Flow cytometric detection and procoagulant activity of circulating canine platelet-derived microparticles

Sarah E. Helmond, BVSC; James L. Catalfamo, PhD; Marjory B. Brooks, DVM

Objective—To measure platelet membrane–derived microparticle (PMP) content and thrombin-generating capacity of canine plasma subjected to specific processing and storage conditions.

Animals—31 clinically normal dogs (19 males and 12 females).

Procedures—Citrate-anticoagulated blood samples obtained from each dog were centrifuged at 2,500 X g to isolate platelet-poor plasma (PPP), then PPP was centrifuged at 21,000 X g to isolate microparticle-free plasma (MPF) and microparticle-enriched plasma (MPEP). Whole blood and paired samples of fresh and frozen-thawed PPP, MPF, and MPEP were dual labeled for flow cytometric detection of membrane CD61 (constitutive platelet antigen) and annexin V (indicating phosphatidylserine externalization). Platelets and PMPs were enumerated with fluorescent, size-calibrated beads. Thrombin generation in fresh and frozen-thawed PPP, MPF, and MPEP was measured via kinetic fluorometric assays configured with low tissue factor and low phospholipid concentrations.

Results—Initial centrifugation yielded PPP with <0.5% the platelets of whole blood, with median counts of 413 PMPs/µL for males and 711 PMPs/µL for females. Sequential centrifugation resulted in a 10-fold concentration of PMPs in MPEP and virtually depleted PMPs from MPF. Thrombin generation depended on PMP content, with median endogenous thrombin potential of 0, 893, and 3,650 nmol•min for MPF, PPP, and MPEP, respectively. Freeze-thaw cycling caused significant increases in PMP counts and phosphatidylserine externalization.

Conclusions and Clinical Relevance—Canine PMPs were major determinants of thrombin-generating capacity; preanalytic variables influenced plasma PMP content. Processing conditions described here may provide a basis for characterization of PMPs in clinical studies of thrombosis in dogs. (Am J Vet Res 2013;74:207–215)
Accurate quantitation and characterization of microparticles are technically challenging because of their small size. In particular, the optimal method to isolate and enumerate PMPs remains a subject of debate.17–20 Preanalytic variables, including blood collection technique, centrifugation conditions, washing steps, and sample storage time and temperature, all influence the PMP content of plasma samples.4,20,21 Flow cytometry is the technique most commonly used to study PMPs; however, sensitivity varies among instruments, and there currently are inherent limitations in the ability of many first-generation cytometers to resolve the size or membrane antigens of the smallest particles.19,21

Functional assays that measure plasma procoagulant activity provide an alternate approach to assess the biological relevance of circulating microparticles in various clinical populations. Commercial reagents and assay protocols have recently become available to measure the in vitro thrombin-forming capacity of plasma samples on the basis of cleavage of a fluorogenic thrombin substrate.22–24 Thrombin-generation assays are configured with tissue factor and phospholipid reagents that trigger activation of coagulation factors and subsequent production of thrombin in re-calified plasma. The resultant graph of the rate and extent of thrombin formation over time is referred to as a thrombogram. Variables measured in TGAs include the lag time for initial thrombin formation, the maximum concentration of thrombin generated, and a summation of the overall thrombin-generating capacity (referred to as ETP, which is the area under the thrombogram). The presence of activated platelets and PMPs in plasma influences thrombogram variables by providing a catalytic surface for assembly of the tenase and prothrombinase coagulation complexes that promote rapid formation of large amounts of thrombin. The use of reagents with a low phospholipid concentration increases thrombogram sensitivity to the activated platelet or PMP content of test plasmas.

Because of the importance of preanalytic and analytic variables, clinical application and interpretation of PMP counts and thrombogram variables require detailed information on sample collection and assay methods. A relative increase in PMPs has been described in dogs with immune-mediated thrombocytopenia,23 and a decrease in PMPs has been reported in dogs with hereditary platelet procoagulant deficiency.26–28, however, to the authors' knowledge, no studies have been conducted to examine via both quantitative and functional assays the circulating PMP in plasma obtained from healthy dogs. The purpose of the study reported here was to evaluate a protocol for blood collection, processing, and flow cytometric enumeration of PMPs in canine plasma, relate PMP content to endogenous thrombin-generating capacity, characterize the range of plasma PMPs and thrombogram variables in healthy dogs, and compare the number of PMPs and thrombin generation in fresh and frozen plasma samples. We hypothesized that condition and proportion of PMPs that expressed membrane phosphatidylserine in plasma fractions would influence thrombin-generating capacity.

