Platelet activation in a population of critically ill dogs as measured with whole blood flow cytometry and thromboelastography

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OBJECTIVE
To determine whether critically ill dogs had increased platelet activation and whether the proportion of activated platelets correlated with severity of illness.

ANIMALS
82 dogs in the intensive care unit of a veterinary teaching hospital and 24 healthy control dogs.

PROCEDURES
Flow cytometry with monoclonal mouse anti-human CD61 and CD62 antibodies in resting and ADP-treated samples and kaolin-activated thromboelastography were used to compare platelet activation in blood samples of critically ill and control dogs. Serum antithrombin, von Willebrand factor, fibrinogen, and activated protein C concentrations; prothrombin time (PT); and activated partial thromboplastin time (aPTT) were measured. Revised survival prediction index, acute patient physiology and laboratory evaluation, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome scores were used to estimate severity of illness. Severity of illness scores and platelet activation measurements were compared with survival time and duration and cost of hospitalization.

RESULTS
Critically ill and control dogs had no differences in platelet activation for non–ADP-treated samples measured. Critically ill dogs had significantly increased platelet activation in response to 2, 6, and 10μM ADP. Critically ill dogs had significantly increased maximum amplitude, α angle, and global clot strength and significantly decreased clot formation time. Critically ill dogs had significantly increased fibrinogen concentration, PT, and aPTT and significantly decreased antithrombin concentration. Survivors and nonsurvivors had similar flow cytometry and thromboelastography values. Three dogs developed macrothrombosis.

CONCLUSIONS AND CLINICAL RELEVANCE
In this study, critically ill dogs had hyperreactive platelets, which may have contributed to a high incidence of hypercoagulability in this patient population. (Am J Vet Res 2015;76:328–337)

Platelet activation has been reported for a variety of conditions in dogs, such as IMHA, nephrotic syndrome, parvoviral enteritis, heartworm disease, lymphosarcoma and other malignancies, and sepsis.[1-8] In humans, excessive platelet activation promotes thromboembolic events and potentiates inflammatory reactions that can lead to sepsis, septic shock, and MODS.[9-13] Once platelets are activated and tethered to the site of endothelial injury, they recruit and activate neutrophils through the action of cell-adhesion receptors, thereby potentiating both the inflammatory and coagulation response.[14] In addition, activated platelets release microparticles into the circulation. These microparticles are small, are highly mobile in the microvasculature, have notable procoagulant activity, and may potentially contribute to thrombus formation or disseminated intravascular coagulation.[15-18]

Systemic inflammation contributes to a hypercoagulable state, likely because of effects attributable to expression of cytokine-mediated tissue factor on the surface of inflammatory cells and on activated endothelium.[19] Hypercoagulability is characterized by an increased MA for thromboelastography, which suggests that platelets may play a central role in this procoagulant state.[20] The potentially devastating effects of inflammation and hypercoagulability are described in a retrospective study[21] of dogs that died or were euthanized because of complications of IMHA. In that study,[21] necropsy findings in 28 of 34 (83%) dogs included hepatocellular necrosis and infarcts in the spleen, kidneys, liver, lungs, or heart. In addition, the severity of lesions was correlated with the degree of

ABBREVIATIONS

APPLE	Acute patient physiology and laboratory evaluation
ICU	Intensive care unit
IMHA	Immune-mediated hemolytic anemia
MA	Maximum amplitude
MAP	Mean arterial blood pressure
MFI	Median fluorescence intensity
MODS	Multiple organ dysfunction syndrome
SIRS	Systemic inflammatory response syndrome
SPI2	Revised survival prediction index score
SpO$_2$	Oxygen saturation as measured by pulse oximetry

leukocytosis, which suggests an association between the inflammatory response and hypercoagulability. Given that activated platelets strongly contribute to inflammation, thrombosis, sepsis, MODS, and disseminated intravascular coagulation in a variety of diseases and syndromes, excessive platelet activation may be a consistent finding in critically ill dogs, and therapeutic options to control platelet activation could impact morbidity and mortality rates of these dogs.

Flow cytometry with a variety of fluorescent-labeled antibodies has been used to measure platelet activation in plasma or whole blood of dogs.[1-7,22-24] Studies on the use of flow cytometry have focused on specific disease states or conditions, such as Scott syndrome,[22] IMHA,[7,8] endocardiosis,[23] inflammation, or sepsis,[4] or on racing Greyhounds after exercise.[25] Of these, only the study[4] of dogs with SIRS and sepsis had a study population that contained some critically ill dogs. In humans, flow cytometry has been used to detect excessive platelet activation in critically ill patients with coronary artery disease,[26] congestive heart failure,[27] sepsis,[13] and MODS.[10,11,28] Similarly, studies[16,17] of humans have used flow cytometry to confirm platelet microparticle release in diseases or conditions such as neoplasia, acute coronary syndrome, immune thrombocytopenic purpura, multiple sclerosis, diabetes mellitus, uremia, and systemic lupus erythematosus, which supports a major role of platelets in critical illness.

