were significantly higher ( $P = .001$ ) than day 0 values when adjusted for drugs. The box and whiskers plot demonstrates the median (line), interquartile range (box), and total range (whiskers)

### 3.2 | Platelet function analysis

Before commencement of the study, the mean PFA-100 closure time using the collagen/ADP cartridge was  $68.6 \pm 18.1$  seconds (mean  $\pm$  standard deviation), and all dogs had results within previously reported reference ranges.<sup>21,22</sup> Mean PFA-100 closure times using the collagen/epinephrine cartridge are presented in Table 1. When compared with day 0 (pretreatment), there was a significant ( $P = .012$ ) increase in closure time on day 7 of leflunomide administration. There were no significant differences in closure times between days 0 and 7 for the remaining immunosuppressive agents ( $P = .220$ ).

### 3.3 | Viscoelastometry (SonoClot)

ACT, ClotRate, and platelet function results are presented in Table 1. For all immunosuppressive agents, there were no significant differences in any of the three measurements between days 0 and 7 ( $P = .340$ ).

### 3.4 | Urine 11-dehydro-thromboxane B<sub>2</sub>

Urine 11-dTXB<sub>2</sub>-to-creatinine ratios for all immunosuppressive agents on days 0 and 7 are represented in Figure 1. Urine could not be collected from one dog on day 0 before azathioprine treatment and from one dog on day 7 during mycophenolate administration. There was not a significant day by medication interaction ( $P = .419$ ) or medication effect ( $P = .614$ ). However, compared with day 0, there was a significant ( $P = .001$ ) increase in the 11-dTXB<sub>2</sub>-to-creatinine ratio on day 7, when adjusted for the effect of medication. During cyclosporine, leflunomide, and prednisone administration, the urine 11-dTXB<sub>2</sub>-to-

creatinine ratio increased in 8, 7, and 6 dogs, respectively. (Supplemental Figures 1-5).

### 3.5 | Urine 6-keto-prostaglandin F<sub>1 $\alpha$</sub>

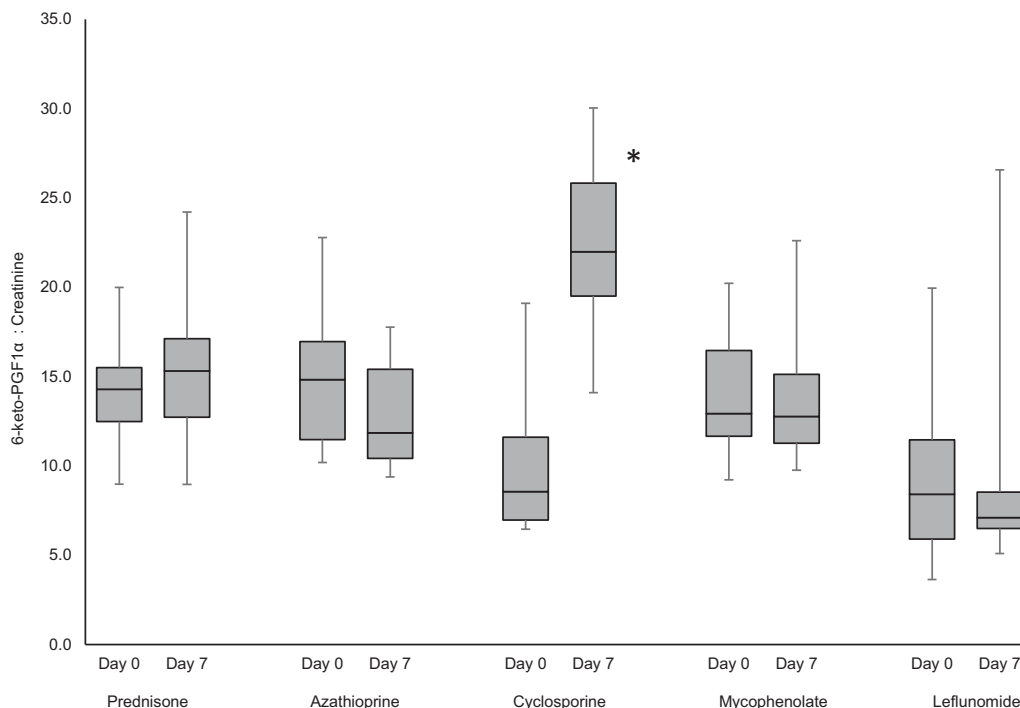
Urine 6-keto-PGF<sub>1 $\alpha$</sub> -to-creatinine ratios for all medications on days 0 and 7 are represented in Figure 2. Compared with day 0, there was a significant ( $P < .001$ ) increase in the 6-keto-PGF<sub>1 $\alpha$</sub> -to-creatinine ratio on day 7 during cyclosporine administration. Compared with day 0, there were no significant differences from pretreatment in the 6-keto-PGF<sub>1 $\alpha$</sub> -to-creatinine ratio for the remaining immunosuppressive medications ( $P = .935$ ).

