

Assessment of platelet activation in dogs with naturally occurring acute pancreatitis

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Abstract

Recent studies have demonstrated that platelets are crucial in inflammation and immune responses. Alteration in platelet activity impact morbidity and survival due to the formation of intravascular clots or bleeding resulting from impaired platelet function. Aiming to evaluate changes in platelet activity in dogs with naturally occurring acute pancreatitis (AP), we identified differences in quantitative and morphological platelet indices, as well as the ratio of platelets expressing P-selectin on their surface (activated platelet ratio; APR) between dogs with naturally occurring AP and healthy dogs. The study included 9 client-owned dogs with AP (the AP group) and 28 client-owned healthy dogs (the control group) from several animal hospitals. Dogs with AP were diagnosed based on compatible clinical signs, increased serum canine pancreatic lipase concentrations, and/or abdominal ultrasonographic findings consistent with AP. In this multi-institutional case-control study, platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelet large cell ratio (P-LCR), and plateletcrit (PCT) were measured using complete blood count. Additionally, fluorescence-activated cell sorting was used to measure the APR. There were no statistically significant differences in MPV, PDW, P-LCR, PLT, or PCT between dogs with acute pancreatitis and healthy controls. APR was statistically significantly higher in the AP group ($P = 0.013$). In conclusion, dogs with naturally occurring AP showed increased APR, suggesting that platelet activation may be involved in the pathophysiology of the disease, although conventional platelet indices did not differ significantly between groups.

Keywords: complete blood count, cluster of differentiation 62P, fluorescence-activated cell sorting, platelet index, P selectin

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Introduction

Acute pancreatitis (AP) is a reversible inflammatory condition if triggering factors or underlying diseases do not persist. Histologically, it is characterized by edema, neutrophil infiltration, and necrosis without irreversible changes (fibrosis or exocrine atrophy) (Ettinger *et al.*, 2024).

The pathophysiology of AP remains incompletely understood. Premature activation of trypsinogen constitutes a primary initiating factor (Mansfield, 2012). Recently, research involving trypsinogen knock-out mice has revealed that premature activation of trypsinogen and nuclear factor- κ B occurs both independently and concurrently in the initial stages of AP (Dawra *et al.*, 2011).

Although platelets are considered to be involved in maintaining vascular integrity and hemostasis, recent studies have demonstrated that they are also crucial in inflammation and immune responses (Gawaz *et al.*, 2005; Yuri Gasparyan *et al.*, 2011; Thomas and Storey, 2015; Scherlinger *et al.*, 2023). In both human and veterinary medicine, alterations in platelet activity impact morbidity and survival due to the formation of intravascular clots or bleeding (de Laforcade, 2012).

Immature platelets are larger and more reactive than mature platelets. Corresponding changes in platelet indices include increased mean platelet volume (MPV), platelet distribution width (PDW), large platelet cell ratio (L-PCR), and plateletcrit (PCT). In human medicine, MPV, PDW, P-LCR, and PCT are associated with platelet activity (Thompson *et al.*, 1982; Giacomini *et al.*, 2003; Braekkan *et al.*, 2010; Chu *et al.*, 2010; Vagdatli *et al.*, 2010; Budak *et al.*, 2016; Sushma and Rangaswamy, 2017). In veterinary medicine, various diseases are associated with changes in platelet indices (Moritz *et al.*, 2005; Yilmaz *et al.*, 2008; Schwartz *et al.*, 2014; Llewellyn *et al.*, 2017; Hosseinpour *et al.*, 2022).

Platelet activity can also be assessed by flow cytometry, which evaluates the expression of cell surface protein (Paniccia *et al.*, 2015). When platelets are activated, P-selectin (CD62P), stored in α -granules of resting platelets, translocates to the platelet surface (Eppihimer *et al.*, 1996). Various studies employed this method to assess platelet function in dogs (Moritz *et al.*, 2003, 2005; Weiss and Brazzell, 2006; Wills *et al.*, 2006; Dudley *et al.*, 2017).

