

Procoagulant Microparticles in Dogs with Immune-Mediated Hemolytic Anemia

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Background: Studies of some human prothrombotic diseases suggest that phosphatidylserine-positive (PS+) and tissue factor-positive (TF+) microparticles (MPs) might play a role in the pathogenesis of thrombosis or serve as biomarkers of thrombotic risk.

Hypothesis/Objectives: To determine if circulating levels of PS+MP and procoagulant activity (PCA) associated with PS+MPs and TF+ MPs are increased in dogs with IMHA.

Animals: Fifteen dogs with primary or secondary IMHA and 17 clinically healthy dogs.

Methods: Prospective case-controlled observational study. Circulating PS+MPs were measured by flow cytometry. PCA associated with PS+MPs and TF+MPs was measured by thrombin and Factor Xa generating assays, respectively.

Results: Circulating numbers of PS+MPs were not significantly higher in dogs with IMHA [control median 251,000/ μ L (36,992–1,141,250/ μ L); IMHA median 361,990/ μ L (21,766–47,650,600/ μ L) $P = .30$]. However, PS+MP PCA [control median 2.2 (0.0–16.8) nM PS eq; IMHA median 8.596, (0–49.33 nM PS eq) $P = .01$] and TF+MP PCA [control median 0.0, (0.0–0.0 pg/mL); IMHA median 0.0; (0–22.34 pg/mL), $P = .04$] were increased. Intravascular hemolysis, which we showed might increase PS+ and TF+MP PCA, was evident in 3 of 5 dogs with PS+MP PCA and 2 of 4 dogs with TF+MP PCA higher than controls. Underlying disease in addition to IMHA was detected in 1 of 5 dogs with PS+PCA and 3 of 4 dogs with TF+MP PCA higher than controls.

Conclusions and Clinical Importance: TF+ and PS+MP PCA is increased in some dogs with IMHA. Further studies that determine if measuring TF+ and PS+ MP PCA can help identify dogs at risk for thrombosis are warranted.

Key words: Auto-immune hemolytic anemia; Hemolysis; Microvesicle; Phosphatidylserine; Procoagulant; Tissue factor.

Immune-mediated hemolytic anemia (IMHA) is an important cause of illness and death in dogs.¹ Thromboembolic disease is a common complication that affects survival.¹ Activation of platelets and coagulation have been reported in dogs with IMHA, but the mechanisms of thrombosis have not been thoroughly investigated.^{1–4} Drugs targeting platelets or coagulation have been used for thromboprophylaxis, although it is not known which, if any, are effective.¹ Studies using individually adjusted doses (IAD) of unfractionated heparin are encouraging.⁵ However, IAD heparin

Abbreviations:

MP	microparticle
PCA	procoagulant activity
IMHA	immune-mediated hemolytic anemia
IVH	intravascular hemolysis
PS	phosphatidylserine

requires monitoring of anti-Xa activity which can be expensive and time consuming. No biomarkers that predict thrombotic risk in individual dogs have been identified to help determine which dogs should receive thromboprophylaxis. Further research investigating mechanisms of thrombosis might help identify therapeutic agents that target pathologic pathways, and identify clinically useful biomarkers.

Recent research suggests that circulating microparticles (MPs) might play a role in pathologic thrombosis or serve as biomarkers of thrombotic risk for several diseases in people.^{6–17} MPs are derived from blebbing of the plasma membrane of many cell types, and are released upon cell activation or injury and cleared primarily by the reticuloendothelial system.^{6,7,18,19} Activation and injury of cells also cause plasma membranes to lose phospholipid asymmetry and expose anionic phospholipids, such as phosphatidylserine (PS), on their surface. PS exposure provides a negatively charged docking site for tenase and prothrombinase complexes of the coagulation cascade.²⁰ Platelet MPs exposing PS are 50- to 100-fold more procoagulant than activated platelets.²¹

Some evidence suggests that PS-positive MPs (PS+MPs) from platelets, RBCs, and other cells are involved in the pathogenesis of thrombosis in hemolytic and polycythemic disease states.^{12,22} Platelet MP

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numbers are elevated in dogs with IMHA; however, PS exposure has not been examined.⁴ Damaged and senescent RBCs also express PS on their surface and form procoagulant MPs.^{9,23,24} Triggers for increased RBC microvesiculation include complement attack and oxidative injury, conditions that are present in dogs with IMHA.^{6,25} In addition to expressing PS, RBC MPs scavenge nitric oxide and impair vasodilation.²⁶ Thus, PS+MPs derived from red cells and platelets might contribute to thrombosis in IMHA.