### 3.6 | Additional tests of hemostasis

Platelet count, PT, and aPTT results are presented in Table 1. Compared with day 0, there was a significant ( $P < .001$ ) decrease in the PT value on day 7 for the prednisone group. Compared to day 0, there were no significant differences in the PT on day 7 for the remaining immunosuppressive medications ( $P = .910$ ). For all medications, there were no significant differences between days 0 and 7 for the platelet count and aPTT ( $P = .151$ ).

## 4 | DISCUSSION

In dogs with IMHA, cyclosporine is widely used as an adjunctive treatment to corticosteroids.<sup>8</sup> As has been reported in humans, cyclosporine, especially at the time of peak blood drug concentrations, will increase synthesis of thromboxane in dogs in a dose-dependent



**FIGURE 2** Urine 6-keto-prostaglandin  $F_{1\alpha}$  : creatinine ratios seen in 8 dogs treated with 5 immunosuppressive medications for 7 days. The box and whiskers plot demonstrates the median (line), interquartile range (box), and total range (whiskers). Sample points labeled \* illustrate significant differences on day 7 ( $P < .001$ ) compared with corresponding day 0 values

manner.<sup>11,12</sup> Our study demonstrated there was a significant increase in thromboxane synthesis after drug administration.

Additional studies in a greater number of animals would be required to further explore the potential effects of leflunomide and prednisone on thromboxane synthesis in dogs. Unlike cyclosporine, increases in thromboxane with prednisone and leflunomide were inconsistent between dogs, and some dogs had a decrease in thromboxane production. Given, the known anti-inflammatory properties of prednisone, the increase in thromboxane concentration was unexpected, because glucocorticoids are known to inhibit the conversion of arachidonic acid into eicosanoids, which would be expected to lead to a reduction in prostaglandin and thromboxane synthesis. The administration of a glucocorticoid is associated with an increase in thromboxane synthesis in dogs.<sup>30</sup>

Thromboxane  $A_2$  increases blood stasis and promotes hemostasis by initiating vasoconstriction and causing platelet activation. Platelets are the most important source of thromboxane  $A_2$ , although other cells, such as leukocytes and endothelial cells, can also synthesize this eicosanoid.<sup>27</sup> Once synthesized, thromboxane  $A_2$  is rapidly converted to several stable metabolites, such as 11-dTXB<sub>2</sub> and 2,3-dinor-thromboxane B<sub>2</sub>, that are eliminated in the urine and considered to be reliable markers of systemic thromboxane  $A_2$  production.<sup>27,28,31</sup> Because only about 30% of dogs have platelets that are sensitive to the effects of thromboxane,<sup>32,33</sup> drug-induced thromboxane synthesis would not be expected to reliably activate platelets in all dogs. Thromboxane, even if it does not affect all canine platelets, could however still stimulate vasoconstriction and predispose to thrombus formation.

