

## STANDARD ARTICLE

# Platelet number and function in response to a single intravenous dose of vincristine

Erin C. Allen<sup>1</sup> | Jaime L. Tarigo<sup>2</sup> | Dana N. LeVine<sup>3</sup> | Jamie P. Barber<sup>4</sup> | Benjamin M. Brainard<sup>1</sup> 

<sup>1</sup>Department of Small Animal Medicine and Surgery, University of Georgia College of Veterinary Medicine, 2200 College Station Road, Athens, Georgia 30602

<sup>2</sup>Department of Pathology, University of Georgia College of Veterinary Medicine, 2200 College Station Road, Athens, Georgia 30602

<sup>3</sup>Department of Veterinary Clinical Sciences, Iowa State University College of Veterinary Medicine, 1809 S. Riverside Drive, Ames, Iowa 50011

<sup>4</sup>Department of Infectious Diseases, University of Georgia College of Veterinary Medicine, 501 DW Brooks Drive, Athens, Georgia 30602

## Correspondence

Benjamin M. Brainard, Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, University of Georgia, 2200 College Station Road, Athens, GA 30602, USA.

Email: brainard@uga.edu

## Funding information

Veterinary Emergency and Critical Care Society Foundation

## Abstract

**Background:** Vincristine might increase circulating platelet numbers but the functional capacity of these newly released platelets is unknown.

**Objective:** To evaluate and compare the functionality of mature and immature (reticulated) platelets after a single intravenous dose of vincristine in dogs.

**Animals:** Ten healthy purpose-bred dogs.

**Methods:** Dogs prospectively received a single IV injection of 0.02 mg/kg vincristine or 0.9% saline. Before and after treatment on days 3, 5, and 7, platelets (resting and after thrombin stimulation) were assessed by flow cytometric determination of P-selectin (CD62P) expression. Reticulated platelets were distinguished using thiazole orange (TO) staining.

**Results:** Relative to saline, vincristine administration increased platelet count from day 0 to day 7 ( $225 \pm 58$  to  $273 \pm 65 \times 10^3/\mu\text{L}$ , vs  $299 \pm 76.4$  to  $214 \pm 20 \times 10^3/\mu\text{L}$ ,  $P = .01$ ) and increased percentage of reticulated platelets from day 0 to day 5 ( $3.9 \pm 1.5\%$  to  $6.1 \pm 1.6\%$ ,  $P = .02$ ). On all days, reticulated platelets had greater resting expression of CD62P than did mature platelets ( $49.6 \pm 4\%$  vs  $10.2 \pm 1\%$ ,  $P \leq .001$ ). Across all days, CD62P expression by reticulated platelets in the vincristine and saline-treated groups was not different when unstimulated ( $P = .7$ ) or after thrombin stimulation ( $P = .33$ ).

**Conclusions and Clinical Importance:** Reticulated platelets released in response to vincristine administration function similarly to mature platelets.

## KEYWORDS

immune-mediated, reticulated platelet, thrombocytopenia, thrombocytopenia

**Abbreviations:** ADP, adenosine diphosphate; hIVIG, human intravenous immunoglobulin; ITP, immune-mediated thrombocytopenia; PRP, platelet-rich plasma; TO, thiazole orange.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2021 The Authors. *Journal of Veterinary Internal Medicine* published by Wiley Periodicals LLC on behalf of American College of Veterinary Internal Medicine.

## 1 | INTRODUCTION

Immune thrombocytopenia (ITP), a common cause of severe thrombocytopenia in dogs,<sup>1</sup> is an immune-mediated disorder resulting in increased platelet destruction and likely decreased platelet production due to antibody and cell-mediated destruction of platelets and

megakaryocytes.<sup>2-5</sup> The case fatality rate associated with ITP in dogs ranges from 10% to 30% and death can be a sequela of thrombocytopenic bleeding.<sup>6,7</sup> The immediate therapeutic goal for ITP is a rapid increase in platelet count to >30 000 to 50 000/ $\mu$ L, as platelet counts above this range are rarely associated with spontaneous bleeding.<sup>7</sup> The first-line treatment of choice for ITP in both dogs and people is immunosuppression with corticosteroids,<sup>6,8</sup> and adjunctive therapies can include additional immunomodulatory agents such as human intravenous immunoglobulin (hIVIg) or vincristine.

Vincristine is a vinca alkaloid that results in an increase in circulating platelet count through several hypothesized mechanisms including stimulation of thrombopoiesis, acceleration of fragmentation of megakaryocytes, impairment of the phagocytosis of platelets by macrophages, and interference with antiplatelet antibody formation and binding.<sup>7,9-12</sup> Adverse effects are uncommon but can include vomiting, diarrhea, peripheral neuropathy, and neutropenia.<sup>7,8</sup> In dogs with ITP, vincristine is most commonly administered as a single IV injection of 0.02 mg/kg, which is 1/10th of the vincristine dose commonly used in cancer treatment.

Vincristine administration increases circulating platelet count in healthy and sick animals.<sup>7,9,13</sup> A single IV dose of vincristine in conjunction with prednisone therapy resulted in a more rapid increase in platelet count to >40 000/ $\mu$ L and a shorter duration of hospitalization relative to dogs receiving prednisone alone.<sup>7</sup> Additionally, a prospective study comparing the use of prednisone and either vincristine or hIVIg in dogs with ITP revealed that both adjunctive therapies resulted in similar blood transfusion requirements, hospitalization times, and survival.<sup>9</sup>

Despite evidence that the use of vincristine increases the number of circulating platelets, it is unclear if these newly released platelets are fully functional. One study found decreased platelet aggregation in dogs with lymphoma after a single dose of vincristine,<sup>14</sup> whereas a prospective study in clinically healthy dogs did not find any change in platelet aggregation after administration of a single dose of vincristine.<sup>13</sup>

There are few prospective studies evaluating the function of platelets in dogs with ITP, as the majority of function assays (eg, aggregometry, platelet function analysis) require platelet counts >100 000/ $\mu$ L. Flow cytometry, however, can rapidly analyze large numbers of cells, even in dogs with cytopenias. Activation responses of platelets can be assessed with flow cytometry by identifying surface expression of P-selectin (CD62P),<sup>15-17</sup> a marker of alpha granule release. Concomitant staining with thiazole orange (TO) can also differentiate between immature (reticulated) platelets and mature platelets<sup>18</sup> and thus enables the assessment of both mature and immature platelet function in response to vincristine administration.

