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# Immunoglobulin G and phosphatidylserine in regenerative and nonregenerative immune-mediated anemias of dogs

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## Abstract

**Background:** Although precursor-targeted immune-mediated anemia (PIMA) is thought to be caused by immune targeting of erythroid precursors (nucleated RBCs, nRBCs), its pathogenesis is unknown. Immunoglobulin G (IgG) or phosphatidylserine (PS) may promote nRBC destruction in PIMA.

**Hypothesis:** Dogs with PIMA have increased nRBC IgG and PS, and dogs with immune-mediated hemolytic anemia (IMHA) have increased RBC PS compared to healthy dogs.

**Animals:** Blood from 20 healthy dogs and from dogs with IMHA (11) or other (non-IMHA) conditions (9), and marrow aspirates with or without blood from 10 healthy dogs and from dogs with PIMA (17) or other (non-IMHA, non-PIMA) conditions (7).

**Methods:** Marrow nRBC stages were separated by density gradient. Flow cytometry was used to assess the percentage of RBCs or nRBCs with increased IgG or PS.

**Results:** Red blood cell (RBC) IgG positivity was increased in 9/11 IMHA dogs and 0/9 non-IMHA dogs. Red blood cell PS positivity was increased in 10/11 IMHA dogs and 2/9 non-IMHA dogs. Five of 17 PIMA dogs had increased nRBC IgG positivity in mid- or late-stage fractions, whereas all 7 non-PIMA dogs were negative. Mid- and late-stage erythroid precursor PS was significantly higher in PIMA dogs compared to healthy dogs. Five of 14 PIMA dogs had increased RBC IgG positivity.

**Conclusions:** Immunoglobulin G and PS may promote destruction of nRBCs in PIMA dogs; PS may promote destruction of RBCs in IMHA dogs.

**KEYWORDS**
bone marrow, erythroid maturation arrest, flow cytometry, immune-mediated hemolytic anemia, ineffective erythropoiesis, precursor-targeted immune-mediated anemia

**Abbreviations:** AnV-Ca, annexin buffer with $CaCl_2$; AnV-Ca-free, annexin buffer without $CaCl_2$; BSA, bovine serum albumin; C3, complement protein 3; DEA, dog erythrocyte antigen; Hct, hematocrit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IMHA, immune-mediated hemolytic anemia; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; nRBC, nucleated red blood cell (erythroid precursor); PIMA, precursor-targeted immune-mediated anemia; PS, phosphatidylserine; RBC, red blood cell.

## 1 | INTRODUCTION

Precursor-targeted immune-mediated anemia (PIMA) has been described in dogs with nonregenerative anemia and evidence of ineffective erythropoiesis, and although it has been suggested that its pathogenesis may relate to immune targeting of erythroid precursors,[1-7] this pathogenesis has not been established. Suspicion of an immune-mediated mechanism is based on: (a) apparent responses to glucocorticoid treatment[1-6] and relapses when tapering it,[2,3,5,6] (b) phagocytosis of intact erythroid precursors,[1,2,4,8] and (c) in some cases, association with concurrent evidence of immune-mediated hemolytic anemia (IMHA), such as spherocytosis, red blood cell (RBC) agglutination, or Coombs' test positivity,[1,2,4-7] or with other immune-mediated diseases such as immune-mediated thrombocytopenia (IMT).[4-6] However, dogs diagnosed with PIMA typically respond slowly to immunosuppressive treatment,[1-6] some do not respond at all,[1,6] and many have no evidence of concurrent IMHA,[3,5,6] leaving unanswered questions about the mechanism(s) of anemia in these dogs.

Phagocytosis of intact erythroid precursors (rubriphagocytosis) by normal-appearing macrophages is found in bone marrow samples from most dogs with PIMA.[9] This phagocytosis is usually selective for the erythroid lineage and for certain stages of erythroid development, with rubriphagocytosis of different stages associated with different bone marrow patterns.[10] This observation suggests a role for phagocytosis in the ineffective erythropoiesis and nonregenerative anemia in these dogs. The different bone marrow patterns seen in these dogs may reflect a spectrum of disease caused by a single mechanism, or they may result from multiple mechanisms.

Phagocytosis by macrophages may be promoted by several cell membrane mediators, including immunoglobulin G (IgG), complement, or phosphatidylserine (PS). Although the role of antibodies in the pathogenesis of IMHA is well demonstrated, membrane exposure of PS through oxidative stress[11,12] and an apoptotic-like mechanism[12] have been suggested to contribute to RBC destruction in dogs and mice[13] with IMHA. However, we are not aware of studies that have identified increased RBC PS in dogs with IMHA. Eryptosis, which is apoptosis of mature RBCs, is characterized by cell shrinkage, cell membrane scrambling, and PS exposure triggering RBC phagocytosis by macrophages, and it is 1 of the mechanisms involved in turnover of senescent RBCs.[14] Apoptosis of nRBCs also occurs, and increased PS exposure of bone marrow-derived nRBCs was demonstrated in association with IgG positivity in a human patient with reticulocytopenic immune-mediated anemia.[15] Reports of PS on nRBCs of PIMA dogs are lacking.

Our goal was to assess whether or not IgG, PS, or both were increased on erythroid cells of dogs with immune-mediated anemias. We hypothesized that PS exposure on RBCs and erythroid precursors is increased in dogs with IMHA and PIMA, respectively, and that nRBC IgG is increased in dogs with PIMA just as RBC IgG typically is increased in dogs with IMHA.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

In this prospective, cross-sectional study, RBCs from residual blood of client-owned dogs suspected to have IMHA and admitted to our hospital from October 2013 through November 2014 were assessed for IgG and PS. Red blood cells from residual blood of client-owned unhealthy dogs without evidence of immune-mediated anemia and collected within 24 hours of samples from suspected IMHA samples were also tested for IgG and PS to evaluate the specificity of results for IMHA. Inclusion criteria for IMHA consisted of a clinical diagnosis of IMHA associated with regenerative anemia and at least 1 of the following findings with no other explanation: (a) spherocytosis, (b) erythrocyte agglutination, and (c) Coombs' test positivity. Unhealthy non-IMHA dogs were defined as any sick dogs with no clinical suspicion or hematologic evidence of immune-mediated anemia, including the absence of spherocytosis, RBC agglutination, and Coombs' positivity.