Tissue factor-positive MPs (TF+MPs) might also contribute to thrombosis or act as markers of thrombotic risk. Tissue factor is the primary cellular initiator of blood coagulation. TF triggers thrombus formation by activating the coagulation cascade.^{27,28} Circulating TF+MP numbers and PCA are increased in some prothrombotic inflammatory, neoplastic, and hemolytic diseases in people.^{10,13,15,29} PCA induced by TF+MPs has been associated with thrombosis in some studies of human patients with sickle cell crisis and pancreatic cancer.^{11,17} Factors that induce TF expression, including proinflammatory cytokines, free heme, and hypoxia, are present in IMHA.^{1,24,30,31} TF+MPs are released primarily from activated monocytes, and cancer cells.^{6,17} Indeed, TF mRNA expression is increased and the concentration of cytokines associated with monocyte/macrophage activation are elevated in blood from dogs with IMHA.^{32,33} Therefore, TF+MPs might also be increased. To date, there have been no studies on TF+MPs in dogs with IMHA.

In addition to the possibility of increased production of MPs because of the presence of hemolysis, platelet activation, and activation of TF-expressing cells, therapies might also increase MP levels in dogs with IMHA. For example, MP formation occurs during storage of blood products.^{34,35} Some immunosuppressive agents such as cyclosporine also increase MP formation.³⁶ Splenectomy has been associated with increases in circulating MPs, possibly because of decreased clearance.^{37–39}

The purpose of this pilot study was to determine if circulating levels of PS+MP and PCA associated with PS+ and TF+MPs are increased in dogs with IMHA.

Materials and Methods

Study Design

Prospective case-controlled observational study.

Study Population

Dogs presenting to a specialty referral hospital for evaluation of hemolytic anemia were screened for IMHA. IMHA (primary or secondary) was confirmed by documenting the presence of anemia (Hct<33%) with spherocytosis, autoagglutination, or a positive Coombs test. Medication history was noted at the time of enrollment. Medical records were reviewed retrospectively for documentation of underlying disease. For the control group, clinically healthy control dogs were recruited from students and faculty of Western University of Health Sciences College of Veterinary Medicine. A lack of historical medical problems, normal physical

examination, and a lack of anticoagulant or antiplatelet medications were required for inclusion into the control group. This study was approved by the Western University of Health Sciences Institutional Animal Care and Use Committee. Written informed owner consent was obtained for each dog.

Sample Acquisition and Handling

We found no difference in the number of PS+MP when 23- or 21-gauge (G) needles were used to draw blood from peripheral veins in dogs. Briefly, venous blood was atraumatically drawn from a peripheral vein (cephalic or lateral saphenous) with a 23-G butterfly needle directly into a 1.8-mL vacutainer tube containing 3.2% sodium citrate for 5 healthy dogs. The procedure was repeated with a 21-G butterfly needle using the opposite limb. MPs were enumerated by flow cytometry. There was no difference between PS+MP and formation (21-G 4,906+/- 1206; 23-G 2710+/- 1448 PS+ MPs/ μ L, $P = .44$). Therefore, 23-G needles were used in the present study for the dog's comfort.

Venous blood was atraumatically drawn from a peripheral vein (cephalic or lateral saphenous) with a 23-G butterfly catheter directly into two 1.8-mL vacutainer tubes containing 3.2% sodium citrate. One sample was submitted to Antech Diagnostics for CBC, coagulation (PT, PTT D-Dimer, and fibrinogen levels), and heartworm (*Dirofilaria immitis*) antigen testing. The other sample was transported to the laboratory with minimal agitation in an upright position, transferred to microcentrifuge tubes and centrifuged at $2,500 \times g$ for 15 minutes at room temperature. The supernatant was carefully removed so as not to disturb the buffy coat and spun again at $2,500 g$ for 15 minutes. The MP containing platelet-free plasma (PFP) was carefully removed leaving a small amount (approximately 100 μ L) of PFP and any remaining platelet pellet undisturbed. The collected PFP was immediately frozen at -80°C . The time of blood sampling to processing was less than 1 hour. All samples were analyzed within 1 year of freezing.

This centrifugation protocol is currently recommended for obtaining PFP for microparticle research in people.⁴⁰ We verified that this centrifugation protocol also effectively removes platelets from dog blood by a highly sensitive platelet detection protocol.⁴⁰ Two 1.8-mL vacutainer tubes containing 3.2% citrate were filled with blood from the cephalic vein of each of 2 dogs with a 23-G butterfly catheter. PFP was isolated by the above protocol, 500 μ L from each of the 4 PFP samples were placed in a disposable chamber mounted with a filter card and spun at $350 g$ for 10 minutes by using a Cytospin 2 centrifuge.^a The supernatant was absorbed by the filter card, and cells were deposited on the slide through a hole in the filter paper. The 4 slides were stained with Giemsa, and 10 high-powered fields ($1,000\times$) on each slide were examined for platelets. The cytospin centrifugation and platelet enumeration were performed by an experienced technologist (STAT VETERINARY LABORATORY San Diego CA). No platelets were seen on any field for any sample. Each of the 2 dogs had a normal circulating platelet count (375,000 and 241,000 per μ L of EDTA anticoagulated whole blood, respectively). This suggests that platelet contamination in the samples used for microparticle detection was minimal, and the protocol of 2 sequential $2,500 \times g$ spins for 15 minutes effectively removes platelets from canine blood.