In contrast to conventional plasma-based coagulation assays, thromboelastography is a whole blood assay that evaluates the dynamic process of coagulation in real time, from the initiation of coagulation through clot formation and fibrinolysis.[20] Thromboelastography determines clot strength, speed of clot formation, and rate of clot dissolution through fibrinolysis.[29] Thromboelastography has been used in veterinary medicine to confirm hypercoagulability in diseases and conditions, such as IMHA,[30,31] immune-mediated thrombocytopenia,[32] neoplasia,[33] parvovirus infection,[5] congenital portosystemic shunts,[34] hyperadrenocorticism,[35] protein-losing nephropathy,[36] protein-losing enteropathy,[37,38] and disseminated intravascular coagulation.[39] Currently, the authors are not aware of any direct evidence in the veterinary literature that conclusively provides a link between hypercoagulable states identified with thromboelastography and increased platelet activation in naturally occurring disease states. Given the relationship between inflammation and coagulation, it is possible that dogs with SIRS or MODS may have a higher degree of platelet activation and hypercoagulability.

Diagnosis-independent scoring systems (SPI2[40] and APPLE[41]) have been developed that can be used to help clinicians predict survival outcome in critically ill dogs. The SPI2 predicts survival outcome in critically ill dogs through multivariate regression of MAP, respiratory rate, creatinine concentration, albumin concentration, age, PCV, and reason for admission (medical vs surgical treatment).[40] The APPLE scoring system predicts survival outcome by assigning scores to creatinine concentration, WBC count, albumin concentration, Spo_2, total bilirubin concentration, mental status, age, respiratory rate, presence or absence of free fluid (in the abdomen, thorax, or pericardium), and lactate concentration. Increases in APPLE scores correlate with a higher probability of death.[41]

Determining a positive relationship between platelet activation and hypercoagulability would improve the understanding of hemostatic changes in critically ill dogs and help guide targeted treatment for such dogs. The purpose of the study reported here was to use the SPI2 and APPLE as well as scoring systems for the measurement of SIRS and MODS to compare severity of illness, platelet activation, and hypercoagulability in a population of critically ill dogs. We hypothesized that critically ill dogs would have a greater degree of platelet activation (as measured by use of flow cytometry), compared with results for healthy dogs, and that critically ill dogs would have evidence of hypercoagulability (as assessed by means of thromboelastography). We also hypothesized that the degree of platelet activation would correlate with disease severity, with the greatest platelet activation in dogs with SIRS, MODS, or illness severity scores suggestive of more profound illness.

Materials and Methods

Animals

Critically ill dogs admitted to the Tufts University Cummings School of Veterinary Medicine ICU between September and December 2010 and from March through May 2011 were considered eligible for inclusion. Dogs were enrolled if they had metabolic disease (diabetes mellitus or hyperadrenocorticism), hepatic or renal disease, immune-mediated disease (IMHA or immune-mediated thrombocytopenia), respiratory disease (pneumonia or pleural space disease), cardiovascular disease (valvular disease, cardiomyopathy, or heart failure), sepsis, or severe trauma. Exclusion criteria included age < 1 year, treatment with clopidogrel[a] or aspirin within 7 days before or after admission, treatment with heparin within 30 days before admission, treatment with corticosteroids within 48 hours before or after admission, blood transfusion within 7 days before or after admission, admission to the ICU strictly to enable observation for seizures, and admission to the ICU as a result of a lack of cage space in hospital wards. Twenty-four healthy (control) dogs of various ages and breeds, without a history of underlying illness or injury, were recruited for the study. Control dogs were owned by faculty, staff, and veterinary students. Control dogs were not age-matched to the critically ill dogs. Client consent was obtained for all dogs enrolled in the study. The study was approved by the Tufts Cummings School of Veterinary Medicine Clinical Sciences Review Committee.

Assessment procedures

Within 24 hours after critically ill dogs were admitted to the ICU, they were stratified into groups ac-

cording to severity of disease on the basis of the SPI2 and APPLE score.[40,41] The SPI2 was calculated on the basis of MAP, respiratory rate, serum creatinine concentration, serum albumin concentration, PCV, age, and reason for admission (medical treatment [score, 1] vs surgical treatment [score, 0]). The SPI2 was calculated as logit P and then solved for P, which represented the patient's probability of survival.[40] The APPLE score was calculated on the basis of lactate concentration, creatinine concentration, albumin concentration, total bilirubin concentration, total WBC count, SpO_2, age, mentation score, fluid score, and respiratory rate. Each of these variables was assigned a numeric score; maximum total potential score was 80, with higher scores representing a higher risk of death.[41]

Scores for SIRS and MODS were recorded during the first 24 hours of hospitalization. The SIRS score was calculated by assigning a score of 1 for each of the following categories, if present: rectal temperature < 37.51°C or > 39.17°C, heart rate > 140 beats/min, respiratory rate > 40 breaths/min, or WBC count > 16,000 WBCs/μL or < 6,000 WBCs/μL or > 3% band cells.[42] Minimum SIRS score was 0, and maximum SIRS score was 4. Dogs with a score ≥ 2 were considered to have SIRS.[43] The MODS score was used to classify organ system dysfunction for the coagulation, respiratory, cardiovascular, renal, and hepatic systems. Coagulation dysfunction was considered to have occurred if the platelet count was < 100,000 platelets/μL or if the partial thromboplastin time or activated partial thromboplastin time was > 25% above the upper limit of the respective reference ranges. Renal dysfunction was considered to have occurred if the creatinine concentration was > 2.0 mg/dL with no evidence of pre-renal or postrenal azotemia (< 0.5 mg/dL change in subsequent creatinine concentration measured after treatment). Respiratory dysfunction was considered to have occurred if supplemental oxygen or mechanical ventilation was required during hospitalization. Cardiovascular dysfunction was considered to have occurred if a dog had evidence of congestive heart failure, had hypotension that was not responsive to fluid administration, or was hospitalized for treatment of cardiac arrhythmias. Hepatic dysfunction was considered to have occurred if the total bilirubin concentration exceeded 0.5 mg/dL.[42]