The specific aims of this study were to evaluate and compare circulating count and functional capacity of reticulated and mature platelets after administration of a single IV dose of vincristine. Flow cytometry was used to determine resting and thrombin-activated platelet P-selectin expression. Our hypotheses were that a single IV dose of vincristine would result in a significant increase in platelet count relative to a similar IV dose of 0.9% saline (control), and that platelet function would not be significantly different in the vincristine-induced newly released reticulated platelets relative to mature platelets.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and study design

Ten healthy purpose-bred research dogs housed in accordance with regulations of the Institutional Animal Care and Use Committee (IACUC) were studied. A complete blood count (Advia 2120i, Bayer, Missouri) and platelet function analysis using an ADP-collagen cartridge in an automated platelet function analyzer (PFA-100, Siemens Healthcare Diagnostics, Tarrytown, New York) were performed as a screening tool to ensure adequate platelet numbers and function before entrance into the study. Minimum platelet count and maximal platelet closure time for enrollment were  $160 \times 10^3/\mu$ L and 90 seconds, respectively. Based on a sample size calculation derived from pilot experiments, 8 dogs received IV 0.02 mg/mg vincristine (Hospira, Lake Forest, Illinois) (vincristine group) and 2 dogs received a similar volume of 0.9% saline (Hospira, Lake Forest, Illinois) (control group). The vincristine or saline was administered via a newly placed IV catheter, which was removed within 1 hour of use. Before and after treatment, CBC's and flow cytometry were performed to assess platelet number and function, as described below.

Whole blood (15.5 mL) was collected via jugular venipuncture with a 19 gauge needle at approximately the same time on day 0 (day of treatment administration but before the treatment was administered) and days 3, 5, and 7 after saline or vincristine administration. This blood was anticoagulated using EDTA (for CBC analysis) or acid citrate dextrose (ACD-A) (in a ratio of 1 : 9 citrate to blood, for aggregometry and flow cytometric analyses). Platelet count was performed via automated CBC with manual verification of platelet count. All analyses were performed within 4 hours of blood collection.

### 2.2 | Platelet-rich plasma preparation

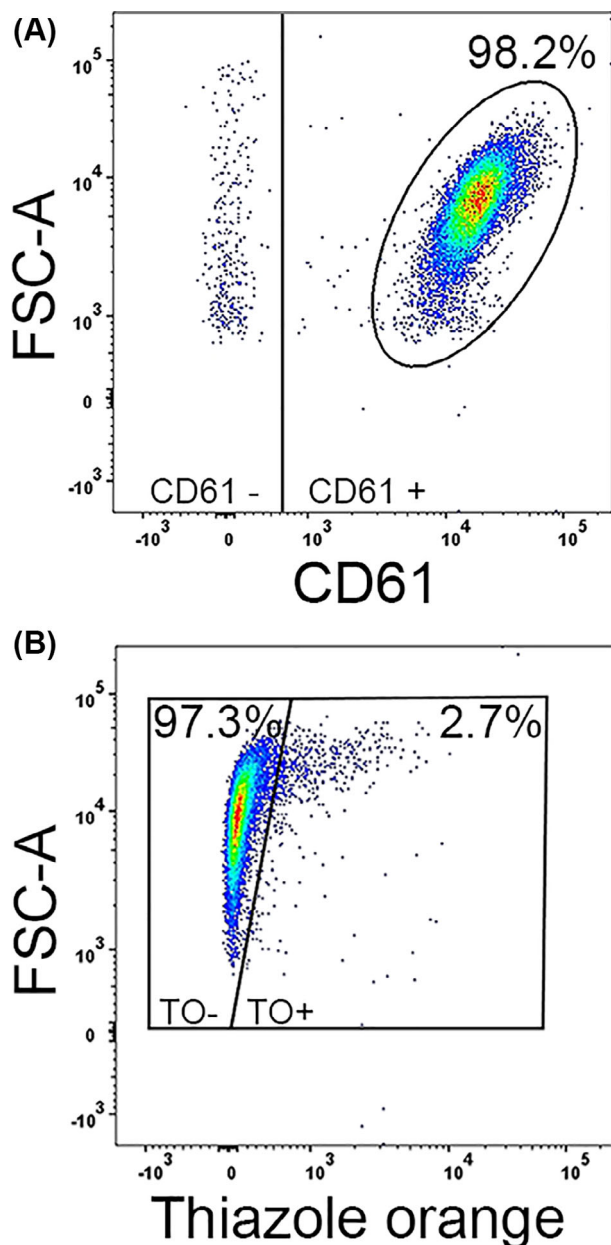
For the preparation of platelet-rich plasma (PRP), 10 mL of ACD-A-anticoagulated whole blood was transferred into a 15 mL conical tube. PRP was prepared via centrifugation at 275g for 5 minutes at 24°C with no brake applied, after which the supernatant (PRP) was removed. This process was repeated a second time to allow for collection of additional PRP. The platelet count of the final PRP product was between 250 000 and 300 000/ $\mu$ L.

### 2.3 | Flow cytometry

Platelet activation state was determined by binding of a monoclonal antibody against P-selectin, mouse anti-human CD62P-PE (clone AC1.2, BD Biosciences, San Jose, California) to detect membrane-expressed P-selectin reflecting alpha granule release. Platelet activation state was assessed in both resting and in vitro activated platelets. An irrelevant isotype-matched antibody mouse IgG1-PE (clone MOPC-2, BD Pharmingen, San Jose, California) was used to demonstrate positive and specific staining for CD62P. A monoclonal antibody against

glycoprotein IIIa, mouse anti-human CD61-Alexa Fluor 647 (clone F11, Bio-Rad, Hercules, California), was used as a platelet identifier. The commercially available antibody clones for CD61 and CD62P have been used in previous flow cytometry studies to identify canine platelet antigens.<sup>15,17,19</sup> Reticulated platelets were identified using TO binding (UltraPure Grade AS, AnaSpec, Fremont, California) coupled with forward scatter gating (Figure 1). Titration studies were performed to determine optimal concentrations for all antibodies and TO.