Flow Cytometry

Thirty microliter of PFP was incubated with 10- μ L Annexin V (AnnV)-FITC (BD) for 30 minutes in the dark at room temperature. Plasma samples were diluted in 470- μ L flow cytometry buffer with or without calcium (140 mM NaCl, 10 mM Hepes, 5 mM CaCl_2) just before running. Guidelines defining MP populations in dogs have not been determined. Therefore, the upper limit for the

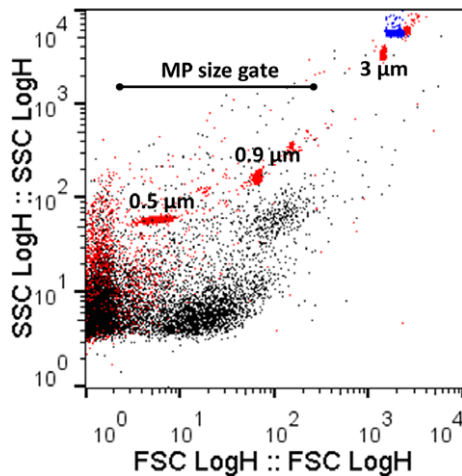


Fig 1. Microparticle (MP) gating strategy. MPs were defined as PS-positive, MP-sized events. Megamix beads (red) are shown with corresponding bead size indicated. The bead population is overlaid on total events of an example dog plasma sample (black) containing counting beads (blue). The MP size gate extended from the minimum detection limit of the Stratidigm S1000Ex flow cytometer (0.1–0.2 μm) until just above the 0.9- μm sized bead population as described in *Materials and Methods*.

MP size gate was determined using Megamix beads^b and extended slightly beyond 0.9 μm to avoid bisecting a uniform population observed in some samples in accordance with the International Society of Thrombosis and Hemostasis Guidelines for human MPs (Fig 1).⁴¹ MPs were defined as AnnV+ events within the MP size gate and counted using cytoCount beads^c. Samples were analyzed on a Stratidigm Ex1000^d flow cytometer.

PCA Associated with PS+MP

Levels of thrombin generation associated with PS+MPs were measured in PFP utilizing a PS capture and prothrombinase complex chromogenic thrombin generation assay (Zymuphen MP-Activity; Aniara) according to the manufacturer's instructions (Fig 2). MP concentration was determined by generating a standard curve using samples with known PS+MP concentrations provided in the test kit and reported as nM PS equivalents (eq).

PCA Associated with TF+MPs

Microparticles were pelleted from 100 μL of PFP (thawed on ice) by centrifugation (20,000 $\times g$ for 15 minutes at 4°C) and washed twice with HBSA buffer as previously described.^{42,43} Total Factor Xa (FXa) and TF-dependent FXa generation was determined by a chromogenic assay. In this assay, procoagulant activity associated with generation of FXa is measured using a chromogenic FXa substrate after adding FVIIa and FX to the MPs (Fig 3A). Antibody directed against TF is added to determine how much of the activity is specifically generated by the presence of TF in the sample (Fig 3B). The assay was performed as previously described for human samples, with a slight modification for use with canine blood.^{42,43} Specifically, 1 μg of goat IgG^c or 1 μg of goat polyclonal anti-human TF antibody were used in each 100- μL reaction volume comprised of 50- μL washed MPs and 50 μL of human FX and FVIIa (the final concentration of FX

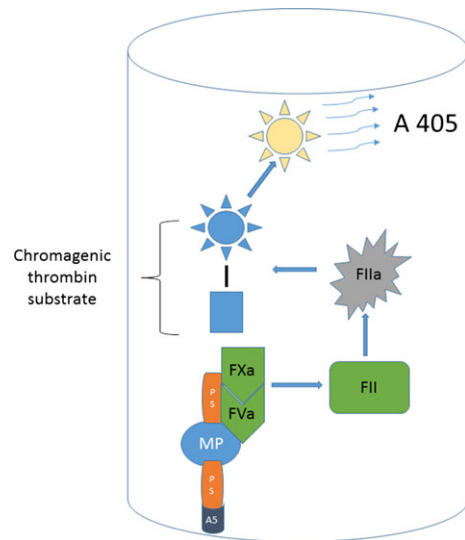


Fig 2. Detection of PCA associated with PS+MPs (Zymuphen MP-Activity Aniara). PS+MPs are captured by streptavidin and biotinylated annexin V (A5) bound to a well. FXa, FVa and prothrombin (FII) are added. In the presence of PS, FXa-FVa activates prothrombin to thrombin (IIa), which in turn acts on a chromogenic thrombin substrate. Absorbance is measured at 405 nm (A405). There is a direct relationship between the concentration of phospholipids and thrombin generation.

was 150 nM and FVIIa was 4.88 nM). This substitution was made because the mouse anti-human TF monoclonal antibody used in the human assay did not inhibit PCA induced by canine TF in preliminary experiments. However, we found that the goat polyclonal anti-human TF antibody at this concentration inhibits 60–100% of PCA associated with TF+MPs in platelet-rich plasma or PFP isolated from canine whole blood incubated with LPS *in vitro* (data not shown).

