Assessment of changes in hemostatic markers in Cavalier King Charles Spaniels with myxomatous mitral valve disease

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Objective—To evaluate markers of hemostasis and their relationship to the degree of mitral regurgitation (MR) and platelet function in Cavalier King Charles Spaniels (CKCSs) with myxomatous mitral valve disease.

Animals—76 clinically healthy CKCSs and 24 control dogs.

Procedure—All dogs underwent echocardiographic examination; various hemostatic, hematologic, and biochemical variables were evaluated in blood. The CKCSs were allocated to 1 of 3 groups on the basis of MR severity. In 8 control dogs and 8 CKCSs, plasma von Willebrand factor (vWF) multimer analysis was performed.

Results—Compared with control dogs, plasma fibrinogen concentration was higher in all CKCSs and related to left ventricular end diastolic diameter and left atrial-to-aortic root ratio among all CKCSs. The activated partial thromboplastin times and plasma D-dimer concentration were similar among the 4 groups. Plasma vWF concentration was lower in CKCSs with moderate to severe MR, compared with that of CKCSs with no MR and control dogs. There was a relationship between plasma vWF concentration and platelet function in CKCSs but not in control dogs. In 4 CKCSs with moderate to severe MR and low plasma vWF concentration, amounts of vWF high-molecular-weight multimers (HMWMs) were low.

Conclusions and Clinical Relevance—In CKCSs, MR appeared to be associated with a low plasma vWF concentration and likely a loss of vWF HMWMs (possibly through their destruction via shear stress to the blood). The importance of the changes in plasma fibrinogen concentration and the thromboembolic risk in dogs with MR remain to be investigated. (*Am J Vet Res* 2004;65:1644–1652)

Myxomatous mitral valve disease (**MMVD**) is the most prevalent heart disease in dogs.[1] It is characterized by a myxomatous degeneration of the valve that may result in substantial **mitral regurgitation (MR)** in some, but not all, dogs. **Mitral valve prolapse (MVP)** is a fundamental feature of MMVD[2-4]; in many ways, MVP in dogs is similar to primary MVP in humans.[5] **Cavalier King Charles Spaniels (CKCSs)** are predisposed to MMVD, and most CKCSs develop a systolic murmur as a result of MR before they reach 10 years of age.[2,6]

In dogs, changes in the hemostatic system have been reported in association with disease syndromes such as parvoviral enteritis,[7] gastric dilatation-volvulus,[8] and hemangiosarcoma.[9] Many humans with cardiovascular disease have hypercoagulation, and the changes in the coagulation system are often associated with atherosclerosis. In cardiovascular abnormalities in which atherosclerosis is not regarded as an underlying feature, such as primary MVP and aortic valve disease, hypercoagulability and increased risk of stroke have been reported.[10] Because of the diseased cardiac valves, regions of increased turbulence and shear stress develop that can activate platelets and coagulation factors in the blood and damage the vascular endothelium.[11]

The process of normal physiological hemostasis is dependent on collaboration between the hemostatic system and the endothelium, which maintains vascular integrity and normal vascular function. Changes in markers of hemostatic dysfunction would therefore theoretically be accompanied by a change in markers of endothelial dysfunction. Recently, a decreased plasma concentration of nitric oxide metabolites was identified in CKCSs with MR, compared with the concentration in clinically normal dogs without MR, which indicates that dogs with MR have endothelial dysfunction[12]; therefore, other and more easily measured markers of endothelial dysfunction would be relevant to study in dogs with MR. Compared with clinically normal individuals, an increase in plasma **von Willebrand factor (vWF)** concentration has been shown to be a consistent marker of endothelial dysfunction in humans[13,14] and vWF concentration is one of the few endothelial dysfunction markers that can be measured readily in canine plasma. Fibrinogen is a clotting fac-

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tor, an inflammatory marker, and an independent risk factor for thrombosis in humans with cardiovascular disease.[15,16] Plasma D-dimer is a fibrin degradation product and a marker of increased fibrin production or increased fibrinolysis. An increase in the plasma concentration of D-dimer has been reported in humans[17] with cardiovascular disease and recently in dogs with thromboembolic disease,[18,19] compared with concentrations in unaffected individuals. The **activated partial thromboplastin time (APTT)** and **prothrombin time (PT)** are measures of the overall coagulation process.