Fluid assessment and mentation scores were assigned within 24 hours after admission for use in calculation of the APPLE score.[41] Fluid assessment scores were obtained by use of ultrasonographic evaluation of the thorax and abdomen to determine the presence of peritoneal, pericardial, or pleural effusion.[44] A score of 1 was assigned to each compartment that contained free fluid. Thus, a dog with free fluid in the pleural, pericardial, and peritoneal spaces was assigned a score of 3. Mentation scores were assigned as follows: 0, normal mentation; 1, able to stand unassisted and responsive but dull; 2, able to stand with assistance and responsive but dull; 3, unable to stand and responsive but dull; and 4, unable to stand and unresponsive.

The SpO_2 was measured for each critically ill dog. The pulse oximeter probe was placed on the nonpigmented part of the upper lip of each dog, and SpO_2 was recorded for as long as the appropriate arterial waveform and heart rate were detected.

Survival to discharge from the hospital, total cost of hospitalization, and duration of hospitalization were determined through review of electronic medical records. To determine whether these data points could be used as surrogate indicators for the degree of hypercoagulability in this patient population, they were compared with results for flow cytometry and thromboelastography and other coagulation variables for dogs hospitalized in the ICU.

Vital signs (heart rate, respiratory rate, and rectal temperature) were recorded for both critically ill and healthy control dogs. Blood pressure was measured with an appropriately sized cuff via Doppler ultrasonographic or oscillometric techniques. For each measurement, systolic and diastolic measurements were obtained. The MAP was calculated as follows: MAP = Diastolic blood pressure + 1/3(systolic – diastolic blood pressure).

For most dogs, venipuncture was performed with an 18- or 20-gauge needle to enable collection of 10 mL of blood. When a blood sample was collected through a central venous catheter, the first 6 mL of blood removed from the catheter was discarded prior to collection of the 10-mL sample. Blood collection tubes were filled in the following order: 5.4 mL was placed in 2 sodium citrate tubes, 2 mL was placed in a sodium EDTA tube, and 2 mL was placed in a serum separator tube. The sodium citrate tubes were used for flow cytometry; determination of von Willebrand factor, activated protein C, and antithrombin concentrations; and measurement of partial thromboplastin time and activated partial thromboplastin time. The sodium EDTA tube was used for a CBC, platelet count, and measurement of fibrinogen concentration. The serum separator tube was used for a serum biochemical analysis.

Flow cytometry

Several flow cytometry protocols[22,24,45-49] were evaluated during the design phase of the study. Ultimately, a citrated whole blood protocol[24] was modified and used to measure platelet activation. Concentrations chosen for all antibodies and agonists were selected on the basis of reported[22,24,46,47] consistent and measurable platelet activation.

Antibody buffers were prepared in 50-mL aliquots that contained final concentrations of 10mM HEPES buffer, 0.15M NaCl, and 0.5% bovine serum albumin. Modified HEPES-Tyrode buffer was prepared in 100-mL aliquots that contained final concentrations of 0.14M NaCl, 2.7mM KCl, 1mM $MgCl_2$, 0.25M glucose, 10mM HEPES buffer, 0.5% bovine serum albumin, 0.4mM $NaHPO_4$, and 12mM $NaHCO_3$. Monoclonal mouse anti-human CD61, clone Y2/51, conjugated to allophycocyanin fluorochrome[b] was used to detect

the platelet population. The antibody used to specifically detect activated platelets through P-selectin expression was a monoclonal mouse anti-human CD62-P, clone 1E3, conjugated to phycoerythrin.[c] Negative isotype control antibodies included normal monoclonal mouse anti-human IgG_{2a} conjugated to phycoerythrin[c] and normal mouse anti-human IgG_1 conjugated to allophycocyanin.[b] Paraformaldehyde buffer (1%) was prepared by dilution of a stock solution of 30% paraformaldehyde.

Fresh aliquots of all buffers were prepared each week. Antibody buffer was mixed with antibodies and, when appropriate, ADP such that the final concentrations of the anti-CD61, CD62-P, and IgG_{2a}-phycoerythrin antibodies were 50 μg/mL and that of IgG_1-allophycocyanin was 100 μg/mL. Final concentration of ADP in the activated reaction buffers was 2, 6, and 10μM. Platelet activation in blood samples from critically ill and control dogs was measured with and without in vitro activation with 2, 6, or 10μM ADP.