**Materials and Methods**

**Animals**—Healthy 1- to 12-year-old dogs owned by staff and students at the Cornell University College of Veterinary Medicine were included in the study. Dogs were enrolled in the study on the basis of results of a preliminary physical examination, CBC, serum biochemical analysis, and coagulation panel consisting of activated partial thromboplastin time, prothrombin time, fibrinogen concentration, antithrombin activity, protein C activity, and D-dimer concentration. Informed consent was obtained from owners for use of their dogs. The study was conducted in compliance with a protocol approved by an institutional animal care and use committee.

**Blood collection and processing**—Blood was collected by careful cephalic venipuncture with 21-gauge, 1.5-inch tubing butterfly catheters and gentle manual venous occlusion for minimal stasis. Blood samples (6 mL) anticoagulated with EDTA were collected directly into evacuated tubes, and citrate-anticoagulated blood samples (8 mL) were collected directly into plastic syringes containing one-tenth volume of 3.8% sodium citrate. Citrated blood samples were stored at room temperature (approx 21°C) until processing, with all fractionation for storage and initial analyses completed within 3 hours after collection. Blood samples collected into EDTA-containing tubes were submitted for automated CBC determination. An aliquot (50 µL) of citrated whole blood was retained for flow cytometry evaluations, and the remainder of each sample was processed through sequential centrifugation steps to prepare PPP, MPF, and MPEP. The first centrifugation was 2,500 × g at room temperature for 20 minutes. The supernatant PPP was carefully harvested such that a 1-cm layer was allowed to remain above the pelleted packed cells. An aliquot (300 µL) of PPP was submitted for coagulation assays, a second aliquot (500 µL) was used in flow cytometry and TGAs, and a third aliquot (200 µL) was stored frozen at –70°C for later analysis. The remaining PPP (2 mL) was divided equally into two 1.5-ml tubes and centrifuged at 4°C for 20 minutes at 21,000 × g, and the supernatant MPF was carefully harvested to leave the pelleted microparticles undisturbed. The pellets were then resuspended in a standard volume (400 µL) of autologous MPF to make MPEP. One set of MPF and MPEP samples (1,600 and 200 µL/sample, respectively) was analyzed immediately, and the second set was stored frozen at ~70°C for up to 6 months before analysis. The frozen aliquots of PPP, MPF, and MPEP were thawed once in a 37°C water bath and placed on wet ice until used for flow cytometry or TGA.

**TGA**—The TGAs were performed with a single lot of a commercially available kit; assays were conducted in accordance with the manufacturer’s instructions. The thrombin calibrators and test samples were assayed in duplicate in wells of a microtiter plate. Each well contained 40 µL of sample (PPP, MPF, or MPEP) or calibrator. Test samples were analyzed after the addition of a proprietary trigger reagent that contained a low concentration of phospholipid micelles and approximately
2pM recombinant human tissue factor in combination with a fluorogenic substrate–calcium solution. Fluorescence intensity was recorded with a spectrophotometer at 1-minute intervals for 120 minutes, with excitation and emission wavelengths of 360 and 460 nm, respectively. Thrombin concentration was calculated with a thrombin standard and the manufacturer's software application of the kit.

Sample labeling for flow cytometry—Platelets and PMPs were identified on the basis of forward- and side-scatter properties and expression of the constitutive platelet membrane antigen CD61 (glycoprotein IIIa). Samples were double-labeled with annexin V to detect membrane phosphatidylserine exposure. All labeling reactions were performed in 5-mL polystyrene tubes in a labeling buffer containing final concentrations of 2mM calcium and 0.4mM gly-pro-arg-pro-NH₂. The total reaction volume (100 µL) consisted of 2 µL of sample (whole blood and MPEP) or 5 µL of sample (PPP and MPF) and 10 µL of each fluorescent label (1:50 dilution of stock CD61-phycoerythrin and 1:30 dilution of stock annexin V–fluorescein isothiocyanate). After incubation in the dark for 15 minutes, the re-

