Plasma and serum serotonin concentrations and surface-bound platelet serotonin expression in Cavalier King Charles Spaniels with myxomatous mitral valve disease

Signe E. Cremer DVM, PhD
Annemarie T. Kristensen DVM, PhD
Maria J. Reimann DVM
Nynne B. Eriksen DVM
Stine F. Petersen DVM
Clara B. Marschner DVM
Inge Tarnow DVM, PhD
Mark A. Oyama DVM
Lisbeth H. Olsen DVM, DVSc

OBJECTIVE
To investigate serum and plasma serotonin concentrations, percentage of serotonin-positive platelets, level of surface-bound platelet serotonin expression (mean fluorescence intensity [MFI]), and platelet activation (CD62 expression) in platelet-rich plasma from Cavalier King Charles Spaniels with myxomatous mitral valve disease (MMVD).

ANIMALS
Healthy dogs (n = 15) and dogs with mild MMVD (18), moderate-severe MMVD (19), or severe MMVD with congestive heart failure (CHF; 10).

PROCEDURES
Blood samples were collected from each dog. Serum and plasma serotonin concentrations were measured with an ELISA, and surface-bound platelet serotonin expression and platelet activation were determined by flow cytometry.

RESULTS
Dogs with mild MMVD had higher median serum (746 ng/mL) and plasma (33.3 ng/mL) serotonin concentrations, compared with MMVD-affected dogs with CHF (388 ng/mL and 9.9 ng/mL, respectively), but no other group differences were found. Among disease groups, no differences in surface-bound serotonin expression or platelet activation were found. Thrombocytopenic dogs had lower serum serotonin concentration (482 ng/mL) than nonthrombocytopenic dogs (731 ng/mL). In 26 dogs, a flow cytometry scatterplot subpopulation (FSSP) of platelets was identified; dogs with an FSSP had a higher percentage of serotonin-positive platelets (11.0%), higher level of surface-bound serotonin expression (MFI, 32,068), and higher platelet activation (MFI, 2,363) than did dogs without an FSSP (5.7%, 1,230, and 1,165, respectively). An FSSP was present in 93.8% of thrombocytopenic dogs and in 29.5% of nonthrombocytopenic dogs.

CONCLUSIONS AND CLINICAL RELEVANCE
A substantive influence of circulating serotonin on MMVD stages prior to CHF development in Cavalier King Charles Spaniels was not supported by the study findings. An FSSP of highly activated platelets with pronounced serotonin binding was strongly associated with thrombocytopenia but not MMVD.

ABBREVIATIONS
CD61-APC  Allophycocyanin-conjugated anti-CD61 antibody
CD62-PE  Phycoerythrin-conjugated anti-CD62 antibody
CHF  Congestive heart failure
FSSP  Flow cytometry scatterplot subpopulation
iLVIDd  Indexed end-diastolic left ventricular internal dimension
IQR  Interquartile range (25th to 75th percentile)
LA:Ao  Left atrium-to-aortic root ratio
MFI  Mean fluorescence intensity
MMVD  Myxomatous mitral valve disease
Serotonin-PECy7  Phycoerythrin and cyanin–conjugated anti-serotonin antibody
PRP  Platelet-rich plasma

High plasma concentrations of serotonin or serotonin agonism, as seen in carcinoid syndrome, and with use of serotonergic medications, respectively, is associated with increased risk of valvular heart disease in humans.1–4 For dogs, there is emerging evidence of an association between serotonin and naturally occurring MMVD.5–11 Serum serotonin concentrations in dogs with MMVD and in healthy Cavalier King Charles Spaniels, a dog breed predisposed to MMVD, are higher than concentrations in healthy dogs of other breeds.6 In addition, the highest serum serotonin concentrations are found among dogs in the early stages of disease.11 Approximately one-third of Cavalier King Charles Spaniels have inherited nonclinical thrombocytopenia with presence of macroplatelets,12–14 an interesting finding, considering that platelets store circulating serotonin.15 The clinical importance of thrombocytopenia in Cavalier King Charles Spaniels is unknown,
and no association with severity of MMVD has been found, to our knowledge.

In people, MMVD has been associated with increased risk of thromboembolic events,16–22 and a similar risk is suspected in dogs.23,24 In Cavalier King Charles Spaniels with MMVD, the hemostatic system has been thoroughly evaluated.25–28 Of special interest is the finding of low plasma von Willebrand factor concentration, particularly pronounced in later stages of MMVD, due to loss of high-molecular weight monomers.20,27 as described for humans with high-shear cardiac conditions.29,30 High-molecular weight monomers of von Willebrand factor are important in the activation of platelets subjected to high shear stress,31 which could indicate less platelet activation in later stages of MMVD.

Platelets subjected to shear stress release serotonin.32 Given the high serum serotonin concentration in early stages of MMVD, a scenario of decreased platelet activation in late-stage MMVD is intriguing.11 Therefore, we hypothesized that platelet activation and resultant serotonin release would be more pronounced in dogs with earlier MMVD stages than in those with late-stage disease. However, very low plasma serotonin concentrations are maintained, compared with the platelet storage pool of serotonin, because platelets store > 99% of systemic serotonin.15 Markedly high plasma serotonin concentrations have so far been documented only for human cases of carcinoid heart disease, where the clearance mechanisms for serotonin become overwhelming.2 However, serotonin can also be retained on the surface of platelets after platelet activation, as seen in people with highly coagulant platelets known as coated platelets.33 Platelet surface-bound serotonin could therefore serve as a valuable source of local platelet serotonin availability in individuals with MMVD.

