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Platelet hyperresponsiveness and increased platelet-neutrophil aggregates in dogs with myxomatous mitral valve disease and pulmonary hypertension

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Abstract

Background: Pulmonary hypertension (PH) in dogs with myxomatous mitral valve disease (MMVD) is caused by increased pulmonary venous pressure. Thrombosis, vascular remodeling, and vasoconstriction mediated by platelets could exacerbate PH.

Hypothesis: Dogs with PH will exhibit a hypercoagulable state, characterized by increased platelet activation, platelet-leukocyte, and platelet-neutrophil aggregate formation.

Animals: Eleven dogs (≥3.5 kg) diagnosed with MMVD and PH and 10 dogs with MMVD lacking PH.

Methods: Prospective cohort ex vivo study. All dogs underwent echocardiographic examination, CBC, 3-view thoracic radiographs, and heartworm antigen testing. Severity of PH and MMVD were assessed by echocardiography. Viscoelastic monitoring of coagulation was assessed using thromboelastography (TEG). Platelet activation and platelet-leukocyte/platelet-neutrophil interactions were assessed using flow cytometry. Plasma serotonin concentrations were measured by ELISA.

Results: Unstimulated platelets from dogs with MMVD and PH expressed more surface P-selectin than MMVD controls ($P = .03$). Platelets from dogs with MMVD and PH had persistent activation in response to agonists. The number of platelet-leukocyte aggregates was higher in dogs with MMVD and PH compared with MMVD controls ($P = .01$). Ex vivo stimulation of whole blood resulted in higher numbers of platelet-neutrophil aggregates in dogs with MMVD and PH ($P = .01$). Assessment of hypercoagulability based on TEG or plasma serotonin concentrations did not differ between groups.

Abbreviations: 5HT, serotonin; ACVIM, American College of Veterinary Internal Medicine; Ao, aorta; CKCS, Cavalier King Charles Spaniel; ELISA, enzyme-linked immunosorbent assay; FI, female intact; FS, female spayed; IQR, interquartile range; LA, left atrium; LA/Ao, left atrium to aorta ratio; LVIDdN, left ventricular internal diastolic diameter indexed to body weight; MA, maximum amplitude; MC, male castrated; MFI, median fluorescence intensity; MMVD, myxomatous mitral valve disease; MR, mitral regurgitation; NETs, neutrophil extracellular traps; PA, pulmonary artery; PA/Ao, pulmonary artery to aorta ratio; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PLA, platelet-leukocyte aggregate; PNA, platelet-neutrophil aggregate; PRP, platelet rich plasma; RA, right atrium; RAA, right atrium area; RPAD index, right pulmonary artery distensibility index; RV, right ventricle; RVAd, diastolic right ventricular area; RVFWd, right ventricular free wall diastolic diameter; SP, split time; TAPSE, tricuspid annular plane systolic excursion; TEG, thromboelastography; TRPG, tricuspid regurgitation pressure gradient; TRV, maximum tricuspid regurgitation velocity; TRV, tricuspid regurgitant jet velocity

Conclusion and Clinical Importance: Platelet hyperresponsiveness and increased platelet-neutrophil interaction occur in dogs with MMVD and PH, suggesting that platelets play a role of in the pathogenesis of PH. Clinical benefits of antiplatelet drugs in dogs with MMVD and PH require further investigation.

KEYWORDS
clinical pathology, hematology, immunothrombosis, platelet function, pulmonary thromboembolism, respiratory tract, serotonin

1 | INTRODUCTION

Pulmonary hypertension (PH) represents an abnormally increased pressure within the pulmonary circulation caused by increased pulmonary vascular resistance, augmented pulmonary blood flow, or increased pulmonary venous pressure.[1-3] Pulmonary hypertension is hemodynamically categorized into pre-capillary PH and post-capillary PH. Pre-capillary PH is caused by pulmonary arterial vasoconstriction or pulmonary vascular disease secondary to respiratory disease or hypoxia, cardiac shunts, thromboembolic disease, heartworm disease or other unknown causes.[1,2,4-6] Post-capillary PH occurs secondary to increased pulmonary venous pressure secondary to left-sided heart disease, which over time might cause pulmonary vascular changes and progression to combined postcapillary and pre-capillary PH in dogs, also known as reactive PH.[1-4,7,8] The most common cause of post-capillary PH in dogs is myxomatous mitral valve disease (MMVD).[2,7,8] Pulmonary hypertension is a debilitating clinical disorder in dogs because it is associated with exercise intolerance, respiratory distress, syncope and, occasionally, right-sided congestive heart failure.[3,5,9,10] Pulmonary hypertension is also associated with increased risk of death in dogs with MMVD.[3,7,8]

Despite the high morbidity and mortality associated with PH, therapeutic options in dogs remain limited. A recent study determined that the 30-day mortality rate of PH in dogs with respiratory disorders was 32% despite sildenafil treatment and improvement in estimated pulmonary arterial pressures.[10] These findings suggest that decreasing pulmonary pressures alone is insufficient to decrease morbidity and mortality in a subset of dogs with PH. Thrombotic pulmonary arteriopathy, characterized by the presence of thrombotic lesions within the pulmonary vasculature, is a common pathologic finding in dogs with pre-capillary PH. The presence of complex constrictive lesions with evidence of thrombosis in pulmonary arteries suggests that thrombosis may play a causative role in the pathogenesis of PH.[11] Current guidelines in human medicine recommend antithrombotic drugs for patients with certain types of PH such as idiopathic, heritable, and drug- or toxin-induced.[1,4,12] Additional treatments such as antiplatelet or anticoagulant medications might improve survival in these dogs. However, given limited understanding of the role of coagulation and innate immunity in the pathogenesis of PH, little is known regarding the indications and benefits of antithrombotic treatment in dogs. Characterizing the role of immunothrombosis in PH pathogenesis would help guide clinicians in implementing appropriate use of antithrombotic treatments in affected dogs.