---

Figure 1—Flow cytometry gates and threshold boundaries for characterizing platelets and platelet-derived microparticles obtained from healthy dogs. A—Dot plot for a citrated whole blood sample containing fluorescent 7.7-µm counting beads. Data were acquired for each sample to obtain 2,500 events within the counting bead gate. The fluorescent beads within their bead gate are delineated (oval). Notice the RBC events (at 10⁷ on the forward-scatter axis; arrow) and platelet events (at 10⁵ on the forward-scatter axis; arrowhead). B—Forward-scatter distribution histogram of 0.9-µm beads. A forward-scatter boundary that encloses 100% of the bead events used to define the forward-scatter upper limit of the microparticle gate and lower limit of the platelet gate is indicated (horizontal bar). C—Dot plot of a citrated whole blood sample with microparticle and platelet gates. The forward scatter of 0.9-µm beads is used to delimit the x-axis boundary between the microparticle and platelet gates. The forward scatter of the RBC population defines the platelet gate upper x-axis boundary, and fluorescence of a CD61-phycocerythrin–conjugated antibody (CD61-PE) defines the y-axis boundary. D—Dual fluorescence analysis dot plot of the microparticle gate. Events in quadrant 1 (Q1) and quadrant 2 (Q2) are positive results for the CD61 marker, which represents PMPs. The microparticles in Q2 also bind annexin-V, which indicates phosphatidylserine expression, whereas quadrant 3 (Q3) contains nonplatelet microparticles that express phosphatidylserine. Events in quadrant 4 (Q4) represent debris, machine noise, and microparticles that did not bind either fluorescent label. The number below each quadrant label is the percentage of total events within that microparticle gate. AnnV = Annexin V. FITC = Fluorescein isothiocyanate. FL1-H = Fluorescence intensity height of signal. FSC-H = Forward-scatter height of signal. MP = Microparticle. PE = Phycoerythrin.
actions were quench diluted by the addition of 1.5 mL (whole blood and MPEP) or 0.5 mL (PPP and MPF) of labeling buffer. A higher volume of buffer was used for whole blood and MPEP in an attempt to reduce coincidence counting. To express platelet and microparticle counts in absolute numbers, fluorescent counting beads\textsuperscript{c} with an established concentration of 51,118 \times 10\textsuperscript{3} particles/µL were added to each tube (ratio of 30 µL of beads to 0.5 mL of quench-dilute buffer).

Flow cytometry data collection and analysis—Samples were analyzed via a cytometer\textsuperscript{d} with the manufacturer’s software\textsuperscript{a} and a calibration bead set for daily standardization of forward- and side-scatter and fluorescence parameters. Data were acquired with logarithmic gain settings; after initial optimization and compensation, the same settings were used throughout the study. An acquisition gate for each sample was set to collect 2,500 counting beads on the basis of their forward-scatter and fluorescence properties (Figure 1).

A forward-scatter upper threshold for microparticles was defined by a histogram display of fluorescent bead counts in absolute numbers, fluorescent counting incidence counting. To express platelet and microparticle counts in absolute numbers, fluorescent counting events less than the 0.9-µm forward-scatter threshold, and a platelet gate was drawn to enclose CD61-positive events greater than the 0.9-µm threshold. Microparticles were differentiated from debris and machine noise on the basis of their membrane expression of CD61 or annexin V. The absolute number of platelets or microparticles per microliter of sample was calculated via the following equation: (number of platelet or microparticle events in test sample/number of counting beads) \times (number of beads in 30 µL/vol of test sample), with the volume of test sample equal to 2 µL (whole blood and MPEP) or 5 µL (PPP and MPF). The proportion of total PMPs (all CD61-positive events \leq 0.9 µm) that expressed phosphatidylserine (events positive for both CD61 and annexin V \leq 0.9 µm) was also determined for each sample.

Statistical analysis—Data were assessed for normality via the Shapiro-Wilk test. Because data for many variables were nonparametric, median and range were calculated for the dogs’ age, CBC results, coagulation profile results, flow cytometry counts, and TGA variables. Differences between blood fractions in cell and PMP counts and TGA variables and differences between males and females were compared via the Wilcoxon rank sum test. Wilcoxon signed rank tests were used to compare platelet and microparticle counts and TGA variables between paired fresh and frozen-thawed samples from each dog. Correlations of the