The objectives of the study reported here were to investigate serum and plasma serotonin concentrations, percentage of serotonin-positive platelets, level of surface-bound platelet serotonin expression (MFI), and platelet activation (CD62 expression) in platelet-rich plasma from Cavalier King Charles Spaniels with various stages of MMVD and assess the influence of thrombocytopenia on those variables.

Materials and Methods

Animals

The study was approved by the Danish Inspectorate for Animal Experimentation. Sixty-four privately owned Cavalier King Charles Spaniels ≥ 4 years of age were prospectively recruited from a research database associated with the University of Copenhagen. Informed owner consent was obtained, and all dogs underwent clinical examination, blood sample collection, and echocardiography. Exclusion criteria were systemic treatment with medications not including heart failure medications; systemic disease identified by clinical examination and results of a CBC and serum biochemical analysis; and pregnancy, lactation, or presence of estrus determined by clinical signs and evaluation of a vaginal smear. Dogs eligible for the study were classified as healthy or having MMVD. The dogs were matched by sex and grouped on the basis of echocardiographic estimation of mitral valve regurgitation relative to the size of the left atrium14,35 as follows: < 20% (control group), 20% to 50% (mild mitral valve regurgitation [mild MMVD group]), > 50% (moderate-severe mitral valve regurgitation [moderate-severe MMVD group]). The remaining dogs were those with clinical CHF due to severe MMVD (severe MMVD-CHF group). Diagnosis of CHF was based on clinical signs, findings of echocardiographic examination and thoracic radiography, and responsiveness to diuretic therapy.

Echocardiography

Echocardiographic examinations were performed by the same two observers (MJR or LHO) according to a standardized echocardiographic protocol as previously described.36 An ultrasound echocardiographic unit with 3S (second harmonic settings, 1.7 to 5.4 MHz) and 5S (second harmonic settings, 2.5 to 5.0 MHz) transducers was used and M-mode, 2-D, and color-flow Doppler data were recorded. Echocardiographic recordings were measured with software by the same observer (LHO).

Blood sample collection and handling

A blood sample (17 mL) was collected by 1 of 2 observers (SEC or LHO) from a jugular vein of each dog. Venipuncture was performed with a 21-gauge butterfly needle, and blood was collected in tubes containing 3.2% sodium citrate (9 mL) for flow cytometric analyses, manual platelet count, and ELISA; in tubes containing EDTA (4 mL) for CBC and manual platelet count; and in tubes without anticoagulant (4 mL) for serum biochemical analysis and later ELISA.

Platelet-rich plasma and platelet-poor plasma were prepared as previously described.37 For manual platelet counts, 20 µL of EDTA-stabilized blood and 20 µL of PRP were added to 380 µL of stromatolytic solution and counted as previously described.14 Platelet-poor plasma and serum samples were stored at –80°C for the serotonin ELISA. All samples were analyzed within 7 months after collection.

Enzyme-linked immunosorbent assay

Serotonin concentrations of serum and plasma samples were measured with a serotonin ELISA kit. The kit was previously validated for use in dogs.8,11 All samples were analyzed in duplicate, and 2 interplate controls for serum and plasma were included on each plate. Results were expressed as the mean value of the duplicate measurements.

Flow cytometric analyses

The protocol for flow cytometric analyses was modified from that of Tarnow et al. Flow cytomet-
ric analyses were performed on samples of phorbol-myristate-acetate–activated PRP. For maximal PRP activation, 480 µL of PRP was thoroughly mixed with 20 µL of phorbol-myristate-acetate (1,000 µM) at a final concentration of 40µg/mL, and then incubated at 37°C for 20 minutes. Ten microliters of phorbol-myristate-acetate–activated PRP was incubated in parallel in 4 tubes, with 10 µL of selected antibodies. Mouse monoclonal anti-human CD61-APC† (final concentration, 1 µg/mL) identified platelets. Mouse monoclonal anti-human CD62-PE‡ (final concentration, 2 µg/mL) as isotype control. Rabbit polyclonal anti-human serotonin-PECy7§ detected surface-bound platelet serotonin. The tubes contained the following antibody mixtures: tube 1, CD61-APC and IgG2a-PE; tube 2, CD61-APC and CD62-PE; tube 3, CD61-APC, CD62-PE, and serotonin-PECy7; and tube 4, none (negative control). Platelet-rich plasma and antibody mixtures were incubated for 20 minutes in the dark at room temperature (approx 21°C). Fixation of each sample with 400 µL of HEPES-saline (0.9% NaCl) solution with 1% formaldehyde ended the incubation, and samples were analyzed by means of flow cytometry within 30 minutes after fixation.