Platelet-rich plasma from each dog was added to platelet flow buffer (137 mM NaCl, 4 mM KCl, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM MgCl-

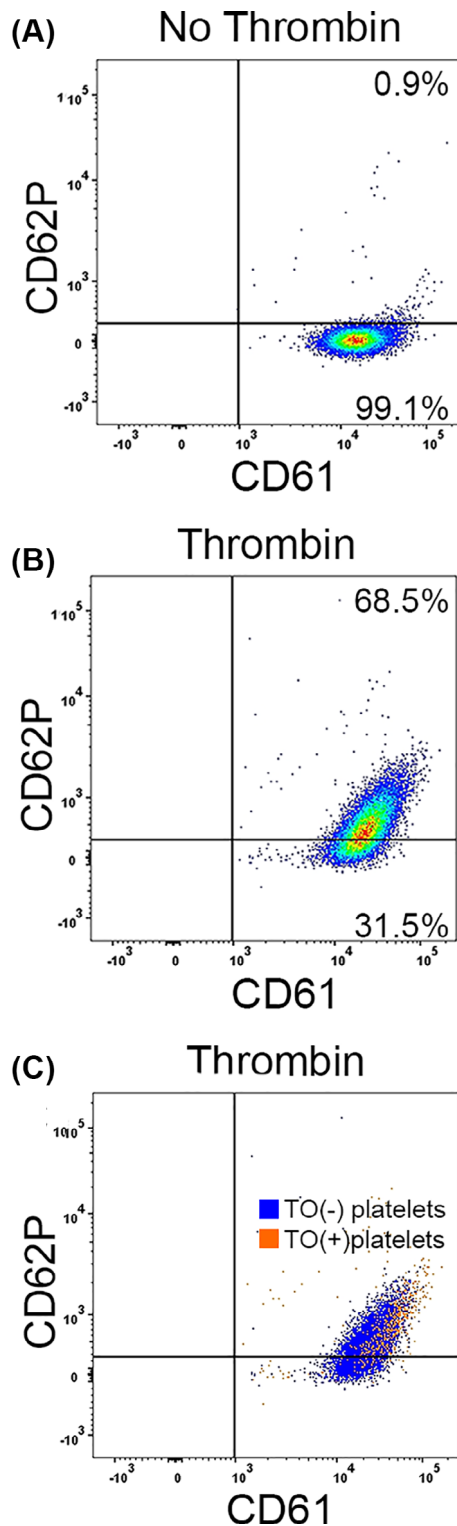


**FIGURE 1** Flow cytometric evaluation of reticulated platelets in canine platelet-rich plasma (PRP). Platelets were identified based on diffracted light scatter and CD61 positivity (indicated as the circled population in A) and then analyzed for incorporation of thiazole orange to identify reticulated platelets (B)

6H<sub>2</sub>O, 0.1% glucose, 0.2% bovine serum albumin, 10 mM HEPES) to achieve a final concentration of 5000 platelets/ $\mu$ L. Buffer contained 20 mM Gly-Pro-Arg-Pro amide (Sigma Aldrich, St. Louis, Missouri) to prevent platelet aggregation. For platelet activation, bovine thrombin (Sigma Aldrich, St. Louis, Missouri) diluted in HEPES buffered saline (10 mM HEPES, 150 mM NaCl) at a final concentration of 1 U/mL, or an equal volume of HEPES buffered saline as a control, were added and PRP was incubated for 15 minutes at room temperature (24°C). After this incubation, primary antibodies were added, and samples were incubated in the dark for 20 minutes at 24°C. Antibody incubations included CD61-A647 (0.45  $\mu$ g/mL) and CD62P-PE (0.55  $\mu$ g/mL). Mouse IgG1-PE isotype control (0.55  $\mu$ g/mL) was included as a negative control for CD62P. After antibody labeling, samples were incubated in the dark with TO (0.35  $\mu$ g/mL) for 30 minutes at 24°C and fixed immediately afterward in 1% ultra-pure formaldehyde (Polysciences Inc, Eppelheim, Germany) for 15 minutes at 24°C in the dark followed by the addition of 150  $\mu$ L of platelet flow buffer. Flow cytometric data including 20 000 putative single platelets per sample were acquired on an LSRII flow cytometer using FACSDiva v.6.1 software (BD Biosciences, San Jose, California) within 1 hour of fixation for all samples at all time points. A digital compensation matrix including all fluorochromes was established and applied during data acquisition to compensate for spectral overlap. For identification of platelets using anti-CD61-A647 and forward (diffracted) and side (refracted) light scatter, an appropriate threshold and gate were set to acquire the maximum number of platelets while minimizing noise and debris. Data were analyzed using commercial software (FlowJo software v.10.3, BD Biosciences, San Jose, California). Mature and immature/reticulated canine platelets were identified using a previously established gating strategy including both forward scatter and TO staining (Figure 1).<sup>20-22</sup> This gating strategy was applied to correct for the increased size of reticulated platelets relative to mature platelets affecting their fluorescence. A threshold of positivity for CD62P staining was defined based on the fluorescence of the isotype-PE-labeled platelets (<1%) as previously described.<sup>15</sup> This threshold was confirmed as biologically relevant when applied to the no thrombin control sample data. A sample of gating is provided in Figure 2 for resting and activated platelets from a typical dog.