Generation of Hemolyzed Blood Samples

TF+MPs were generated by incubating 2 mL of whole blood anticoagulated in 3.2% Na citrate with LPS (10 $\mu\text{g}/\text{mL}$,⁵) for 5 hours at 37°C.⁴⁴ To hemolyze the samples, untreated or LPS-activated whole blood was aspirated and flushed vigorously 5 times through a 26-G needle with a syringe. PS+MP and TF+MP PCA were measured as described above.

Statistical Analysis

Comparisons of continuous variables were made using the Mann-Whitney *U*-test. Comparisons of categorical variables were made using the Fisher's exact test. Bivariate correlations were done using the Spearman Rho test. A paired *t*-test was used to test for differences in MP formation because of needle size. Statistical analyses were performed by the statistical software program.^g

Results

Signalment and Medication History

Seventeen control and 15 dogs with IMHA were included in the study. There was no difference in age between the dogs with IMHA and control dogs

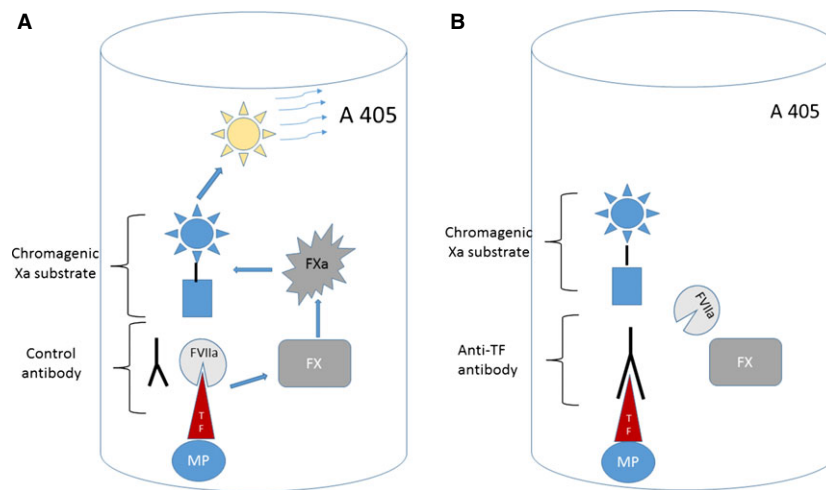


Fig 3. Detection of PCA associated with TF+MPs. **(A)** Total Xa PCA is measured by adding FVIIa, FX and control antibody to a well containing MPs pelleted from PFP by high speed centrifugation. FXa is generated in the presence of TF because of activation of FX by extrinsic tenase (TFVIIa). FXa then cleaves a chromogenic Xa substrate. Absorbance is read at 405 nm (A405) and compared to a standard curve. **(B)** TF-dependent PCA is determined by the replacement of control antibody with a TF-specific antibody.

(control; median 4.0 range 0.25–11 years; IMHA; median 5.0 range 2–13 years; $P = .14$). There were 4 neutered males, 10 spayed females, 1 intact female, and 2 intact males in the control group. There were 9 neutered males, 4 spayed females, 1 intact female, and 1 intact male in the IMHA group. There was no difference in sex distribution between control and IMHA groups ($P = .16$). There were 7 mixed breed dogs, 3 English springer spaniels, 2 German shorthaired pointers, and 1 each of Pointer, Heeler, Australian cattle dog, rat terrier and beagle dog in the control group. The IMHA group consisted of 5 mixed breed dogs (2 were Cocker spaniel mixes), 2 Cocker spaniels, and 1 each of the following breeds; Bernese mountain dog, Dachshund, Labrador retriever, Schnauzer, Maltese, Papillion, poodle, and Welsh corgi.

Before sampling, 2 dogs with IMHA received 1 dose of aspirin, 8 received dexamethasone or prednisone, and 4 received azathioprine. One dog received a transfusion 24 hours before sampling. Five had not received any medication before sampling.