Prostacyclin, which primarily originates from the vascular endothelium, inhibits platelet activation and causes vasodilation, thereby countering the prothrombotic properties of thromboxane.<sup>34</sup> In a manner similar to thromboxane  $A_2$ , prostacyclin is also rapidly converted to stable metabolites such as 6-keto-PGF<sub>1 $\alpha$</sub> , and excreted in the urine.<sup>35</sup> Our study demonstrated that urinary prostacyclin concentration is significantly increased in dogs receiving immunosuppressive doses of cyclosporine. This increase in prostacyclin synthesis could potentially counteract and minimize the prothrombotic effects of cyclosporine-induced thromboxane synthesis.

In our study, similar to previous studies,<sup>11,12</sup> the administration of cyclosporine to healthy dogs was not associated with a change in measures of platelet function such as aggregometry or PFA-100 analysis, or measures of secondary hemostasis such as PT, PTT, and viscoelastometry. These results suggest that any increase in thromboxane was either insufficient to alter platelet function, or that the concurrent increase in prostacyclin synthesis negated any platelet activating tendencies of cyclosporine. Given the results of our study, the use of cyclosporine and the potential associated induction of thromboxane synthesis in dogs with IMHA might not be a major risk factor for thromboembolism. However, because our study was conducted in healthy dogs and not IMHA patients, there might still be a potential increased predisposition to thromboembolism in dogs with IMHA receiving cyclosporine, especially in hypercoagulable dogs receiving inadequate anticoagulant treatment. The concurrent use of low-dose aspirin (1 mg/kg PO q24h) with cyclosporine eliminates cyclosporine-induced thromboxane synthesis while still preserving the antiplatelet effects of aspirin in healthy dogs.<sup>12</sup>

When dogs were administered leflunomide, there was a decrease in ADP-induced platelet aggregation and prolongation of PFA-100 closure times by day 7, suggesting this medication might have antiplatelet properties. An *in vitro* study involving mice, a leflunomide metabolite analog, LFM-A13, inhibited platelet shape change, degranulation, and aggregation after stimulation with collagen as an agonist.<sup>36</sup> There are currently no published studies that have investigated the effects of leflunomide metabolites on platelet aggregation in dogs.

In our study, by day 7 of administration of prednisone, PT was statistically shorter than pretreatment values, but remained within the normal reference interval. A decrease in PT below reference interval has been associated with a greater predisposition to thrombus formation in dogs, but it is not certain if a decrease in PT that does not drop outside of reference ranges is associated with the same predisposition.<sup>37</sup> There were no significant changes in any of the remaining hemostatic indices that were measured during prednisone administration. The results of our study are in contrast to previous studies that have demonstrated changes in multiple indices of hemostasis during prednisone treatment.<sup>16,17</sup> In one previous study, in contrast to our study, platelet aggregation with ADP as an agonist increased when dogs were administered immunosuppressive dosages of prednisone.<sup>17</sup> Unfortunately, a direct comparison between this previous study and our study cannot be made because differences in the aggregometry protocol and ADP concentration used do not provide consistent conditions to allow assessment of the effects of prednisone on platelet aggregation. Previous studies utilizing thromboelastography have suggested that healthy dogs became hypercoagulable while receiving glucocorticoid treatment.<sup>16,18</sup> Although thromboelastography was not used in our study, a very similar method, viscoelastometry, was used, and no significant differences before and after treatment with prednisone were detected. A potential explanation for the difference between the previous studies and our study is that thromboelastography could be more sensitive than viscoelastometry to the effects of glucocorticoids on hemostatic indices and clot strength. Additional research would be required to determine the exact relationship between prednisone and predisposition to thrombosis in dogs.