We therefore hypothesized that dogs with MMVD and PH would exhibit a global hypercoagulable state, characterized by increased platelet activation, platelet-leukocyte and neutrophil aggregate formation, and increased serotonin concentrations when compared with MMVD dogs without PH. To test this hypothesis, our primary objective was to evaluate platelet activation, serotonin concentrations, platelet-neutrophil and platelet-leukocyte interactions in MMVD dogs with and without PH. Our secondary objective was to assess global coagulation in MMVD dogs with and without PH using thromboelastography (TEG).

2 | MATERIALS AND METHODS

2.1 | Study population

All owners provided informed written consent before study enrollment. The study procedures were approved by the University of California Davis Institutional Animal Care and Use Committee (protocol # 21471).

2.1.1 | Study group

To be eligible for inclusion into the MMVD and PH group, dogs had to be ≥3.5 kg, diagnosed with MMVD (American College of Veterinary Internal Medicine Stages B2, C, or D) and PH by echocardiography (defined below) based on an echocardiography performed for clinical purposes. Affected dogs were excluded if diagnosed with a left-to-right cardiac shunt (PH associated with increased blood flow), they lacked right heart remodeling or failure (PH caused by isolated post-capillary PH), or if a mass or other compressive lesion on the pulmonary artery (PA) was identified. Although thrombocytopenia ($<150 \times 10^9$/mL) was an exclusion criterion, no dogs with PH were excluded because of thrombocytopenia. Dogs receiving corticosteroids, anticoagulants such as fractionated heparin, unfractionated heparin, direct PO anticoagulants and drugs known to affect platelet function such as aspirin, clopidogrel and non-steroidal anti-inflammatory drugs were excluded.[13-16] Dogs that had received phosphodiesterase-5 inhibitors within 1 week of enrollment also were excluded. Dogs with echocardiographic (visible thrombus within the main pulmonary artery) or radiographic evidence (oligemia and Westermark signs)[17,18] of pulmonary thrombosis or thromboembolism also were excluded.

Once enrolled, dogs underwent transthoracic echocardiographic examination for study purposes, a CBC (HM5, Abaxis, Zoetis, Parsippany-Troy Hills, New Jersey), 3-view thoracic radiographs, and Dirockek canine heartworm antigen ELISA testing (Zoetis, Parsippany-Troy Hills, New Jersey) at the authors' institution. All radiographs were reviewed by board-certified radiologists. Additional diagnostic tests were performed at the discretion of the attending clinicians.

2.2 | Echocardiography

All dogs underwent a standard echocardiographic examination consisting of 2-dimensional and Doppler echocardiography by board-certified cardiologists or cardiology residents under direct supervision of a board-certified cardiologist. All echocardiographic measurements were acquired and interpreted based on recently published references.[19-21] All echocardiographic assessments and measurements were performed by a study investigator (L. Duler or L. Visser). Echocardiographic techniques are described in Supplemental Text 1.

Dogs diagnosed with MMVD were categorized as stage A, B1, B2, C, and D according to the ACVIM consensus guidelines for the diagnosis and treatment of MMVD in dogs.[22] Dogs with MMVD stage A and B1 were excluded. Dogs with normal left atrial (LA) or left ventricular size or both on pimobendan were not excluded if previously diagnosed with MMVD stage B2 by echocardiography at the authors' institution.

All dogs with MMVD and PH enrolled in the study had high probability of PH according to ACVIM consensus statement guidelines.[2] Briefly, PH was diagnosed using echocardiography and included a tricuspid regurgitation jet velocity (TRV) >3.4 m/s (pressure gradient >46 mmHg) with at least 1 of the following findings involving remodeling of the right heart or pulmonary artery or both: (a) right ventricular (RV) enlargement (end-diastolic RV area indexed to body weight >1.33 $cm^2/kg^{0.62}$)[21] with systolic septal flattening; (b) enlargement of the pulmonary artery (PA/Ao >1.0)[21] or right pulmonary artery distensibility index <30%[23]; or (c) right atrial enlargement (RA area indexed to body weight >0.76 $cm/kg^{0.71}$).[21] Right ventricular outflow tract obstruction was ruled out in all dogs. Dogs with a TRV ≤ 3.0 m/s lacking the aforementioned criteria for right heart or PA remodeling were categorized as controls.

2.2.1 | Blood collection

After echocardiographic examination, blood was collected from either the jugular or cephalic vein using an 18G or 21G needle and syringe. Blood was immediately aliquoted to tubes containing 3.2% trisodium citrate or lithium-heparin. Citrated blood samples for TEG and platelet function assays were analyzed within 60 minutes after collection.

2.2.2 | Evaluation of platelet activation

To assess surface expression of platelet P-selectin, platelet rich plasma was generated from citrated whole blood (centrifugation at 300 × *g*, 5 minutes, acceleration 1, no brakes) then standardized to 1×10^7 cells/mL with Tyrode-HEPES buffer (pH 7.2, dextrose 5 mM, no divalent cations). After stimulation with 20 μM adenosine diphosphate (ADP; Sigma-Aldrich, St. Louis, Missouri) or 0.005 U/mL thrombin (Haematologic Technologies, Essex Junction, Vermont; 15 minutes, 37°C), P-selectin (CD62P) and beta-3 integrin (CD61) were labeled and analyzed as previously described.[24] All cell preparations before fixation were performed under sterile conditions. Platelet activation was measured as the percentage of P-selectin positive platelets and P-selectin density as median fluorescence intensity (MFI). Platelet responsiveness to agonists was calculated using the formula:

$$\text{MFI fold change} = \log 10\left(\frac{\text{MFI activated}}{\text{MFI resting}}\right).$$

2.2.3 | Detection of platelet-leukocyte and platelet-neutrophil aggregate formation

Leukocyte concentration in citrated whole blood was first standardized to 1×10^6 cells/mL with Tyrodes-HEPES (pH 7.2, 5 mM dextrose) and placed at 37°C under gentle rocking for 30 minutes. Diluted whole blood then was treated with 10 μM ADP or 0.005 U/mL bovine α-thrombin (15 minutes, 37°C). Untreated samples served as resting samples. Leukocytes and neutrophils were labeled with mouse anti-canine CD18 antibodies conjugated to Alexa Fluor 647 (1:40; Clone CA1.4E9, Bio-Rad, Hercules, California) and anti-canine neutrophil antibody conjugated to fluorescein isothiocyanate (1:200; CADO48A, Monoclonal Antibody Center, Washington State University, Washington), respectively. Platelet integrin beta3 was labeled with phycoerythrin-conjugated mouse anti-human monoclonal antibodies to CD61 (Clone:VI-PL2, eBioscience, San Diego, California). After incubation for 40 minutes at 37°C under gentle rocking, erythrocyte lysis and fixation of the remaining blood cells were performed (30 minutes, room temperature, 1×, FACS Lysing Solution, BD Biosciences, San Jose, California). Samples were stored at 4°C and analyzed by cytometer within 7 days.