Citrated whole blood samples were allowed to sit undisturbed (ie, rest) in a tube rack for 30 minutes at room temperature (approx 23°C). Modified HEPES-Tyrode buffer was used to dilute the blood to a concentration of 1:10. Then, 20 μL of diluted blood was added to tubes that contained 450 μL of modified HEPES-Tyrode buffer at room temperature. An aliquot (20 μL) of the appropriate antibodies with or without ADP buffer was added to each tube. Samples were mixed gently and incubated for 20 minutes at room temperature in the dark, which was followed by the addition of 600 μL of 1% paraformaldehyde fixation solution. Tubes were placed on ice and analyzed within 4 hours.

Flow cytometry was performed with a flow cytometer[d] calibrated to detect platelets by use of a threshold gate of 800, which effectively excluded all cells not labeled by the CD61 antibody specific to resting or activated platelets. Each run sampled 10,000 events by use of a gate that included platelets and excluded other types of cells on the basis of forward- and side-scatter characteristics. Flow cytometer software[e] measured MFI of the CD62-P–phycoerythrin signal and the percentage of total platelets expressing P-selectin (ie, percentage of cells that recorded CD62-P–phycoerythrin positive events) for cells segregated by use of calibration to detect cells that bound CD62-P–phycoerythrin antibody on activated platelet surfaces. Data analysis was performed by personnel at the University of Massachusetts Flow Cytometry Laboratory by use of the flow cytometer software.[f] Both MFI and percentage of positive CD62-P events were measured for all sample replicates of each dog.

Thromboelastography

A disposable thromboelastography cup and pin was fitted to the thromboelastograph.[g] For blood samples collected through an indwelling IV catheter, a disposable thromboelastography cup and pin system impregnated with heparinase was used. An aliquot (20 μL) of $CaCl_2$ was added to overcome effects of citrate-anticoagulated blood. After samples were allowed to sit at room temperature for 30 minutes, 1 mL of citrated whole blood was added to a kaolin hemostasis tube.[g] Tubes were inverted gently 5 times. Then, 340 μL of kaolin-activated whole blood was added to each cup containing $CaCl_2$, which initiated the reaction. A central pin was lowered into the reaction cup and held in suspension by a torsion wire. The cup was gently oscillated at an angle of 4.75° for 10-second cycles over 30 to 60 minutes. Formation of fibrin between the cup and pin generated tracings from which R (reaction time), α angle, K (clot formation time), MA, and G (global clot strength) were determined.[20] On the basis of G, dogs were classified as hypercoagulable (> 7,200 dynes/s), normocoagulable (3,200 to 7,200 dynes/s), or hypocoagulable (< 3,200 dynes/s).[39]

Statistical analysis

Statistical analysis was performed with a commercial statistical software program.[h] Values of $P \leq 0.05$ were considered significant. The Wilcoxon signed rank test was used to compare median differences of the percentage of positive events and MFI of CD62-P events between resting and ADP-activated platelets within the control and critically ill groups. The Mann-Whitney U test was used to compare results of all laboratory data, flow cytometry data, and thromboelastography data between the control and critically ill groups. The Kruskal-Wallis test was used to compare results of all laboratory data, flow cytometry data, and thromboelastography data between critically ill dogs stratified into groups on the basis of SPI2, APPLE score, SIRS score, MODS score, reason for admission (medical treatment vs surgical treatment), duration of hospitalization, or cost of hospitalization. Dogs were stratified into subgroups on the basis of the interquartile range distribution for each measure. A Spearman correlation test was used to correlate SPI2, APPLE score, cost of hospitalization, and duration of hospitalization with the degree of platelet activation and to correlate thromboelastography results with the degree of platelet activation. Sample size calculations based on results of a 2006 study[7] indicated that 80 dogs would provide adequate statistical power (α = 0.05 and power > 80%) to reject the null hypothesis.

Results

Eighty-two critically ill dogs were enrolled in the study. The 82 dogs represented 39 breeds, including 16 mixed-breed dogs, 7 Golden Retrievers, 4 Standard Poodles, 4 German Shepherd Dogs, 3 Doberman Pinschers, 3 Pugs, 3 Mastiffs, 3 English Bulldogs, 2 Dachshunds, 2 Boxers, 2 Beagles, 2 Labrador Retrievers, 2 English Sheepdogs, 2 German Shorthaired Pointers, 2 West Highland White Terriers, and 2 American Pit Bull Terriers; 23 other breeds were represented by 1 dog each. There were 37 spayed females, 4 sexually intact females, 32 castrated males, and 9 sexually intact males. Mean age was 7.86 years. The 24 control dogs represented 12 breeds, including 8 mixed-breed

dogs, 4 Golden Retrievers, 2 Labrador Retrievers, and 2 American Pit Bull Terriers; 8 breeds were represented by 1 dog each. There were 12 spayed females, 3 sexually intact females, 7 castrated males, and 2 sexually intact males. Mean age was 5.54 years. There was no significant difference in age between healthy control and critically ill dogs.

Of the 82 critically ill dogs, 53 were hospitalized in the ICU for medical treatment and 29 were hospitalized for surgical treatment. Of the 29 dogs hospitalized for surgical conditions, 10 did not undergo surgery because of client financial constraints or a poor prognosis. Overall, 21 dogs were hospitalized because of known or suspected neoplasia that resulted in critical illness. Within the medical treatment group, cardiac disease (n = 11), renal disease (6), neurologic disease (6), hepatic disease (5), pneumonia (5), pulmonary hypertension (3), immune-mediated disease (2), hypoadrenocorticism (2), and pancreatitis (2) were the most common causes for hospitalization. Two dogs within the medical treatment group had lethargy and fever of unknown origin for which no cause was determined. Within the surgical treatment group, neoplasia (n = 6), sepsis (5), trauma (4), nontraumatic hemoperitoneum (4), gastric-dilatation or gastric dilatation-volvulus syndrome (3), and lung lobe torsion (2) were the most common reasons for surgery.

