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A Remote Assay for Measuring Canine Platelet Activation and the Inhibitory Effects of Antiplatelet Agents

M. Dunning, J. May, J. Adamany, S. Heptinstall, and S. Fox

Background: Antiplatelet medications are increasingly used in dogs. Remote analysis of platelet activity is challenging, limiting assessment of antiplatelet drug efficacy.

Hypothesis/Objectives: To evaluate a method used in humans for stimulation and remote analysis of canine platelet activity.

Animals: Forty-five dogs of various ages without a coagulopathy or thrombocytopenia. Six were receiving antiplatelet medication.

Methods: Prospective observational study. Platelets were stimulated with combinations of arachidonic acid (AA) and epinephrine (Epi) or adenosine diphosphate (ADP) and the thromboxane A_2-mimetic U46619 (U4). PAMFix was added to the blood samples to facilitate delayed analysis of platelet activity. Activity was assessed by flow cytometric measurement of surface P-selectin (CD62P) expression.

Results: Canine platelets could be stimulated with both AA/Epi and ADP/U4. The levels of P-selectin were significantly greater than paired, unstimulated samples ($P < 0.001$). Inhibition of P-selectin expression occurred after this stimulation by adding antiplatelet drugs in vitro. The efficacy of antiplatelet drugs in samples from treated dogs was also measurable ex vivo using this method. Delayed analysis of platelet activity at time points up to 22 days demonstrated excellent correlation between respective mf values at each time point ($r^2 = 0.92$, $P < 0.0001$).

Conclusions and Clinical Importance: This study evaluated a new method to remotely assess canine platelet activity. It shows that PAMFix can be used for this purpose. This provides opportunities to interrogate the inhibitory action of antiplatelet drugs in clinical settings.

Key words: Aspirin; Clopidogrel; Delayed analysis; P-selectin.

Altered platelet activity influences morbidity and survival in dogs and cats as well as humans, due to activated platelets contributing to intravascular clot formation.[1,2] Dogs with immune-mediated hemolytic anemia (IMHA), hyperadrenocorticism, those receiving exogenous corticosteroids, protein-losing nephropathies (PLN), various neoplastic and infectious diseases, pancreatitis, and enteropathies are at increased risk of thromboembolic complications from altered platelet activity.[3–10]

Antiplatelet medication is often prescribed to reduce the likelihood of clot formation.[2,11,12] Despite antiplatelet medication being considered by many veterinarians as standard of care, the choice of agent and the effective dose in individual animals remain unclear. The recommended antithrombotic dose of aspirin has increased over time, with most recent evidence recommending 2 mg/kg daily.[13–15] There is increasing evidence that aspirin is variably efficacious in dogs, which raises concerns over a single dose rate.[13–15] Alternative antiplatelet medications include the $P2Y_{12}$ antagonists, such as clopidogrel. The optimum dose of clopidogrel in individual dogs remains unclear, and at least in humans, there is considerable variation in efficacy between individuals.[16] In addition to unclear dosing strategies, assessing the efficacy of any antiplatelet medication is

Abbreviations:

AA	arachidonic acid
ADP	adenosine triphosphate
ASA	acetylsalicylic acid
ANOVA	one-way analysis of variance
BID	twice daily dosing
Cang	Cangrelor
Epi	epinephrine
HCM	hypertrophic cardiomyopathy
IMHA	immune-mediated hemolytic anemia
Mf	median fluorescence
PLN	protein-losing nephropathy
U4	U46619
UK	United Kingdom

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The clinical samples were acquired at a single specialist referral center - Pride Veterinary Centre, Derbyshire, UK, and the measurements were performed in the Platelet Research Laboratory in the Division of Clinical Neuroscience at the Queen's Medical Centre, University of Nottingham, UK.

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challenging, largely revolving around the absence of major thrombotic events.[12,17] This approach has clear limitations as a surrogate marker. Smaller thrombotic events could still occur in individuals and contribute to long-term morbidity.

Due to inherent problems with activation and survival of platelets in whole blood ex vivo, functional testing must occur shortly after sample acquisition. Platelet activity is therefore almost impossible to assess remotely. This provides a major impediment to in-clinic analysis. Limited attempts at remote platelet analysis in dogs have highlighted several problems, precluding commercial application.[18–20] In addition to inherent problems in studying platelet physiology, equipment to test platelet function is expensive and requires a high level of technical expertise to operate. Similar problems are encountered with remote analysis of human platelets.[21–23]

Preliminary studies using the fixative paraformaldehyde, for analyzing canine platelet activity, suggested this could have clinical utility for delayed analysis.[24] A method for remotely testing platelet activity has recently been developed for humans, based on the use of PAMFix[a] (Platelet Solutions Ltd, Nottingham, UK). This method measures P-selectin to quantitate platelet activity after activation in vitro with specific agonists or combinations of agonists.[25] The PAMFix fixative terminates activation and stabilizes the platelets for subsequent flow cytometric analysis.[23,25] The level of P-selectin expression remains stable for at least 9 days.[25] This provides a robust method for remote testing of platelet activity.[21] Residual platelet activity seen via measuring P-selectin in patients with acute coronary syndromes treated with clopidogrel is associated with poor cardiovascular outcomes.[26] This suggests P-selectin measurement is a good surrogate marker of therapeutic efficacy and supports inadequate inhibition of platelet activity as important in patient outcome.