## 2.4 | Platelet aggregometry

For all dogs receiving vincristine, optical platelet aggregometry was performed on days 0, 3, 5, and 7 (Model 700, Chrono-Log Corporation, Havertown, Pennsylvania). Aliquots of PRP were maintained at 37°C and maintained in suspension by a siliconized stir bar (1200 rpm). Once baseline light transmission was established, aggregation was induced with either 10  $\mu$ M ADP (Chrono-Log Corporation, Havertown, Pennsylvania) or 20  $\mu$ g/mL collagen (Chrono-Log Corporation, Havertown, Pennsylvania) and the increase in light transmission compared to autologous platelet-poor plasma was recorded for 7 minutes. Percent aggregation and



**FIGURE 2** Flow cytometric analysis for detection of activated platelets in canine platelet-rich plasma (PRP) after activation with thrombin. CD62P expression on all unactivated (A), all activated (B) and on activated thiazole orange positive (TO+) reticulated and thiazole orange negative (TO-) nonreticulated platelets (C)

the slope of the aggregation curve were calculated and recorded by a dedicated computer program (Aggrolink, Chrono-Log Corporation, Havertown, Pennsylvania).

## 2.5 | Statistical analysis

Statistical analysis was performed using a commercially available software (SigmaStat, version 3.5). The statistical difference between treatment and time for parameters such as TO+ platelet number and percent antibody binding for both TO+ and TO- platelets was assessed using a 2-way ANOVA. The average difference between the activation in the TO+ group vs the TO- platelets was compared using *t* tests or Wilcoxon signed rank test, as appropriate for the data distribution. Aggregometry values were compared between days using a 1-way ANOVA. A *P*-value of  $<.05$  was considered significant, and corrections for multiple comparisons using a Bonferroni correction were employed where relevant. Power analysis focused on an increase in platelet numbers after vincristine administration was performed, targeting an increase of  $150 \times 10^3$  in the vincristine-treated group (based on pilot data). With 8 vincristine-treated dogs, and 2 saline-treated dogs, accepting an alpha of .05, the calculated power was 0.815.

## 3 | RESULTS

### 3.1 | Animals

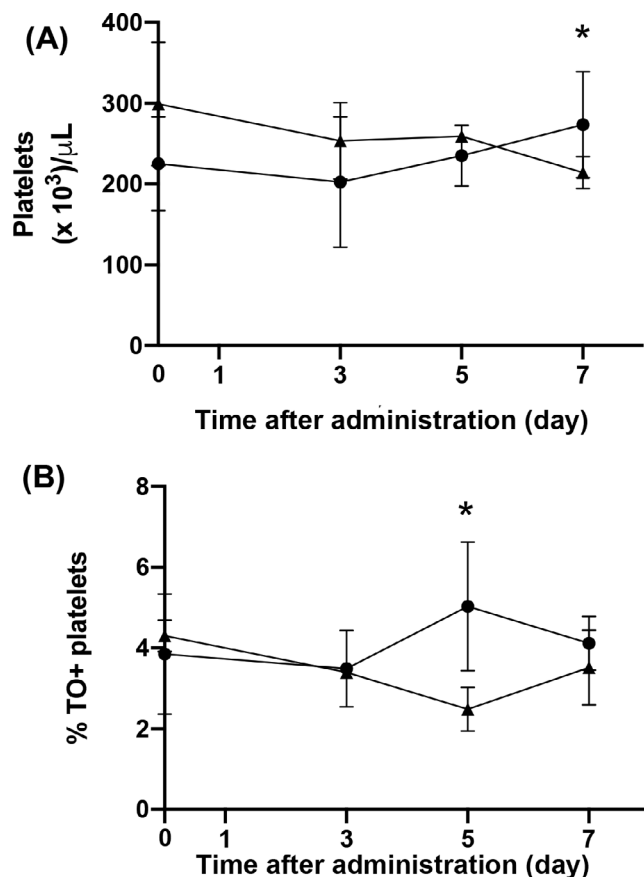
Ten healthy purpose-bred adult research dogs were prospectively enrolled in the study. All dogs were housed in the same AAALAC-approved facility and fed a balanced diet, and no dogs were receiving any medications. Breeds included 6 Beagle dogs and 4 mixed-breed hounds. All dogs were between 1 and 2 years of age and mean body weight was  $16.66 \pm 8.02$  kg. Body weight was not significantly different between treatment groups ( $P = .44$ ).

Mean platelet count at enrollment (day 0) was  $225 \pm 58 \times 10^3/\mu\text{L}$  for dogs in the vincristine group, and was  $299 \pm 76.4 \times 10^3/\mu\text{L}$  for dogs in the control group, which was not significantly different ( $P = .16$ ). No dog was leukopenic or neutropenic at the time of enrollment.

Platelet function testing via platelet function analysis (PFA-100, using the ADP-collagen cartridge) performed at enrollment to the study on day 0 showed a mean closure time of  $63.1 \pm 10.08$  seconds (reference range, 52-86 seconds). There was no difference in closure times between treatment groups ( $P = .41$ ).

### 3.2 | Treatment

The mean vincristine dose given to the dogs in the vincristine group was  $0.347 \pm 0.179$  mg IV. One dog developed evidence of phlebitis (erythema, pain on palpation, edema) at the catheter site, and extravasation of vincristine was suspected. These adverse effects were mild and self-limiting with symptomatic treatment. No adverse effects were noted in any dog in the placebo group.



**FIGURE 3** Changes in platelet count (A) and percentage of TO+ (reticulated) platelets (B) in dogs treated with vincristine (circle) or saline (triangle) between day 0 and day 7. \* Indicates significant difference in vincristine-treated dogs from day 0 value,  $P < .05$

### 3.3 | Cell counts

Vincristine administration resulted in an increased platelet count from day 0 to day 7 ( $225 \pm 58$  vs  $273 \pm 65 \times 10^3/\mu\text{L}$ ,  $P = .01$ ), and an increased percentage of reticulated platelets from day 0 to day 5 ( $3.9 \pm 1.5\%$  to  $6.1 \pm 1.6\%$ ,  $P = .02$ ) (Figure 3). There was no significant difference in platelet count for dogs in the vincristine treatment group between any other time points. There was no significant change in the total platelet count or the percent of reticulated platelets in the saline group over the course of the study ( $P = .24$  and  $.54$ , respectively).