Overall, 61 (74.4%) dogs survived to discharge from the hospital. Seventeen (20.7%) dogs were euthanized during the course of hospitalization because of a poor prognosis or acute decline in condition during hospitalization. No dogs were euthanized strictly because of the cost of care. Four (4.9%) dogs died during hospitalization as a result of severity of illness. Two dogs that survived to discharge were euthanized

within 2 weeks after leaving the ICU. Dogs were hospitalized in the ICU for a median of 3 days (range, 1 to 11 days) and at a median cost of $2,751 (range, $357 to $12,243).

Dogs were scored for SIRS and MODS on a scale of 0 to 4, as described previously. Forty-one dogs had a SIRS score $\geq$ 2 (score of 2 for 30 dogs and score of 3 for 11 dogs); thus, SIRS was diagnosed. Eight dogs were excluded from SIRS scoring because they lacked a laboratory WBC count. The most common diseases or conditions associated with SIRS included confirmed or suspected sepsis (n = 11), neoplasia (10), congestive heart failure (3), acute kidney injury (3), dilated cardiomyopathy (2), hypoadrenocorticism (2), and liver failure (2). Most dogs did not have evidence of MODS. Thirty-three dogs had no evidence of organ dysfunction, and 32 dogs had dysfunction in only 1 organ system. Eleven dogs had dysfunction in 2 or 3 organ systems; thus MODS was diagnosed. Diseases or conditions for the 11 dogs with MODS included pericardial effusion with or without a visible cardiac mass (n = 3), acute kidney injury (2), metastatic neoplasia (2), IMHA (1), congestive heart failure (1), pulmonary hypertension (1), and pyothorax (1).

Macrothromboembolic disease was detected in 3 dogs during surgery, diagnostic imaging, or necropsy. One dog had a large mass in the left atrium and fully occlusive thrombus at the bifurcation of the abdominal aorta and femoral arteries and was euthanized shortly after emergency admission. This dog had the greatest degree of platelet activation in a resting sample as measured with flow cytometry. The other 2 dogs had visible thrombi that were detected during surgery: one dog had small thrombi throughout the pleural cavity attributable to pyothorax, and the other had multiple thrombi throughout the spleen secondary to hemangiosarcoma.

Table 1—Median (range) thromboelastography values for critically ill dogs and healthy control dogs.

| Variable | Critically ill dogs | | Control dogs | | |
	n	Median (range)	n	Median (range)	P value*
K (min)	80	1.85 (0.80–17.90)	24	2.60 (1.80–4.80)	< 0.001
R (min)	82	5.50 (2.40–9.90)	24	4.85 (2.70–10.30)	0.164
α angle (°)	82	63.95 (19.30–82.50)	24	57.85 (38.70–65.30)	0.002
MA (mm)	82	64.85 (12.50–86.90)	24	54.05 (47.90–61.40)	< 0.001
G (dynes/s)	82	9,228.6 (711.2–33,140.6)	24	5,888.1 (0–7,966.0)	< 0.001

*Values were considered significant at $P \leq 0.05$.
G = Global clot strength. K = Clot formation time. R = Reaction time.

Table 2—Median (range) flow cytometry values for blood samples from critically ill dogs and healthy control dogs.

| | Positive events (%) | | | | | MFI (arbitrary units) | | | | |
| | Critically ill dogs | | Control dogs | | | Critically ill dogs | | Control dogs | | |
Variable	n	Median (range)	n	Median (range)	P value*	n	Median (range)	n	Median (range)	P value*
Resting†	78	0.57 (0.0–7.65)	24	0.30 (0.0–5.38)	0.230	78	306 (121–975,000)	24	523 (130–5,654)	0.723
2μM ADP	76	5.76 (0–81.10)	24	3.20 (0–14.70)	< 0.001	76	916 (123–70,700)	24	508 (0–21,500)	< 0.001
6μM ADP	78	21.05 (0.01–91.30)	24	15.60 (0.0–42.5)	0.028	78	1,818 (110–23,800)	24	782 (258–22,700)	< 0.001
10μM ADP	82	51.90 (0–91.20)	17	28.70 (0.34–50.90)	0.263	72	2,256 (187–31,700)	21	1,080 (426–3,733)	< 0.001

†Represents unstimulated whole blood.
***See** Table 1 for remainder of key.

Thromboelastography classified dogs as hypercoagulable (n = 56), normocoagulable (19), and hypocoagulable (7), as assessed on the basis of G. The hypercoagulable group consisted of dogs with sepsis (n = 9), acute kidney injury (5), pulmonary hypertension (3), and pancreatitis (2), whereas none of the normocoagulable or hypocoagulable dogs had these diseases or conditions. Pneumonia, trauma, and congestive heart failure were represented most frequently among hypercoagulable and normocoagulable dogs, whereas cardiac disease, nontraumatic hemoperitoneum, and neoplasia were detected in all dogs regardless of coagulation status. These groups were not of sufficient size to allow statistical comparisons.