Underlying Disease Screening in Dogs with IMHA

All dogs were screened for infection with *Dirofilaria immitis* as incentive to join the study. One of the 15 dogs with IMHA was occult antigen positive and microfilaremic. Nine of 15 dogs had additional vector-borne disease screening; 7 were tested for antibody to *Borrelia burgdorferi*, 6 were tested for antibody to *Ehrlichia canis* and *Rickettsia rickettsii*, and 3 were tested using PCR for circulating DNA from *Anaplasma* sp., *Babesia* sp., *Bartonella* sp., *Ehrlichia* sp., *Mycoplasma* sp., *Neorickettsia risticii*, and *Rickettsia rickettsii*. All tests were negative. Abdominal ultrasound was performed in 8 of 15 dogs; 1 dog had a small focal liver mass, 1 dog had evidence of hepatomegaly and mild pancreatitis. One dog had mild bilateral adrenomegaly,

and a slightly enlarged gall bladder with echogenic debris in the lumen, 1 dog had mild gall bladder distension with slight thickening of the gall bladder wall, and 1 had slightly decreased corticomedullary junction in both kidneys and a small renal infarct. Abdominal ultrasound was unremarkable in 3 dogs. Stage C valvular heart disease was diagnosed in 1 dog based on history, thoracic radiographs, abdominal ultrasound, and echocardiogram. Two additional dogs had unremarkable thoracic radiographs.

Hematologic and Coagulation Variables

Results for CBC and coagulation testing were available for 13 of 15 dogs with IMHA and 17 control dogs. The hematocrit was significantly lower, and total white cell, neutrophil, and monocyte counts were significantly higher in dogs with IMHA. There was no difference in platelet or lymphocyte count between groups (Table 1). PT and PTT were significantly longer, and fibrinogen levels were significantly higher in dogs with IMHA. There was no difference in frequency of increases in D-dimer levels between the 2 groups (Table 1).

Circulating Microparticle Analysis

PS+MPs were enumerated in 14 of 15 IMHA dogs and 17 of 17 control dogs by flow cytometry. The median number of PS+MPs was higher in dogs with IMHA versus control dogs, although the difference was not statistically significant (control; median 251,000/ μ L range 36,992–1,141,250/ μ L; IMHA; median 361,990/ μ L, range 21,766–47,650,600/ μ L, $P = .30$) (Fig 4).

Dogs with IMHA had significantly elevated PS+PCA (control; median 2.2, range 0–16.8 nM PS eq; IMHA; median 8.596, range 0–49.33 nM PS eq, $P = .01$). This difference was mostly attributable to high values in 3 dogs. Two additional dogs with IMHA had PS+PCA

Table 1. Hematologic and coagulation variables in dogs with IMHA and control dogs.

Variable	Control n = 17 Median (Range)	IMHA n = 13 Median (Range)	P Value
Hematocrit %	49 (34–55)	14 (7–23)	.0001*
Platelet count × 1000/μL ^a	235 (174–369)	250 (48–583)	.06
WBC × 1000/μL	7.4 (4.5–12.4)	22.1 (7.6–70.9)	.0001*
Neutrophils × 1000/μL	4.7 (2.7–7.8)	18.2 (5.9–53.2)	.0001*
Lymphocytes × 1000/μL	2.4 (1.0–4.0)	1.1 (0.3–6.9)	.21
Monocytes × 1000/μL	0.296 (0.124–680)	1.4 (0.2–8.5)	.0005*
PT (reference range 8–12 s)	6.6 (6.0–7.4)	7.2 (6.1–10.9)	.03*
APTT ^b (reference range 10–25 s)	10 (<10.0–12.4)	14.2 (10–23.6)	.0001*
Fibrinogen (reference range 150–400 mg/dL)	159 (120–263)	580 (167–844)	.0001*
Elevated D-dimer	1/17	2/13	.56

*Statistically significant difference.

^aIn 4 control and 2 IMHA dogs, platelet count was estimated as “adequate”. A value of 250,000/μL was used for calculations in these samples.

^bTwo control dogs had an APTT estimate of less than 10 seconds. These were assigned a value of 10 for calculations.

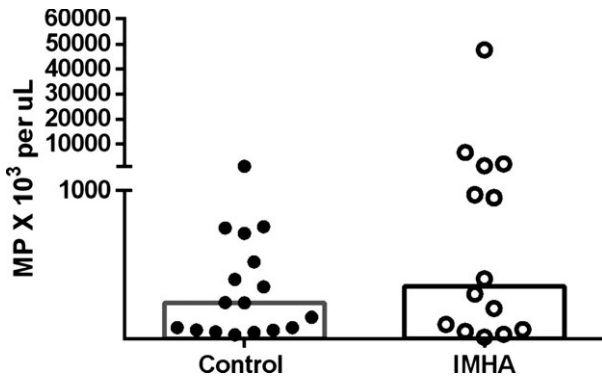


Fig 4. Flow cytometric enumeration of Annexin V binding circulating MP in IMHA (n = 14) and control (n = 17) dogs. Data are expressed as the number of events X 10³/μL. The median is indicated by the bars (P = .30).