In human medicine, several studies evaluated the association between AP and platelet activation. One study found significant differences in MPV, P-LCR, PDW, and proportion of platelets expressing P-selectin on their surface (activated platelet ratio; APR) between the onset and remission stages (Mimidis *et al.*, 2004). Subsequent research evaluated the functional state of platelets in mild and severe AP, revealing differences based on disease severity (Osada *et al.*, 2012).

In veterinary medicine, few studies evaluated platelet activation in AP, providing inconsistent results. In a study on dogs with experimentally induced AP, platelet aggregation decreased (Jacobs *et al.*, 1986). In contrast, another study observed increased platelet aggregability (Łukaszyk *et al.*, 1989). Recently, a study has evaluated hemostatic dysfunction in naturally occurring AP patients by

thromboelastography, identifying a hypercoagulable state (Nielsen *et al.*, 2019). However, to date, no studies have evaluated both changes in platelet indices and P-selectin expression in dogs with AP.

This study aimed to determine differences in platelet indices and P selectin expression between dogs with naturally occurring AP and healthy dogs. We hypothesized that platelet activity would be increased in naturally occurring AP patients, increasing APR, MPV, PDW, P-LCR, and PCT.

Materials and Methods

Animals: Dogs that presented to the hospital and subsequently received a clinical diagnosis of AP were included in the AP group. The clinical diagnosis of AP was defined by meeting two criteria: (1) exhibiting clinical symptoms such as vomiting, anorexia, and abdominal pain within 48 hours and (2) having a canine pancreatic lipase (Vcheck cPL 2.0, Bionote Corp.) level ≥ 400 μ g/L or showing changes related to AP on abdominal ultrasound (pancreatic enlargement, hypoechoic changes in the pancreatic parenchyma, or hyperechoic changes in the peripancreatic mesentery).

The exclusion criteria for the study included (1) cases with thrombocytopenia (platelet count $< 100 \times 10^9$ /L confirmed by smear examination); (2) cases with anemia (hematocrit $< 30\%$) (Callan and Giger, 2001); (3) a history of medication affecting platelet function (calcium channel blockers, beta-blockers, fish oil supplementation, or corticosteroids) within the last 7 days (Knight *et al.*, 1997; Rose *et al.*, 2008; Westgarth *et al.*, 2018); (4) concurrent diseases known to alter platelet function (immune-mediated hemolytic anemia, chronic kidney disease, tumors, or myxomatous mitral valve disease) (McNiel *et al.*, 1997; Clancey *et al.*, 2009; Ridyard *et al.*, 2010; Dudley *et al.*, 2017).

Patients presenting for annual health checkups or neutering surgeries were included in the control group if their history and physical examination showed no abnormalities with normal complete blood count (CBC) values, including white blood cells, hematocrit, and PLT. If clinically indicated, canine pancreatic lipase was measured.

The dogs' owners provided informed consent for participation and data publication. The ethical committee of our institutions (2024-0089) approved all animal studies.

Assessment of platelet indices and P-selectin expression: Blood samples were collected from the dog's jugular vein for analysis. CBC evaluations were conducted using blood samples, and fluorescence-activated cell sorting (FACS) analysis was performed on those who consented to additional blood collection.

Evaluation of platelet indices: For CBC analysis, 0.5 mL of blood was collected into EDTA tubes (Microtainer® K2 EDTA Tube 0.5 mL, Becton Dickinson and Company). Among the CBC parameters, PLT, MPV, PDW, P-LCR, and PCT were compared between the groups.

Detection of activated platelets: Three mL of whole blood was placed in plain tubes (5 mL Snap Tubes, SPL LIFE SCIENCES) containing 0.6 mL acid citrate dextrose as an anticoagulant. The collected blood was centrifuged at room temperature for 7 min at 1,000 rpm to obtain platelet-rich plasma. Subsequently, platelet-rich plasma samples underwent another centrifugation step at room temperature for 7 min at 500 rpm to remove any remaining erythrocytes. The platelet-rich plasma was centrifuged at 2,000 rpm with platelet suspending buffer (129 mM NaCl, 10.9 mM sodium citrate, 8.9 mM sodium bicarbonate, 1g/L glucose, 10 mM Tris, 2.8 mM KCl, 0.8 mM KH₂PO₄, 0.05% BSA) for 7 min to isolate platelets.