Different platelet abnormalities have been identified in dogs with MMVD and in CKCSs in particular because this breed also has a high prevalence of inherited macrothrombocytopenia.[20,21] In CKCSs, an increased platelet reactivity has been detected in dogs without macrothrombocytopenia.[22] In addition, a decreased platelet function has been identified in dogs with moderate to severe MR.[23,24] Thus, it has been speculated that platelet function plays a role in the pathogenesis of MMVD and MR.[22,24] Because platelets are intimately linked to the coagulation system, platelet function should be considered in studies of the coagulation system.

The purpose of the study reported here was to evaluate markers of hemostasis and their relationship to the degree of MR and to platelet count and function in CKCSs with MMVD. We hypothesized that CKCSs with MMVD may have endothelial dysfunction and alterations in markers of hemostasis, compared with healthy dogs, and that such changes might perhaps reflect hemodynamic effects and shear stress associated with MR.

Materials and Methods

Animals—Seventy-six client-owned CKCSs (median age, 4.3 years; 25th to 75th percentiles, 2.3 to 6.0 years) with no clinical signs associated with MR and 24 client-owned clinically normal control dogs (median age, 3.9 years; 25th to 75th percentiles, 2.4 to 5.4 years) were included in the study. Among the CKCSs, there were 38 males (2 of which were neutered) and 38 females; among the control dogs, there were 14 males (3 of which were neutered) and 10 females (1 of which was neutered). The CKCSs underwent cardiac examination consecutively from January 2002 to February 2003 as part of an MMVD screening program to be approved for breeding. In addition to breeding stock, older dogs owned by breeders were examined to monitor the progression of their mitral valve disease. The control dogs were owned by staff of the Department of Animal and Veterinary Basic Sciences of the Royal Veterinary and Agricultural University and their relatives; a dog was included in the study if no signs of heart disease, other systemic disease, or bleeding tendencies at clinical examination; the results of serum biochemical analyses and a CBC were within reference ranges; no heart murmur was detected via cardiac auscultation; and it had a regurgitant jet occupying ≤ 15% of the left atrial area detected echocardiographically. Informed consent was obtained from owners of all dogs.

On the basis of sizing the regurgitant jets relative to the left atrial area, the CKCSs were allocated to 1 of 3 groups according to their degree of MR: dogs with no or minimal MR (jet size, ≤ 15%), dogs with mild MR (jet size, > 15% and ≤ 50%), and dogs with moderate to severe MR (jet size, > 50%).

For all dogs, the examination comprised an interview with the owner and evaluation of the dogs, including collection of blood samples, physical examination, and echocardiographic examination (performed in that sequence). Food was withheld from all dogs for approximately 12 hours prior to the examination. None of the dogs were sedated for any part of the examination, and the owners were present to keep the dogs calm. None of the dogs had a history or signs of congestive heart failure, a bleeding disorder, or any other systemic disease; furthermore, none of the dogs had received any medication for the last 2 months prior to inclusion in the study. None of the dogs had hematologic or serum biochemical abnormalities (except for some CKCSs that had low platelet counts).

Blood sample collection and handling—Blood was collected by the same investigator (HDP) with careful venipuncture of the jugular vein into tubes containing no anticoagulant, 3.2% sodium citrate, or EDTA. For each dog, serum was used for biochemical analyses; the citrated blood sample was used for platelet function analysis (whole blood), a hemostatic profile (APTT, PT, and fibrinogen and D-dimer concentrations), measurement of vWF concentration, and multimer analysis (plasma); and blood with EDTA was used for a CBC and a manual count of platelets. Specimens of plasma and serum were prepared by centrifugation at 3,100 × *g* for 10 minutes within 1 hour of blood collection and portioned into aliquots. Sera were stored at 5°C and analyzed within 24 hours. Plasma samples were stored at –80°C until analysis (within 1 year). Whole blood samples with sodium citrate or EDTA were stored at 21°C and processed within 4 hours.