Critically ill dogs had significant increases in MA, α angle, and G and significantly lower K, compared with values for control dogs. In addition, R was prolonged in critically ill dogs, but not significantly, compared with the result for control dogs **(Table 1)**. There were no differences in thromboelastographic variables among critically ill dogs when subclassified on the basis of SIRS scores, MODS scores, reason for admission (medical treatment vs surgical treatment), or cost or duration of hospitalization.

Critically ill dogs had significant increases in platelet reactivity to ADP, as measured by both the percentage of positive events and MFI for the CD62-P (P-selectin) antibody signal, compared with results for control dogs. There were no significant differences between the proportion of circulating activated platelets in critically ill dogs, compared with that of healthy control dogs, in samples that were not stimulated with ADP, although critically ill dogs had both a higher percentage of positive events and greater MFI for the P-selectin signal. Critically ill dogs had significant increases in platelet activation when whole blood samples were stimulated with 2, 6, or 10μM ADP for measurements of MFI or percentage of positive events **(Table 2)**. There were no differences in P-selectin expression in resting samples or samples stimulated with ADP among critically ill dogs classified on the basis of SIRS scores, MODS scores, or reason for admission (medical treatment vs surgical treatment).

Flow cytometry data were visually examined **(Figure 1)**. No significant correlations were identified between MA or G and any measure of platelet activation assessed by use of flow cytometry.

Expression of P-selectin was compared between control dogs and critically ill dogs identified on the basis of G as hypercoagulable (n = 52), normocoagulable (20), or hypocoagulable (7).

Dogs identified as hypercoagulable and normocoagulable had significant increases in percentage of positive events when samples were stimulated with 2, 6, or 10μM ADP but did not differ in percentage of positive events or MFI for resting (unstimulated) samples. There were no significant differences in percentage of positive events or MFI between these hypercoagulable and normocoagulable dogs. No statistical comparisons were performed for hypocoagulable dogs because of the small sample size of this group.

When critically ill dogs were stratified into subgroups on the basis of SPI2, APPLE score, SIRS score, or MODS score or by cost or duration of hospitalization, dogs stratified by APPLE score had differences in the percentage of positive events for blood stimulated with 10μM ADP. Correlation analysis revealed a weak but significant negative relationship ($R = -0.347$; $P = 0.012$) between APPLE score and platelet activity for blood stimulated with 10μM ADP. When stratified into groups on the basis of interquartile range for APPLE scores, dogs with the lowest APPLE scores (< 22) had significantly greater platelet activation for blood stim-
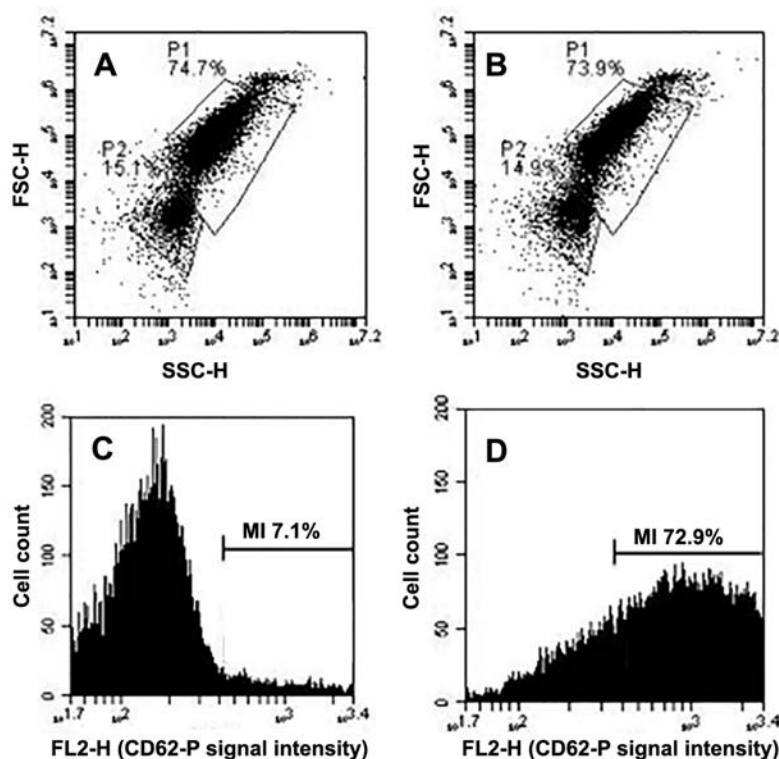

Figure 1—Representative flow cytometry output for a critically ill dog. A—Scatterplot of a resting (nonstimulated) whole blood sample. On the basis of forward-scatter (FSC-H) and side-scatter (SSC-H) properties, the majority of platelets are in gate P1. B—Same scatterplot as panel A after the whole blood sample was stimulated with 10μM ADP. The MFI for CD62-P–phycoerythrin is determined from platelets within gate P1. C—Histogram for a resting whole blood sample that indicates counts of activated platelets detected at the FL2-H sensor, which detects the phycoerythrin fluorochrome tagged to the CD62-P antibody that binds to activated platelets. Notice the gated area in which activated platelets would be detected (M1), which is detecting 7.1% of total activated platelets. D—Same histogram as in panel C after the whole blood sample was stimulated with 10μM ADP. Notice the profound increase in the percentage of activated platelets (72.9%) within the M1 gate.