Then, washed platelets were centrifuged, followed by resuspension in ice-cold phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS) and 1% sodium azide. The samples were blocked with ice-cold PBS containing 3% BSA and labeled with CD62P primary antibody (Psel.KO.2.7, Bio-Rad Laboratories) for 30 min at 4°C in the dark. The samples were washed repeatedly in ice-cold PBS and labeled with FITC-conjugated secondary antibody (FITC-labeled horse anti-mouse IgG, Vector Laboratories) in 3% BSA/PBS for 30 min at 4°C in the dark. After repeated washing with ice-cold PBS, the samples were resuspended in ice-cold PBS, 3% BSA, and 1% sodium azide. The

samples were fixed in 4% paraformaldehyde and stored until analysis. Flow cytometry was performed using a FACS (FACS Calibur flow cytometer, Becton Dickinson), with data analyzed using CellQuest software (Becton Dickinson Immunocytometry System). Results were expressed as the percentage of CD62P+ platelets, representing the APR (Fig. 1).

Statistical analysis: Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp.). Chi-squared tests and independent sample t-tests were conducted to test the homogeneity of subjects between the groups. Normality tests using the Kolmogorov-Smirnov and Shapiro-Wilk tests indicated that non-parametric tests were suitable for CBC, while parametric tests were appropriate for P-selectin expression. Differences in PLT, MPV, P-LCR, PDW, and PCT between the groups were analyzed using the Mann-Whitney U test, and differences in P-selectin expression rates were analyzed using the independent sample t-test. Values were reported as mean $\pm$ standard deviation (SD). A *P*-value of ≤ 0.05 indicated statistical significance. Graphs were generated using GraphPad Prism 10.2.3 (GraphPad Software Inc.).