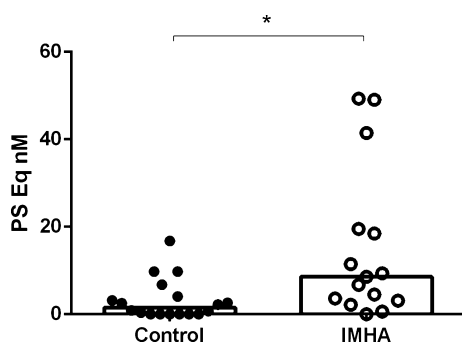


Fig 5. PCA associated with PS+MPs in IMHA (n = 15) and control (n = 17) dogs. Data are expressed as nM PS equivalents (Eq). The median is indicated by the bars (P = .01). *=statistically significant.

slightly higher than the control dogs (Fig 5). 3 IMHA dogs with PS+MP PCA above controls had evidence of intravascular hemolysis (IVH) (18.47, 41.48 and 49.07 nM PS eq).

Both total FXa generation and TF-dependent FXa generation (TF+MP PCA) were significantly higher in

dogs with IMHA as compared to control dogs. None of the control dogs had detectable total FXa generation (median 0.0 range 0.0–0.0), whereas 5 of 15 dogs with IMHA had detectable total Xa generation (median 0.0 range 0–22.3 pg/mL P = .02) (Fig 6A). None of the control dogs had detectable TF+PCA (median 0.0 range 0.0–0.0), whereas 4 of 15 dogs with IMHA had increased levels (median 0.0; range 0–22.34 pg/mL, P = .04) (Fig 6B). All of the total FXa generation was TF dependent for 1 dog and none of the total FXa generation was TF dependent for another. The other 3 dogs had both TF-dependent and TF-independent FXa generation. The dog with the highest TF-dependent activity associated with MPs was infected with *Dirofilaria immitis*, was microfilaremic, and had IVH (22.34 pg/mL). TF-dependent activity associated with MPs was slightly elevated in a dog with suspected mild pancreatitis (0.074 pg/mL), and moderately increased in a dog with bilateral adrenomegaly and a mildly enlarged gall bladder who had received a transfusion (0.677 pg/mL). No underlying disease was detected in the 4th dog with elevated TF-dependent PCA (0.347 pg/mL), although this dog had IVH.

We determined the effect of hemolysis on PCA associated with PS+MP and TF+MPs. Mechanical hemolysis increased basal PS+PCA in PFP derived from LPS-treated whole blood (Fig 7A). Mechanical hemolysis also augmented TF-dependent and TF-independent FXa generation in the MP fraction isolated from LPS-treated whole blood (Fig 7B,C).

For dogs with IMHA, total FXa generation and TF-dependent FXa generation was not correlated with red cell count, platelet count, white cell count, neutrophil count, monocyte count, or coagulation variables (data not shown).

Posthoc analysis of dogs with IMHA receiving glucocorticoids versus those who did not reveal a difference in PS+MPs PCA (glucocorticoids; n = 8, median 14.98, range 2.18–49.33; no glucocorticoids; n = 7, median 3.6, range 0–41.48; P = .12). Two of 4 dogs with increased TF-dependent FXa generation had received glucocorticoids.

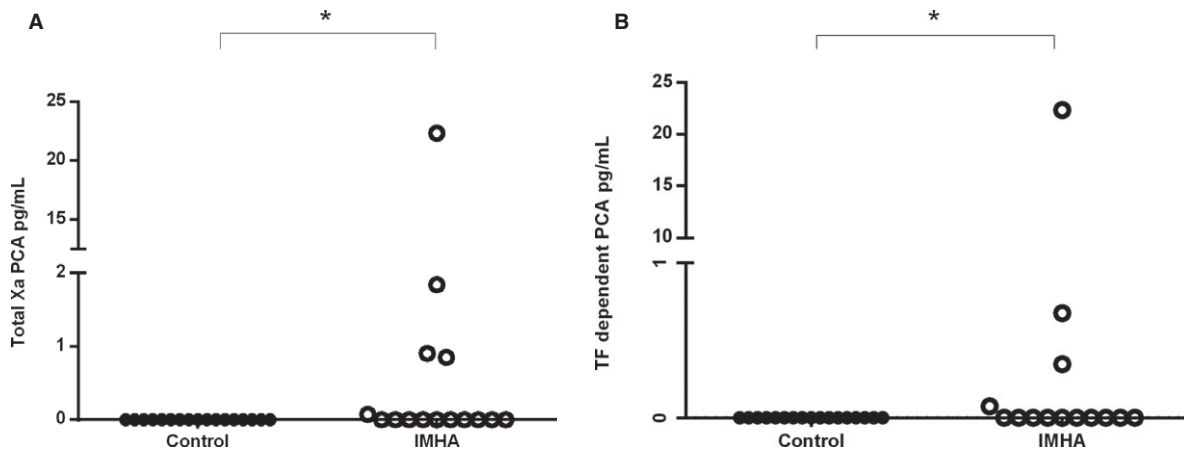


Fig 6. Tissue factor-associated PCA in IMHA (n = 15) and control dogs (n = 17). **A)** Total Xa MP PCA (P = .02). **B)** TF-dependent MP PCA (P = .04). *=statistically significant.