---

Table 1—Median and range values for Hct, leukocyte count, platelet count, and coagulation assay results for 31 healthy dogs.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (%)</th>
<th>Range</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct</td>
<td>51</td>
<td>41–60</td>
<td>41–60</td>
</tr>
<tr>
<td>WBCs (\times 10\textsuperscript{3} cells/µL)</td>
<td>7.65</td>
<td>5.7–10.3</td>
<td>5.7–14.2</td>
</tr>
<tr>
<td>Platelets (\times 10\textsuperscript{3} cells/µL)</td>
<td>257</td>
<td>191–475</td>
<td>186–545</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>11.2</td>
<td>9.8–12.3</td>
<td>10.0–17.0</td>
</tr>
<tr>
<td>PT (s)</td>
<td>14.5</td>
<td>10.4–16.8</td>
<td>11.0–19.0</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>310.5</td>
<td>227–478</td>
<td>147–479</td>
</tr>
<tr>
<td>Antithrombin activity (%)</td>
<td>100</td>
<td>80–132</td>
<td>65–145</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>132</td>
<td>0–245</td>
<td>0–245</td>
</tr>
<tr>
<td>Protein C activity (%)</td>
<td>92</td>
<td>75–120</td>
<td>75–135</td>
</tr>
</tbody>
</table>

APTT = Activated partial thromboplastin time. PT = Prothrombin time.

Table 2—Median and range values for platelet and PMP counts in samples of citrated whole blood obtained from 31 healthy dogs.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Platelet count (platelets/µL)</th>
<th>PMP count (microparticles/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrated whole</td>
<td>193,692 (112,338–333,057)</td>
<td>9,294 (3,165–131,624)</td>
</tr>
<tr>
<td>PPP</td>
<td>313 (44–1,332)</td>
<td>505 (151–2,522)</td>
</tr>
<tr>
<td>MPF</td>
<td>120 (34–363)</td>
<td>108 (36–150)</td>
</tr>
<tr>
<td>MPEP</td>
<td>2,831 (400–2,872)</td>
<td>1,850 (223–5,630)</td>
</tr>
</tbody>
</table>

Table 3—Median and range values for thrombogram variables in plasma fractions separated via centrifugation from 31 healthy dogs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak thrombin (nmol)</th>
<th>Lag time (min)</th>
<th>ETP (nmol·min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median* Range</td>
<td>Median* Range</td>
<td>Median* Range</td>
</tr>
<tr>
<td>PPP</td>
<td>16 0–146</td>
<td>17 9–41</td>
<td>893 0–3,387</td>
</tr>
<tr>
<td>MPF</td>
<td>0 0–11</td>
<td>42 20–67</td>
<td>0 0–426</td>
</tr>
<tr>
<td>MPEP</td>
<td>107 11–261</td>
<td>10 5–16</td>
<td>3,650 345–5,210</td>
</tr>
</tbody>
</table>

*Median values for this variable differ significantly (P < 0.001; Wilcoxon rank sum test) among the 3 sample types.
number of PMPs and phosphatidylserine expression for various plasma fractions and TGA variables were determined via Spearman rank tests. Descriptive statistics were compiled with functions integrated in a spreadsheet application, and comparisons and correlations were performed with a data analysis and statistical software program.

Results

Study population—Thirty-two dogs were recruited in the interval from January to June 2010, including 20 males (19 neutered) and 12 females (9 neutered). Dogs comprised 9 Labrador Retrievers, 4 mixed-breed dogs, 3 Beagles, 3 Dalmatians, 2 Staffordshire Bull Terriers, 2 Australian Shepherd Dogs, 2 English Springer Spaniels, and 1 each of Doberman Pinscher, Boxer, American Eskimo Dog, Golden Retriever, Great Dane, Husky, and Schnauzer. One dog (4-year-old neutered male Husky) was excluded because of abnormal findings on the CBC (eosinophilia) and a deviation of >1 second from the reference interval for clotting time (17-second prolongation of activated partial thromboplastin time). The 31 dogs included in the study ranged from 1 to 9 years of age (median age, 5 years). Results for CBC and coagulation panel analyses were summarized (Table 1).

Platelet and PMP counts in blood and plasma fractions—The platelet and PMP counts per microliter of sample measured via cytometry in citrated blood and derived plasma fractions were summarized (Table 2). The initial centrifugation step removed most of the platelets from the supernatant plasma, which resulted in a median residual platelet count in PPP <0.5% that of the starting whole blood. The second (high-speed) centrifugation step removed any residual platelets from the supernatant MPF fraction and concentrated them in the MPEP. Platelet counts in PPP and the resultant MPEP were significantly correlated ($r = 0.85; P < 0.001$), which indicated a quantitative recovery of intact platelets in the PMP pellet.