Residual bone marrow and blood samples from client-owned dogs for which a bone marrow aspirate was assessed as part of their diagnostic evaluation from April 2012 through December 2014 were analyzed for nRBC IgG and RBC IgG, respectively; samples of dogs assessed after October 2013 also were tested for PS. The following data were collected: signalment, clinical history and presenting complaint, bone marrow aspirate (and core, when available) findings, relevant diagnostic test results, clinical diagnosis and therapeutic regimen, and clinical outcome with emphasis on hematologic response. Clinical and diagnostic data were reviewed to identify dogs with clear diagnoses of PIMA and dogs that clearly did not have PIMA (non-PIMA dogs). Inclusion criteria for PIMA dogs were as follows: (a) unexplained persistent nonregenerative or inappropriately regenerative anemia (on the day of bone marrow collection, all had hematocrit [Hct] ≤ 29%, inadequate erythroid regeneration for the degree of anemia, and documented anemia or anemia-related clinical signs for at least 5 days), (b) ineffective erythropoiesis, as evidenced by persistent anemia with erythroid hyperplasia or by erythroid maturation arrest or left shift with rubriphagocytosis, (c) lack of erythroid dysplasia, and (d) a clinical diagnosis of PIMA. At our institution, dogs with nonregenerative anemias but evidence of IMHA (eg, agglutination, spherocytosis, hyperbilirubinemia) are typically treated for IMHA without bone marrow assessment to investigate for evidence of concurrent PIMA, so our study focused on dogs with PIMA and without IMHA, a group for which further diagnostic testing may be of benefit. Inclusion criteria for non-PIMA dogs were the same as described for unhealthy non-IMHA dogs, with the addition of: (a) no evidence of erythroid precursor targeting, including absence of ineffective erythropoiesis, and (b) a clear clinical diagnosis of a condition other than PIMA. Dogs were excluded from the study if they had any evidence of neoplastic cells in bone marrow samples, or if the cell yield from bone marrow aspirates was insufficient for flow cytometric assessment.

A control population for IgG and PS on RBCs consisted of 20 clinically and hematologically healthy adult blood donor dogs. Similarly,

IgG was assessed on erythroid precursors from 10 clinically and hematologically healthy adult colony dogs (5 from our institution and 5 from a research facility), and PS was assessed for 5 of these dogs (all from our institution). Animal care and sample collection procedures from all healthy colony dogs were performed in compliance with the Institutional Animal Care and Use Committees of the institutions involved in this study.

## 2.2 | Blood and marrow samples: RBC and nRBC isolation and testing for IgG

Erythrocytes and nRBCs were isolated, prepared, and tested for surface-associated IgG as previously described[16] (detailed methods in Supporting Information). Briefly, blood and marrow samples were anticoagulated with ethylenediaminetetraacetic acid. Blood from client-owned dogs was tested on the day of collection or stored at 4°C and tested the next day. For each animal, marrow was tested within 60 minutes of collection, and 3 marrow fractions enriched in early-, mid-, and late-stage nRBCs were obtained by gradient separation. Cell suspensions were prepared from washed RBCs or nRBCs for testing.

Erythrocytes were identified based on forward and side scatters, and nRBCs were identified based on labeling for CD18 and staining for LDS751 (Supporting Information). Positivity for IgG was assessed based on the number of % positive events above a cutoff determined using an irrelevant antibody for each sample.

## 2.3 | RBC testing for PS

Erythrocytes were prepared for PS analysis by washing 20 μL of RBC suspension with annexin buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), pH 7.4; filter sterilized) and then resuspending and incubating the RBCs in the dark for 15 minutes in 100 μL of incubation buffer mixed with 2 μL of 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester-conjugated annexin V (annexin V-FLUOS, catalog number 11988549001, Roche Diagnostics, Indianapolis, Indiana) in the presence of calcium (AnV-Ca). Cells were then resuspended in 400 μL of annexin buffer and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, California) using CellQuest Pro software (Becton-Dickinson).

Controls consisted of: (a) RBCs from a healthy dog incubated with AnV-Ca (negative control for PS), (b) PS-positive control RBCs incubated with AnV-Ca (positive control for PS), (c) RBCs from each tested patient incubated as described above, but in the absence of annexin V-FLUOS (control for RBC autofluorescence), and (d) RBCs from each tested patient incubated as described above, but with a calcium-free annexin control buffer (annexin buffer without $CaCl_2$) used in every step (AnV-Ca-free) to control for nonspecific binding of annexin, because annexin binding to PS requires calcium.[17] Phosphatidylserine-positive control cells were made by twice washing 10 μL of packed RBCs from a healthy dog with a NaCl solution containing bovine serum albumin (BSA; 147.5 mM NaCl, 2.5 mM KCl, 5 mM D-glucose, 20 mM HEPES, 0.1% BSA, and 0.025% sodium azide; pH 7.5), resuspending the cell pellet into 800 μL of the same solution without BSA or sodium azide, and incubating 200 μL of this RBC suspension with 2 μL of 10 mM 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium (BzATP, Sigma-Aldrich, St. Louis, Missouri) stock solution at 37°C for 4 hours.[18] Erythrocytes were then washed and resuspended in 200 μL of annexin buffer for use. Phosphatidylserine-positive control RBCs were stable for at least 2 weeks.

Phosphatidylserine positivity was determined by percent positive events as follows: control RBCs from each tested dog were incubated with AnV-Ca-free and used to set a visual marker to include the main population of RBCs in the fluorescence histogram. This resulted in 0.1% to 0.7% positive events outside the main peak. Those test RBCs incubated with AnV-Ca that had greater fluorescence were considered PS-positive events. This strategy was applied to results of 20 healthy dogs, and cutoffs for PS positivity were calculated based on mean % positive events plus 3 SDs. Patients with % positive events above the cutoff determined based on healthy dogs were considered positive for PS on RBCs (Figure S1). Immunoglobulin G and PS results from same-day and next-day patient samples were interpreted using same-day and next-day reference cutoffs, respectively.