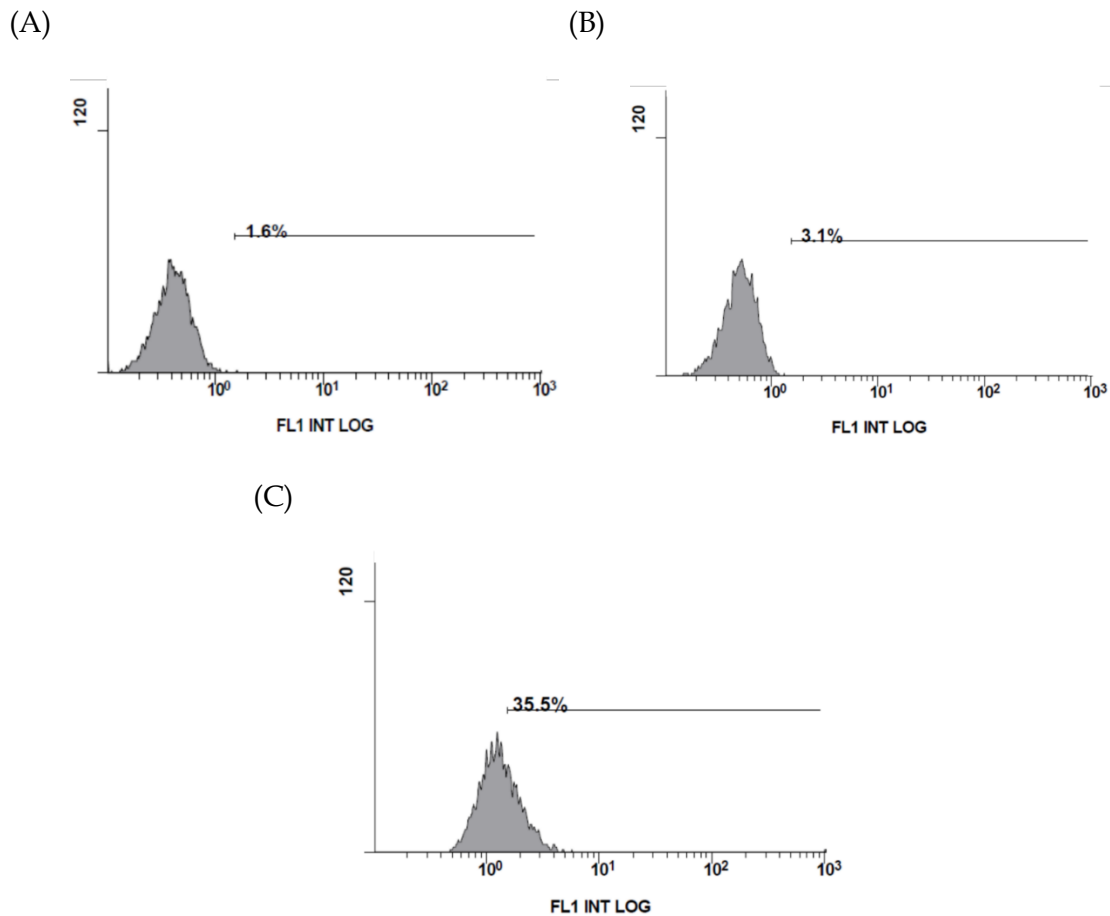


Figure 1 Histogram of fluorescence (FL1) of platelets labeled with anti-CD62P antibody. The percentage of CD62P-positive platelets is defined as APR and expressed as %. The X-axis represents fluorescence intensity, and the Y-axis represents cell counts. (A) Isotype control. (B) Platelets from a healthy dog. (C) Platelets from a dog with AP. AP, acute pancreatitis; APR, activated platelet ratio.

Results

Animals: The study was conducted on patients who visited four hospitals: A, B, C, and D.

The study included 28 dogs in the control group and 9 dogs in the AP group. Table 1 presents the average age and gender distribution of each group. Pre-study homogeneity between the two groups was confirmed.

The breeds of patients included in the AP group were Pomeranian and Toy Poodle (each 3/9, 33.3%), Chihuahua, French Bulldog, and Mongrel (each 1/9, 11.1%). The breeds of patients included in the control group were Mongrel (9/28, 32.1%), Maltese (6/28, 21.4%), German Shepherd and Beagle (3/28, 10.7%), Toy Poodle (2/28, 7.1%), Bichon Frise, Cocker Spaniel, Greyhound, Jindo, and Pomeranian (each 1/28, 3.6%).

Table 2 shows the inclusion criteria satisfied by patients classified into the AP group. Upon

presentation, clinical symptoms included vomiting in 8/9 (88.9%) patients, abdominal pain and borborygmi in 2/9 (22.2%) patients each, and diarrhea, lethargy, anorexia, and weight loss in 1 (11.1%) patient each. At presentation, 3/9 (33.3%) patients exhibited two symptoms, and 2/9 (22.2%) patients exhibited three symptoms. In one of the dogs (AP 4), abdominal ultrasonography revealed diffuse hypoechogenicity of the pancreatic parenchyma accompanied by hyperechoic changes in the surrounding mesentery, findings consistent with acute pancreatitis.

Assessment of platelet indices and P-selectin expression: All 37 dogs included in the study underwent CBC analysis. Among these, owners of 15 dogs from the control group and 9 dogs from the AP group consented to additional blood collection for FACS analysis and were subsequently tested.

Table 1 Test of homogeneity.

		Group		P value
		Control (n = 28)	AP (n = 9)	
Sex	Male	4 (14.3%)	0 (0.0%)	0.504
	Castrated male	7 (25.0%)	4 (44.4%)	
	Female	5 (17.9%)	1 (11.1%)	
	Spayed female	12 (42.9%)	4 (44.4%)	
	Age	3.71 ± 3.01	5.22 ± 3.53	0.215

Note: Data are presented as mean ± standard deviation. AP, acute pancreatitis.

Table 2 Inclusion criteria satisfaction for the acute pancreatitis group.