2.2.4 | Flow cytometry analysis

Gating strategies and detection of platelet P-selectin expression were performed as described (Figure 2).[24] P-selectin expression in the presence or absence of agonists was measured as percentage (%) of CD62P-positive events out of 10 000 platelets or MFI. Flow cytometric detection of circulating platelet-leukocyte aggregates (PLA) and platelet-neutrophil aggregates (PNA) is described in Figure 1. Additional gating strategy and immunodetection of leukocytes and neutrophils are detailed in Supplemental Text 1. Platelet-leukocyte aggregates and PNA were quantified as the percentage of total leukocytes or neutrophils that was positive for the platelet-specific marker, CD61. Flow cytometry data were analyzed using commercially available software (FlowJo, Treestar, Ashland, Oregon).

2.2.5 | Thromboelastography

Viscoelastic assessment of coagulation was evaluated by TEG (TEG5000, Haemonetics, Boston, Massachusetts) in accordance with published consensus standards.[25] All blood samples were recalcified and activated with kaolin according to manufacturer's instructions. Heparinase cups were used in dogs that received saline flushes containing heparin. All tests were run by a single trained technician and reference intervals for TEG variables were previously established at our institution. Dogs were considered hypercoagulable if they had ≥2 of the following: R < 2 minutes, K < 1 minute, G > 8100 dynes/cm^2, maximum amplitude (MA) >62 mm or alpha angle >74°.

2.2.6 | Plasma serotonin concentrations

Plasma serotonin concentrations were measured using a commercially available serotonin ELISA that was previously validated in domestic animal species[26] (Enzo Life Sciences, Framingale, New York). The ELISA sensitivity for plasma was 0.239 ng/mL (range, 0.49-500 ng/mL).

2.3 | Statistical analysis

Sample size calculation was performed using commercially available software (STATmate 2.0, GraphPad, San Diego, California). Based on preliminary data generated from healthy dogs and 3 dogs with PH, a sample size of 10 dogs per group would yield statistical power of 80% to detect an estimated detectable difference of 40% to 50% increase in platelet activation and platelet-leukocyte formation with alpha error probability set at 0.05. The model used for sample size calculation was a 2-tailed t-test for unpaired data.

D'Agostino-Pearson test and QQ plots were used for normality testing of continuous data. Normally distributed data are presented as mean ± SD and were evaluated using an unpaired t-test. Nonparametric data are presented as median and interquartile range (IQR) and were compared using Mann-Whitney test. Fisher's exact test was used to evaluate the proportion of dogs that were hypercoagulable within each group as detected by TEG. Statistical significance was set at P < .05. Correlations between platelet responsiveness, PLA/PNA formations to agonists and severity of MMVD, measured as LA/Ao long-axis, LA/Ao short-axis and left ventricular end diastolic diameter normalized for body weight (LVIDdN), were calculated using Pearson correlation with Bonferroni's adjustment, for which a value of P < .02 was considered significant. All statistical tests were performed using commercially available software (Prism 9.0, GraphPad, San Diego, California).

3 | RESULTS

Twenty-one dogs, including 11 dogs with MMVD and PH and 10 dogs with MMVD without PH, were enrolled between July 2020 and May 2022. Demographic data are summarized in Table 1. Mean age and body weight of the study population were 11.45 ± 1.60 years old and 8.56 ± 5.51 kg, respectively. Study groups were relatively homogenous, with body weight (P = .001) being the only significantly different variable between the 2 groups. None of the dogs had evidence of left-sided congestive heart failure at the time of enrollment or was in respiratory distress requiring supplemental oxygen therapy. Six dogs (4 MMVD PH and 2 MMVD) had radiographic evidence of lower airway disease (P = .6). Other co-morbidities included systemic hypertension in the PH group, and intervertebral disc disease, idiopathic epilepsy and osteoarthritis in the non-PH group. Leukocyte count was significantly higher in MMVD PH dogs compared with MMVD controls (9.93 × 10^9/L; IQR, 9.18-10.97 vs 7.6 × 10^9/L; IQR, 6.53-10.2; P = .04), which was characterized by increased monocyte count (MMVD PH dogs = 0.58 × 10^9/L; IQR, 0.49-0.72 vs MMVD control