Flow cytometric analyses were performed with a fluorescence-activated cell sorting system† and results were processed with software† by an observer (SEC) who was unaware of the identity and health status of each dog. All instrument settings including compensation control, threshold, and photomultiplier tube voltages were modified from Tarnow et al§§ and optimized on non–Cavalier King Charles Spaniels with unknown macroplatelet status by the same observer (CBM). Accordingly, the protocol was set up to measure normal-size platelets in whole blood samples from non–Cavalier King Charles Spaniels. Briefly, compensation controls for individual APC and PE filters were performed with single-stained whole blood samples by use of CD61-APC and CD62-PE, respectively. Threshold was set at 200, and photomultiplier

Table 1—Age, weight, sex, echocardiographic data, Hct, platelet counts, and presence or absence of thrombocytopenia and a platelet FSSP in healthy (control) Cavalier King Charles Spaniels and those with various stages of MMVD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 15)</th>
<th>Mild MMVD (n = 18)</th>
<th>Moderate-severe MMVD (n = 19)</th>
<th>Severe MMVD-CHF (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>4.67 (4.25–6.83)</td>
<td>8.16 (4.50–8.75)</td>
<td>8.41 (8.00–9.33)</td>
<td>10.00 (9.25–11.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>9.00 (8.20–10.60)</td>
<td>9.75 (8.70–10.20)</td>
<td>9.50 (8.50–10.90)</td>
<td>8.85 (8.50–10.30)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Mitral valve regurgitation (%)</td>
<td>10 (5–10)⁵,6,7</td>
<td>40 (30–50)⁶,7</td>
<td>80 (70–100)⁵,6,7</td>
<td>100 (100–100)⁵,6,7</td>
</tr>
<tr>
<td>LA:Ao</td>
<td>1.3 (1.2–1.4)⁵,6</td>
<td>1.3 (1.2–1.5)⁵,6</td>
<td>1.5 (1.4–1.6)⁵,6</td>
<td>1.9 (1.8–2.1)⁵,6</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>7.0 (6.2–7.6)</td>
<td>7.0 (6.0–8.0)</td>
<td>7.2 (6.4–8.4)</td>
<td>7.0 (6.8–7.8)</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>8.6 (8.2–9.0)</td>
<td>8.2 (7.4–9.6)</td>
<td>9.4 (8.8–10.6)</td>
<td>9.2 (8.6–9.8)</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>29.2 (26.8–33.0)</td>
<td>27.9 (27.3–31.8)</td>
<td>36.4 (32.8–39.8)</td>
<td>41.1 (38.8–42.6)⁸,9</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>19.6 (17.8–22.4)</td>
<td>20.5 (19.0–23.2)</td>
<td>24.2 (22.6–26.6)</td>
<td>24.6 (23.2–28.6)⁸,9</td>
</tr>
<tr>
<td>iLVIDd (mm)</td>
<td>15.3 (14.1–17.1)</td>
<td>14.8 (14.3–15.4)</td>
<td>18.7 (17.8–20.4)</td>
<td>21.0 (18.9–22.5)⁸,9</td>
</tr>
<tr>
<td>Fractional shortening</td>
<td>0.32 (0.26–0.38)</td>
<td>0.28 (0.22–0.31)</td>
<td>0.35 (0.29–0.39)</td>
<td>0.33 (0.31–0.44)</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>39.3 (38.2–44.5)</td>
<td>39.7 (35.5–41.2)</td>
<td>40.4 (37.3–42.9)</td>
<td>37.7 (32.6–45.2)</td>
</tr>
<tr>
<td>Platelet count (× 10⁴ plates/μL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-anticoagulated blood</td>
<td>105 (64–240)</td>
<td>236 (157–287)</td>
<td>226.5 (125–317)</td>
<td>112.9 (90–417)⁹</td>
</tr>
<tr>
<td>PRP</td>
<td>201 (39–287)</td>
<td>243 (89–308)</td>
<td>229 (54–393)⁹</td>
<td>124 (52–508)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>4</td>
<td>2⁹</td>
<td>3⁹</td>
</tr>
<tr>
<td>No</td>
<td>8</td>
<td>14</td>
<td>16⁹</td>
<td>6⁹</td>
</tr>
<tr>
<td>FSSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Easily identified</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Presence questionable</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
<td>5</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Continuous variables are listed as median (IQR).

†Lack of manual platelet count and inadequate blood sample account for missing data for 1 dog in the moderate-severe MMVD and 1 dog in the severe MMVD-CHF groups, respectively.

IVSd = End-diastolic interventricular septum. IVSs = End-systolic interventricular septum. LVIDd = End-diastolic left ventricular internal dimension. LVIDs = End-systolic left ventricular internal dimension.

Within a row, values with different superscript letters are significantly (P < 0.001) different from the value for the control; mild MMVD; moderate-severe MMVD; or severe MMVD-CHF group. Within a row, values with different superscript letters are significantly (P < 0.05) different from the value for the control; mild MMVD, moderate-severe MMVD, or severe MMVD-CHF group.

Dogs (n = 62) were grouped on the basis of echocardiographic findings including estimation of mitral valve regurgitation relative to the size of the left atrium: < 20% (control group), 20% to 50% (mild MMVD group), > 50% (moderate-severe MMVD group), and clinical CHF due to severe MMVD (severe MMVD-CHF group). Dogs were considered thrombocytopenic when the anticoagulated blood platelet count was < 100,000 × 10⁴ platelets/mL. Flow cytometry revealed the presence of a platelet FSSP in some dogs.
tube voltages were predetermined from analyses on 5 healthy non-Cavalier King Charles Spaniels by double staining of single whole blood samples with CD61-APC and CD62-PE and setting the photomultiplier tube for CD61-APC events. Ten thousand events (platelets) were recorded, and gates were set to identify platelets according to size (forward scatter), granularity (side scatter), and surface expression of CD61-APC. The P1 gate identified normal-size platelet populations solely according to forward scatter and side scatter. The P2 gate included events in the P1 gate with additional positive CD61-APC fluorescence. The P3 gate included events in P2 with additional positive CD62-PE fluorescence, accepting up to 1% of isotype controls in the positive population. The P4 gate included events in P2 with additional positive serotonin-PECy7 fluorescence, defining the positive population where the nonspecific signal in the PECy7 filter ended. Drift in fluorescence was checked daily with flow cytometry calibration particles.