The aims of this study were to evaluate the use of the PAMFix platelet fixative method in dogs and to establish whether this technique could enable us to determine the efficacy of canine antiplatelet drug therapy.

Materials and Methods

Animals and Blood Collection

Blood samples were obtained from 45 dogs of various ages and breeds presented to a single specialist referral center over 16 months. These dogs were presented for a variety of reasons (none for investigation of abnormalities in blood clotting), and all blood samples were obtained by jugular venipuncture by one of the authors (MD or JA) as part of the routine diagnostic investigations; the residual blood was used for this study. Dogs were included in the study as a "treated" group if they were receiving antiplatelet medications. All owners gave consent for the residual blood to be used in this research. Blood was obtained in accordance with the guidelines from the School of Veterinary Medicine and Science, approved by the University of Nottingham's Ethical Review Board. Dogs subsequently identified to have thrombocytopenia (a platelet count <150 $\times$ 10^9/L) were excluded from the study. None of the animals were receiving medications that would significantly alter platelet activity other than those on clopidogrel[b] (mean daily dose - 2.4 mg/kg (range 1.4-3.3)) or aspirin[c] (mean daily dose - 1.5 mg/kg (range 0.55-2)) which were part of the study. A standard volume of blood was drawn and anticoagulated immediately using trisodium citrate dihydrate (3.13% w/v) at a ratio of 1 part anticoagulant to 9 parts blood. Total tube volumes were either 0.5 mL or 1 mL, with accurate filling of each blood tube. Processing and fixation of the samples was always performed within 2 hours of collection.

Treatment of Blood Samples

Within 2 hours of acquisition, the blood was re-warmed to 37°C before activating the platelets. Aliquots of the blood (88 μL) were incubated with the required platelet agonist (12 μL) for 5 min. The fixing agent, PAMFix (200 μL), was added and the sample mixed. The fixed samples were then stored at room temperature awaiting flow cytometric analysis. Analysis was always performed within 7 days of fixation, usually within 1-3 days. Platelet stimulation was carried out using separate conditions designed to specifically interrogate antiplatelet drugs used as therapy in both humans and dogs. This method is used for this purpose in human patients.[23] The flow cytometer used in this study was a Becton Dickinson FACSCanto II operating with FACSDiVa software using a 96-well HTS unit for high throughput. Eight peak rainbow beads were used before each analysis to ensure correct operation of the flow cytometer and reduce any fluctuation in fluorescence intensity measured. The protocol used for staining of platelets in preparation for flow cytometry was as follows: for each sample, one well of a 96-well plate was prepared by adding 10 μL of a mixture of a 1 : 20 dilution of CD61-PE antibody and a 1 : 8 dilution of CD62P-FITC antibody in saline. To a further well, 10 μL of a mixture of a 1 : 20 dilution of CD61-PE antibody and a 1 : 8 dilution of IgG-FITC was added as a control. The prepared plate was covered with parafilm and stored at 2-8°C before use. The samples to be analyzed were mixed until all the sedimented red cells were re-suspended. 5 μL of the sample was added to the appropriate well containing the antibodies and incubated for 20-30 minutes in the dark at 2-8°C. Following incubation, 0.2 mL of FACSflow was added to each well and the plate placed on the HTS of the flow cytometer; 3,000 CD61-positive platelet events were recorded, and CD62P was quantitated as the Median fluorescence (mf) values for the whole population of CD61 positive cells. An IgG control provided a baseline mf for nonspecific fluorochrome binding. Condition A is designed to investigate the effects of the cyclooxygenase inhibitor aspirin. This condition uses a mixture of arachidonic acid and epinephrine (AA/Epi) for platelet stimulation; the final concentrations in the blood were 0.5 mM and 100uM, respectively. Condition C is designed to investigate the efficacy of the $P2Y_{12}$ antagonist clopidogrel. This condition uses a mixture of adenosine diphosphate (ADP) and the thromboxane A_2 mimetic: U46619 (U4) for platelet stimulation; the final concentrations in the blood were 10uM and 1uM, respectively. These combinations of stimulants were chosen after investigation of a number of potential agents and combinations of agents to identify conditions for optimal stimulation of P-selectin expression in human blood.[23] The control condition (B) uses physiological saline in which no stimulation is expected to occur. EDTA was always included with the agonist to prevent platelet aggregation after stimulation. All reagents were supplied by Platelet Solutions Ltd (Nottingham, UK) in kit form.