The whole blood and recovered plasma fractions contained a broad range of PMPs (Table 2). Although slightly lower counts were detected for males, the median PMP counts in whole blood were not significantly different ($P = 0.14$) from the supernatant MPF fraction ($8,954$ microparticles/$\mu$L for males vs $11,888$ microparticles/$\mu$L for females). However, there were significant differences ($P = 0.046$) in median PMP count for the PPP fraction ($413$ microparticles/$\mu$L for males vs $711$ microparticles/$\mu$L for females), and this difference was also significant ($P = 0.020$) in MPEP (median MPF, $1,070$ microparticles/$\mu$L for males vs $2,730$ microparticles/$\mu$L for females). Overall, PMP counts in whole blood and PPP had a weak correlation ($r = 0.42; P = 0.017$), compared with the correlation for the recovery of PMPs from the PPP fraction after high-speed centrifugation.

![Figure 3](https://via.placeholder.com/150)

Figure 3—Box-and-whisker plots of the effects of a freeze-thaw cycle on peak thrombin concentration (A), lag time (B), and ETP (C) in thrombograms generated for paired aliquots of fresh and frozen-thawed PPP, MPF, and MPEP obtained from 31 healthy dogs. Each circle represents results for 1 dog. Each box represents the interquartile range, the horizontal line in each box represents the median, the whiskers extend to the farthest observations within 1.5 times the interquartile range, the plus sign indicates an outlier (>1.5 but <3 times the interquartile range), and triangles represent extreme outliers (>3 times the interquartile range). Median values differ significantly for the various sample types ($P < 0.001$ for peak thrombin concentration and ETP; $P < 0.05$ for lag time; Wilcoxon signed rank tests).
(PMPs in PPP vs PMPs in MPEP; $r = 0.70; P < 0.001$). The platelet count and PMP count in PPP were significantly correlated ($r = 0.78; P < 0.001$); however, there was not a significant correlation ($r = -0.10; P = 0.722$) between platelet count and PMP count in whole blood.

**Thrombin-generating activity of plasma fractions**—The thrombin-generating capacity and resultant thrombograms of the 3 plasma fractions differed markedly (Figure 2). Thrombin generation and ETP were almost 4-fold as high in the MPEP fractions, compared with results for PPP, and were virtually undetectable in the MPF fractions. Consequently, there were significant (P < 0.001 for all comparisons) differences among the 3 sample types for the median values of peak thrombin concentration, lag time, and ETP (Table 3). However, the thrombogram variables did not differ significantly between males and females for any plasma fraction.

**Effects of a freeze-thaw cycle on number of PMPs and thrombin-generating activity**—Plasma fractions that were subjected to a freeze-thaw cycle had quantitative and qualitative differences in PMP count and TGA results (Figure 3; Table 4). The frozen-thawed PPP and MPEP aliquots had significantly higher PMP counts than did the paired fresh samples. The platelet count in fresh PPP was significantly ($P < 0.001$) correlated with the PMP count in frozen-thawed PPP ($r = 0.96$) and MPEP ($r = 0.80$). The freeze-thaw cycle also significantly increased the percentage of PMPs with externalized phosphatidylserine. After freezing and thawing, the median values for annexin V–positive PMPs increased to > 90% in both the PPP and MPEP fractions. In addition, the median peak thrombin concentrations for frozen-thawed PPP (42nM) and MPEP (175nM) and median ETP for these sample types (2,115 and 4,638 nmol/min, respectively) were significantly ($P < 0.001$ for all comparisons) higher than the corresponding values for the fresh sample types. The freeze-thaw cycle also caused a significant decrease in median lag time for PPP (18 minutes) and MPEP (9 minutes), compared with the median lag time for fresh PPP and MPEP.

**Correlation between phosphatidylserine expression on PMPs and TGA variables**—The number of PMPs with externalized phosphatidylserine was generally associated with an increase in peak thrombin concentration, decrease in lag time, and high ETP in both fresh and frozen-thawed plasma fractions (Table 5). There were significant positive correlations for phosphatidylserine externalization with peak thrombin concentration in PPP and MPEP and with ETP in frozen PPP. The strongest positive relationship was found for the peak thrombin concentration in the fresh MPEP samples.