Flow cytometric results from tube 3 (CD61-APC, CD62-PE, and serotonin-PECy7) were used in the data analyses and included platelet binding percentage and level (MFI) of anti-CD62 and anti-serotonin antibodies. As anti-serotonin antibody increased platelet activation, results from tube 2 were compared with those from tube 3 and the difference in CD62 activation was assessed.

**Statistical analysis**

Response variables (serotonin concentration in serum and plasma, platelet expression of serotonin measured as a percentage of serotonin-positive platelets and
level of binding of anti-serotonin antibody, and platelet expression of p-selectin measured as a percentage of CD62-positive platelets and level of binding of anti-CD62 antibody) were analyzed by means of multiple linear regression analyses.\textsuperscript{1,39} Explanatory variables, including disease group, age, sex, body weight, Hct, and presence of thrombocytopenia (ie, platelet count in anticoagulated blood samples, < 100,000 × 10\(^3\)/mL) were included in the analyses and removed via backward selection. In addition, LA:Ao and iLVIDd were analyzed in replacement of disease group. The final model was checked for normality and homogeneity and log or square root transformed if requirements were not fulfilled. Post hoc \( t \) test analyses with Tukey-Kramer adjustment were applied. When normality and homogeneity were not achievable, a univariate nonparametric Kruskal-Wallis test with Wil-

Figure 2—Surface-bound platelet serotonin and CD62 expression in activated platelets in the healthy Cavalier King Charles Spaniels and those with various stages of MMVD that were or were not thrombocytopenic in Figure 1. Flow cytometric analyses were performed to determine the percentages of serotonin- and CD62-positive platelets and level of expression of surface-bound platelet serotonin and CD62 (MFI). A—Percentage of serotonin-positive platelets for dogs in the control group (n = 15), mild MMVD group (16), moderate-severe MMVD group (16), and severe MMVD-CHF group (10). B—Level of serotonin-binding expression for dogs in the control group (n = 15), mild MMVD group (16), moderate-severe MMVD group (16), and severe MMVD-CHF group (10). Notice the distinct 2-layer effect with a subpopulation of platelets showing high serotonin binding. C—Percentage of CD62-positive platelets for dogs in the control group (n = 15), mild MMVD group (16), moderate-severe MMVD group (16), and severe MMVD-CHF group (10). D—Level of CD62 expression for dogs in the control group (n = 15), mild MMVD group (16), moderate-severe MMVD group (16), and severe MMVD-CHF group (10). See Figure 1 for remainder of key.
coxon rank sum scores was used. Difference in platelet activation before and after anti-serotonin antibody addition was tested by means of a Student t test. Explanatory variables were tested for group differences by means of univariate ANOVA or Fisher exact test. All analyses were performed in statistical software. Values of \( P \leq 0.05 \) were considered significant. Unless stated otherwise, results are reported as median (IQR).

### Results

#### Animals

Two of the 64 Cavalier King Charles Spaniels were excluded because of estrus, leaving 62 dogs in the study. No dogs were excluded on the basis of results of CBCs or serum biochemical analyses. Of the 62 dogs, the EDTA-anticoagulated blood sample and the sample without anticoagulant were not obtainable from 1 dog with severe MMVD and CHF; additionally, for 1 dog with moderate-severe MMVD, the manual platelet count was not performed. Overall, there were 15 healthy dogs, 18 dogs with mild MMVD, 19 dogs with moderate-severe MMVD, and 10 dogs with severe MMVD and CHF. Descriptive statistics based on health status classification were summarized (Table 1).

#### Serum serotonin concentration

For 1 sample from a dog with mild MMVD, the measurement was deemed unreliably low (62 ng/mL) and was excluded from statistical analyses. Median serum serotonin concentration was 591 ng/mL (IQR, 449 to 800 ng/mL) in the control group (\( n = 15 \)), 746 ng/mL (IQR, 651 to 868 ng/mL) in the mild MMVD group (17), 638 ng/mL (IQR, 482 to 761 ng/mL) in the moderate-severe MMVD group (19), and 388 ng/mL (IQR, 299 to 651 ng/mL) in the severe MMVD-CHF group (9). There was a significant \( (P = 0.04) \) difference in serum serotonin concentration between dogs with mild MMVD and those with severe MMVD and CHF (Figure 1). In dogs with thrombocytopenia (ie, platelet count in anticoagulated blood samples, < 100,000 \( \times 10^3/\text{mL} \); \( n = 16 \)), median serum serotonin concentration was 482 ng/mL (IQR, 399 to 651 ng/mL); in nonthrombocytopenic dogs (\( n = 44 \)), median serum serotonin concentration was 731 ng/mL (IQR, 569 to 929 ng/mL), which was significantly \( (P = 0.01) \) greater. Thrombocytopenia remained a significant (all \( P \leq 0.01 \)) factor regardless of disease variable (disease group, iLVIDd, and LA:Ao) included in analyses as explanatory variables. Age was negatively associated with serum serotonin concentration when either LA:Ao \( (P = 0.05) \) or iLVIDd \( (P = 0.02) \) was used in analyses as an explanatory variable.