Studies to investigate the effects of adding aspirin or another $P2Y_{12}$ receptor antagonist cangrelor[d] were also carried out on the blood samples. Clopidogrel cannot be used for this purpose as it is a prodrug requiring activation in vivo. Here anticoagulated blood was pre-incubated with aspirin (100 μM) or cangrelor (1 μM) and then activated with the appropriate agonists, as before fixation. After fixation and storage, platelets were analyzed by flow cytometry. Specifically, the amount of P-selectin on the surface of the platelets was measured as a means of quantitating the level of platelet activation. This was expressed as median fluorescence (mf). An anti-CD61[e] antibody was used to positively identify the platelet population, and 3,000 platelet events were collected. The P-selectin expression on this platelet population was measured using an anti-CD62P[f] antibody. This antibody to P-selectin has been validated in dogs.[24,27] The CD61 and CD62P antibodies used had good selectivity for canine platelets. To ascertain the stability of the samples after activation and fixation, the same fixed samples were stored and re-analyzed at 4-7 days. After this initial testing, all samples were stored at 4°C pending re-analysis at 20-22 days. Measurements were made at either 1-3 days (n = 45), 4-7 days (n = 30), or 20-22 days (n = 30) after fixation.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism 6. Tests for normality were performed on each group (D'Agostino and Shapiro-Wilk Tests). Individual datasets were compared using the Student's *t*-test. For multiple comparisons with parametric datasets, the one-way analysis of variance (ANOVA) was performed, and for nonparametric datasets, the Kruskal-Wallis test was performed to test for independence. Dunn's multiple comparisons test was used to analyze differences between specific groups. For all data, averages are expressed as median with interquartile range with significance set at $P < 0.05$.

Results

Blood samples were obtained from untreated dogs (not undergoing treatment with an antiplatelet drug or anticoagulant) (n = 39). Samples were also obtained from dogs receiving treatment with an antiplatelet drug (aspirin or clopidogrel) (n = 6).

Untreated Dogs

Aliquots of blood samples from 39 untreated dogs were studied under the three separate conditions (B, A, and C). In addition, two further conditions were investigated: condition A+ASA where platelets were stimulated as per condition A, in the presence of aspirin (acetylsalicylic acid, ASA) and condition C+Cang where platelets were stimulated as per condition C, in the presence of the $P2Y_{12}$ antagonist cangrelor. In these assays, aspirin and cangrelor were added to the blood ex vivo. The results are shown in Figure 1.

The 39 untreated dogs were presented for a variety of reasons, summarized (along with their signalment, treatment, and P-selectin mf under each condition) in supplementary online material, Table S1. There was no significant difference in the mf values in relation to the signalment, reasons for initial presentation to the veterinarian or the medications administered for any dogs.

Without stimulation (condition B), the median P-selectin mf was 177 (120-296). Thirty-five of the 39 samples tested were <500. Four values were > 500 but <1,000. Values <500 were deemed to reflect minimal P-selectin on the unstimulated platelets. In those dogs (n = 4) with mf >500, there were no common conditions

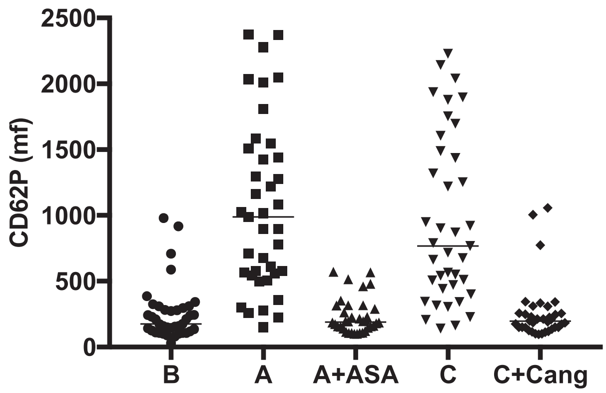

Fig 1. Platelet P-selectin (CD62P) expression as measured by median fluorescence (mf), in 39 dogs not receiving any antiplatelet therapy. Results were obtained after no stimulation (B = saline control), stimulation under condition A (AA/Epi) in the absence of (A) and presence of aspirin (A + ASA) and stimulation under condition C (ADP/U4) in the absence of (C) and presence of cangrelor (C + Cang) (—— represents the median CD62P (mf) value)

or signalment details to explain this result. None of these dogs were receiving any medications at the time of sampling, and their presenting conditions were as follows: hypoadrenocorticism, otitis externa, perineal herniation, and low-grade mast cell tumor.