**Discussion**

In the present study, we used a standard protocol for processing citrated blood to characterize PMPs in plasma samples obtained from healthy dogs. We found that centrifugation and storage conditions affected plasma PMP content, healthy dogs had a wide range of PMP counts, and thrombin generation in an assay system with a low phospholipid concentration depended on the presence of PMPs and was influenced by the proportion of PMPs with externalized phosphatidylserine. The purpose of the initial centrifugation was to generate platelet-depleted plasma that retained its PMP content. Inadequate centrifugation (speed or time) results in cellular contamination of plasma fractions; however, excessive centrifugation may cause ex vivo platelet or leukocyte activation, mechanical cell frag-
mentation, or sedimentation of PMPs. These conditions may spuriously increase or decrease PMP counts in the recovered plasma and may not provide a representative sample of endogenous circulating PMPs.\textsuperscript{20,21,29} The initial centrifugation we performed did not appear to activate or fragment platelets; however, it did not effectively sediment all platelets from PPP because some intact platelets were recovered in the MPEP fraction. These residual platelets influenced the analyses subsequently performed on frozen-thawed PPP. The second (high-speed) centrifugation of PPP effectively removed any residual platelets from MPF; consequently, we found few differences in PMP content between the fresh and frozen-thawed MPF fractions.

Despite consistent collection, centrifugation, and analyses of blood samples throughout the study, we found a wide range of PMP counts in the healthy dogs. Although analysis of whole blood prevents artifacts formed by centrifugation, the whole blood PMP counts among dogs were no less variable than were PMP counts in PPP. The initial centrifugation of whole blood did not concentrate PMPs in the supernatant PPP, and the PMP content of these 2 sample types was not strongly correlated. The highly cellular whole blood milieu may have caused clumping and coincidence counting, thereby reducing the accuracy of PMP counts. The relatively high PMP counts in whole blood may have been attributable, in part, to ex vivo platelet activation during the interval between blood collection and cytometric analysis of whole blood. Blood collection tubes that contain citrate and specific platelet inhibitory agents (citrate-theophylline-adenosine-dipyridamole) minimize platelet activation in whole blood during storage. However, a comparison of human whole blood mixed with citrate versus citrate-theophylline-adenosinedipyridamole anticoagulant and stored for 1 to 2 hours revealed no significant differences in PMP content.\textsuperscript{29} Alternatively, the relatively low PMP counts we observed in PPP may have been caused by their removal during the initial centrifugation step. Regardless of sample type, the apparent broad range for the number of PMPs in healthy dogs will require careful consideration of control subjects for studies of PMPs for various pathological conditions.

In humans, subjects are matched on the basis of gender between plasma PMP counts are higher in healthy women than in healthy men.\textsuperscript{18,30} Fluctuations in total numbers of PMPs and the proportion of annexin V-positive microparticles have also been related to the phase of the menstrual cycle in women.\textsuperscript{30} Although we found that female dogs generally had higher PMP counts than did male dogs, the small numbers and inclusion of few sexually intact females (and males) in our study precluded subgroup analyses to evaluate hormonal influences on canine PMP counts.

In the present study, we used plasma fractions with various PMP contents to test the influence of PMPs on thrombin generation in dogs. The TGA trigger reagent contained tissue factor that initiated coagulation in concert with endogenous factor VIIa in the test plasma. This reagent also contained phospholipid; however, the phospholipid concentration was designed to be suboptimal for supporting coagulation complex assembly in human plasma.\textsuperscript{24} Therefore, thrombin generation in the assay mixture was dependent on microparticles in the test plasma to supply sufficient phospholipid to generate active tenase and prothrombinase complexes. The 3 sample types evaluated (PPP, MPF, and MPEP) yielded markedly different thrombograms. Samples of MPF generated virtually no thrombin, whereas PMP-enriched MPEP rapidly generated high peak thrombin concentrations with high ETP, compared with results for the PPP samples. Analysis of these results indicated that centrifugation conditions affect the PMP content of canine plasma and thereby influence the rate and extent of thrombin generation in a low-phospholipid TGA. Although beyond the scope of the present study, the content of tissue factor and phospholipid in the trigger reagent could be manipulated to maximize thrombin generation in canine plasma in a manner independent of or sensitive to the presence of microparticles.