No dog became anemic at any time point in the study. Throughout the course of the study, 4/10 dogs were leukopenic (WBC  $<5500/\mu\text{L}$ ) or neutropenic (segmented neutrophils  $<2900/\mu\text{L}$ ) at least 1 time point, and all were in the vincristine group. Two of the 4 dogs that developed neutropenia or leukopenia were  $<15$  kg, and 2 of the 4 dogs were  $>15$  kg. Within dogs that became neutropenic, the neutropenia was diagnosed on day 5 in 1 and on day 7 in the other 3. No dog developed a neutropenia  $<1200$  neutrophils/ $\mu\text{L}$ , and no dog developed any clinical signs consistent with immune compromise at any time point over the course of the study.

### 3.4 | Flow cytometry

There was no difference between treatment and control groups in the percentage of CD62P expression in resting platelets, with the exception of day 7, where the percent of platelets that were CD62P+ was greater in the saline group ( $25.9 \pm 24\%$  vs  $9.5 \pm 3.4\%$ ,  $P = .02$ , Figure 4A). After thrombin activation (Figure 4B), there was no difference between groups at any time point ( $P = .46$ ).

There was no difference in the degree of CD62P expression in TO+ platelets between the dogs treated with vincristine and those treated with saline for either resting ( $P = .7$ ) (Figure 5A) or activated ( $P = .33$ ) platelets (Figure 5B). Similar to the total platelet data, the frequency of resting TO- platelets that were CD62P+ was higher in the saline group on day 7 ( $P = .03$ ), but this was not seen for activated platelets ( $P = .18$ ) (Figure 5A,B). When TO+ platelets were compared to TO- platelets without regards to treatment group, TO+ platelets had a higher resting expression of CD62P (mean of  $49.6 \pm 7\%$ ) compared to TO- platelets (mean of  $10.2 \pm 2\%$ ), on all days ( $P \leq .001$ ) (Figure 6).

### 3.5 | Aggregometry

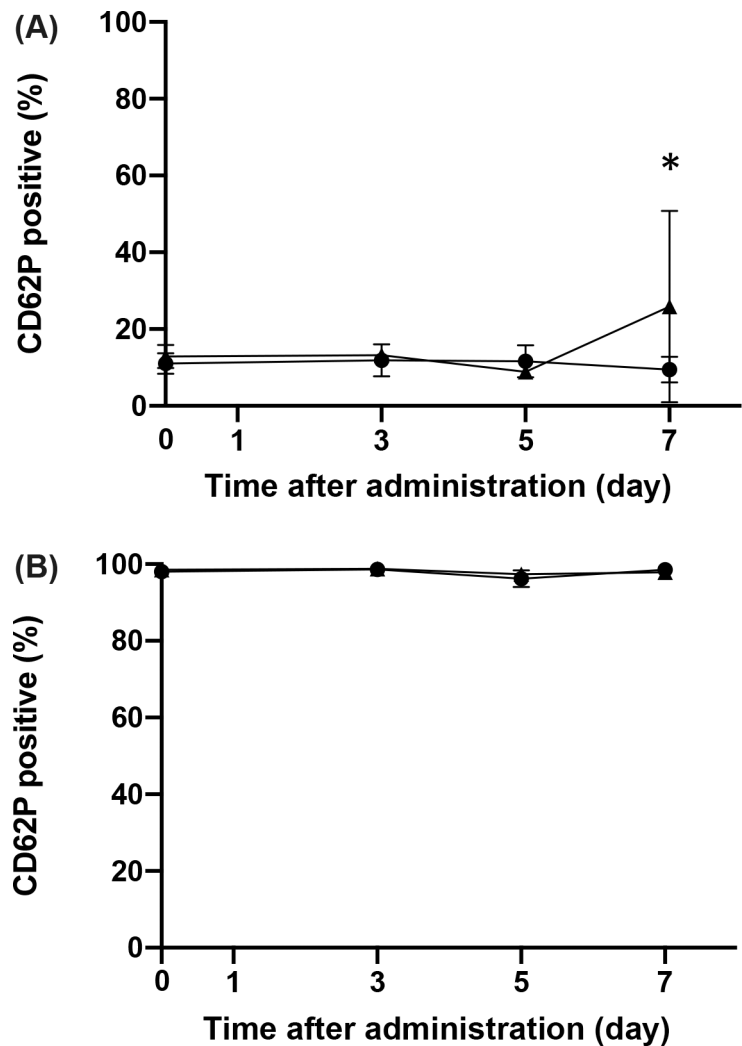
In the vincristine treated dogs, there was no change over time in maximal aggregation to either ADP ( $68 \pm 8\%$ ;  $P = .14$ ) or collagen ( $57 \pm 15\%$ ;  $P = .38$ , see Figure S1). Likewise, there was no change in the slope of aggregation after either ADP ( $84 \pm 4$ ;  $P = .74$ ) or collagen ( $65 \pm 8$ ;  $P = .38$ ) activation.

## 4 | DISCUSSION

The results of this study show that a single IV dose of vincristine results in a significant increase in platelet count relative to saline, and that not only do the newly released platelets demonstrate a higher activation state at rest than mature platelets, but vincristine administration did not result in a significant difference in expression of cell-surface markers of activation in stimulated platelets (TO+ or TO-). While the current study focuses on the investigation of healthy dogs, the techniques described can now be applied to thrombocytopenic dogs.

A single IV dose of vincristine resulted in a significant increase in platelet numbers relative to administration of IV 0.9% saline. This finding is consistent with a previous study, which reported an increase in circulating platelet count in response to vincristine administration in healthy dogs.<sup>13</sup> However, the present study additionally focused on the quantification and functionality of the newly released platelets. The percentage of reticulated platelets in the vincristine group significantly increased by day 5 of the study, which is slightly different than a previous report, which documented a maximal increase in total platelet count 8 days after vincristine administration.<sup>13</sup> The total platelet count as reported by an automated analyzer was significantly increased in the vincristine group on day 7, which is consistent

**FIGURE 4** Comparison of dogs receiving vincristine ( $n = 8$ , circles) or saline ( $n = 2$ , triangles) on day 0 for CD62P expression in resting platelets (A) and in platelets after activation with 1 U/mL thrombin (B). \* $P < .03$  between treatment groups



with this prior report.<sup>13</sup> In a prior report that described the use of vincristine with prednisone in dogs with ITP, dogs receiving vincristine experienced an increase in platelet count  $4.9 \pm 1.1$  days after vincristine dosing, and spent a shorter duration of time in the hospital,<sup>7</sup> so the latency period seen in the current study for an increase in platelet numbers is within clinically relevant expectations.