Stimulation using condition A (AA/Epi) gave a range of P-selectin values and a median mf of 988 (557-1508). This was significantly different to the unstimulated condition B ($P < 0.0001$). In 32/39 dogs, the mf was >500. In 19/39 dogs, values increased to >1,000; in 13/39 dogs, the values were between 500 and 1,000. In 7/39 dogs, values remained <500. In these dogs, there were no common underlying conditions, treatment or signalment details (Table 1). Thus, in most dogs (32/39), stimulating platelets led to a marked increase in P-selectin. The inhibitory effects of aspirin on platelet function were interrogated by adding aspirin in vitro to Condition A (A+ASA) (Figure 1). Adding aspirin to the blood sample decreased the median mf value to 191 (142-310). There was no significant difference between A+ASA and the unstimulated condition B. This result confirms that AA/Epi-induced P-selectin expression detects inhibitory effects of aspirin on canine platelets.

Stimulation under condition C (ADP/U4) gave a median mf of 768 (443-1489), which was significantly greater than condition B ($P < 0.0001$). In 28 of 39 dogs, values were >500. In 14 of 39 dogs, the values were >1,000. A further 14 dogs had mf values 500-1,000. In 11 of 39 dogs, values remained <500. In these dogs, there were no common underlying conditions, treatment or signalments (Table 1). Thus, in most dogs (28/39), stimulating platelets with ADP/U4 caused a moderate-to-marked increase in P-selectin expression. The inhibitory effects of $P2Y_{12}$ antagonists on platelet function were interrogated by adding cangrelor in vitro to condition C (C+Cang) (Figure 1). Adding cangrelor decreased the median mf value to 196 (149-257). In all cases, the final values were similar the condition B mf values, for the respective samples. There was no significant difference between C+Cang and condition B. In 3 dogs, the mf values for C+Cang were >500, these dogs also had a condition B mf value >500 (Figure 2). However, their respective A and C stimulated mf values were greater than conditions C+Cang and B, indicating submaximal platelet activity in the resting samples.

There were 5 dogs whose mf values were <500 under both conditions A and C (Table 1). Whilst these dogs failed to demonstrate an mf >500 poststimulation, the mf values for conditions A and C remained significantly above condition B ($P = 0.005$ and $P = 0.05$, respectively). After the addition of inhibitors under conditions A+ASA and C+Cang, the mf was reduced (Figures 3A, B). The subsequent mf values were similar to those in condition B; a trend toward significance was noted ($P = 0.06$). After a review of these dog's details, there were no common presenting conditions, treatment or signalment features.

Treated Dogs

Blood was obtained from six dogs receiving antiplatelet drugs. The dogs were receiving antiplatelet medication for the following reasons: IMHA (×1), PLN (×2), iliac thrombus (×1), pulmonary artery thrombus (×1), forelimb thrombus (×1). Three dogs were receiving clopidogrel alone, two both aspirin and clopidogrel and one aspirin alone. One dog receiving clopidogrel alone was sampled on multiple occasions. Blood samples from the treated dogs were interrogated under conditions B (saline), A (AA/Epi), and C (ADP/U4) (Figure 4). Under condition B, P-selectin mf was low for all but one sample, indicating minimal background platelet activity as seen in the untreated group. The dog which had a high mf in the unstimulated sample was suffering with PLN and was receiving aspirin at a dose of 0.55 mg/kg q24 h. Stimulation under condition A greatly increased P-selectin in the dogs not receiving aspirin but minimally increased P-selectin in 2 of 3 samples from dogs receiving aspirin. The sample that remained >500 despite aspirin was from the PLN dog with a high condition B mf; the other two were receiving 1.2 mg/kg and 2 mg/kg and suffered a pulmonary artery thrombus and iliac thrombus, respectively. Despite this sample, there was no significant difference between the mf between condition B and condition A in dogs receiving aspirin. Condition C P-selectin values remained low for all seven clopidogrel treated samples, indicating good inhibition of platelet activity. The sample from the dog receiving aspirin and not clopidogrel demonstrated a substantial increase in P-selectin as expected.