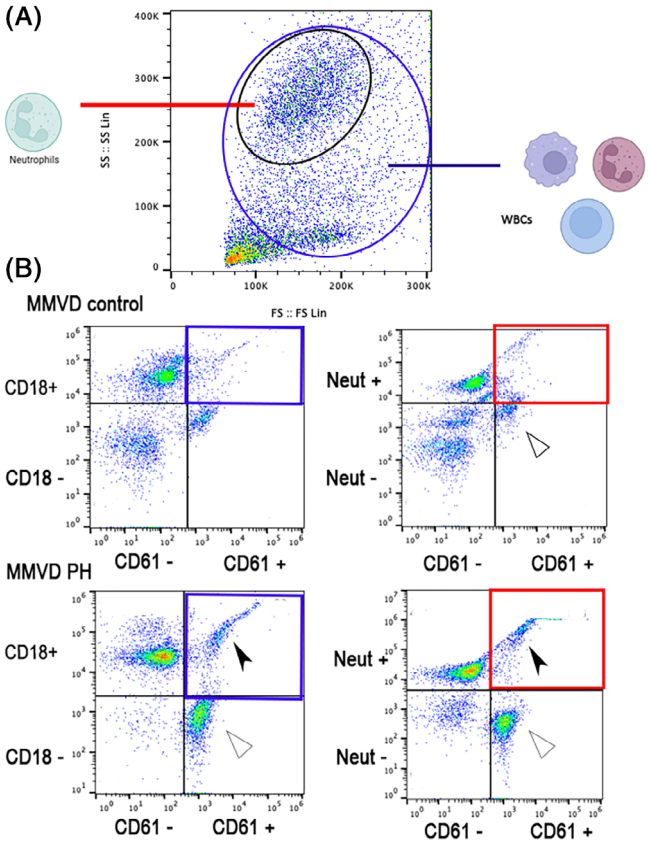

FIGURE 1 Representative scatter plots of whole blood on flow cytometry in dogs. (A) Gating of neutrophils and white blood cells (WBCs) was established by side and forward scatter properties (neutrophils outlined black, WBCs outlined blue) on linear scale to exclude non-leukocyte and debris. (B) Additional gating of WBCs and neutrophils was determined by immunodetection of CD18 and neutrophil marker, respectively. Quantities of platelet leukocyte aggregates (PLA, blue square) and platelet neutrophil aggregates (PNA, red square) were determined by the presence of CD61 on leukocytes (CD18+) or neutrophils. Note the marked increase in PLA and PNA (arrowheads) in a dog with MMVD and PH after stimulation with thrombin compared with a control dog with MMVD without PH. Open arrowhead indicates platelet aggregates and binding to other leukocytes.

TABLE 1 Summary of patient signalment, medications, and clinical signs.

	MMVD and PH group (n = 11 dogs)	MMVD without PH group (n = 10 dogs)
Demographic data		
Age (years)	11.89 ± 1.72	11.10 ± 1.45
Sex	6 MC, 5 FS	4 MC, 5 FS, 1 FI
Body weight	5.36 ± 1.18	11.77 ± 6.48
Breeds	Chihuahua (4), Maltese (2), Cocker spaniel (1), Pekinese (1), Jack Russel terrier (1), Pomeranian (1), Bichon Frise (1)	Shih Tzu (2), Beagle (2), Chihuahua (1), Cavalier King Charles spaniel (1), Cocker spaniel (1), Staffordshire terrier (1), Dachshund (1), Mixed breed (1)
Medications		
Diuretics (furosemide, torasemide)	7	0
RAAS-modulators (ACE inhibitor, spironolactone)	6	3
Pimobendan	8	5
Hydrocodone	2	1
No medication	1	4
Clinical signs		
Cough	11	4
Tachypnea or respiratory efforts	4	0
Syncope	5	0
Exercise intolerance	2	0
RCHF (ascites, hepatomegaly)	2	0
Comorbidities		
Airway disease	4	2

Note: All data is represented as mean and SD.
Abbreviations: FI, female intact; FS, female spayed; MC, male castrated; MMVD, myxomatous mitral valve disease; PH, pulmonary hypertension; RCHF, right-sided congestive heart failure.

dogs = 0.3 × 10^9/L; IQR, 0.26-0.35; $P = .007$). MMVD PH dogs also had lower erythrocyte count (6.24 × 10^{12}/L; IQR, 5.34-6.68) compared with MMVD control dogs (6.78 × 10^{12}/L; IQR, 6.46-8.28, $P = .03$) and a lower hematocrit (MMVD PH dogs = 39.26%; IQR, 33.38-43.78) compared with MMVD control dogs (46.43%; IQR, 43.02-53.10; $P = .01$). None of the biochemistry variables was significantly different between groups. Regarding echocardiographic variables, the left atrium to aorta ratios (LA/Ao) performed from the right parasternal long ($P = .0004$) and short ($P = .001$) axes and LVIDdN ($P = .0004$) were significantly different between groups. Echocardiographic findings are summarized in Table 2.

3.1 | Platelet activation

Flow cytometry indicated that platelets from dogs with MMVD and PH not only had persistent platelet activation, but also showed augmented activation in response to platelet agonists when compared with MMVD control samples. When evaluating unstimulated (resting) platelets, the number of P-selectin positive platelets was similar between the 2 groups (MMVD PH: 16.8%; IQR, 6.7-19.0 vs MMVD Control: 10.8%; IQR, 6.4-23.0; $P = .7$; Figure 2A). However, dogs with MMVD and PH had platelets with increased surface P-selectin density compared with MMVD dogs without PH (MFI: 5543; IQR, 2571-9241 vs 2342; IQR, 2073-3286; $P = .03$; Figure 1). In vitro stimulation of platelets from MMVD PH dogs with thrombin led to higher surface P-selectin expression, both in numbers (17.4% positive; IQR, 11.7-37.1) and surface density (MFI: 15 721; IQR, 10 663-35 046) compared with resting platelets ($P = .03$, $P = .02$, respectively; Figure 2). Only dogs with PH were shown to have increased surface expression of P-selectin, characterized by high P-selectin density, in response to ADP (MFI_{ADP}: 7281 ± 4543 vs $MFI_{resting}$: 5847 ± 3208; $P = .01$). When comparing P-selectin density (MFI) in activated platelets between dogs with PH and those without PH, dogs with PH had significantly higher P-selectin density compared with control dogs (MFI_{ADP}: 3100 ± 1310; $P = .03$).

Activated platelets in MMVD PH dogs with a marked response to agonists were characterized by high surface density of P-selectin and decreased surface expression of beta-3 integrin (CD61) as shown in representative scatter dot plots in Figure 2B.

3.2 | Platelet-leukocyte and platelet-neutrophil aggregate formation

The number of PLA in unstimulated whole blood was significantly higher in dogs with MMVD and PH compared with dogs in the MMVD control group (MMVD PH: 14.8%; IQR, 10.7-28.9 vs MMVD:

TABLE 2 Summary of echocardiographic findings.