#### Plasma serotonin concentration

Median plasma serotonin concentration was 15.3 ng/mL (IQR, 6.9 to 43.1 ng/mL) in the control group (\( n = 15 \)), 33.3 ng/mL (IQR, 9.8 to 69.4 ng/mL) in the mild MMVD group (18), 14.7 ng/mL (IQR, 8.2 to 32.0 ng/mL) in the moderate-severe MMVD group (19), and 9.9 ng/mL (IQR, 8.2 to 13.3 ng/mL) in the severe MMVD-CHF group (10). There was a significant \( (P = 0.004) \), overall \( P = 0.002 \) difference in plasma serotonin concentration between dogs with mild MMVD and those with severe MMVD and CHF (Figure 1). For all disease variables (disease group \( P = 0.01 \), LA:Ao \( P = 0.03 \), and iLVIDd \( P = 0.02 \)), there was a decrease in plasma serotonin concentration with increasing age. For iLVIDd \( P = 0.01 \), there was also a negative association between plasma serotonin concentration and Hct \( P = 0.03 \).

#### Flow cytometric analyses

All samples were fixed within 80 minutes after blood sample collection and flow cytometric analysis.
was performed within 2 hours after blood sample collection. Results from 5 dogs (2 dogs with mild MMVD and 3 dogs with moderate-severe MMVD) were excluded owing to technical problems performing the flow cytometric analyses.

**Surface-bound platelet serotonin expression**—There were no group differences \((P = 0.86)\) in percentage of serotonin-positive platelets, with a median of 9.8\% (IQR, 6.6\% to 15.5\%) in the control group \((n = 15)\), 7.3\% (IQR, 5.7\% to 11.2\%) in the mild MMVD group \((16)\), 8.7\% (IQR, 5.8\% to 15.1\%) in the moderate-severe MMVD group \((16)\), and 10.1\% (IQR, 7.4\% to 12.9\%) in the severe MMVD-CHF group \((10); Figure 2\). In regard to level of serotonin-binding expression, no group differences \((P = 0.67)\) were found, with a median MFI of 26,581 (IQR, 925 to 36,508) in the control group \((n = 15)\), 9,699 (IQR, 1,090 to 28,774) in the mild MMVD group \((16)\), 8,986 (IQR, 1,396 to 31,970) in the moderate-severe MMVD group \((16)\), and 25,951 (IQR, 1,254 to 34,852) in the severe MMVD-CHF group \((10)\). Of note, a distinct 2-layer effect was present, with some platelets having high serotonin binding. Both per-

---

**Figure 4**—Surface-bound platelet serotonin and CD62 expression in activated platelets in the healthy Cavalier King Charles Spaniels and those with various stages of MMVD that were or were not thrombocytopenic in Figure 2. Dogs were grouped on the basis of an easily identified FSSP (FSSP-1), questionable presence of an FSSP (FSSP-2), or absence of an FSSP (FSSP absent). Flow cytometric analyses were performed to determine the percentages of serotonin- and CD62-positive platelets and level of surface-bound platelet serotonin and CD62 expression (MFI). A—Percentage of serotonin-positive platelets for dogs identified as FSSP-1 \((n = 26)\) or FSSP-2 \((17)\) and FSSP absent \((14)\). B—Level of serotonin-binding expression for dogs identified as FSSP-1 or FSSP-2 and FSSP absent. Notice the distinct 2-layer effect with a subpopulation of platelets showing high serotonin binding. C—Percentage of CD62-positive platelets for dogs identified as FSSP-1 or FSSP-2 and FSSP absent. D—Level of CD62 expression for dogs identified as FSSP-1 or FSSP-2 and FSSP absent. See Figure 1 for remainder of key.
Percentage of serotonin-positive platelets and level of serotonin-binding expression were significantly (all \( P \leq 0.001 \)) higher in dogs with thrombocytopenia, regardless of disease variable tested.

**Platelet activation**—The percentage of CD62-positive platelets did not differ significantly (\( P = 0.10 \)) among groups, with a median of 78.8% (IQR, 57.7% to 89.8%) in the control group (\( n = 15 \)), 90.0% (IQR, 77.7% to 95.1%) in the mild MMVD group (16), 89.3% (IQR, 85.3% to 93.8%) in the moderate-severe MMVD group (16), and 89.9% (IQR, 63.0% to 91.8%) in the severe MMVD-CHF group (10; Figure 2). In regard to level of CD62 expression, no group differences (\( P = 0.07 \)) were found, with a median MFI of 1,325 (IQR, 904 to 2,505) in the control group (\( n = 15 \)), 1,527 (IQR, 1,172 to 2,004) in the mild MMVD group (16), 1,571 (IQR, 1,318 to 2,222) in the moderate-severe MMVD group (16), and 1,452 (IQR, 1,025 to 3,237) in severe MMVD-CHF group (10). The level of CD62 expression was significantly (all \( P < 0.001 \)) higher in dogs with thrombocytopenia, regardless of disease variable tested.