## 2.4 | Erythroid precursor testing for PS

Erythroid precursor testing for PS was done by applying the same method used for RBCs but assessing $10^6$ bone marrow-derived nucleated cells from each Percoll fraction. After resuspending cells in 400 μL of annexin buffer, cells were mixed with 160 μL of LDS751 working solution[19] and incubated for 20 minutes to approximately 1 hour in the dark before flow cytometric analysis. Erythroid precursors were identified based on weak CD18 labeling and strong LDS751 staining. Negative and positive PS controls were the same as those used for RBCs, and nucleated cells from Percoll fractions were additionally incubated with LDS751 to test for autofluorescence and nonspecific annexin binding. Phosphatidylserine positivity was determined by % positive events as follows: erythroid precursors from each Percoll fraction of each dog were incubated with AnV-Ca-free and used to set a marker with 2% positive events based on dot plot distributions of gated nRBC regions. All test sample events with fluorescence greater than this cutoff were considered PS-positive (Figure S2). Because PS positivity was high on erythroid precursors compared to RBCs, effects of processing and time were individually assessed on unfractionated marrow nucleated cells by testing them immediately and 5.5 hours later, the duration of the assay. No effects of processing on PS results were detected.

## 2.5 | Statistical analysis

Comparisons involving RBC IgG and PS among ≥3 groups were performed using Kruskal-Wallis followed by Dunn's multiple comparison

tests. Comparisons between healthy dogs tested for IgG on same-day or next-day were done using a paired *t* test, and comparisons between healthy dogs tested for PS on same-day or next-day samples were done using a Wilcoxon matched-pairs signed rank test. A 2-tailed Mann-Whitney test was used to assess differences in erythroid precursor PS between PIMA and healthy dogs. Statistical analyses were performed using statistical software (GraphPad Prism, GraphPad Software Inc, San Diego, California), and differences were considered to be significant at $P < .05$.

## 3 | RESULTS

### 3.1 | RBC IgG and PS in IMHA

Eleven IMHA and 9 non-IMHA dogs were assessed. All IMHA dogs had Hct < 33% (reference interval, 40%-55%). Of the non-IMHA dogs, 1 was not anemic, and the other 8 had regenerative (4 of 8; Hct low-high: 30%-35%) and nonregenerative (4 of 8; Hct low-high: 28%-39%) anemias. Flow cytometric results indicated that 9 of 11 IMHA and 0 of 9 non-IMHA dogs were positive for IgG (Figure 1). The median % positive events for IgG in IMHA and non-IMHA dogs were 46.8% (low-high: 2.6%-100%) and 4.4% (low-high: 2.2%-8.2%), respectively. One of the 2 IgG-negative IMHA dogs had been treated with prednisone for 4 days before blood was collected. No significant difference was found in RBC IgG for healthy dogs tested on the day of collection or the day after collection, so same-day results were used for group comparisons with sick animals (cutoff for RBC IgG positivity: 9.4% positive events). Dogs with IMHA had significantly higher RBC IgG results than healthy (same-day and next-day) and non-IMHA dogs, but no statistical difference was found between healthy and non-IMHA dogs.

Erythrocyte PS positivity was found in 10 of 11 IMHA dogs and in 2 of 9 non-IMHA dogs (Figure 2). The median % positive events for PS in IMHA and non-IMHA dogs were 1.9% (low-high: 0.8%-5.4%) and 0.6% (low-high: 0.2%-1.8%), respectively. One of the 2 PS-positive non-IMHA dogs had 0.9% positive events, no anemia, and a diagnosis of chronic renal disease, whereas the other had 1.8% positive events, mild nonregenerative anemia (Hct = 35%), and a diagnosis of immune-mediated neutropenia (IMN). No significant difference was found in RBC PS for healthy dogs tested on the day of collection or the day after, so same-day results were used for group comparisons with sick animals (cutoff for RBC PS positivity, 0.7% positive events). Phosphatidylserine results for dogs with IMHA were significantly different from those of healthy dogs, but PS results for non-IMHA dogs were not significantly different from those of either IMHA dogs or healthy dogs tested on the same day.

### 3.2 | IgG and PS on erythroid precursors and RBCs in PIMA

Bone marrow samples from 40 dogs were analyzed, and after review of case materials for each, 17 PIMA and 7 non-PIMA dogs were identified (Figure S3). Of the 16 remaining dogs, 1 was excluded because of high nonspecific binding of erythroid precursors, 1 had diagnostically inadequate bone marrow samples, 3 were excluded

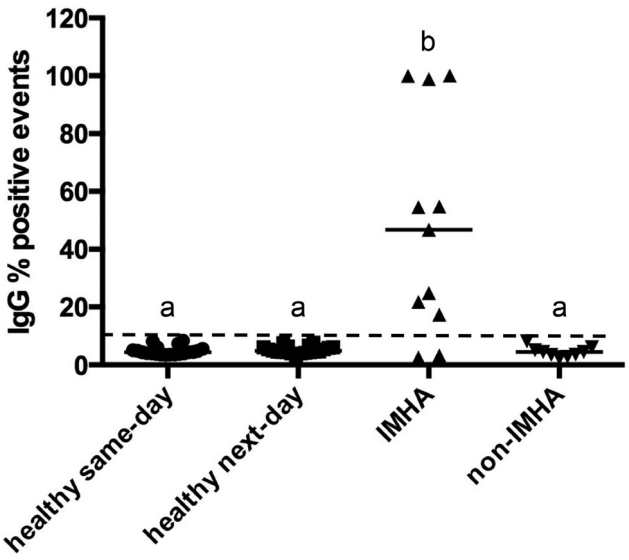


**FIGURE 1** Immunoglobulin G (IgG) positivity on RBCs from healthy dogs (n = 20) and dogs with IMHA (n = 11) or without IMHA (non-IMHA, n = 9). Red blood cells from healthy dogs were tested on the day of and 1 day following sample collection. Horizontal bars represent group medians and the dashed line represents the cutoff for IgG positivity based on healthy dogs tested on the day of collection. [a]No significant difference, and [b]Significantly different ($P < .05$). IMHA, immune-mediated hemolytic anemia; RBCs, red blood cells