Delayed Analysis of Platelet Activity

The experiments above were conducted within 1-3 days of fixation. In addition, we performed

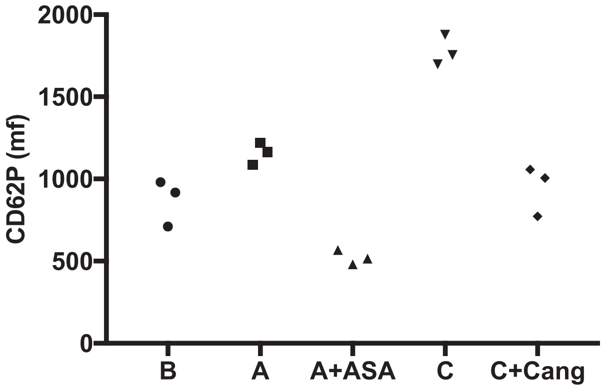

Fig 2. Platelet P-selectin (CD62P) expression as measured by median fluorescence (mf), in 3 dogs with C+Cang values >500. Results were obtained after no stimulation (B = saline control), stimulation under condition A (AA/Epi) in the absence of (A) and presence of aspirin (A + ASA) and stimulation under condition C (ADP/U4) in the absence of (C) and presence of cangrelor (C + Cang).

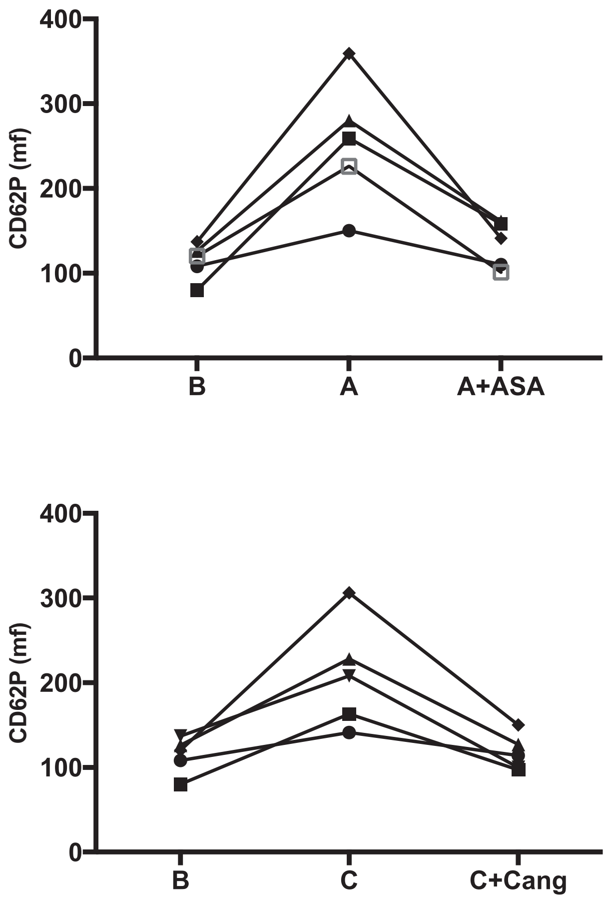

Fig 3. Data from dogs with condition A and C values <500 before and after inhibition with aspirin (A+ASA) and cangrelor (C+Cang). Unstimulated condition B included to demonstrate the respective similarities between this condition and A+ASA and C+Cang. Increase mf in conditions A and C can be seen above condition B with reductions in the respective mf values after the addition of aspirin (A+ASA) and cangrelor (C+Cang) back to the baseline condition mf values.

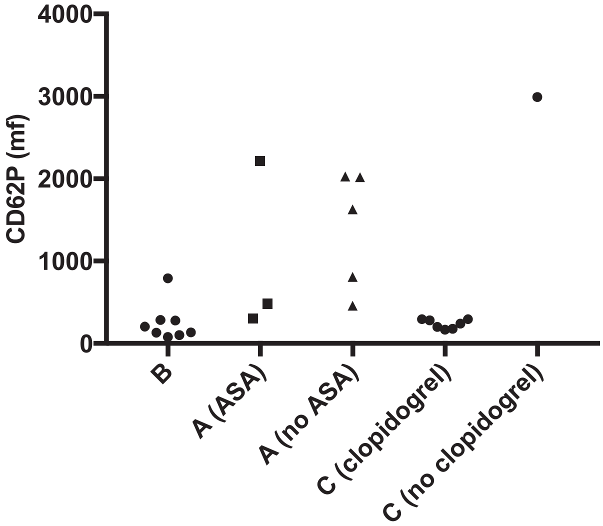

Fig 4. Platelet P-selectin (CD62P) expression as measured by mf, in six dogs receiving antiplatelet therapy (either aspirin (ASA) alone, clopidogrel alone, or a combination of both). Five dogs were receiving clopidogrel and two were also receiving aspirin, one was only receiving aspirin. Results were obtained after no stimulation (B = saline control), stimulation under condition A (AA/Epi), and stimulation under condition C (ADP/U4).

measurements (n = 30) 4-7 days after stimulation and fixation and after 20-22 days (n = 30). There was an excellent positive linear correlation between P-selectin expression on samples fixed with PAMFIX after 1-3 days and 4-7 days ($r^2 = 0.92$, $P < 0.0001$) and after 1-3 days and 20-22 days ($r^2 = 0.93$, $P < 0.0001$) (Figure 5).