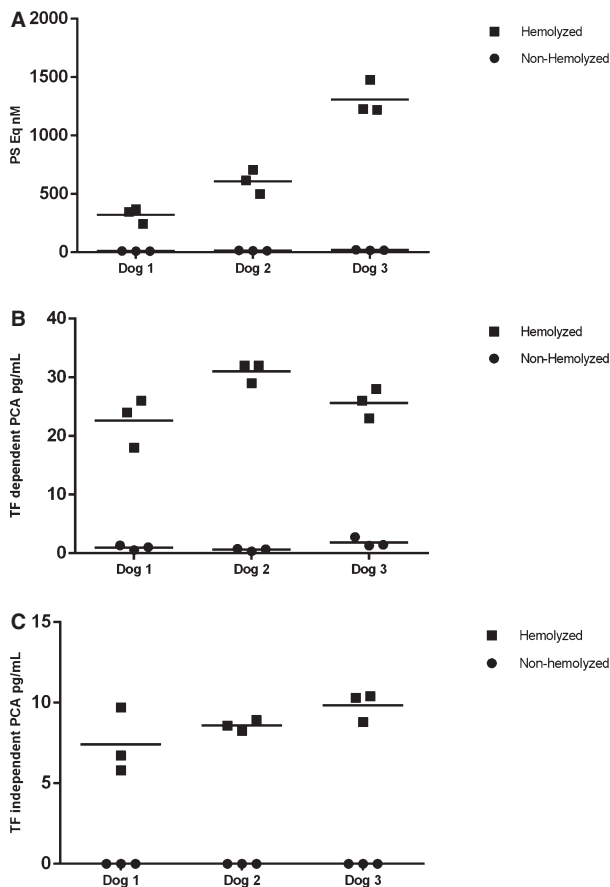


Fig 7. The effect of hemolysis on PCA associated with PS+MP and TF+MP. PS+MP PCA, TF-dependent and TF-independent PCA were measured in PFP isolated from blood that was mechanically hemolyzed in vitro after phlebotomy. Blood samples from each of 3 dogs were assayed in triplicate. The mean PCA is represented by the bar. For the TF+MP PCA assays, whole blood was incubated with LPS before hemolysis and PFP isolation as described in *Materials and Methods*. **(A)** PS+ PCA in PFP from mechanically hemolyzed and nonhemolyzed blood. **(B)** TF-dependent and **(C)** TF-independent PCA associated with the MP fraction isolated from nonhemolyzed and mechanically hemolyzed blood.

Discussion

In this study, we found that some dogs with IMHA had increased PCA associated with PS+MPs and TF+MPs. We did not determine the cellular origin of MPs in this study. However, the pathophysiologic state of dogs with IMHA would theoretically support formation of PS+MPs because of the presence of red cell destruction and platelet activation; and TF+MPs because of the presence of monocyte activation and conditions supporting endothelial cell activation.^{1,4,33} No studies of MPs in people with IMHA have been performed. However, there is some evidence MPs play a role in the pathophysiology of thrombosis in other inflammatory hemolytic diseases in people. Sick cell crisis is similar to IMHA in that it is associated with a proinflammatory, hypercoagulable state, hemolysis, endothelial dysfunction, and thrombosis.^{13,45} PS+MPs derived from RBCs and platelets, and TF+MPs from monocytes and endothelial cells are elevated during sickle cell crisis.^{13,29} Increased PCA is associated with both PS+MP and TF+MP in human patients with sickle cell disease, and TF+MP PCA is associated with acute chest syndrome in these human patients.^{11,29,39} However, TF+MPs are not increased in people with paroxysmal nocturnal hemoglobinuria.⁴⁶ Therefore, generalized conclusions about MPs and thrombotic risk in proinflammatory hemolytic disease cannot be made.

It is noteworthy that PS+MP PCA and TF+MP PCA was above the control range in only 5 of 15 and 4 of 15 dogs with IMHA, respectively (Figs 5 and 6B). Not all dogs with IMHA are in a hypercoagulable state or form thromboemboli.^{1,2,47} Whether or not thrombosis is more likely in a dog with increased MP PCA requires further study.