**Effect of anti-serotonin antibody on platelet activation**—In activated PRP, addition of anti-serotonin antibody caused a general increase in percentage of CD62-positive platelets (3.5%; IQR, 1.1% to 9.9%; \( P < 0.001 \)), but not level of CD62 expression (MFI, 12; IQR, -157 to 273; \( P = 0.07 \)). There were no group differences for percentage change in CD62 expression (\( P = 0.91 \)) or MFI (\( P = 0.13 \)).

**FSSPs**

In 26 dogs (Table 1), an easily identified FSSP of highly activated platelets was identified after addition of anti-serotonin antibody. The platelets in the FSSP had the same forward and side scatter as other platelets but had a decrease in anti-CD61 antibody binding level (Figure 3). The FSSPs were graded as easily identified (FSSP-1; \( n = 26 \)), questionable (FSSP-2; 17), or absent (14). There was no significant (\( P = 0.92 \)) difference in presence of FSSPs among the 4 health status groups (Table 1), but an easily identified FSSP was significantly (\( P < 0.001 \)) more common among dogs with thrombocytopenia (93.8%) than among dogs without thrombocytopenia (29.5%). To specifically evaluate the effect of an FSSP, thrombocytopenia was substituted for FSSP in multiple linear regression analyses.

**Surface-bound platelet serotonin expression**—Significant differences among groups with (FSSP-1 and FSSP-2) or without FSSPs were detected for both percentage of serotonin-positive platelets (\( P = 0.001 \)) and level of serotonin-binding expression (\( P < 0.001 \); Figure 4). Percentage of serotonin-positive platelets was significantly (\( P = 0.001 \)) higher in the FSSP-1 group (11.0%; IQR, 8.9% to 15.5%), compared with findings for the group without FSSPs (5.7%; IQR, 3.3% to 6.9%). Percentage of serotonin-positive platelets in the FSSP-2 group (7.4%; IQR, 6.0% to 11.7%) did not differ from that in either the FSSP-1 group (\( P = 0.13 \)) or group without FSSPs (\( P = 0.20 \)). Level of serotonin-binding expression was significantly (\( P < 0.001 \)) higher in the FSSP-1 group (MFI, 32,068; IQR, 27,190 to 40,393) than in the FSSP-2 group (MFI, 1,706; IQR, 812 to 17,373) or the group without FSSPs (MFI, 1,230; IQR, 845 to 2,063), with no difference (\( P = 0.49 \)) between findings for the FSSP-2 group and the group without FSSPs.
CD62 expression—Among the FSSP groups, there was no difference (P = 0.27) in percentage of CD62-positive platelets, with a median of 90.7% (IQR, 74.1% to 93.4%) in the FSSP-1 group, 88.7% (IQR, 78.8% to 94.3%) in the FSSP-2 group, and 82.0% (IQR, 65.5% to 88.6%) in the group without FSSPs (Figure 4). In regard to level of CD62 expression, there was a significant (P < 0.001) difference among FSSP groups. More specifically, the FSSP-1 group had significantly higher level of CD62 expression (MFI, 2,563; IQR, 1,458 to 2,903) than did the FSSP-2 group (MFI, 1,441; IQR, 1,097 to 1,678; P < 0.001) and the group without FSSPs (MFI, 1,165; IQR, 903 to 1,356; P < 0.001).

With the exceptions of thrombocytopenia and platelet count, none of the other descriptive variables were significantly different among the FSSP groups (data not shown). Platelet-rich plasma platelet count, anticoagulated blood platelet count (Figure 5), and serum serotonin concentration (Figure 6) were significantly (all P < 0.001) lower in the FSSP-1 group, compared with findings in the FSSP-2 group and group without FSSPs. Median serum serotonin concentration was 520 ng/mL (IQR, 411 to 651 ng/mL) in the FSSP-1 group (n = 24), 766 ng/mL (IQR, 579 to 897 ng/mL; 17) in the FSSP-2 group (P < 0.001), and 792 ng/mL (IQR, 737 to 983 ng/mL; 14) in the group without FSSPs (P < 0.001).

Discussion

Results of the present study reflected to some extent only the previously established association of high serum serotonin concentrations among Cavalier King Charles Spaniels with mild MMVD.11 Interestingly, the results indicated that plasma serotonin concentration follows the same disease-group pattern as that for serum serotonin concentration. Although serum and plasma serotonin concentrations in dogs with mild MMVD were higher than findings in dogs with severe MMVD and CHF, no differences in either variable were found among any other health status groups. Moreover, no association was found with regard to surface-bound platelet serotonin expression or platelet activation and MMVD severity. Surprisingly, a subpopulation of highly activated platelets with high surface-bound serotonin expression that was strongly associated with thrombocytopenia was identified.