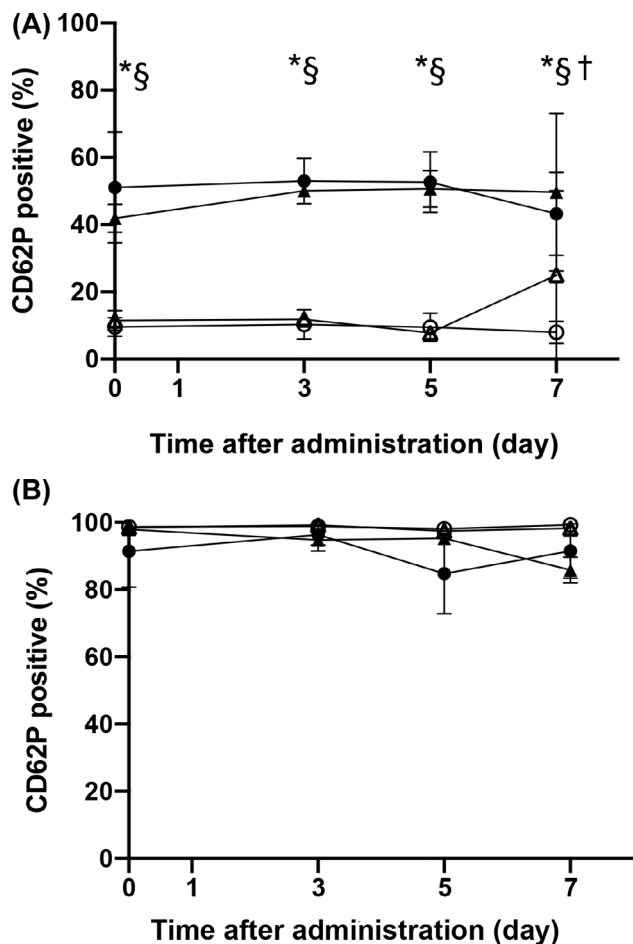
Newly released reticulated platelets appear to demonstrate a higher activation state at rest than mature platelets. When TO<sup>+</sup> platelets were compared to mature platelets without regard for treatment group, TO<sup>+</sup> platelets had a higher resting expression of CD62P than mature platelets. This might be protective against bleeding. This is supported clinically in human ITP patients where the fraction of immature platelets is inversely correlated with bleeding score.<sup>23</sup>

Vincristine administration did not result in a significant difference in expression of cell-surface markers of activation in thrombin-stimulated (activated) platelets. Relative to saline, reticulated platelets released in response to vincristine have the same resting and activated CD62P expression, suggesting that the function of the newly released, immature platelets was not impacted by vincristine administration. When all platelets were evaluated as a group, dogs receiving vincristine demonstrated decreased CD62P expression at rest on one

of the days of the study (day 7). However, this difference was no longer seen once the platelets underwent activation with thrombin, again suggesting the capacity for appropriate functionality of platelets in dogs receiving a single IV dose of vincristine.

A previous report in humans demonstrated that reticulated platelets have a significantly higher capacity to increase the expression of platelet activation markers compared with mature platelets when stimulated with ADP.<sup>24</sup> Although we did not assess response to ADP, this data combined with ours, suggests that reticulated platelets have a higher resting activation state than mature platelets, and in fact might be able to respond more robustly to stimulation by some agonists than mature platelets.

Previous studies evaluating platelet function in response to vincristine administration have yielded conflicting results. Similar to the current report, 1 study found that platelet aggregation in dogs after vincristine administration to healthy dogs was normal.<sup>13</sup> In contrast to our study, Grau-Bassas et al found decreased platelet aggregation in dogs with lymphoma after a single dose of vincristine.<sup>14</sup> However, in this study, dogs were administered an antineoplastic dose of vincristine ( $0.5 \text{ mg/m}^2$ , IV) rather than the lower dose traditionally utilized for dogs with immune thrombocytopenia ( $0.02 \text{ mg/kg}$ , IV), and



**FIGURE 5** Comparison of dogs receiving vincristine ( $n = 8$ , circles), or saline ( $n = 2$ , triangles) on day 0 for CD62P expression in resting (A) and activated (B) platelets that were positive (closed symbols) or negative (open symbols) for thiazole orange (TO) staining. \*  $P < .03$  between TO+ and TO- platelets in vincristine-treated dogs, §  $P < .01$  between TO+ and TO- platelets in saline-treated dogs. † Indicates higher CD62P expression in TO- platelets in the saline group compared to the vincristine-treated group,  $P = .03$