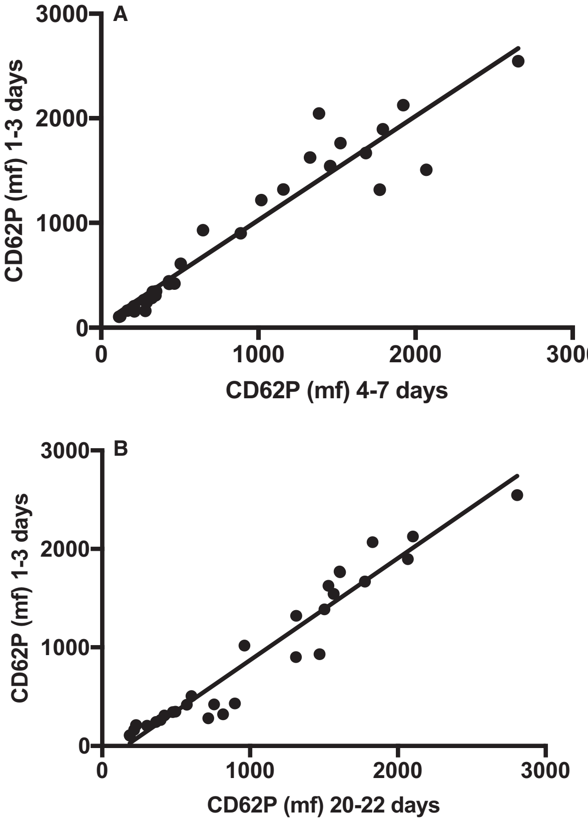

Fig 5. **(A)** The correlation between measures of P-selectin (CD62P) on activated platelets in blood samples from dogs measured under conditions B, A, or C 1-3 days and 4-7 days after activation and fixation ($r^2 = 0.92$). **(B)** The correlation between measures of P-selectin (CD62P) on activated platelets in blood samples from dogs measured under conditions B, A, or C 1-3 days and 20-22 days after activation and fixation ($r^2 = 0.93$).

Test Reproducibility

Blood samples were obtained from one dog on three separate occasions over 12 months. The dog was receiving clopidogrel. The P-selectin mf was very similar, demonstrating activation or lack of, on each occasion (Figure 6). The results would lead to similar clinical interpretation of the dogs' platelet activity on each occasion.

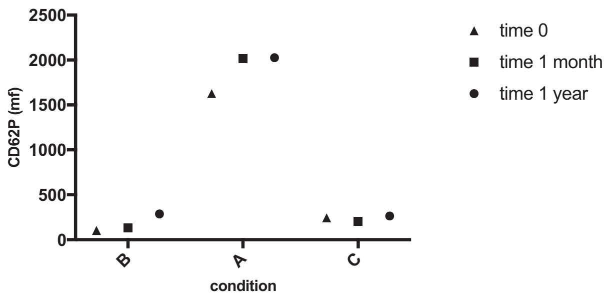

Fig 6. Individual measurements of P-selectin (CD62P mf) for one dog studied on 3 different occasions under conditions B, A, and C. The subsequent time points were 1 month and 1 year after initial sampling (time 0). No significant difference exists between the mf values at each time point ($P = 0.19$).

Discussion

We have herein provided preliminary evidence that a remote assay measuring platelet activity, used with human blood can provide similar valuable information with canine blood.

Stimulating canine platelets with AA/Epi caused a significant increase in P-selectin expression compared with unstimulated samples. Adding aspirin to the blood ex vivo inhibited this increase. Similarly, stimulating platelets with ADP/U4 caused a significant increase in P-selectin compared with unstimulated samples. This increase in P-selectin was inhibited by the $P2Y_{12}$ antagonist cangrelor when added ex vivo *in* the majority of cases. These results are very similar to those obtained with human blood.[25–29] The results from 2 of 3 dogs receiving aspirin indicated good inhibition of platelet activity. However, mf values in this cohort were higher than unstimulated values with one value substantially higher. This observed difference could indicate suboptimal inhibition of platelet activity by aspirin in these dogs, perhaps reflecting inadequate dosing[g].[13,14,15,30]

Despite these differences, there was no significant difference between conditions A and C. The failure to reach significance could be due to the small numbers in the treated dog group and represent a Type II statistical error. A larger sample size would help determine if this effect was real. The results from dogs receiving clopidogrel showed good inhibition of platelet activity with no clear increase in mf over baseline in any dogs, suggesting, in this cohort at least, the dose was adequate.