Because laboratory analysis immediately after collection is rarely feasible in clinical settings, we investigated the effects for storage of frozen PPP. A study\textsuperscript{21} of human PMPs revealed that a delay in the initial centrifugation of whole blood beyond 2 to 3 hours after collection increases the PMP content of PPP samples. We centrifuged whole blood within 2 hours after collection to eliminate this preanalytic variable and then compared the effects of a freeze-thaw cycle on the paired plasma fractions. We found that frozen-thawed PPP had lower residual platelet counts than did the corresponding fresh PPP samples and also had a concomitant increase in PMP. The direct correlation between initial platelet count in fresh PPP and the PMP content of the frozen-thawed samples indicated that intact platelets did not survive the freeze-thaw process, and their membrane fragments were subsequently detected as PMPs. In addition to the effects on PMP count, we found that a freeze-thaw cycle increased the proportion of PMPs with externalized phosphatidylserine, from a median of approximately 40% phosphatidylserine-positive PMPs in fresh PPP to 90% phosphatidylserine-positive PMPs in frozen-thawed PPP. The expected ratios of PMPs with positive and negative results for annexin V binding in healthy humans differ widely\textsuperscript{14,16} and span the median values observed for both sample types in the study reported here. Considering that preanalytic processing is also highly variable among reports in the human literature, this important property of PMPs requires further examination. Although the strength of association varied among sample types and individual thrombogram variables, we found that increased numbers of PMPs with externalized phosphatidylserine contributed toward enhanced thrombin generation in both fresh and frozen-thawed samples. In preliminary experiments, we found that virtually all PMPs were depleted from the PPP of some dogs when the speed or time of the initial centrifugation step was increased. Therefore, further refinement of centrifugation conditions is required to retain PMPs while eliminating residual platelets and the variability their fragmentation causes in analyses of frozen PPP.

The present study did not address the inherent limitations of flow cytometry for analyzing PMPs. In particular, the differences between
early- and late-generation models and among cy-tometer manufacturers influence size discrimina-tion on the basis of forward-scatter parameters and the interference of background electronic noise. The cytometeru available for use in the pres-ent study could not fully resolve 0.5-µm beads recently recommended for standardization of PMP enumeration in a study of humans.13 It has also been suggested that calibration beads may not be reli-able for creating size-based gates because of differ-ences in scattering from smooth bead surfaces versus complex cellular surfaces.19,21 Measurement of PMPs on the basis of molecules of bound fluorochrome, rather than as absolute event counts, has been advocated as another means for comparing results among laborato ries.13 This approach requires establishing calibration curves that relate the intensity of soluble fluorochrome molecules to fluorescent-conjugated antibodies directed against platelet-specific markers. In most human standardization studies, antibodies di-rected against the glycoprotein Ib-V-IX complex (eg, CD42a and CD42b) are recommended as constitu-tively expressed platelet antigens to distinguish PMPs from other cell-derived microparticles. To the authors’ knowledge, commercial antibodies that cross-react from other cell-derived microparticles. To the authors’ knowledge, commercial antibodies that cross-react with the canine glycoprotein Ib-V-IX complex are not available for comparable PMP enumeration in dogs.21

An additional limitation of the present study was the use of CD61 as the sole platelet membrane–specific marker. This led to an inability to differentiate platelet membrane fragments from intact PMPs in the frozen-thawed PPP samples. Phalloidin is a cyclic peptide that binds f-actin, a platelet contractile protein located on the inner membrane surface of platelets. Multicolor analyses that include phalloidin along with outer plate-let membrane markers have been suggested as a means to distinguish damaged platelet membrane fragments from intact cell membrane vesicles and PMPs.15 The damaged platelet membranes will be double positive, binding both phalloidin and a constitutive platelet outer membrane marker, whereas PMPs will only express the outer membrane antigen. This approach is poten-tially applicable to studies of canine PMPs, providing variations in centrifugation conditions cannot elimi-nate all residual platelets from plasma.

In the present study, there were broad ranges in circulating PMP counts of healthy dogs in both whole blood and plasma samples. The PMP content in plasma greatly influences thrombin generation in a low-phos-photidyl TGA. Storage of frozen plasma samples that contain residual platelets will result in platelet fragmen-tation and increased thrombin generation, compared with results for analysis of fresh plasma. The sample collection and assay protocols in this study provide a basis for further optimization to assess the pathophysi-o logic relevance of circulating PMPs and thrombin gen-eration in various disease states of dogs.

References

18. Robert S, Poncelet P, Lacroix R, et al. Standardization of plate-