In this study, we included dogs with both primary and secondary IMHA and did not exclude dogs with medical problems unrelated to IMHA. The dog with the highest TF+MP PCA and modestly increased PS+MP PCA was heartworm positive and microfilaremic. Mild and moderate increase in TF+MP PCA without increase in PS+MP PCA was observed in 1 dog

with mild pancreatitis, and 1 dog with bilateral adrenomegaly and mild gall bladder enlargement, respectively. Inflammation and endothelial damage are associated with *D. immitis* and *Wolbachia* spp. infection and pancreatitis, and thrombosis is associated with heartworm disease, pancreatitis, and hyperadrenocorticism.^{48–50} Therefore, underlying disease processes could have contributed to MP formation in these dogs. No other dog with PS+MP (n = 3) or TF+MP PCA (n = 1) higher than the control range had evidence of underlying disease. However, the presence of undetected underlying disease cannot be ruled out because the diagnostic work up varied between dogs because of financial constraints of the owner and clinician preference.

Three of 15 dogs with IMHA had evidence of IVH based on the presence of marked hemoglobinemia. This prevalence is consistent with other studies of dogs with IMHA.⁵¹ Profound hemoglobinuria confirmed IVH in 1 dog. Urinalysis was not performed for the other 2. An atraumatic phlebotomy protocol was utilized and hemolysis was not observed in any control samples. Therefore, iatrogenic hemolysis as a cause for the marked hemoglobinemia is unlikely.

All of the dogs with IVH had increased PCA. Two of them had TF+MP PCA and PS+ MP PCA higher than the control range, whereas the other had elevated PS+ PCA only. There is a strong correlation between intravascular hemolysis and levels of circulating MPs in people with sickle cell disease.³⁹ Furthermore, complement-mediated red cell destruction increases RBC microvesiculation and PCA.⁵² In this study, we found that mechanical hemolysis augmented both PS- and TF-mediated PCA. Increased exposure of PS and other negatively charged phospholipids that facilitate binding of tenase (FIXa/FVIIIa) and prothrombinase (FXa/FVa) complexes could explain the augmented activity seen with hemolysis in these assays.^{52,53} Further study is required to determine whether RBC membrane fragments or MP formation associated with IVH facilitate thrombosis in vivo.

Drug and other treatments might affect MP levels by a number of mechanisms.^{34–36,38} We found no significant increase in PS+MP PCA in the 8 dogs with IMHA which had received glucocorticoids compared to the 7 who did not, but the sample size was small. Only 4 dogs received azathioprine, 2 dogs received aspirin and 1 dog received a transfusion before sampling. Therefore, larger studies are required to determine the effect of specific treatments on MP levels.

In contrast to PCA associated with PS+MPs, we did not find a significant increase in PS+MP number by flow cytometry in dogs with IMHA. This might be explained by the fact that flow cytometry is less sensitive in detecting small MPs than functional assays of MP PCA.^{7,43,54} Importantly, most PCA associated with MPs can be attributed to small MPs.⁵⁵ In addition, 2 of 5 dogs with increased TF-independent FXa generation did not have increased PS-associated PCA. This is consistent with other studies suggesting phospholipids other than PS play a role in PCA associated with membranes.⁵³ It is possible that PCA associated with

exposed phospholipids other than PS could augment and increase PCA associated with PS+MPs, without an observed increase in PS+MP number.

Several preanalytical variables affect formation of MPs. The most important are delay between sample collection and centrifugation, agitation of samples after collection, and centrifugation speed.⁴⁰ Substantial effort was made to limit these and other preanalytical variables from affecting the results of this study. Most clinical studies of MPs necessitate freezing of MPs before analysis.⁴⁰ Freeze/thawing can affect MP formation, although these effects are more pronounced when platelet poor, rather than PFP samples are used.^{43,56} PFP was used in this study, and control and IMHA samples were treated identically. However, it is possible that freeze/thawing might have affected MP number and PCA values. Additional limitations of this study include its small size, lack of comprehensive underlying disease screening, and standardized treatment and monitoring protocols. We also did not determine the cellular origin of MPs. Therefore, conclusions regarding the potential role of MPs in the pathophysiology of thrombosis in idiopathic and secondary IMHA and whether they can serve as markers for thrombotic risk cannot be made. However, in this study we have demonstrated that some dogs with IMHA have increased PCA associated with PS+MPs and TF+MPs in peripheral blood. Based on these results, larger studies with comprehensive underlying disease screening and standardized medical treatment to determine whether MPs can be used as markers of thrombotic risk, and studies to determine the cellular origin of MPs in dogs with IMHA are warranted.

Footnotes

- ^a Shandon Southern Instruments, Inc., Sewickley, PA.
- ^b Biocytex, Merseille, France
- ^c Dako, CA
- ^d Stratedigm, CA
- ^e Santa Cruz Biotech Dallas TX
- ^f Sigma Aldrich, St. Louis, MO
- ^g Graphpad Prism Version 6.0 San Diego CA

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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