Analytical procedures—A CBC and serum biochemical analyses were performed for each dog. The hemostatic profile (including assessment of APTT, PT, and plasma fibrinogen and D-dimer concentrations) and plasma vWF concentration were assessed by use of an automated chemical analyzer.[a] Assessment of the APTT involved the use of synthetic phospholipids and silica to trigger coagulation.[b] The PT and fibrinogen concentration were evaluated simultaneously by use of a rabbit brain calcium thromboplastin.[c] Light scattering before and after clot formation was recorded by use of the automated chemical analyzer, and the fibrinogen concentration was calculated by use of these values and a calibration curve. Concentrations of D-dimer[d] and vWF[e] were measured via a turbidometric immunoassay. A pooled sample of plasma from 13 to 15 clinically normal dogs without evidence of bleeding was analyzed together with the samples. The D-dimer concentration was only determined in 8 control dogs and 43 CKCSs (consecutively examined during the period January 2002 to June 2002) because preliminary results indicated that there was no difference in D-dimer concentration between control dogs and CKCSs.[f] Plasma samples were thawed at 37°C in a waterbath immediately before analysis and centrifuged at 3,000 × *g* for 5 minutes (to avoid remnants of cryoprecipitate in plasma after thawing); the supernatants were used for analysis.

Manual counts of platelets were performed by use of a stromatolytic agent[g] and a counting chamber, as previously described.[20] The fixed platelets were counted in duplicate on the day of the examination. A platelet function analyzer[h] was used to assess platelet function according to the manufacturer's instructions; this device has been described in detail.[25] In brief, the platelet function analyzer measures platelet function in vitro by aspirating citrated whole blood from a test cartridge through a 150-µm-diameter aperture in a membrane coated with a combination of collagen and adenosine diphosphate as the platelet aggregation agonist. A platelet plug closes the aperture in the membrane, and the instrument monitors the time required for complete occlusion (closure time measured in seconds). The cutoff time of the instrument is 300 seconds (ie, nonclosure is reported if the

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cutoff time exceeds 300 seconds). We used a combination of collagen and adenosine diphosphate as the agonist in our assessments of closure time because findings of other studies[24,26] have indicated that the use of collagen and epinephrine as the agonist (which is used in studies of human platelets) does not consistently cause platelet aggregation and adhesion in canine blood samples. Closure times from 8 control dogs and 55 CKCSs included in this study have been reported previously.[24]

Analysis of the multimeric composition of vWF—Multimeric profiles of vWF were performed in 8 control dogs and 8 CKCSs. The dogs were selected on the basis of their plasma vWF concentration and degree of MR: 4 control dogs with plasma vWF concentration of 78% to 132%; the 4 control dogs with the lowest plasma vWF concentrations (ie, 54% to 69%); 4 CKCSs with no or minimal MR and with plasma vWF concentrations of 101% to 127%; and the 4 CKCSs with the lowest plasma vWF concentrations (45% to 54%) among the dogs with moderate to severe MR (ie, those dogs with a regurgitant jet size of 55% to 90%). A pooled sample of plasma from 8 to 10 clinically normal dogs (these dogs were not selected for individual sample analysis) without evidence of bleeding was included in each western blot analysis.

Citrated plasma samples were analyzed by use of **SDS-agarose gel electrophoresis (SDS-AGE)**, western blot analysis, and immunoperoxidase detection. The technique has been described in detail.[27] In brief, SDS-AGE was performed in a 1% separating gel prepared by use of high-gelling-temperature agarose[i] dissolved in gel buffer (0.05M Tris-HCl and 0.1% SDS; pH, 8.35) with a 0.75% stacking gel (high-gelling-temperature agarose dissolved in gel buffer).

Plasma samples were diluted 1:4 in sample buffer (0.01M Tris, 1mM EDTA-Na_2, 8M urea, 2% SDS, and bromophenol blue; pH, 8.0); 350 µL of stacking gel was added, and the diluted sample was heated for 20 minutes at 60°C. A 40-µL volume was pipetted into each well, and horizontal electrophoresis was performed in electrophoresis buffer (0.05M Tris, 0.394M glycine, and 0.1% SDS; pH, 8.4) at a constant current of 12.5 mA/gel (duration, approx 16 hours). Resolved protein was transferred to a polyvinylidene fluoride[j] membrane via electrotransfer at 33 V for 6.5 hours (2 gels). After transfer, the polyvinylidene fluoride membrane was blocked in 0.5% bovine serum albumin diluted in 0.1M PBS solution with 0.5% Tween 20 (pH, 7.4) at 4°C for 16 to 18 hours. Following blocking, the membrane was washed in PBS-Tween 20 solution and incubated with horseradish peroxidase-conjugated polyclonal rabbit anti-human vWF antibody[k] diluted 1:500 in PBS-Tween 20 solution. After a 2-hour incubation at 21°C, the membrane was washed 5 times in PBS-Tween 20 solution (5 to 10 min/wash). The vWF multimers were visualized by use of 0.05% 3,3-diaminobenzidine HCl in 0.05M Tris-HCl with hydrogen peroxide. The vWF multimer profiles were evaluated qualitatively by a person who was unaware of the source of the plasma samples with regard to the identity of the dogs and their clinical findings.