The present study revealed that serum and plasma serotonin concentrations followed the same pattern in relation to MMVD progression in Cavalier King Charles Spaniels. All groups had fairly large variation in serum and plasma serotonin concentrations; this was especially pronounced for plasma serotonin concentration in the control and mild MMVD groups. The much higher plasma serotonin concentration in these groups was especially interesting, considering that these dogs were likely developing MMVD or were in the early stage of MMVD. A recent study43 revealed no differences in plasma and platelet serotonin concentrations between healthy dogs predisposed to MMVD and dogs with MMVD, whether clinical or subclinical, which indicated no involvement of plasma and platelet serotonin in the progression of MMVD. However, as the part of circulating serotonin freely available to mediate local effects, high plasma serotonin concentrations in some dogs in the control and mild MMVD groups of the present study adds to the speculation regarding long-term consequences of high circulating serotonin concentrations in dogs with minimal or mild disease. It is possible that high plasma and platelet serotonin concentrations might represent a familial trait among some Cavalier King Charles Spaniels that could lead to early onset of MMVD, which occurs frequently in this breed. It is unknown whether plasma serotonin concentrations of the magnitude detected in the present study are sufficient to induce early valvular changes in dogs; nevertheless, it is an intriguing theory, given that serotonin is speculated to activate valvular interstitial cells via induction of transforming growth factor-β signaling.5,6,10,44,45 However, people with carcinoid heart disease secondary to high plasma serotonin concentration have plasma serotonin concentrations approximately twice as high as the highest concentration measured in the present study. The lack of differences between any other MMVD groups prior to development of CHF moreover demonstrates the uncertainties as to whether serotonin is involved in the early progression of MMVD, and it is plausible that the lower serotonin concentrations among dogs with severe MMVD and CHF perhaps is a consequence of CHF. Follow-up studies of young dogs with and without high plasma serotonin concentrations are needed to assess the clinical relevance of plasma serotonin concentrations in the range measured in the dogs of the present study.

In contrast to 2 previous studies8,11 in which there was no association between serum serotonin concentration and platelet size and count, the present study revealed a lower serum serotonin concentration in dogs with thrombocytopenia (eg, platelet count < 100,000 X 10^3 platelets/mL), compared with concentration in nonthrombocytopenic dogs. The discrepancy between the findings of present study and those other studies8,11 could be explained, partly, by the slightly different means of classification of dogs as having or not having thrombocytopenia. In the previous studies,8,11 platelet size affected the grouping criteria. In the present study, dogs were categorized as having thrombocytopenia based only on a platelet count < 100,000 X 10^3 platelets/mL. This platelet count has been identified as the cutoff under which most macroplatelets are found.47 The importance of lower serum serotonin concentrations among Cavalier King Charles Spaniels with thrombocytopenia is unknown. It has been shown that Cavalier King Charles Spaniels with macroplatelets have normal internal platelet morphology48 and the same plateletcrit as Cavalier King Charles Spaniels with normal platelet size.49 Assuming that the thrombocytopenic dogs of the present study had the same plateletcrit as the nonthrombocytopenic dogs, it appears from the present study’s findings...
that, in regard to serotonin, thrombocytopenic dogs have a lower serotonin concentration in relation to total platelet volume. Further studies of exact measurements of platelet serotonin content in macroplatelets versus normal-size platelets are needed.

In the present study, surface-bound platelet serotonin expression was measured as a marker of the dogs’ circulating serotonin availability. In this respect, platelet activation (CD62 expression) was addressed simultaneously. Among the health status groups, there were no differences in either the percentage of serotonin-positive platelets or level of serotonin-binding expression (MFI), and the data did not support involvement of surface-bound platelet serotonin in the progression of MMVD. Also, for CD62 expression, there was no association of either percentage of platelet activation or level of expression with disease progression. With regard to the percentage of platelet activation, this finding was in agreement with that of a previous Cavalier King Charles Spaniels study. The present study data did not support decreased platelet activation as the cause of lower circulating serotonin concentrations in dogs with severe MMVD and CHF; however, only a single activation marker was used and no platelet function tests were applied.

In contrast to the findings for health status groups, dogs across all health status groups with thrombocytopenia had a significantly higher percentage of serotonin-positive platelets, higher level of serotonin-binding expression, and higher level of CD62 expression, compared with findings for nonthrombocytopenic dogs. Also, the information gained from addressing level of CD62 expression (MFI) versus the percentage of CD62-positive platelets was striking. Although little or no differences were found from assessment of the percentage of serotonin- or CD62-positive platelets among health status groups, assessment of level of serotonin binding or CD62 expression revealed a more detailed picture. The previously described 2-layer effect was very pronounced for platelet serotonin-binding expression and present for level of platelet CD62 expression (Figure 2). In other words, it was visually apparent that some platelets had very high-level CD62 expression and high-level serotonin binding. These findings indicated that some dogs had the potential for very high-level platelet activation and additionally retention of large amounts of surface-bound platelet serotonin. In addition, a large proportion of these dogs were thrombocytopenic. Perhaps this reflects a compensatory mechanism of increased p-selectin and serotonin expressions on individual platelets as a result of lower platelet number. A better understanding of platelet activation might be gained by addressing platelet volume (vs count) and by use of multiple activation markers.

An interesting discovery was the presence of an FSSP on flow cytometry scatterplots for some dogs, which was strongly related to thrombocytopenia. In addition, dogs with an easily identified FSSP had a higher percentage of serotonin-positive platelets, much higher level of serotonin-binding expression, and higher CD62 expression, compared with dogs that had no FSSP. The only distinctive characteristic of dogs with FSSPs was the large proportion that had thrombocytopenia. Given that most macroplatelets are typically found in this group, an appealing explanation would be a platelet size and binding relationship. However, macroplatelets were not included in the flow cytometric analysis in the present study. Information on serotonin and CD62 expressions of macroplatelets might add valuable information regarding their presence in FSSPs among thrombocytopenic dogs. Also, inclusion of non-Cavalier King Charles Spaniels breeds could provide information as to whether this finding is breed specific.