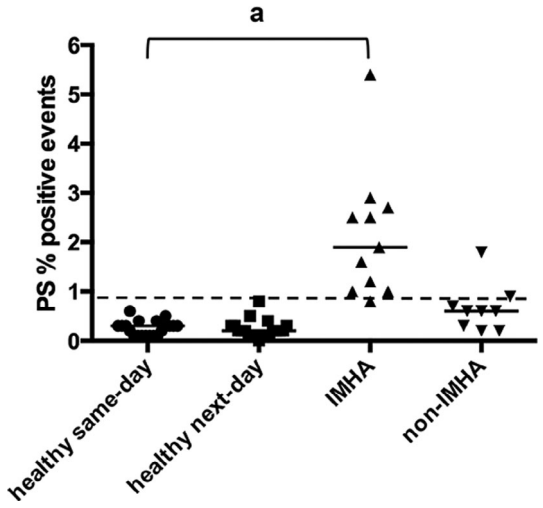


**FIGURE 2** Phosphatidylserine (PS) positivity on RBCs from healthy dogs (n = 20) and dogs with IMHA (n = 11) or without IMHA (non-IMHA, n = 9). Red blood cells from healthy dogs were tested on the day of and 1 day following sample collection, and no significant difference was found in PS positivity. Horizontal bars represent group medians and the dashed line represents the cutoff for PS positivity based on healthy dogs tested on the day of collection. [a]Significantly different ($P < .05$). IMHA, immune-mediated hemolytic anemia; PS, phosphatidylserine; RBCs, red blood cells

because of suspected cytopenias secondary to phenobarbital treatment,[20] the mechanism of which has not been identified, and 11 were classified as unclear cases because they did not clearly fulfill criteria for either PIMA or non-PIMA groups. Unclear cases consisted of dogs with moderate to severe anemia (Hct < 30%) and erythroid left shift, rubriphagocytosis, or both, which suggested a possible immune-mediated reaction against erythroid precursors, but PIMA criteria were not met for a variety of reasons. For some of these dogs, anemia was too mild (Hct ≥ 30%) or not persistent, or a clinical diagnosis of PIMA was not made by the attending clinician. For others, there was nonselective rather than selective phagocytosis that involved the erythroid lineage, or unexplained anemia and erythroid hypocellularity, both of which could have resulted from an immune-mediated component against erythroid precursors. Clinical diagnoses for unclear dogs were IMN (2 of 11), IMT (1 of 11), hemophagocytic syndrome secondary to ehrlichiosis (1 of 11), chronic large bowel diarrhea (1 of 11), and unknown (6 of 11). The dogs with unknown diagnoses had suspected immune-mediated cytopenias (n = 3), suspected hemophagocytic syndrome or other inflammatory disease (n = 2), or pancytopenia that resolved without treatment (n = 1).

All 35 dogs classified as PIMA, non-PIMA, or unclear were tested for IgG, and 10 of those were also tested for PS on erythroid precursors (6 PIMA, 2 non-PIMA, and 2 unclear). Clean gating of early erythroid precursors in the top Percoll fraction was problematic in certain clinical samples with a relatively large number of lymphocytes or early myeloid cells, so only the mid and bottom Percoll fractions were used to assess IgG and PS. Because of insufficient cells, not every fraction could be assessed for each dog. Of the 35 IgG-tested dogs, the bottom fraction was assessed in 34, and of the 10 PS tested dogs, the mid and bottom fractions were assessed in 9 and 8 dogs, respectively.

Cutoffs for nRBC IgG positivity based on 10 healthy dogs were 19.1% and 18.6% positive events in the mid and bottom fractions, respectively. No statistical differences were found among groups of dogs classified as healthy, PIMA, non-PIMA, or unclear regarding IgG positivity on nRBCs. However, when compared to fraction-specific cutoffs for healthy dogs, 5 of 17 PIMA dogs were positive for IgG, including 3 in the mid fraction (low-high: 25.7%-57.6% positive events) and 5 in the bottom fraction (low-high: 19%-59.5% positive events), whereas IgG positivity was not found in any of the 7 non-PIMA dogs (mid fraction low-high: 2.2%-14.7% positive events; bottom fraction 1.6%-17.6% positive events) (Figure 3). Two of the 12 IgG-negative PIMA dogs had been on immunosuppressive treatment for 8 (prednisone) to 24 (mycophenolate) months, and 2 had received prednisone in the 5 days before bone marrow collection. Additionally, 4 of 11 unclear cases were positive for IgG, including 3 in the mid fraction (low-high: 21.2%-33.2% positive events) and 3 in the bottom fraction (low-high: 26.8%-36.4% positive events). One of these dogs had pancytopenia and rubriphagocytosis with potential immune-mediated destruction of all cell lineages. Rubriphagocytosis was not detected in the other 3 dogs, 1 with pancytopenia suspected to be secondary to recent bone marrow injury, 1 with leptospirosis but evidence of secondary hemophagocytic syndrome, and 1 with unexplained nonregenerative anemia associated with IMT.

Cutoffs for nRBC PS positivity based on 5 healthy dogs were 24.8% and 49.8% positive events in the mid and bottom fractions, respectively. When comparing groups for PS on erythroid precursors, PIMA dogs (n = 5) were statistically different from healthy dogs (n = 5) for both the mid and bottom Percoll fractions, but there were too few (n = 2) non-PIMA dogs to compare statistically (Figure 4). When compared to fraction-specific cutoffs for healthy dogs, 5 of 6 tested PIMA dogs were positive, 4 in the mid fraction (low-high: 36.9%-77.8% positive events) and 3 in the bottom fraction (low-high: 64.6%-70.0% positive events). Neither of the 2 non-PIMA dogs were positive for PS; 1 of the 2 unclear dogs was positive for PS (33.7% positive events) in the mid fraction. This dog had pancytopenia suspected to be secondary to recent bone marrow injury.

Erythrocyte IgG was assessed in 14 of 17 PIMA dogs, 6 of 7 non-PIMA dogs, and in 9 dogs with unclear diagnoses; positivity was found for 5 of 14 PIMA (9.5%, 10.2%, 16.0%, 76.9%, and 99.4% positive events), 2 of 6 non-PIMA (9.9% and 23.1% positive events), and 2 of 9 unclear (11.0% and 23.8% positive events) dogs. Three of the 5 PIMA dogs with IgG positivity on RBCs were also IgG positive on nRBCs, and 1 of them was also Coombs' test positive. Phosphatidylserine was assessed on the RBCs of 7 PIMA, 2 non-PIMA, and 4 unclear dogs, and all of them yielded negative results, including 1 PIMA and 1 non-PIMA dog that each had IgG-positive RBCs (76.9% and 23.1% positive events, respectively).