	MMVD and PH group (n = 11)	MMVD without PH (n = 10)	*P*-value
TRV (m/s)	4.20 ± 0.57	N/A	N/A
TRPG (mmHg)	70.56 ± 20.35	N/A	N/A
RVAd (cm^2)	3.5 ± 1.56	3.12 ± 1.40	.9
Septal flattening	N = 7	N = 0	N/A
PA/Ao	1.1 ± 0.15	0.77 ± 0.09	.3
RAA (cm^2)	3.81 ± 2.57	2.51 ± 1.45	.7
RPAD (%)	22.8 ± 8.06	37.2 ± 5.11	.4
TAPSE (mm)	12.96 ± 3.28	11.08 ± 0.66	.1
LA/Ao Sx	2.65 ± 0.63	1.66 ± 0.21	.001*
LA/Ao Lx	4.25 ± 1.03	2.71 ± 0.45	.0004*
LVIDdN	2.08 ± 0.34	1.75 ± 0.42	0.0004*
Type I PA velocity flow profile	N = 1	N = 10	N/A
Type II PA velocity flow profile	N = 4	N = 0	N/A
Type III PA velocity flow profile	N = 6	N = 0	N/A
RVFWd (mm)	4.45 ± 1.44	3.25 ± 0.89	.8

Abbreviations: LA/Ao Lx, long axis left atrium to aorta ratio; LA/Ao Sx, short axis left atrium to aorta ratio; LVIDdN, left ventricular internal diastolic diameter indexed to body weight; PA/Ao, pulmonary artery to aorta ratio; RAA, right atrium area; RPAD index, right pulmonary artery distensibility index; RVAd, diastolic right ventricular area; RVFWd, right ventricular free wall diastolic diameter; TAPSE, tricuspid annular plane systolic excursion; TRPG, tricuspid regurgitation pressure gradient; TRV, maximum tricuspid regurgitation velocity.
*P-value < .05.

7.8%; IQR, 6.4-10.7; $P = .01$; Figure 3A). Ex vivo treatment of whole blood with thrombin or ADP resulted in significantly higher amounts of PLA in dogs with MMVD and PH compared with MMVD controls (thrombin: MMVD PH: 24.50% ± 13.9 vs MMVD: 12.77% ± 5.6; $P = .02$; ADP: MMVD PH: 26.0% ± 15.7 vs MMVD: 14.4% ± 6.1; $P = .04$; Figure 3A).

When assessing the amount of PNA in unstimulated whole blood, dogs with MMVD and PH had significantly higher numbers of PNA (21.26%; IQR, 11.4-37.0) compared with dogs without PH (9.6%; IQR, 7.7-14.0; $P = .01$; Figure 3B). Similar to the platelet activation data, ex vivo treatment of whole blood with thrombin led to significantly higher amounts of PNA in dogs with MMVD and PH compared with those without PH (MMVD PH = 42.85%; IQR, 12.3%-47.6% vs MMVD = 13.30%; IQR, 9.2-17.8; $P = .03$), as shown in Figure 3B. Similarly, treatment of whole blood with ADP resulted in higher numbers of PNA in dogs with MMVD and PH (38.6% ± 29.7) compared with MMVD control dogs (15.4% ± 5.0; $P = .03$; Figure 3B). Ex vivo treatment of whole blood with thrombin or ADP led to a significant increase in PNA compared with resting (unstimulated) samples in dogs with MMVD and PH ($P = .02$) but not in control dogs ($P > .05$).

3.3 | Thromboelastography

Kaolin-activated TEG was performed in 15 dogs (9 MMVD controls, 6 MMVD with PH). Using the criteria of hypercoagulability, 7/9 (77.8%) dogs in the MMVD control group were hypercoagulable compared with 5/6 (83.3%) in the MMVD PH group ($P = 1.0$). When comparing each TEG variable, none of the variables was significantly different between the 2 groups (Table 3).

3.4 | Plasma serotonin concentrations

Plasma samples from 10 MMVD controls and 8 MMVD PH dogs were available for serotonin analysis by ELISA. Overall, no difference was noted between the 2 groups (MMVD: 31.2 pg/mL; IQR, 12.1-71.1 vs MMVD PH: 22.7 pg/mL; IQR, 13.4-34.2; $P = .63$).

3.5 | Correlations

Platelet responsiveness to ADP, measured as MFI fold change (FC; log 10), did not correlate significantly with MMVD severity based on echocardiographic measurements (Figure S1A). Platelet responsiveness to thrombin was correlated with LVIDdN ($r^2 = 0.24$; $P = .03$; Figure S1B). Platelet-leukocyte aggregates and PNA formation in response to ADP or thrombin did not correlate with MMVD severity ($P > .02$; Figure S2A,B).

4 | DISCUSSION

We demonstrated that dogs with MMVD and PH have enhanced platelet activation, PLA and PNA compared with dogs with MMVD that lack PH. Using flow cytometry, we found that dogs with MMVD and PH not only had more activated platelets, but their platelets also were primed to readily respond to physiologic agonists.

Platelet priming occurs because of exposure to molecules that either modulate their inhibitory mechanisms or potentiate activating pathways, resulting in unrestrained and amplified intracellular response upon subsequent activation by physiological agonists. Evidence