Echocardiographic evaluation—Echocardiography was performed under continuous ECG monitoring with an echocardiograph,[l] as previously described.[24] Jet size, MVP severity, **left ventricular end-diastolic diameter (LVEDD)**, and left atrial and aortic root diameters were assessed from the video-recorded echocardiograms by an observer (HDP) who was unaware of the dogs' identities or their clinical findings. The severity of MVP was assessed as previously described.[28] In brief, the maximal protrusion (in increments of 0.5 mm) of the anterior and posterior leaflets of the mitral valve and the coaptation point of the 2 leaflets into the left atrium during systole were measured; MVP severity was defined as the mean of these 3 measurements. Regurgitant jet size was estimated as the percentage of the left atrial area (assessed by eye to the nearest 5%) that was occupied by the largest jet. The measurement of LVEDD was made coincidentally with the beginning of the QRS complex. The diameters of the left atrium and the aortic root were measured at the end of ventricular systole, when the left atrium was at its maximal diameter. The **left atrial-to-aortic root (LA:Ao)** ratio was calculated. The LVEDDs and LA:Ao ratios reported here are the means of values obtained during 5 consecutive heart cycles.

Statistical analyses—Differences in the measured variables among the 4 groups of dogs (ie, 3 groups of CKCSs and the control group) were analyzed by use of a 1-way ANOVA. When the overall *F* test of the ANOVA was significant, least-squares means were compared by use of *t* tests with a Tukey-Kramer adjustment for multiple comparisons. The Kruskal-Wallis rank-sum test was used to compare APTT among the groups because transformation failed to yield a Gaussian distribution. In CKCSs, the influences of various factors on plasma vWF and fibrinogen concentrations were evaluated by use of multiple stepwise linear regression with sex as the dummy variable and age, weight, platelet count, and a disease-related variable (ie, regurgitant jet size, MVP severity, LVEDD, and LA:Ao ratio) as possible predictors. The disease-related variables were entered separately in the models because of the high degree of dependence among these variables. Before analysis, LVEDD was divided by body weight $(kg)^{0.29}$ to normalize the value to the size of the dog[29]; therefore, weight was not included in those particular analyses. Effect of plasma vWF concentration on closure time (assessed by use of the platelet function analyzer) was evaluated by use of an analysis of covariance with regurgitant jet size as a covariate in CKCSs and control dogs. For each model, the residuals were tested for normality and homogeneity of variation by means of a Shapiro-Wilks test and residual plots, respectively. To obtain normality, a logarithmic transformation was used for plasma fibrinogen and vWF concentrations and a reciprocal transformation was used for plasma D-dimer concentration and closure time. All statistical calculations were performed by use of statistical software.[m] The levels of significance chosen were a value of $P < 0.01$ for the multiple regression analyses and $P < 0.05$ for the remaining analyses. Data are given as medians and 25th to 75th percentiles unless otherwise indicated.

Results

The groups of dogs differed with regard to age, weight, and severity of MVP (**Table 1**). The CKCSs with moderate to severe MR were significantly older than CKCSs with no or minimal MR and control dogs although the CKCSs with moderate to severe MR were older than those with mild MR, this difference was not significant. The mean weight of the control dogs was significantly greater than that of each of the 3 groups of CKCSs. As mentioned, none of the dogs had hematologic or serum biochemical abnormalities (except for some CKCSs that had low platelet counts).

Findings in control dogs—In the control dogs, the ranges of the measured hemostatic variables were as follows: PT, 5.9 to 7.4 seconds; APTT, 10.4 to 12.4 seconds; plasma fibrinogen concentration, 1.01 to 3.01 mg/mL; plasma D-dimer concentration, 0.14 to 0.45 mg/L; plasma vWF concentration, 64% to 167%; closure time (assessed by use of the platelet function analyzer), 45 to 83 seconds; and manual platelet count,

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Table 1—Sex, age, weight, platelet count and function, severity of mitral valve prolapse (MVP), hemostatic variables, and plasma von Willebrand factor (vWF) concentration of 24 clinically normal dogs (control group; n = 24) and 76 untreated Cavalier King Charles Spaniels (CKCSs) with clinically inapparent mitral regurgitation (MR) as a result of myxomatous mitral valve disease.