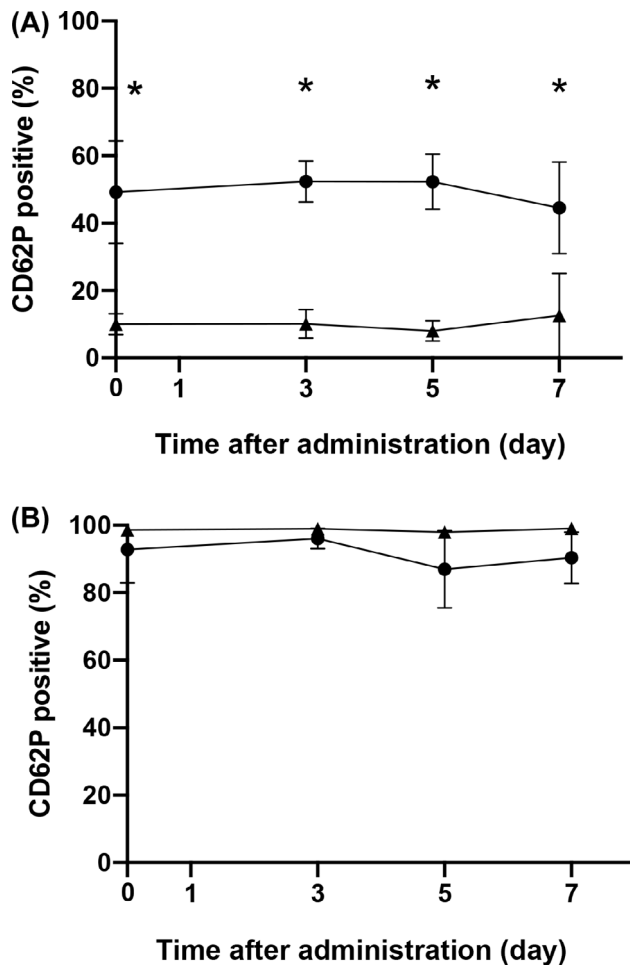
were also undergoing chemotherapy protocols including other anti-neoplastic agents. Additionally, despite normal hematologic parameters with no clinical evidence of bleeding, the bone marrow of dogs with lymphoma undergoing multiagent chemotherapy would be expected to function differently than that of otherwise healthy dogs or dogs with ITP. Consistent with the findings in our study that platelets released in response to vincristine function similarly to mature platelets, 1 previous study found that vincristine administration to dogs with ITP in conjunction with prednisone resulted in a shorter duration of hospitalization relative to dogs receiving prednisone alone, whereas another study reported similar hospitalization times in ITP dogs treated with vincristine and prednisone compared to dogs treated with hVIG and prednisone.<sup>7,9</sup> While the endpoint of these studies of time-to-discharge was a surrogate marker for functionality of platelets, had the platelets released in response to

vincristine not been functional, these dogs would have been expected to demonstrate clinical bleeding thereby lengthening their hospitalization time.

One limitation of the present study is the lack of a comparison between the vincristine and saline groups for a functional assay, aggregometry. Because of the small percentage of reticulated platelets compared to total platelet mass (and because aggregometry of necessity is performed on the total platelet population), it was not anticipated that a large contribution would be seen, but without a comparator group, we can only note that there were no changes in the vincristine-treated dogs despite an increasing percentage of reticulated platelets. Platelet function assessed by turbidimetric aggregometry in 16 clinically healthy dogs that received a single dose of vincristine (0.02 mg/kg, IV) administered concurrently with prednisone was not significantly affected.<sup>13</sup> Additionally, in this same study, they did not find any significant effects on clot retraction or buccal mucosal bleeding times. In the current report, aggregometry within the vincristine-treated group supports the flow cytometric data.

An additional limitation of the present study is that healthy dogs were used to evaluate the response to vincristine administration. These animals likely have normal bone marrows and it might be expected that thrombocytopenic dogs might have a different response to vincristine, especially because (as at least in human ITP), decreased platelet production due to immune destruction of megakaryocytes is well-described.<sup>25,26</sup> While consideration was given to studying clinical dogs with ITP, it has been shown that the cell-surface antibody binding that is central to the pathogenesis of ITP impacts the ability of the platelet-identifying CD61 antibody to consistently bind to the cell.<sup>20</sup> Therefore, owing to potential difficulty with analysis as a result of inconsistent ability to label and identify platelets with antibody, the decision was made to investigate healthy dogs first. Additionally, it has been shown that humans with ITP have differing levels of expression of cell-surface markers of platelet activation as assessed by flow cytometry, which has been proposed as 1 potential mechanism for why humans with similar platelet counts have differences in bleeding severity.<sup>27</sup> As such, utilizing dogs with ITP might have introduced confounding factors in assessing platelet function after vincristine administration. Regardless, the techniques described herein, now validated in healthy dogs, can now be applied to thrombocytopenic dogs.

The decision to include only 2 control dogs in the current study was made based on a sample size calculation using a pilot dog before the commencement of the study. The pilot dog, who satisfied all the same inclusion criteria as study dogs, was administered a single dose of vincristine (0.02 mg/kg, IV), and complete blood counts as well as platelet function analysis and flow cytometric analysis were performed as described for dogs included in the study. Based on the response of this dog, it was found that more than 2 control dogs were not necessary to achieve statistical significance. Additionally, a main focus of this study was comparing to baseline within each dog, rather than comparison between dogs, and as such a larger number of control dogs would not be required. Another limitation in the comparison of P-selectin expression between groups was the use of relatively high



**FIGURE 6** Comparison between TO+ (circles) and TO- (triangles) platelets, irrespective of treatment group, for CD62P expression in resting platelets (A) and platelets after activation with 1 U/mL thrombin (B). \* $P \leq .001$  on all days

concentration of thrombin; this strong stimulus might have obscured possible differences in activation between TO+ and TO- platelets.

An unexpected finding in the present study was that 50% of the dogs in the vincristine group became neutropenic or leukopenic at some time point. The dose of vincristine used in the context of ITP (0.02 mg/kg) is a lower dose than that used for chemotherapy (0.5 mg/m<sup>2</sup>), and myelosuppression is thought to be an uncommon adverse effect.<sup>7-9</sup> However, as animal body weight increases, the mg/kg dosing structure for the ITP-dose of vincristine approaches the antineoplastic dose, and at a body weight >16 kg, the 0.02 mg/kg dosing will exceed the antineoplastic dose of vincristine. While the development of neutropenia in the present study was never <1200/ $\mu$ L and was not associated with clinical evidence of immunosuppression, it might be more relevant in hospitalized dogs, especially those who are already immunosuppressed as part of their therapy. Therefore, dose reduction or selection of a maximum total dose in larger dogs might be warranted.

A single IV dose of vincristine increases both total platelet count as well as the percentage of reticulated platelets. Reticulated platelets

appear to have a higher resting activation state than mature platelets, and demonstrate similar CD62P expression, regardless of treatment group. Fifty percent of dogs that received vincristine in this study became neutropenic or leukopenic at some point during the study, which prompts a clinical suggestion for a maximum dose, especially in larger dogs, where the platelet-releasing dose approaches the chemotherapeutic dose.

#### ACKNOWLEDGMENT

Funding provided by the Veterinary Emergency and Critical Care Society Foundation. Funding source did not have any involvement in the study design, data analysis and interpretation, or writing and publication of the manuscript. Flow cytometric data were collected in the UGA College of Veterinary Medicine Cytometry Core facility.