	Number of symptoms	cPL (ng/mL)	Abdominal ultrasound
AP 1	1	√	
AP 2	1	√	
AP 3	2	√	
AP 4	3		√
AP 5	1	√	
AP 6	3	√	
AP 7	1	√	
AP 8	2	√	
AP 9	2	√	

This table shows the inclusion criteria satisfaction for patients classified into the acute pancreatitis group. Each row represents a patient, with the number of symptoms (vomiting, anorexia, and abdominal pain within 48 hours), and whether they met the criteria of elevated cPL levels and/or positive findings on abdominal ultrasound.

Note: The checkmarks (√) indicate that the respective criteria were satisfied.

AP, acute pancreatitis; cPL, canine pancreatic lipase.

Evaluation of platelet indices: The analyses were conducted using equipment available at each hospital. The instruments used were: (1) XN-V (Sysmex Corporation, Kobe, Japan), (2) ProCyte Dx (IDEXX Laboratories, Westbrook, ME, USA), and (3) Exigo H400 (Boule Medical AB, Spånga, Sweden). Depending on the equipment, some of these parameters were not measured. For device (2), P-LCR was not measured, while for device (3), PDW and P-LCR were not measured.

Table 3 and Figure 2 demonstrate the results of the Mann-Whitney U test comparing platelet indices between the groups.

MPV, PDW, P-LCR, PLT, and PCT did not differ significantly between dogs with acute pancreatitis and healthy controls.

Detection of activated platelets: As a result of analyzing washed platelets by CBC, white and red blood cell counts were not measured, and gating was not set during subsequent FACS analysis.

Table 4 and Figure 3 show the results of the independent sample t-test comparing APR between the groups. APR was statistically significantly higher in the AP group (28.14 ± 28.55 vs. 7.93 ± 5.1 , respectively).