Table 3—Median (range) results of coagulation panel testing for critically ill dogs and healthy control dogs.

| Variable | Critically ill dogs | | Control dogs | | |
	n	Median (range)	n	Median (range)	P value*
Fibrinogen (mg/dL)	36	374 (60–773)	13	164 (126–220)	< 0.001
Prothrombin time (s)	37	8.2 (6.3–11.8)	14	7.5 (6.5–8.7)	0.029
Activated partial thromboplastin time (s)	36	15.4 (9.9–31.5)	14	12.7 (11.0–15.1)	0.007
Antithrombin (%)	37	94.6 (44.5–138.0)	17	124.0 (68.3–139.0)	0.003
von Willebrand factor (%)	36	76.4 (9.2–139.0)	14	90.4 (72.0–132.0)	0.070
Activated protein C (%)	33	100.0 (53.4–183.0)	7	91.4 (36.6–121.0)	0.293

See Table 1 for key.

ulated with 10μM AD, compared with results for all other subgroups. No other flow cytometry or thromboelastography variables were correlated with any measure of illness severity.

Coagulation variables, including fibrinogen concentration, partial thromboplastin time, activated partial thromboplastin time, von Willebrand factor concentration, antithrombin concentration, and activated protein C concentration, were compared between critically ill dogs and healthy control dogs. Critically ill dogs had a significantly higher fibrinogen concentration, prolonged partial thromboplastin time, and activated partial thromboplastin time and a significantly decreased percentage of measurable antithrombin, compared with results for control dogs **(Table 3)**. No differences were found between critically ill dogs stratified on the basis of SPI2, APPLE score, SIRS score, or MODS score or by cost or duration of hospitalization. No correlations were found between coagulation variables and platelet activation or hypercoagulability.

Discussion

The present study provided evidence that platelets in healthy dogs are inherently more resistant to ADP-dependent activation than are platelets from critically ill dogs. This evidence supports the notion that measuring platelet reactivity to exogenous agonists may provide a more reliable assessment of the overall functional status of circulating platelets in sick dogs than analysis of platelet activation in nonstimulated samples. Platelets that are activated in vivo may be rapidly removed from the circulation and may not reflect the true activation status of circulating platelets in sick animals.[50] Activated platelets can rapidly lose surface expression of P-selectin but continue to circulate and function in platelet aggregation, fibrinogen binding, provision of a procoagulant surface, and shedding of procoagulant microparticles.[51] Therefore, addition of a platelet agonist in vitro may be useful in determining platelet hyperreactivity and may be considered a useful indicator of the capacity of circulating platelets to contribute to the prothrombotic state often encountered in patient populations such as dogs hospitalized in an ICU.

P-selectin was used as the marker of platelet activation in this population of dogs. The P-selectin expression for healthy control dogs, as measured by percentage of positive CD62-P events, was similar to that described in veterinary studies[8,22,24,46] that involved use of a whole blood flow cytometry protocol. However, results for maximal median P-selectin expression after activation with 10μM ADP differed from those of 2 other studies[24,46] in which investigators used ADP as a platelet agonist for flow cytometry. Within the study population reported here, there was some variation among subjects with regard to robustness of ADP-dependent platelet activation. The disparity seen with ADP-dependent platelet activation in dogs is likely multifactorial and may be attributable to variation within individuals and study populations or subtle differences in protocols among laboratories. Historically, ADP has been considered a weaker platelet agonist in aggregation evaluations as a result of variable individual responses and inconsistency when used in whole blood assays.[7,50] Agonists such as collagen, thrombin, platelet activating factor, and phorbol myristate acetate are viewed as stronger platelet activators,[50] and some of them have been successfully used as agonists in other veterinary studies.[7,8,24,25,46,47] Future studies that involve more potent agonists may be useful for examining platelet activation in disease states.

Most dogs admitted to the ICU in the present study were hypercoagulable as determined on the basis of G, K, MA, and α angle. These results are similar to findings from previous studies in which investigator found a hypercoagulable state in dogs with severe diseases and conditions such as IMHA,[30,31] immune-mediated thrombocytopenia,[32] protein-losing enteropathy,[37] protein-losing nephropathy,[36] metastatic neoplasia,[33] and disseminated intravascular coagulation.[39] However, no significant correlation was detected between the degree of ADP-stimulated platelet activation and the thromboelastography variables MA or G. It is possible that ADP, as a weak agonist, did not provide a strong enough stimulus to enable effective measurement of platelet hyperreactivity when used at lower concentrations. The MA may also be affected by factors other than platelet function, such as fibrinogen concentration. In the present study, fibrinogen concentration was significantly higher in critically ill dogs and may have contributed to clot strength and therefore the increased MA detected in this group. Fi-

nally, anemia can result in an artifactual increase in MA. Some dogs in this study developed anemia, which may have contributed to the lack of correlation between thromboelastography variables (eg, MA) and ADP-dependent platelet activation.