We reviewed the details of the untreated dogs enrolled in this study and found no relation between signalment, reason for initial presentation, the drugs that were being administered and their resultant P-selectin expression under any condition; 23 of 39 dogs were receiving no medication at the time of sampling, 7 of 39 dogs were presented for elective procedures including neutering and routine health examinations, and in a further 9 of 39 dogs, their reason for presentation was considered to be local rather than systemic disease, for example, rhinitis, urinary incontinence, and low-grade mast cell tumor removal. Taking these groups together, 41% of the dogs had no "systemic" disease or medications. This suggests our cohort is reasonable to provide an initial proof of principle for the methodology.

In the dogs with minimal increase in P-selectin after stimulation in conditions A or C (n = 13): 8 of 13 (62%) of these were presented for routine health checks or neutering and were receiving no medications at the time of sampling. The reasons for the minimal increase therefore cannot be attributed to suppression of platelet activity due to disease or medications. This may represent an anomaly from the sampling process; however, this was standardized as much as possible with an ECVIM-CA diplomate sampling each dog (MD/JA). It is possible that interdog variation in platelet activity accounts for the response. However, given that both conditions A and C test distinct aspects of platelet activity, we feel this unlikely. In addition, and perhaps most importantly, despite the minimal stimulation in this cohort, the P-selectin expression remained above that of the unstimulated condition B. We therefore feel it is unlikely to be an inherent problem with platelet response to agonists; however, further work on this is therefore required.

Four dogs had increased background platelet activity (condition B mf >500). Three were considered systemically well, presented for perineal hernia repair, otitis externa, and low-grade mast cell tumor removal. The increase in mf could indicate increased platelet activity due to their conditions (despite their presentation); given the small numbers, this is difficult to conclude and remains speculation. It is also possible that this resulted from sampling anomalies. Greater numbers would help to determine this point. However, despite the high P-selectin in condition B, the response to conditions A and C in all dogs led to a significant subsequent increase in P-selectin. This indicates the methodology can still interrogate platelet activity in samples with high background P-selectin expression. Three of these dogs also demonstrated limited inhibition in condition C+Cang. However, their respective condition A and C mf substantially increased above their condition B mf. This increase in P-selectin above background was inhibited by cangrelor to the expected extent, indicating background expression of P-selectin did not negate cangrelor's effect. This observation did not reach statistical significance, and we suspect this also represents a type II error.

The impact of sample storage on P-selectin expression is fundamental to the success of this method. Samples were held at room temperature for the first analysis and subsequently stored at 4°C until re-analysis. Data obtained 1-3, 4-7, and 20-22 days after stimulation and fixation were very similar with excellent correlation between the individual samples indicating minimal impact of storage duration or temperature. This would suggest temperatures encountered during transport between clinic and reference laboratory are unlikely impact on the results.

Why is measuring the efficacy of antiplatelet medication in individual dogs necessary, if dose ranges are available? An absence of licensed medications, variable pharmacokinetics, and accepted clinical efficacy of a particular dose create uncertainty. The ability of antiplatelet medications to prevent or reduce thrombotic events is unclear if we are unsure of the effect individual dose. There is robust evidence in the veterinary literature that the dose/efficacy of aspirin is frequently suboptimal.[13,14,30] Whether this applies to clopidogrel is unclear; however, dose adjustments might be necessary in cats undergoing long-term treatment.[30] In humans, interpatient variability in efficacy occurs with both aspirin and clopidogrel.[31] In humans, genetic polymorphisms significantly influence the beneficial and undesirable effects of these medications.[32] This phenomenon is also recognized in dogs carrying the MDR-1 mutation,[33,34] which may impact clopidogrel therapy due to the ABCB1 gene's role in intestinal uptake.[30,35] In humans, genetic polymorphisms lead to significant differences in the efficacy of oral antithrombotic drugs.[16,26,31,36] Clopidogrel requires cytochrome P450 for activation and polymorphisms convey variable clinical efficacy of some $P2Y_{12}$ antagonists.[37,38] Many alleles have been implicated, causing both gain and loss of function.[39–41] This is complicated by other medications, which are also substrates for cytochromes, for example, omeprazole.[42] Dogs receiving clopidogrel and omeprazole had increased concentrations of clopidogrel inactive metabolites although this did not alter platelet activity.[43] The influence of genetic polymorphisms in dogs remains unclear as variations in cytochrome P450-metabolic activity exist between humans, dogs, and cats.[44] $P2Y_{12}$ polymorphisms, which do not cause spontaneous hemorrhage, can still alter the response to medications.[37,38] A small case series reported a novel $P2Y_{12}$ mutation in a family of Greater Swiss Mountain dogs.[45] These dogs were subclinically affected and notable hemorrhage only occurred under specific conditions. New $P2Y_{12}$ antagonists have been developed for humans to circumvent these problems with clopidogrel. Ticagrelor does not require activation[46] and vicagrel uses an alternative pathway for metabolism.[47–49] There are no reports on the use of these new drugs clinically in dogs, although experimental studies exist.[48,50] The group of dogs receiving clopidogrel in this study demonstrated effective suppression of platelet activity, which is encouraging but should not infer an effective dose in all dogs. As in dogs, variable responses occur in humans receiving aspirin.[51–53] Explanations for this include the rate of platelet turnover and frequency of administration (q12h dosing may improve efficacy in humans).[54,55] Point-of-care genetic testing can also aid with individualizing human therapy when available.[56,57] In our small cohort, the aspirin response did vary and requires further evaluation.