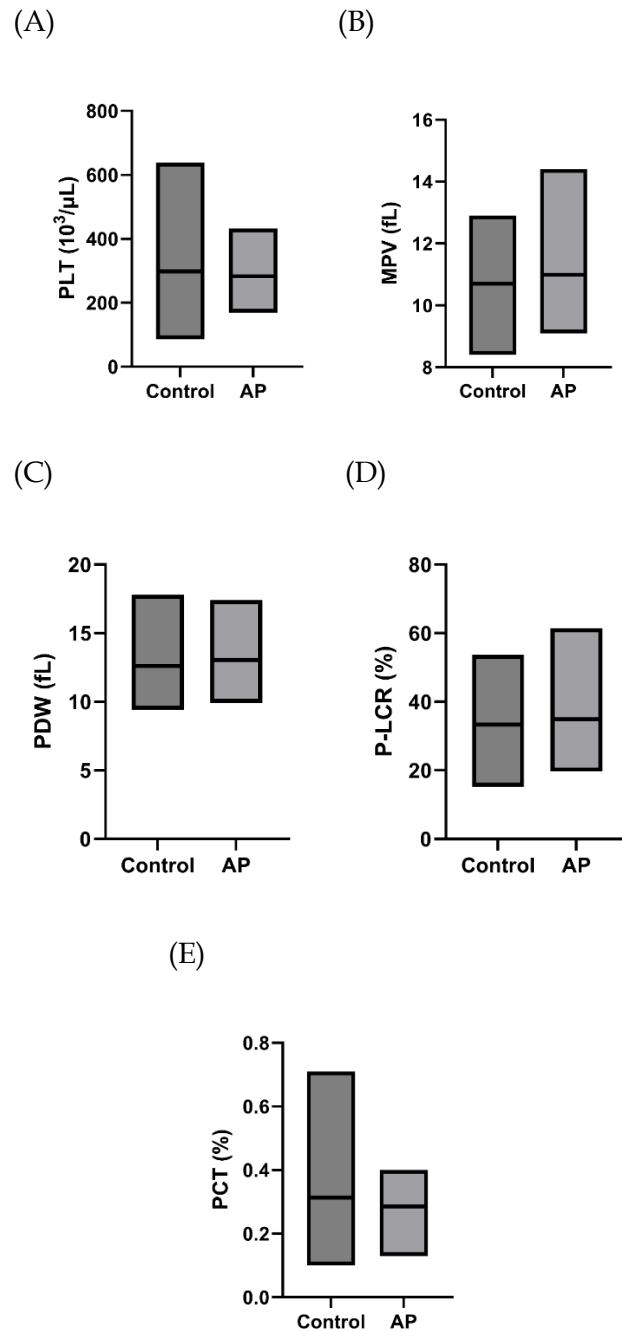


Figure 2 Floating box plots of platelet indices. The floating box plots illustrate the distribution of each platelet index. The lower and upper boundaries of the floating box represent the minimum and maximum observed values, respectively. The line within the box indicates the mean value of the data. (A) PLT, (B) MPV, (C) PDW, (D) P-LCR, (E) PCT. AP, acute pancreatitis; MPV, mean platelet volume; PCT, plateletcrit; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; PLT, platelet count.

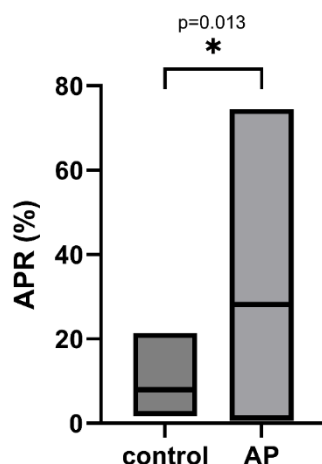


Figure 3 Floating box plots of APR. The floating box plots illustrate the distribution of APR values. The lower and upper boundaries of the floating box represent the minimum and maximum observed values, respectively. The line within the box indicates the mean value of the data. Significantly different results are indicated by * with p values shown above. AP, acute pancreatitis; APR, activated platelet ratio.

Table 3 Differences in platelet index between control and AP groups.

	Group		P value
	Control (n = 28)	AP (n = 9)	
PLT (10 ³ /μL)	297.75 ± 146.12	282.89 ± 86.27	0.887
MPV (fL)	10.70 ± 1.16	10.99 ± 2.00	0.832
PDW (fL)	12.61 ± 1.86	13.03 ± 2.85	0.977
P-LCR (%)	33.46 ± 10.83	34.67 ± 14.9	0.897
PCT (%)	.31 ± .15	.29 ± .07	0.929

Note: Data are presented as mean ± standard deviation.

AP, acute pancreatitis; MPV, mean platelet count; PCT, plateletcrit; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; PLT, platelet count.

Table 4 The difference in APR between control and AP groups.

	Group		P value
	Control (n = 15)	AP (n = 9)	
APR (%)	7.93 ± 5.1	28.14 ± 28.55	0.013

Note: Data are presented as mean ± standard deviation.

AP, acute pancreatitis; APR, activated platelet ratio.

Discussion

AP can often be accompanied by thrombosis and bleeding disorders (Xenoulis, 2015). In human medicine, extensive research has been conducted on platelet activation in patients with AP. However, in veterinary medicine, studies on this subject are limited, providing inconsistent results. We evaluated the quantitative and morphological indices of platelets and APR. There was no significant difference in platelet indices (PLT, MPV, PDW, P-LCR, and PCT) between AP patients and controls, but there was a significant difference in APR.

Studies evaluating platelet activation in human AP showed statistically significant differences in these indices. Considering the high proportion of activated platelets at disease onset, the changes in platelet indices occur due to an increase in immature platelets as a compensatory response of the bone marrow after

the consumption of platelets in the early phase of the disease (Mimidis *et al.*, 2004).

A study in veterinary medicine assessed platelet indices in patients presenting for orthopedic surgery (control group) and patients with AP (32 dogs). When comparing platelet indices between the two groups, PLT, MPV, PDW, and PCT were higher in the AP group, but these differences were not statistically significant (Smith *et al.*, 2014).