Compared with pretreatment values, there were no significant difference with any test of hemostasis for either azathioprine or mycophenolate mofetil. Because both azathioprine and mycophenolate mofetil have similar mechanisms of action, it was expected that these medications would have similar results. The results of our study are similar to previous studies performed in humans that did not identify a significant change in measures of hemostasis during treatment with azathioprine or mycophenolate mofetil.<sup>9,10,38</sup>

There were several limitations to our study. Firstly, healthy dogs with no evidence of hypercoagulability were used in our study. Hypercoagulable dogs with hyperactive platelets might react differently to immunosuppressive medications than healthy animals. Secondly, although a sample size calculation was performed before study initiation, it is possible that with more dogs enrolled in the study, the results would have been different. Thirdly, while our study evaluated the effects of immunosuppressive treatment on measurements of hemostasis after 7 days of drug administration, most clinical patients that

receive immunosuppressive agents require chronic treatment that persists for far longer than 1 week. The effects of the evaluated immunosuppressive agents on the hemostatic system could be different during chronic treatment.

Additional study limitations were associated the platelet function assays used. Firstly, although our study used a number of tests that examined different aspects of hemostasis, there were some assays, such as flow cytometric assessment of markers of platelet activation and measurement of individual clotting factors and von Willebrand factor, which were not included. Additional assays of hemostasis, in combination with utilization of different agonists and agonist concentrations, could have been able to detect more subtle changes in platelet function and coagulation. For example, in our study, we used the collagen/epinephrine PFA-100 cartridge to assess drug-induced platelet dysfunction. The collagen/epinephrine cartridge has a wide reference interval in dogs, and is susceptible to greater biological variation than the collagen/ADP PFA-100 cartridge.<sup>25,26,39</sup> The collagen/ADP cartridge could have detected some changes in platelet function that were not observed with the collagen/epinephrine cartridge, and the collagen/ADP cartridge could be used in future studies to assess the effects of immunosuppressive treatment on platelet function. Secondly, hematocrit was not determined each time blood samples were analyzed. Especially for whole blood analyzers, such as the PFA-100 and SonoClot, a significant decrease or increase in the hematocrit can alter the results of tests of hemostasis. At the beginning of our study, the hematocrits for all dogs were within the reference interval established for our laboratory. During the study, none of the dogs demonstrated clinical signs of anemia, and subsequent hematocrits, performed after the completion of our study, were within normal reference intervals. Although there could have been transient changes in the hematocrit during our study, we believe that there were unlikely to have been changes in hematocrit that were marked enough to have an important impact on our results. Finally, in our study, 40  $\mu$ M of ADP was used for platelet activation, which was the lowest concentration of ADP that consistently yielded at least 50% platelet aggregation in untreated dogs. It is possible that, with the use of a lower ADP concentration, platelet dysfunction associated with immunosuppressive medications could have been detected. However, with the use of lower ADP concentrations, it is also possible that inadequate platelet activation because of insufficient agonist could be mistaken for platelet dysfunction.

Cyclosporine increases thromboxane synthesis in dogs, suggesting that this medication could increase the risk of thromboembolism in hypercoagulable patients such as dogs with IMHA.<sup>11,12</sup> Alternative immunosuppressive agents might therefore be considered because of the perceived safety of these medications in hypercoagulable dogs. Unlike cyclosporine, however, there has been little published regarding the effects of these alternative immunosuppressive agents on the hemostatic system. Our study indicates that none of the immunosuppressive agents evaluated appeared to enhance platelet function or coagulation, although leflunomide did cause a significant decrease in platelet function as measured by aggregometry and PFA-100 analysis. Thromboxane synthesis increased during administration of immunosuppressive

medications. Prostacyclin concentrations increased during cyclosporine treatment, potentially negating any potential prothrombotic tendencies associated with increased concentrations of thromboxane. The results of these in vitro hemostatic tests need to be correlated with the clinical outcomes of dogs with IMHA.

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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

Animal use was approved by the Mississippi State University IACUC and was in compliance with the requirements of a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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