Neither the SPI2 nor APPLE scoring systems were predictive of the proportion of activated platelets or hypercoagulability in dogs in the study reported here. Several factors may explain this finding. First, a minimum SPI2 or APPLE score was not required for study inclusion. Incorporation of a minimum APPLE or SIRS score into the inclusion criteria for enrollment would have narrowed the population to sicker dogs and may have increased the likelihood of detecting changes in hemostasis or platelet activation in the patient population. Additionally, at least 20% of the patient population did not qualify for scoring because they did not have results for the appropriate laboratory tests within 24 hours after admission. As a result, the relationship between these scores and platelet activation did not reflect the entire population. Finally, it is possible that both the SIRS and APPLE scoring systems were inappropriate for use in predicting hypercoagulability in critically ill dogs. Both systems were developed for use in predicting survival to discharge on a population basis and were not intended for use in predicting outcome for individual animals or predicting the amount of hypercoagulability or platelet activation. Future studies could focus on developing a diagnosis-specific scoring system for predicting hypercoagulability and outcome, much as the International Renal Interest Society scoring system is used to assess renal disease outcome.[52] To the authors' knowledge, there currently are no scoring systems in human or veterinary medicine designed specifically for assessing platelet activation or hypercoagulability with clinical outcome.

In the present study, we did not detect a relationship between cost or duration of hospitalization and any measures of platelet activation or hypercoagulability as measured by use of flow cytometry or thromboelastography. Similarly, cost or duration of hospitalization did not correlate with any of the 4 calculable measures of illness severity (SIRS score, MODS score, SPI2, or APPLE score).

The increased fibrinogen concentration, decreased antithrombin concentration, and prolonged partial thromboplastin and activated partial thromboplastin times for critically ill dogs in the present study were within laboratory reference ranges and therefore probably were not clinically relevant. The SIRS and MODS scores also had no relationship to the proportion of activated platelets or degree of hypercoagulability as measured by use of flow cytometry or thromboelastography. Only 41 (50%) dogs of the study population had SIRS, whereas 11 (13%) dogs had MODS. Future studies could focus on screening dogs by use of SIRS or MODS scoring before enrollment. By selecting for dogs with SIRS or MODS, a uniform population of sicker dogs could be evaluated.

The exclusion of dogs that recently received corticosteroids, NSAIDs, or blood products was a major limiting factor for this study. Many dogs with immune-mediated diseases such as IMHA, immune-mediated thrombocytopenia, or inflammatory bowel disease were excluded because of prior corticosteroid administration, despite evidence that they were severely ill. A number of dogs with severe trauma were excluded because the dogs had received NSAIDs prior to referral. Also, severely and acutely hypovolemic dogs that required blood transfusions at the time of admission to our emergency department often received blood products before samples could be collected for flow cytometry or thromboelastography. As a result, the opportunity was lost to include these critically ill dogs that were subsequently admitted to the ICU during the study period.

Although P-selectin is a robust marker for platelet activation, its use as a singular marker in the present study may have limited the ability to detect the true spectrum of activated platelets in critically ill dogs. It has been suggested that P-selectin–positive platelets may be buried in platelet-leukocyte aggregates and may not be detectable with standard flow cytometry assays that use CD62-P as the only marker for platelet activation.[46] Therefore, in vivo increases in platelet activation for nonstimulated blood samples cannot be ruled out despite the lack of significant effects for P-selectin in the study reported here. Platelet-leukocyte aggregates are considered a sensitive marker of platelet activation in humans and have been used in several veterinary studies[8,46] to measure platelet activation in dogs. Platelet microparticles are readily shed from the lipid membrane of activated platelets and contain many of the surface elements necessary for leukocyte activation, platelet activation, and support of coagulation.[16] Platelet microparticles are frequently measured in humans[16] and have been investigated in at least 3 veterinary studies[8,25,46] regarding platelet activation. Additionally, mean platelet concentration, an automated measure of platelet refractive index, has been indicated as a possible method of measuring platelet activation.[50] As platelets activate and degranulate, mean platelet concentration should theoretically decrease. Mean platelet concentration has been associated with platelet activation during nonseptic inflammation in dogs.[47] Future studies should focus on the use of these markers in combination with P-selectin expression to further define the extent of platelet activation in critically ill dogs.

In the present study, a large population of critically ill dogs had increased platelet activation in blood samples in response to stimulation with ADP as measured by P-selectin expression. The same population had evidence of hypercoagulability as measured by use of thromboelastography. Analysis of these results suggested that critically ill dogs have hyperreactive platelets, which may in part contribute to a prothrombotic state. Overall, the severity of illness scoring systems tested (including APPLE score, SPI2, SIRS score,

and MODS score) or cost or duration of hospitalization did not strongly correlate with the proportion of activated platelets or degree of hypercoagulability in this patient population.

Acknowledgments

Footnotes

a. Plavix, Bristol-Myers Squibb, New York, NY.
b. Dako, Glostrup, Denmark.
c. Santa Cruz Biotechnology, Santa Cruz, Calif.
d. Accuri C6 flow cytometer system, BD Accuri Cytometers, Grand Rapids, Mich.
e. C-Flow, BD Accuri Cytometers, Grand Rapids, Mich.
f. FloJo flow cytometry analysis software, version 7.6.4, FlowJo LLC, Ashland, Ore.
g. TEG, Haemoscope, Niles, Ill.
h. SPSS, version 17.0, SPSS, Chicago, Ill.

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