The identification of an FSSP of highly activated platelets with pronounced serotonin surface binding in Cavalier King Charles Spaniels is interesting in light of the presence of a well-established subtype of hyperactivated platelets identified following platelet activation in people. These platelets in humans, referred to as coated platelets, bind procoagulant proteins such as fibrinogen, factor V, phosphatidylserine, thrombospondin, and glycoprotein Ib/IIia with exceptionally high affinity and are capable of very high levels of thrombin generation. Serotonin has been identified as the cross-binding molecule of coated platelets, linking and perhaps anchoring these procoagulant proteins on the platelet surface. In humans, the ability to generate coated platelets is enhanced among those with immature younger platelet populations, characteristic of people with thrombocytopenia. The similarities to the FSSPs detected in the Cavalier King Charles Spaniels with thrombocytopenia in the present study are striking. The clinical relevance of coated platelet generation is unknown, but they have been suggested as a prothrombotic marker. This could indicate an association between increased circulating serotonin concentration and a risk of generating platelets with a yet unknown potential. One feature of coated platelets is the common need for dual agonist stimulation. This is in agreement with the present study findings, insofar as the FSSP platelets were present only after dual stimulation with agonist and anti-serotonin antibody. It has also been described that antibodies can lead to induction of coated platelets. The results of the present study strongly indicated, but did not prove, the presence of coated platelets. Previous studies have identified induction of coated platelets in dogs. Similar studies in Cavalier King Charles Spaniels are warranted, because coated platelets have not been investigated in this breed and the clinical relevance of these findings needs to be elucidated. One might posit the presence of a Cavalier King Charles Spaniel-specific platelet phenotype that generates immature, highly activated platelets to explain the phenomenon of nonclinical thrombocytopenia in this breed. It is reasonable to assume that platelets with these combined and pronounced characteristics would serve a specific purpose, a purpose that may or may not be related to MMVD. If the platelets we identified in Cavalier King Charles Spaniels are indeed...
coated platelets, their purpose seems unrelated to development of MMVD per se.

The study was limited by the inclusion of only normal-size platelets in the flow cytometric analysis, whereas macroplatelets also contributed to the results of the ELISA. Also, inclusion of other breeds of dog with no macrothrombocytopenia could have helped distinguish the discovered platelet subpopulation as breed specific. The fairly small health status group sizes may have limited the power of the study.

Results of the present study indicated that Cavalier King Charles Spaniels with mild MMVD have higher serum and plasma serotonin concentrations than do Cavalier King Charles Spaniels with severe MMVD and CHF. However, those findings do not support involvement of circulating serotonin in the pathogenesis of MMVD because no other group differences were found. Although very high plasma serotonin concentrations were found in some individual dogs with minimal or mild MMVD, it remains speculative whether plasma serotonin concentrations of such magnitude could influence the early progression of MMVD in this breed. No associations were found between MMVD status and either surface-bound platelet serotonin expression or platelet activation. Yet, a subpopulation of highly activated platelets with high serotonin-binding expression was discovered and strongly associated with thrombocytopenia but not with MMVD. This FSSP could be the equivalent of highly procoagulant coated platelets in people and dogs.

Acknowledgments
This manuscript represents portion of a thesis submitted by Dr. Cremer to the University of Copenhagen Faculty of Health and Medical Sciences as a partial fulfillment of the requirements for a Doctor of Philosophy degree.

Supported in part by the research center LIFEPHARM and a PhD grant from University of Copenhagen, Copenhagen, Denmark.

Presented in abstract form at the American College of Veterinary Internal Medicine Forum, Nashville, Tenn, June 2014.

The authors thank Christina Tirsdal Kjempff, Susanne Kronborg, Anne Kirstine Havnsøe Krogh, and Louise Bochsen for technical assistance.

Footnotes
a. Vivid i, GE Health Care, Broendby, Denmark.
b. EchoPAC, PC version 112, GE Health Care, Broendby, Denmark.
c. Mascii Brunelli, Milano, Italy.
d. RES9121, IBL International GMBH, Hamburg, Germany.
e. Clone Y2/51, DAKO Cytomation, Glostrup, Denmark.
f. Clone 1E3, Santa Cruz Biotechnology, Santa Cruz, Calif.
g. Clone Y2/51, DAKO Cytomation, Glostrup, Denmark.
h. RE59121, IBL International GMBH, Hamburg, Germany.
i. FACSDiva, version 5.0.3, BD Biosciences, Albertslund, Denmark.
j. FACSCanto II, BD Biosciences, Albertslund, Denmark.
k. FACSDiva, version 5.0.3, BD Biosciences, Albertslund, Denmark.
l. FACSCanto II, BD Biosciences, Albertslund, Denmark.
m. RCP-50 rainbow particles, Spherotech Inc., Libertyville, Ill.

References
25. Tarnow I, Kristensen AT, Olsen LH, et al. Assessment of changes in

Unauthenticated | Downloaded 11/25/23 01:03 PM UTC