Our results indicate the method developed for humans works for dogs. There may therefore be value in conducting similar pilot studies in other key species, particularly cats. Cats with hypertrophic cardiomyopathy (HCM) frequently receive antiplatelet medications due to the risk of thromboembolism.[17,58–60] Most cats with HCM receive long-term antiplatelet medication and would clearly benefit from regular assessment of drug efficacy. However, a single study in cats suggests clopidogrel is more efficacious in preventing a thromboembolic event.[17] In humans however, evidence exists that aspirin monotherapy is more efficacious in preventing thromboembolism in cardiovascular disease.[61] Given that the optimal dose of aspirin remains unclear, this method when refined would facilitate optimization of therapy. The data presented in this pilot study indicate that canine platelets can be stimulated in vitro using this method which could also help detect defects in platelet activity in dogs that cause excessive bleeding. This would replicate the approach used in humans.[28,29]

Inevitably in a preliminary study, limitations exist. Recruitment for this study was not case controlled; future studies with controlled recruitment will facilitate greater evaluation of whether presenting conditions influence the P-selectin expression following stimulation. The impact of the sampling process was controlled for by condition B; however, sampling individual dogs on subsequent occasions would help interrogate this further. Perhaps most importantly, we need greater numbers of treated dogs at defined drug concentrations. This would help with assessing treatment efficacy and go some way to determining the optimal concentration of antiplatelet medication required to inhibit platelet activity. The impact of systemic inflammation on the results is also of interest given the accepted influence of inflammation on platelet activity. These extended data were not available in this cohort and so further analysis has not been performed, although changes on the CBC (where it was performed) showed no correlation with mf values (data not shown). A further potential limitation is the impact of breed, as has been suggested in previous studies.[62] Whilst not apparent from this study, this is a possible confounder to this methodology which greater numbers from each breed would help to interrogate.

The main focus of these experiments was whether this remote assay could facilitate assessment of the impact of antiplatelet medications on canine platelet activity, and the results obtained were positive in this regard. Consequently, once fully validated, it might be possible for this method to help optimize treatment with either aspirin or clopidogrel in animals. Once refined, the availability of a simple to use test for remote analysis of platelet activity will enable a better understanding of the relationship between the effectiveness of drugs acting as inhibitors of platelet function and clinical outcome; in the same way as has been established in humans.[21–23]

Footnotes

[a] PAMFIX - Platelet Solutions Limited, www.plateletsolutions.co.uk, The Colin Campbell Building, Triumph Road, Nottingham, UK, NG7 2TU.

[b] Clopidogrel, Plavix®, Sanofi UK, One Onslow Street, Guildford, Surrey, UK, GU1 4YS

[c] Aspirin, Aspar, Pharmaceuticals Ltd, London, UK

[d] Cangrelor, The Medicines Company, Parsippany, NJ 07054, USA

[e] CD61 - Bio-Rad, Langford Business Park, Endeavour House, Langford Ln, Kidlington, OX5 1GE

[f] CD62P - Santa Cruz Biotechnology, Inc, Bergheimer Str. 89-2, 69115, Heidelberg, Germany

[g] McLewee N, Archer T, Wills R, et al. Effects of aspirin dose escalation on canine platelet function and urinary thromboxane and prostacyclin levels. J Vet Intern Med 2016;30:1465 (abstract)

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Conflict of Interest Declaration: Stan Heptinstall, Sue Fox, and Jane May are founders of Platelet Solutions Ltd, a spin out company of the University of Nottingham which manufactures PAMFix, one of the reagents used in this investigation. Stan Heptinstall is also a director of the Company.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Case details of the dogs in the untreated group. The respective CD62P mf values are shown in columns B-C+Cang. Results were obtained after no stimulation (B = saline control), stimulation under condition A (AA/Epi) in the absence of (A) and presence of aspirin (A + ASA) and stimulation under condition C (ADP/U4) in the absence of (C) and presence of cangrelor (C + Cang).