Rubriphagocytosis was seen in all 17 PIMA dogs, which, combined with bone marrow findings,[10] allowed characterization of these dogs' conditions as early- (n = 2), mid- (n = 4), or late-stage PIMA (n = 11). Considering only the dogs with positivity for IgG, PS, or both (Table 1), 6 of the 7 PIMA dogs with rubriphagocytosis of late-stage nRBCs had positivity for IgG, PS, or both on nRBCs from the bottom Percoll fraction; the seventh dog did not have sufficient cells to allow for testing of that fraction. Additionally, both dogs with rubriphagocytosis of mid-stage nRBCs had positivity for PS on nRBCs from the mid Percoll fraction. No dogs with positivity for IgG, PS, or both on nRBCs from the mid and bottom Percoll fractions had rubriphagocytosis of early-stage nRBCs.

### 3.3 | Clinical and diagnostic findings in PIMA dogs

Nine PIMA dogs were spayed females, 7 were castrated males, and 1 was an intact male. Their ages ranged from 4 to 13 years old, with most dogs (n = 13) being middle-aged (5-10 years old). Dogs were of the following breeds: mixed (n = 5), dachshund (n = 3), miniature dachshund (n = 1), Labrador retriever (n = 2), Chihuahua (n = 1), German shepherd (n = 1), fox terrier (n = 1), Boston terrier (n = 1), Pembroke Welsh corgi (n = 1), and Havanese (n = 1). One dog had evidence of previous immunologic disease, having been diagnosed with PIMA 25 months before assessment for our study.

Evidence of concurrent RBC targeting was seen in 2 of 17 PIMA dogs: mild spherocytosis in 1 mid-stage PIMA dog, and Coombs' positivity at 20°C to 21°C (but not 37°C) in 1 dog with late-stage PIMA. Only 3 dogs were Coombs' tested because most lacked any evidence of hemolytic disease.

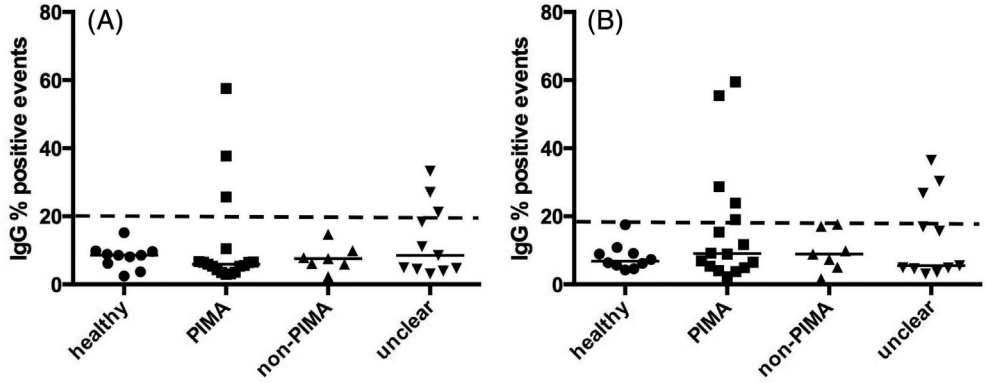


**FIGURE 3** Immunoglobulin G (IgG) positivity on erythroid precursors from the mid (A) and bottom (B) Percoll fractions of bone marrow from healthy dogs (n = 10) and dogs with PIMA (n = 17 for mid, n = 16 for bottom), without PIMA (non-PIMA, n = 7), or with unclear diagnoses (n = 11). Horizontal bars represent group medians and dashed lines represent cutoffs for IgG positivity based on healthy dogs. No statistically significant differences were detected, but increased values were present in some dogs with PIMA or unclear diagnoses that may have had an immunologic component. PIMA, precursor-targeted immune-mediated anemia

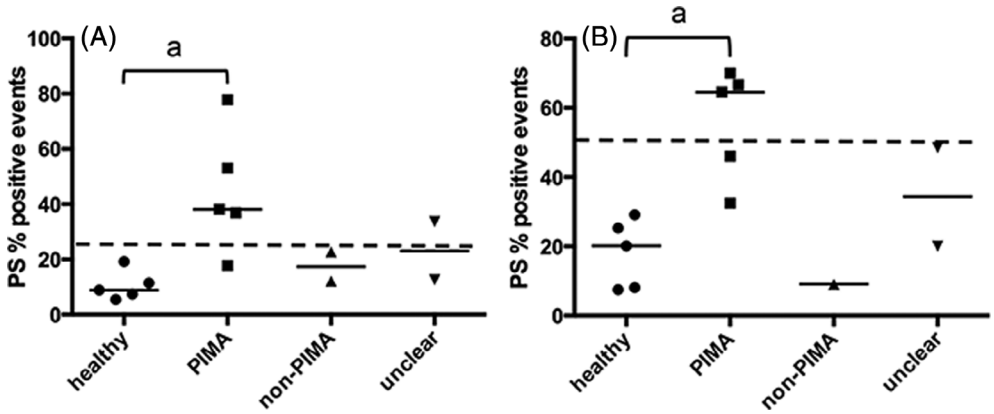


**FIGURE 4** Phosphatidylserine (PS) positivity on erythroid precursors from the mid (A) and bottom (B) Percoll fractions of bone marrow from healthy dogs (n = 5) and dogs with PIMA (n = 5 for mid and bottom), without PIMA (non-PIMA, n = 2 for mid, n = 1 for bottom), or with unclear diagnoses (n = 2). Horizontal bars represent group medians and dashed lines represent cutoffs for PS positivity based on healthy dogs. Dogs with PIMA had greater PS positivity than healthy dogs ($^{a}P < .05$), but dogs without PIMA were too few for statistical comparisons. PIMA, precursor-targeted immune-mediated anemia; PS, phosphatidylserine

Mild to severe collagen myelofibrosis was detected in core biopsy samples of 4 of 17 PIMA dogs. All 4 dogs with myelofibrosis were negative for IgG on nRBCs, and the only myelofibrosis dog tested for PS was positive in both fractions. None of the 12 non-PIMA or unclear dogs assessed had myelofibrosis, but 6 could not be assessed for myelofibrosis because bone marrow core biopsy samples were inadequate (n = 3) or not collected (n = 3).