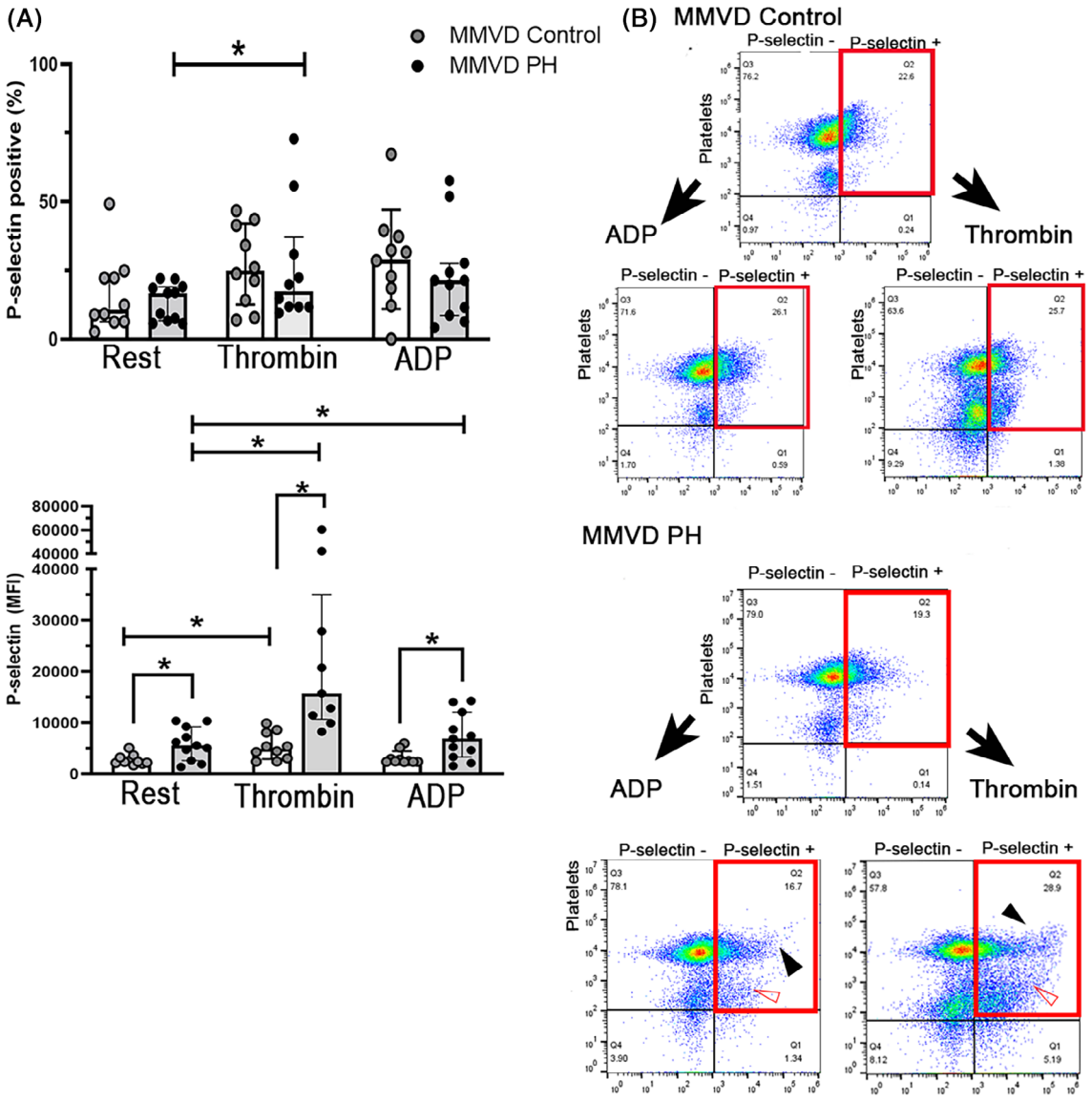

FIGURE 2 Flow cytometry analysis of platelet P-selectin expression in 11 dogs with MMVD and PH, and 10 dogs with MMVD without PH. (A) Platelet activation was measured as percentage (%) positive for P-selectin and surface density of P-selectin, measured as median fluorescence intensity (MFI), before and after in vitro stimulation with thrombin or ADP. Dogs with MMVD and PH had platelets with increased response to thrombin compared with resting platelets. Adenosine diphosphate stimulation also increased surface P-selectin density in dogs with MMVD and PH. P-selectin density in dogs with PH was also higher than those without PH among all treatments. (B) Representative scatter plots of platelets in a MMVD control dog and a dog with MMVD and PH. Platelets were identified by integrin β3 and activated platelets were identified by P-selectin (red box). Although the number of activated platelets were similar between these 2 patients after stimulation with agonists, increased magnitude of activation, characterized by high P-selectin density (black arrowheads) and decreased surface density of beta-3 integrin (red arrowheads) was noted in the dog with PH.*$P < .05$, bars represent mean and error bars represent SD.

suggests that humans and other animals with some types of PH are hypercoagulable.[12,27-37] Increased activation of platelets, which are the primary effector cells of hemostasis and innate immunity, augments vasoreactivity with subsequent architectural changes in the pulmonary vasculature. Similar to previous findings in studies of humans, we found that platelets in dogs with PH were hyperresponsive to thrombin stimulation. A previous study found that humans with pre-capillary PH had increased platelet protease-activated receptor 1, a key receptor responsible for thrombin-mediated platelet activation and aggregation.[38] The expression of this receptor along with platelet P-selectin expression also was associated with thrombocytopenia, indicating that increased platelet activation may result in increased platelet consumption because of microvascular thrombosis. Another possible mechanism of platelet priming in PH and MMVD could be the exposure of circulating platelets to high shear stress because of severe mitral regurgitation causing mechanical activation. Another study found that dogs with heart disease causing turbulent high-velocity blood flow had changes in platelet function.[39] Specifically,