Unlike in human studies, no significant differences were identified in previous veterinary study and the present study. Possible reasons for the discrepancy between human and veterinary studies could be no actual changes in platelet indices, or changes without statistically significant differences due to sample size, pre-analytical factors, or the influence of disease severity. Pre-analytical factors include cell swelling or clumping when blood samples are stored in EDTA (Willard and Tvedten, 2011). Additionally, although jugular venipuncture minimizes collection-induced

platelet aggregation and activation, these issues can still occur during blood collection (Bauer *et al.*, 2011).

In this study, the proportion of platelets expressing P-selectin was defined as the APR, which was significantly higher in the AP group compared to the control group. Studies on humans with AP showed differences in P-selectin expression depending on disease onset and resolution, as well as disease severity (Mimidis *et al.*, 2004; Osada *et al.*, 2012). In veterinary medicine, various studies evaluated platelet activity using P-selectin expression, but no such evaluations have been conducted in dogs with naturally occurring AP (Moritz *et al.*, 2003; Moritz *et al.*, 2005; Weiss and Brazzell, 2006; Wills *et al.*, 2006; Dudley *et al.*, 2017;). According to findings from human studies and the results of the present study, platelet activation may play a role in the pathophysiology of AP as evidenced by increased platelet activation in AP (Mimidis *et al.*, 2004; Osada *et al.*, 2012).

Additionally, in this study, the statistically significant increase in APR in AP patients compared to the control group, coupled with the non-significant changes in platelet indices, suggests that P-selectin expression may be a more sensitive marker for evaluating platelet activation.

This study has several limitations. The study population was small. A larger study population might detect subtle changes in platelet indices or APR in dogs with naturally occurring AP. The small study population also might have caused a type II error. Thus, future research should confirm these findings through larger studies. Another limitation is the use of different hematology analyzers across participating hospitals, with some instruments not providing all platelet indices. This inter-instrument variability may have introduced additional measurement variability and could have reduced the ability to detect subtle differences in platelet indices between groups. A histopathological examination is considered the gold standard for diagnosing AP. However, histopathological examination was not performed in this study. The kit used for cPL measurement was the Vcheck cPL 2.0. Recent studies comparing the results of different cPL measurement devices have found that the Vcheck cPL has a higher proportion of results with an unacceptably high coefficient of variant and exhibits disagreements in interpretation with repeat measurements. Currently, the most precise biochemical marker for pancreatic lipase in dogs is the Spec cPL (Spec cPL ELISA, Texas A&M Gastrointestinal Laboratory, College Station, TX 77843, USA) (Cridge *et al.*, 2020). Therefore, using this assay in future studies may enhance the accuracy of patient selection. Furthermore, this study did not evaluate the severity of AP. Methods for assessing the severity of AP in patients include the APPLE score, the CAPS score, the more simplified APPLEfast score, and the sCAPS score (Hayes *et al.*, 2010; Fabrès *et al.*, 2019). Although severity assessment was not conducted in this study, there were probably no severe cases among study subjects, considering that the included dogs were discharged within 1-2 days following standard treatment for AP (fluid therapy, analgesics, antiemetics, and nutritional support) without long-term hospitalization. Some studies in human medicine

identified differences in platelet activation markers based on disease severity (Osada *et al.*, 2012). As differences in platelet activation based on severity could also occur in dogs with naturally occurring AP, future studies should also evaluate disease severity.

In conclusion, no significant differences were observed in platelet indices (PLT, MPV, PDW, P-LCR, and PCT) between the two groups. However, the APR was significantly higher in the AP group. Thus, platelet activation may be involved in the pathophysiology of AP. Furthermore, the significant increase in APR, coupled with the absence of significant changes in other platelet indices, highlights the potential of APR as a sensitive marker for platelet activation. Based on our findings, future research could explore the use of APR in diagnosing AP and evaluate the effects of antiplatelet therapy on treatment outcomes and survival. Further studies including larger sample sizes, assessment of disease severity, and multiple time points after disease onset, are needed to fully elucidate the role of platelet activation in AP.

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