All PIMA dogs were treated with immunosuppressive doses of prednisone, with or without other immunosuppressive drugs. Fourteen of 17 PIMA dogs achieved remission (n = 10) or responded (n = 4), with remission based on achieving a Hct > 35%, and response based on either an increase in reticulocyte concentration to above the upper reference limit (76 000/μL) or a Hct increment of at least 5 percentage points without transfusion. Three dogs did not respond to treatment; 1 died 90 days after diagnosis and 2 were euthanized 35 and 39 days after diagnosis because of clinical deterioration and suspected neoplasia in 1 of them. Of the 5 IgG-positive PIMA dogs, all responded and at least 4 achieved remission. Of the 5 dogs with increased PS, 4 of 5 responded, with at least 1 of the 4 achieving remission. The only PIMA dog that was positive for both IgG and PS achieved remission.

## 4 | DISCUSSION

Our findings suggest that IgG contributes to PIMA by binding to erythroid precursors in at least a subset of dogs, and that increased PS exposure on RBCs or erythroid precursors may contribute to erythroid cell destruction in dogs with IMHA or PIMA. No relationship between IgG and PS positivity was apparent for PIMA or IMHA dogs, suggesting that IgG and PS could be involved independently of each other in different subsets of affected dogs. Limited availability of bone

**TABLE 1** Relationship between the stage of rubriphagocytosis (RP) reported on bone marrow examination and the Percoll fractions of bone marrow with IgG and/or phosphatidylserine (PS) positivity in PIMA dogs

| Stage of RP | Fraction with IgG positivity | Fraction with PS positivity |
|---|---|---|
| Late | Mid, bottom | ND |
| Late | Bottom | ND |
| Late | Mid, bottom | ND |
| Late | Mid, bottom | Bottom[a] |
| Late | Bottom | None |
| Mid | None | Mid |
| Late | None | Mid, bottom |
| Late | None | Mid[b] |
| Mid | None | Mid and bottom |

Abbreviations: IgG, immunoglobulin G; ND, not done; PIMA, precursor-targeted immune-mediated anemia.
[a]Mid fraction not tested because of insufficient cells.
[b]Bottom fraction not tested because of insufficient cells.

marrow samples from healthy and non-PIMA dogs, especially for PS exposure on nRBCs, was a limitation of our study, warranting further studies using larger populations.

Several findings support the hypothesis that IgG may play a role in the pathogenesis of PIMA in at least some dogs. All non-PIMA dogs were negative for nRBC IgG, all 5 IgG-positive dogs appeared to respond to immunosuppressive treatment, with 4 of them achieving remission, and 3 of the 4 IgG-positive dogs that were tested were also positive for RBC IgG. These findings align with the previous demonstration of a circulating inhibitor of erythropoiesis in the IgG-containing plasma fraction of some dogs with nonregenerative anemia associated with pure red cell aplasia,[21] particularly considering that, in our experience, some dogs interpreted as having pure red cell aplasia have expanded rubriblasts and rubriblast phagocytosis supportive of early-stage PIMA. Involvement of IgG has also been documented in a few human patients with reticulocytopenic immune-mediated anemia, either by detecting plasma or RBC-eluate IgG that was reactive with RBCs and erythroid precursors,[22,23] or by detecting surface-bound IgG on erythroid precursors using flow cytometry.[15]

Although only 2 early-stage PIMA dogs were tested and IgG results were not reported for the top Percoll fractions, the stage of erythroid precursor with IgG positivity matched the stage of rubriphagocytosis in all IgG-positive dogs (all late-stage PIMA), with additional positivity of the mid Percoll fraction in 3 of 5 dogs. Positivity in both fractions could have been caused by targeting of an epitope common to both mid and later erythroid precursors or by imperfect separation of erythroid stages among Percoll fractions. Additionally, the stage of PS positivity also matched the stage of erythroid precursor attack in PS-positive dogs, although the bottom fraction was not tested (insufficient cells) in 1 late-stage PIMA dog that was PS positive in the mid fraction.

Reticulocytes may also be attacked by the immune system as part of the spectrum of immune-mediated anemias in dogs. Although our flow cytometric assay was able to detect reticulocytes with confidence,[16] assessment of these cells for IgG and PS positivity was not possible for PIMA dogs because of the lack of reticulocytosis and therefore a paucity of reticulocytes in the marrow fractions of these dogs.

Negative results for IgG on nRBCs of 12 PIMA dogs may have occurred for several reasons. Four PIMA dogs with IgG-negative nRBCs had received immunosuppressive treatment, which could have resulted in decreased IgG positivity if treatment was effective. As in IMHA, immunoglobulin M (IgM), complement protein 3 (C3), or immunoglobulin A (IgA) rather than IgG may have mediated PIMA in some dogs,[24-27] and negative IgG results may also have occurred in some PIMA dogs because of inadequate assay sensitivity for low amounts of IgG. Additionally, other mediators of phagocytosis or cell-mediated immunity may have been involved.

Erythroid precursor IgG positivity in the 4 dogs with unclear diagnoses is difficult to interpret, but these dogs were included to reflect the reality that definitive diagnoses are not always achievable and to consider how such an assay might provide support for an immunologic mechanism in otherwise unclear cases. Several of these dogs could have had an immunologic component to their anemias. Phagocytosis of multiple cell lines was present in the bone marrow of 2 of these dogs, and it may have reflected immune-mediated disease, although nonselective phagocytosis may occur by nonimmunologic mechanisms. A third dog had concurrent IMT and may have had additional immunologic disease directed at erythroid precursors.