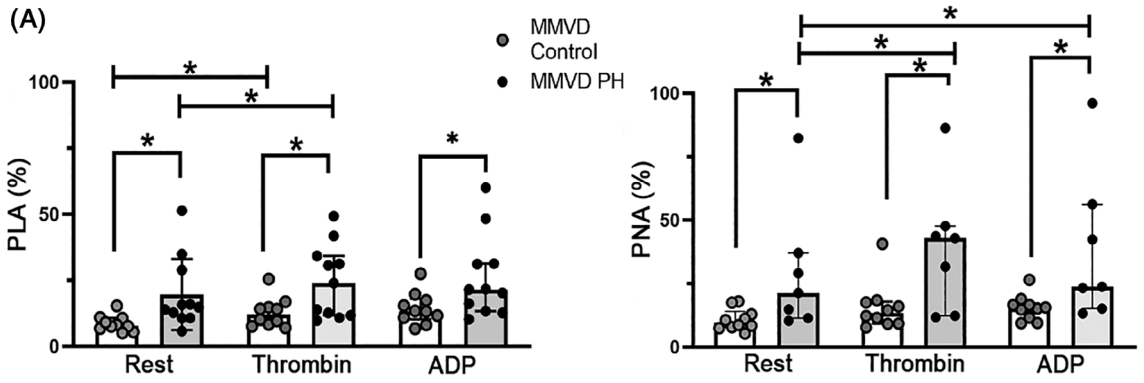

FIGURE 3 Flow cytometry analysis of platelet-leukocyte and platelet-neutrophil aggregates in whole blood in 11 dogs with MMVD, and PH and 10 dogs with MMVD without PH. Platelet-neutrophil aggregates were measured in 7 dogs with MMVD and PH. (A) Platelet-leukocyte aggregates (PLA) and platelet-neutrophil aggregates (PNA), measured as percentages (%) of leukocytes (CD18) and neutrophil positive for platelets (CD61), were evaluated in the absence or presence of thrombin or ADP. Dogs with MMVD and PH had significantly higher numbers of PLA compared with control dogs with MMVD across all treatments. Thrombin stimulation resulted in significantly higher PLA in dogs with MMVD and PH compared with unstimulated (rest) samples. Similarly, dogs with MMVD and PH had high numbers of PNA compared with MMVD controls across all treatments. When compared with rest samples, only whole blood from dogs with MMVD and PH responded to platelet agonists by increasing PNA formation. *$P < .05$, bars represent median and IQR.

Cavalier King Charles Spaniels with mitral valve prolapse had enhanced maximal aggregation response, reflective of increased platelet reactivity. These results were also confirmed in another study.[40] Although the concentration of thrombin utilized in our study is considered low compared to that used in previous studies, it potentially indicated platelet priming and subtle differences in platelet activation not seen in assays such as TEG.

Whether activated platelets play a causative role in the pathogenesis of PH or their presence is secondary to MMVD severity in dogs requires further study. Nevertheless, the presence of increased platelet priming in dogs with MMVD and PH suggests that platelet activation may exacerbate PH beyond its contribution to thrombosis in the pulmonary vasculature. This conclusion is further supported by our findings on TEG, which indicated that dogs with MMVD and PH were not systemically more hypercoagulable than those without PH. In addition to their hemostatic functions, platelets may contribute to the other basic mechanisms of PH pathogenesis such as vasoconstriction and vascular remodeling. Upon activation, platelets release vasoactive mediators such as thromboxane A_2 and serotonin (5HT), both of which are potent vasoconstrictors and can enhance the hypertensive effects of other vasoconstrictors.[41,42] Platelets serve as the largest reservoir of serotonin within the circulation because serotonin is stored in platelet dense granules, which degranulate to release serotonin into the systemic circulation upon activation.[43,44] Given the role of 5-hydroxytryptamine (5HT) in PH pathology, we measured plasma serotonin in dogs with or without PH. However, we did not find any associations between plasma serotonin concentrations and PH in dogs with MMVD. A plausible explanation is that serotonin concentrations were already increased in MMVD control dogs as previously shown that MMVD in dogs was associated with increased 5HT concentrations, 5HT receptor upregulation, autocrine production within the mitral valve leaflets, and down-regulation of 5HT clearance mechanisms.[45] This possibility therefore may minimize the statistical power to detect any significant difference between the 2 groups. It also is likely that increased serotonin only contributes to 1 of the many multifaceted and complex pathways responsible for PH pathology in dogs. Additional studies are required to delineate the role of other vasoactive mediators such as thromboxane in dogs with PH.

Activated platelets also mediate adhesive interactions with circulating leukocytes resulting in PLA formation, an important process known as immunothrombosis.[30-33] Our findings indicate that thrombin activation led to increased PNA formation in dogs with MMVD and PH. Platelet-neutrophil aggregate formation requires firm adhesive interactions between activated platelets and neutrophils. The exact molecular mechanisms of platelet-neutrophil interaction, which is highly species specific, have not been identified in dogs. However, research in mice and humans indicates that stimulation of both cell types is required to upregulate or activate their respective adhesion molecules. In people, neutrophil integrin (MAC-1) once activated, binds to the platelet integrin $\alpha_{IIb}\beta_3$ via fibrinogen and glycoprotein 1bα via von Willebrand factors.[31,34] The adhesive interactions between platelets and neutrophils can synergistically mediate the formation of neutrophil extracellular traps (NETs), which are web-like extracellular chromatin decorated with neutrophil granular enzymes and histones. Neutrophil extracellular traps previously have been characterized in human patients with idiopathic PH in occlusive plexogenic lesions within remodeled vascular walls, perivascular infiltrates and thrombotic lesions.[35,36] In addition to the proliferative lesions induced by NETs, NETs have been shown to be proinflammatory and prothrombotic by activating coagulation pathways, inducing endothelial and platelet activation.[31,34] Because platelet-neutrophil interactions and associated NET formation appear to play a role in the pathologic mechanisms of PH, additional studies are needed in this area because it may present opportunities for novel therapeutic options for dogs with PH.

TABLE 3 Kaolin-activated thromboelastography in 6 dogs with MMVD and PH, and 9 dogs with MMVD without PH.

TEG parameter	MMVD and PH group (n = 6)	MMVD without PH group (n = 9)	*P*-value
R (min)	2.65 (2.12-3.35)	2.20 (1.40-2.75)	.2
Split time (min)	2.40 (1.75-2.85)	1.90 (1.15-2.30)	.2
K (min)	1.05 (0.90-1.65)	1.40 (1.20-1.90)	.1
Alpha-angle (degrees)	75.15 (68.13-75.80)	69.9 (65.85-74.90)	.4
Maximum amplitude (mm)	71.20 (63.28-77.20)	64.40 (60.90-67.80)	.1
G (dynes/cm^2)	12.95 (8.80-16.93)	9.0 (7-85-10.55)	.05

Note: All data are represented as median and interquartile range. *P*-value < .05 is considered significant.
Abbreviations: IQR, interquartile range; MMVD, myxomatous mitral valve disease; PH, pulmonary hypertension; TEG, thromboelastography.