#### 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 Georgia College of Veterinary Medicine IACUC and performed in compliance with institutional guidelines for research on animals.

#### HUMAN ETHICS APPROVAL DECLARATION

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

#### ORCID

Benjamin M. Brainard  <https://orcid.org/0000-0003-4299-2936>

#### REFERENCES

- O'Marra SK, Delafordcade AM, Shaw SP. Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia. *J Am Vet Med Assoc.* 2011;238:346-352.
- Consolini R, Legitimo A, Caparello MC. The centenary of immune thrombocytopenia - part 1: revising nomenclature and pathogenesis. *Front Pediatr.* 2016;4:102.
- LeVine DN, Brooks MB. Immune thrombocytopenia (ITP): pathophysiology update and diagnostic dilemmas. *Vet Clin Pathol.* 2019;48(Suppl 1):17-28.
- Lewis DC, Meyers KM. Studies of platelet-bound and serum platelet-bindable immunoglobulins in dogs with idiopathic thrombocytopenic purpura. *Exp Hematol.* 1996;24:696-701.
- Lewis DC, Meyers KM. Canine idiopathic thrombocytopenic purpura. *J Vet Intern Med.* 1996;10:207-218.
- Putsche JC, Kohn B. Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). *J Am Anim Hosp Assoc.* 2008;44:250-257.
- Rozanski EA, Callan MB, Hughes D, et al. Comparison of platelet count recovery with use of vincristine and prednisone or prednisone alone for treatment for severe immune-mediated thrombocytopenia in dogs. *J Am Vet Med Assoc.* 2002;220:477-481.
- Stirnemann J, Kaddouri N, Khellaf M, et al. Vincristine efficacy and safety in treating immune thrombocytopenia: a retrospective study of 35 patients. *Eur J Haematol.* 2016;96:269-275.



9. Balog K, Huang AA, Sum SO, et al. A prospective randomized clinical trial of vincristine versus human intravenous immunoglobulin for acute adjunctive management of presumptive primary immune-mediated thrombocytopenia in dogs. *J Vet Intern Med.* 2013;27:536-541.
10. Park HJ, Kim JW, Song KH, et al. Application of vincristine-loaded platelet therapy in three dogs with refractory immune-mediated thrombocytopenia. *J Vet Sci.* 2015;16:127-130.
11. Weigert O, Wittmann G, Grutzner S, et al. Vincristine-loaded platelets for immune thrombocytopenia. *Thromb Haemost.* 2010;104:418-419.
12. Weigert O, Wittmann G, Gruetzner S, et al. Successful salvage treatment of chronic refractory immune thrombocytopenia using a simplified method to generate vincristine-loaded platelets. *Thromb Haemost.* 2008;100:705-707.
13. Mackin AJ, Allen DG, Johnston IB. Effects of vincristine and prednisone on platelet numbers and function in clinically normal dogs. *Am J Vet Res.* 1995;56:100-108.
14. Grau-Bassas ER, Kociba GJ, Couto CG. Vincristine impairs platelet aggregation in dogs with lymphoma. *J Vet Intern Med.* 2000;14:81-85.
15. Brooks MB, Randolph J, Warner K, et al. Evaluation of platelet function screening tests to detect platelet procoagulant deficiency in dogs with Scott syndrome. *Vet Clin Pathol.* 2009;38:306-315.
16. Sharpe KS, Center SA, Randolph JF, et al. Influence of treatment with ultralow-dose aspirin on platelet aggregation as measured by whole blood impedance aggregometry and platelet P-selectin expression in clinically normal dogs. *Am J Vet Res.* 2010;71:1294-1304.
17. Tarnow I, Kristensen AT, Krogh AK, et al. Effects of physiologic agonists on canine whole blood flow cytometry assays of leukocyte-platelet aggregation and platelet activation. *Vet Immunol Immunopathol.* 2008;123:345-352.
18. Wilkerson MJ, Shuman W, Swist S, et al. Platelet size, platelet surface-associated IgG, and reticulated platelets in dogs with immune-mediated thrombocytopenia. *Vet Clin Pathol.* 2001;30:141-149.
19. Wills TB, Wardrop KJ, Meyers KM. Detection of activated platelets in canine blood by use of flow cytometry. *Am J Vet Res.* 2006;67:56-63.
20. Bachman DE, Forman MA, Hostutler RA, et al. Prospective diagnostic accuracy evaluation and clinical utilization of a modified assay for platelet-associated immunoglobulin in thrombocytopenic and non-thrombocytopenic dogs. *Vet Clin Pathol.* 2015;44:355-368.
21. Smith R III, Thomas JS. Quantitation of reticulated platelets in healthy dogs and in nonthrombocytopenic dogs with clinical disease. *Vet Clin Pathol.* 2002;31:26-32.
22. Ault KA. The clinical utility of flow cytometry in the study of platelets. *Semin Hematol.* 2001;38:160-168.
23. Greene LA, Chen S, Seery C, et al. Beyond the platelet count: immature platelet fraction and thromboelastometry correlate with bleeding in patients with immune thrombocytopenia. *Br J Haematol.* 2014;166:592-600.
24. Lador A, Leshem-Lev D, Spectre G, et al. Characterization of surface antigens of reticulated immature platelets. *J Thromb Thrombolysis.* 2017;44:291-297.
25. Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). *J Clin Med.* 2017;6:16.
26. Iraqi M, Perdomo J, Yan F, et al. Immune thrombocytopenia: anti-platelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro. *Haematologica.* 2015;100:623-632.
27. Frelinger AL III, Grace RF, Gerrits AJ, et al. Platelet function tests, independent of platelet count, are associated with bleeding severity in ITP. *Blood.* 2015;126:873-879.

#### SUPPORTING INFORMATION

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

**How to cite this article:** Allen EC, Tarigo JL, LeVine DN, Barber JP, Brainard BM. Platelet number and function in response to a single intravenous dose of vincristine. *J Vet Intern Med.* 2021;35(4):1754-1762. <https://doi.org/10.1111/jvim.16169>