In agreement with previous findings,[10] PIMA dogs in our study had rare evidence of RBC targeting (eg, spherocytosis, Coombs' test positivity) based on routine testing, but 5 of 14 tested PIMA dogs were positive for RBC IgG by flow cytometry. This finding indirectly supports IgG-mediated targeting of erythroid precursors as well and raises the question of what erythroid epitopes are being attacked in PIMA. Although it is tempting to suspect antibodies targeting a single epitope shared by RBCs and precursors, potentially via the same antibodies,[23] distinctly different concurrent antibodies to RBCs and erythroid precursors have been documented in a human patient.[22]

The increase in erythroid precursor PS, but not RBC PS, in PIMA dogs compared to healthy dogs suggests that PS may contribute to rubriphagocytosis in at least some dogs. However, rubriphagocytosis in PIMA dogs is characterized by morphologically intact cells within macrophages, and this finding contrasts with phagocytosis of apoptotic-appearing cells as is seen, for example, in the bone marrow of some dogs with myelodysplastic syndrome. Interestingly, in the only such report found, 1 person with reticulocytopenic IMHA and increased erythroid precursor IgG and PS had no morphologic abnormalities of erythroid precursors by light microscopic examination.[15] These findings may in part be explained by reports that PS exposure occurred before morphologic changes on cytologic preparations and before detection of cell death using propidium iodide,[28,29] or by the triggering of PS exposure on cell membranes by nonapoptotic mechanisms.[30] Externalized PS by itself can induce phagocytosis of cells in early apoptosis,[29] supporting the idea that

PS exposure could have mediated rubriphagocytosis of intact-appearing erythroid precursors in PIMA. However, it is unclear what might cause increased PS exposure in PIMA dogs.

The cutoff for PS positivity on nRBCs from bottom Percoll fractions of healthy colony dogs was greater than the other cutoffs in our study (Figure 4). This difference aligns with a previous report of physiologically large amounts of PS on the surface of metarubricytes because of cellular changes that precede nuclear extrusion.[31] The same study also documented that PS concentrates on the cell membrane surrounding protruded nuclei during the final steps of nuclear extrusion and suggested that PS serves as a signal for phagocytosis of extruded nuclei by macrophages.

Increased RBC PS in IMHA dogs supports a potential role for PS and possibly eryptosis in IMHA. Eryptosis has been associated with anemias from a variety of causes including oxidative stress,[14] and evidence of increased oxidative stress and active apoptotic pathways have been reported in some dogs with IMHA.[11,12,14] Alternatively, increased oxidative injury of RBC membranes and PS exposure may be secondary to free iron from hemolysis.[11,32]

Although oxidative stress has not been determined to play an important role in human patients with IMHA,[33] it was shown to exacerbate IMHA in mice that have decreased antioxidant capacity,[34] and IgG binding to RBCs with concurrent oxidative stress or PS exposure has been documented.[13,34,35] Whether increased oxidative stress and PS exposure trigger immunoglobulin binding to RBCs[13] and an autoimmune reaction[34] or, conversely, immunoglobulin binding occurs first and promotes PS exposure on RBCs is not clear.[35] In our study, increased PS positivity of RBCs from 4 IgG-negative dogs, 2 each in the IMHA and non-IMHA groups, suggests that PS exposure can occur independently of IgG binding, although IgG coating may have been below the detection limit of the method, and a different class of immunoglobulin may have been present in the 2 IMHA dogs. Furthermore, 1 IMHA dog that was strongly positive for IgG (100% positive events) was PS negative, and 2 bone marrow-tested dogs had positive IgG but negative PS results on RBCs, suggesting that IgG binding does not necessarily cause PS exposure. An in vitro experiment to assess the possibility that IgG binding causes PS exposure showed that coating RBCs with IgG by DEA1 typing serum did not promote PS exposure or nonspecific binding of annexin V-FLUOS (data not shown).

Increased RBC PS can occur for multiple reasons, so it is not surprising that RBC PS was increased in 2 non-IMHA dogs, 1 nonanemic dog with chronic renal failure and 1 dog with IMN, mild anemia, and mild polychromasia. It is possible that PS exposure accelerated erythrocyte destruction in these dogs. Chronic renal disease has been associated with increased oxidative stress in dogs,[36] as well as with increased oxidative stress and PS exposure in the outer membrane of human RBCs.[14,37] No clear association has been identified between IMN and eryptosis.

Increased RBC PS exposure is 1 of the main mechanisms of thromboembolism in people with thalassemia because exposed PS activates coagulation and promotes a hypercoagulable state.[38] A hypercoagulable state and increased risk of thromboembolism contributing to mortality has been demonstrated in dogs with IMHA[39,40] and in dogs with PIMA.[9] The role of PS exposure in the hypercoagulable state of dogs with immune-mediated anemias is unknown and further studies to clarify this relationship are warranted.

In conclusion, data generated using a novel assay for IgG on canine marrow erythroid precursors suggest that IgG may mediate anemia in at least a subset of PIMA dogs, and increased PS exposure may play a role in IMHA and PIMA in dogs. Phosphatidylserine and IgG findings did not completely parallel each other, so multiple mechanisms may be involved. Additional studies are needed to further assess the pathogeneses of immune-mediated anemias so that patient management can be optimized.

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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

This study was performed in compliance with the MSU IACUC and Charles River Laboratories (former MPI Research) IACUC.

## HUMAN ETHICS APPROVAL DECLARATION

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

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## REFERENCES

1. Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. *J Am Anim Hosp Assoc*. 1987;23:201-204.
2. Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. *J Am Anim Hosp Assoc*. 1980;16:927-931.
3. Weiss DJ, Stockham SL, Willard MD, et al. Transient erythroid hypoplasia in the dog: report of five cases. *J Am Anim Hosp Assoc*. 1982;18:353-359.
4. Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. *J Am Vet Med Assoc*. 1990;197:1045-1048.
5. Scott-Moncrieff JCR, Reagan WJ, Glickman LT, DeNicola D, Harrington D. Treatment of nonregenerative anemia with human γ-globulin in dogs. *J Am Vet Med Assoc*. 1995;206:1895-1900.
6. Stokol T, Blue JT, French TW. Idiopathic pure red cell aplasia and nonregenerative immune-mediated anemia in dogs: 43 cases (1988-1999). *J Am Vet Med Assoc*. 2000;216:1429-1436.