Dogs with advanced stages of MMVD (Stages C and D) were previously found to have increased proinflammatory cytokines such as interleukins and tumor necrosis factor alpha (TNFα) that correlate with disease severity.[37] These findings parallel our observations of increased leukocytes and monocytes in dogs in the PH group. Chronic inflammation with increases in TNFα has been shown to affect thrombopoiesis in humans and mice, causing platelet mitochondrial dysfunction and hyperreactivity.[46] Also, inflammatory cytokines such as interleukin-8 and TNFα activate leukocytes, especially neutrophils, to enhance cellular adhesion. In turn, NETs may further exacerbate proinflammatory functions of leukocytes and platelets causing a vicious cycle of inflammation. An underlying proinflammatory state in some dogs with advanced MMVD likely plays a role in PH pathogenesis, but future clinical studies with severity-matched controls will be needed to elucidate the role of inflammation.

Based on TEG, dogs with MMVD and PH were not significantly more hypercoagulable than MMVD control dogs. The discrepancy between platelet function assays and TEG findings could be explained by the low dose of thrombin in our platelet assay and the small number of dogs tested in each group (type 2 statistical error). Although TEG evaluates global coagulation, which is influenced by clot initiation, strength, and stability,[47,48] kaolin as an activator does not provide a sensitive assessment of the extrinsic pathway, platelet contribution to clot formation, and fibrinolytic activity.[47-49] A previous study showed that aggregometry and flow cytometry were more sensitive indicators of platelet function than were TEG parameters.[50] Thromboelastography with platelet mapping also has been described recently, and was found to reliably identify platelet dysfunction in humans.[49,51,52]

Evidence suggests that antithrombotic drugs may be beneficial in humans with certain types of PH. Current guidelines in human medicine recommend the use of anticoagulants for patients with idiopathic pulmonary arterial hypertension (PAH), hereditary PAH, and drug or toxin-indued PAH.[12,53] The recent trend in human medicine is a shift from warfarin to direct PO anticoagulant administration, despite the paucity of data and efficacy in humans with PAH.[54-56] Direct PO anticoagulants such as rivaroxaban were shown to attenuate right ventricular pressure and right ventricular hypertrophy in rats with experimentally-induced PAH.[57] Although platelet activation also has been reported in humans with PH, the clinical benefits of antiplatelet drugs are unclear. Aspirin, which inhibits platelet production of thromboxane A2, was shown to improve pulmonary vascular remodeling in humans and rats.[58-60] However, it failed to alleviate exercise intolerance in people with precapillary PH in a randomized clinical trial.[61] Several antiplatelet agents also were found to prevent platelet aggregation in experimental models of PH.[62-67]

Interestingly, dogs with MMVD and PH had significantly higher leukocyte and monocyte count, and lower red blood cell count, hemoglobin concentration and hematocrit compared with MMVD dogs without PH. Iron deficiency anemia has been commonly reported in dogs[68,69] and humans with mitral valve disease[70] and appears to be commensurate with disease severity. A previous study found that dogs with congestive heart failure, including 44/72 dogs with MMVD, had significantly increased neutrophils, band neutrophils, and monocyte compared with healthy dogs, although cell counts remained within normal reference range.[71] Additionally, several experimental studies suggest that inflammation may be involved in cardiac remodeling secondary to MMVD.[37,72]

Our study had several limitations. First, PH was diagnosed and graded based on echocardiography, although right-sided heart catheterization is the gold standard to diagnose PH.[2,4,73] However, a previous study found that, if echocardiography and right-heart catheterization are not interchangeable, some echocardiographic data correlated well with cardiac catheterization to diagnose PH.[74] Pulmonary thromboembolism also could not be completely ruled out in our patient population because advanced imaging such as computed tomography and angiography was not performed. Second, the lack of a severity- and size-matched control group of MMVD dogs is a limitation. Hence, the degree of mitral regurgitation may be a contributing factor for increased platelet priming and PLA. A positive and weak correlation between LVIDdN and platelet responsiveness to thrombin suggests that MMVD severity may only be partially responsible for platelet priming, given that PLA and PLN formation did not correlate with MMVD severity in our study. Additional factors such as changes in vascular rheology and systemic inflammation may contribute to platelet-leukocyte interactions in dogs with MMVD and PH. This scenario is likely the case because dogs in the PH group had evidence of chronic inflammation. Third, additional diagnostic tests were not performed to rule out other underlying systemic diseases, especially pulmonary parenchymal or airway diseases that could exacerbate PH in the

dogs with MMVD. Although none of the dogs required hospitalization and oxygen therapy, assessment of oxygenation would help to rule out any underlying respiratory dysfunction that could lead to vascular remodeling and vasoconstriction. Our study sample was relatively small despite a sample size calculation to determine sufficiency in identifying significance in platelet activation. Our limited sample size prevented us from performing subgroup analyses to correlate disease severity with platelet activation and PLA formation. Lastly, in addition to the physiologic agonists such as thrombin that were used to induce platelet activation and PLA and PNA formation, pre-activation of whole blood with phorbol myristate acetate, a potent protein kinase C activator and NETs inducer, would serve as a more definitive positive control for our flow cytometry assay.

5 | CONCLUSION

Dogs with MMVD and PH have increased platelet activation, characterized by hyperresponsiveness to platelet agonists and platelet-neutrophil interaction, which may play a role in PH pathogenesis in dogs. Additional studies are needed to elucidate the cause-and-effect relationship between platelet hyperreactivity and PH in dogs. Antiplatelet treatment may hold promise as an adjunctive therapy in dogs with MMVD and PH, and prospective randomized, placebo-controlled clinical trials are warranted to determine the clinical benefits of antiplatelet treatment in these patients.

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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

Approved by the University of California Davis, Davis IACUC, number 21471.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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

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