7. Weiss DJ. Bone marrow pathology in dogs and cats with non-regenerative immune-mediated haemolytic anaemia and pure red cell aplasia. *J Comp Pathol*. 2008;138:46-53.
8. McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune-mediated anemia: 44 cases (1996–2000) [abstract]. *Vet Pathol*. 2000;37:526.
9. Assenmacher TD, Jutkowitz LA, Koenigshof AM, de A Lucidi C, Scott MA. Clinical features of precursor-targeted immune-mediated anemia in dogs: 66 cases (2004-2013). *J Am Vet Med Assoc*. 2019;255:366-376.
10. Lucidi CA, de Rezende CLE, Jutkowitz LA, et al. Histologic and cytologic bone marrow findings in dogs with suspected precursor-targeted immune-mediated anemia and associated phagocytosis of erythroid precursors. *Vet Clin Pathol*. 2017;46:401-415.
11. Pesillo SA, Freeman LM, Rush JE. Assessment of lipid peroxidation and serum vitamin E concentration in dogs with immune-mediated hemolytic anemia. *Am J Vet Res*. 2004;65:1621-1624.
12. Tan E, Bienzle D, Shewen P, Kruth S, Wood D. Potentially antigenic RBC membrane proteins in dogs with primary immune-mediated hemolytic anemia. *Vet Clin Pathol*. 2012;41:45-55.
13. Lee JM, Chan K, Kan YW, Johnson JA. Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. *Proc Natl Acad Sci U S A*. 2004;101:9751-9756.
14. Lang E, Lang F. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. *Semin Cell Dev Biol*. 2015;39:35-42.
15. van de Loosdrecht AA, Hendriks DW, Blom NR, Smit JW, de Wolf JT, Vellenga E. Excessive apoptosis of bone marrow erythroblasts in a patient with autoimmune haemolytic anaemia with reticulocytopenia. *Br J Haematol*. 2000;108:313-315.
16. Lucidi CA, Gerlach JA, Forney S, Jutkowitz LA, Scott MA. Analytic characterization of flow cytometric assays for detection of immunoglobulin G on canine erythroid cells, including detection of dog erythrocyte antigen 1 on erythroid precursors. *Am J Vet Res*. 2018;79:1123-1132.
17. Tait JF, Gibson D. Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. *Arch Biochem Biophys*. 1992;298:187-191.
18. Sluyter R, Shemon AN, Hughes WE, et al. Canine erythrocytes express the P2X7 receptor: greatly increased function compared with human erythrocytes. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R2090-R2098.
19. Saad A, Palm M, Widell S, Reiland S. Differential analysis of rat bone marrow by flow cytometry. *Comp Haematol Int*. 2000;10:97-101.
20. Bersan E, Volk HA, Ros C, De Risio L. Phenobarbitone-induced haematological abnormalities in idiopathic epileptic dogs: prevalence, risk factors, clinical presentation and outcome. *Vet Rec*. 2014;175:247.
21. Weiss DJ. Antibody-mediated suppression of erythropoiesis in dogs with red blood cell aplasia. *Am J Vet Res*. 1986;47:2646-2648.
22. Mangan KF, Besa EC, Shadduck RK, Tedrow H, Ray PK. Demonstration of two distinct antibodies in autoimmune hemolytic anemia with reticulocytopenia and red cell aplasia. *Exp Hematol*. 1984;12:788-793.
23. Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. *Am J Med*. 1978;65:342-345.
24. Piek CJ, Teske E, van Leeuwen MW, Day MJ. Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia. *Acta Vet Scand*. 2012;54:10.
25. Wilkerson MJ, Davis E, Shuman W, Harkin K, Cox J, Rush B. Isotype-specific antibodies in horses and dogs with immune-mediated hemolytic anemia. *J Vet Intern Med*. 2000;14:190-196.
26. Slappendel RJ. The diagnostic significance of the direct antiglobulin test (DAT) in anemic dogs. *Vet Immunol Immunopathol*. 1979;1:49-59.
27. Day MJ. Serial monitoring of clinical, haematological and immunological parameters in canine autoimmune haemolytic anaemia. *J Small Anim Pract*. 1996;37:523-534.
28. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*. 1995;182:1545-1556.
29. Shiratsuchi A, Osada S, Kanazawa S, Nakanishi Y. Essential role of phosphatidylserine externalization in apoptosing cell phagocytosis by macrophages. *Biochem Biophys Res Commun*. 1998;246:549-555.
30. Takatsu H, Takayama M, Naito T, et al. Phospholipid flippase ATP11C is endocytosed and downregulated following $Ca^{2+}$-mediated protein kinase C activation. *Nat Commun*. 2017;8:1423.
31. Yokoyama T, Etoh T, Udayanga KG, et al. Immunohistochemical detection of phosphatidylserine and thrombospondin on denucleating erythroblasts in rat bone marrow. *J Vet Med Sci*. 2011;73:949-952.
32. Musallam KM, Taher AT, Karimi M, Rachmilewitz EA. Cerebral infarction in β-thalassemia intermedia: breaking the silence. *Thromb Res*. 2012;130:695-702.
33. Garratty G. The James Blundell Award Lecture 2007: do we really understand immune red cell destruction? *Transfus Med*. 2008;18:321-334.
34. Konno T, Otsuki N, Kurahashi T, et al. Reactive oxygen species exacerbate autoimmune hemolytic anemia in New Zealand Black mice. *Free Radic Biol Med*. 2013;65:1378-1384.
35. Attanasio P, Shumilina E, Hermle T, et al. Stimulation of eryptosis by anti-A IgG antibodies. *Cell Physiol Biochem*. 2007;20:591-600.
36. Buranakarl C, Trisiriroj M, Pondeenana S, Tungjitpeanpong T, Jarutakanon P, Penchome R. Relationships between oxidative stress markers and red blood cell characteristics in renal azotemic dogs. *Res Vet Sci*. 2009;86:309-313.
37. Bonomini M, Sirolli V, Settefrati N, et al. Increased erythrocyte phosphatidylserine exposure in chronic renal failure. *J Am Soc Nephrol*. 1999;10:1982-1990.
38. Sirachainan N. Thalassemia and the hypercoagulable state. *Thromb Res*. 2013;132:637-641.
39. Carr AP, Panciera DL, Kidd L. Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. *J Vet Intern Med*. 2002;16:504-509.
40. Kidd L, Mackman N. Prothrombotic mechanisms and anticoagulant therapy in dogs with immune-mediated hemolytic anemia. *J Vet Emerg Crit Care*. 2013;23:3-13.


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