Variable	Control dogs	No or minimal MR (n = 44)	Mild MR (11)	Moderate to severe (21)
Sex (No. of males, No. of females)	14, 10	18, 26	6, 5	14, 7
Age (mo)	47 (25–63)	35 (23–56)	57 (48–61)	68 (58–96) §
Weight (kg)	26.0 (14.3–30.0)	8.5 (7.6–9.4)	8.7 (7.3–11.5)	10.0 (9.1–11.9)
Platelet count (× 10^9/L)	221 (136–235)	170 (81–330)	261 (153–346)	321 (192–467)§
MVP* (mm)	–0.6 (–1.6–0.1)	1.3 (1.2–1.7)	2.2 (1.5–2.5) §	2.6 (2.3–3.3) §‖
PT (s)	6.6 (6.4–6.8)	6.3 (5.9–6.6)	6.4 (6.2–6.6)	6.3 (6.2–6.6)
APTT (s)	10.4 (10.4–10.7)	10.4 (10.4-21.7)	10.4 (10.4–53.7)	10.7 (10.4–34.5)
Plasma fibrinogen (mg/mL)	1.25 (1.10–1.65)	2.07 (1.69–2.42)	1.95 (1.34–3.10)	2.39 (2.12–3.01)
Plasma D-dimer (mg/L)†	0.16 (0.14–0.21)	0.17 (0.15–0.20)	0.19 (0.13–0.32)	0.18 (0.16–0.19)
Plasma vWF concentration (%)	92 (78–128)	99 (79–115)	86 (79–94)	72 (53–87) §
Col+ADP CT (s)	55 (49–65)	54 (51–62)	61 (56–81)	93 (85–132) §‖

With the exception of sex, data are shown as medians (25th to 75th percentiles).

*Severity of MVP was assessed as the mean of the maximal protrusion (in increments of 0.5 mm) of the anterior and posterior leaflets of the mitral valve and the coaptation point of the 2 leaflets into the left atrium during systole. †Plasma D-dimer concentration was determined in only 8 control dogs, 31 CKCSs with no or minimal MR, 4 CKCSs with mild MR, and 9 CKCSs with moderate to severe MR. ‡Value is significantly ($P < 0.05$) different from that of the control group. §Value is significantly ($P < 0.05$) different from that of the CKCSs with no or minimal MR. ‖Value is significantly ($P < 0.05$) different from that of CKCSs with mild MR.

PT = Prothrombin time. APTT = Activated partial thromboplastin time. Col+ADP CT = Closure time measured by use of a platelet function analyzer with collagen and adenosine diphosphate as the combined agonist.

136,000 to 343,000 platelets/μL. Values of the measured hemostatic variables for the pooled sample of plasma from 13 to 15 clinically normal dogs (dogs included in the pooled sample were not included as control dogs in this study) were as follows: PT, 6.3 seconds; APTT, 10.4 seconds; plasma fibrinogen concentration, 1.13 mg/mL; plasma D-dimer concentration, 0.25 mg/L; and plasma vWF concentration, 92%.

Hemostatic profile and plasma vWF concentration—The PTs in CKCSs with no or minimal MR and those with moderate to severe MR were shorter than that of the control dogs. There was no difference in values of APTT among the groups. However, a large variation in APTT was found among the CKCSs (range, 10.4 to 151 seconds), compared with values among the control dogs (range, 10.4 to 12.4 seconds). Nine CKCSs had very prolonged APTTs (> 60 seconds); those dogs were equally distributed among the 3 groups of CKCSs, and both sexes were represented (data not shown). There was no relationship between plasma vWF concentration and APTT in the CKCSs (data not shown). In all CKCS groups, the plasma fibrinogen concentration was significantly greater than that of the control group. There was no difference in plasma D-dimer concentration among the 4 groups. Among the 3 CKCS groups, there was no difference in any of the 4 variables included in the hemostatic profile (ie, APTT, PT, and plasma fibrinogen and D-dimer concentrations). The CKCSs with moderate to severe MR had significantly lower plasma vWF concentration, compared with that of CKCSs with no or minimal MR and control dogs (**Figure 1**).

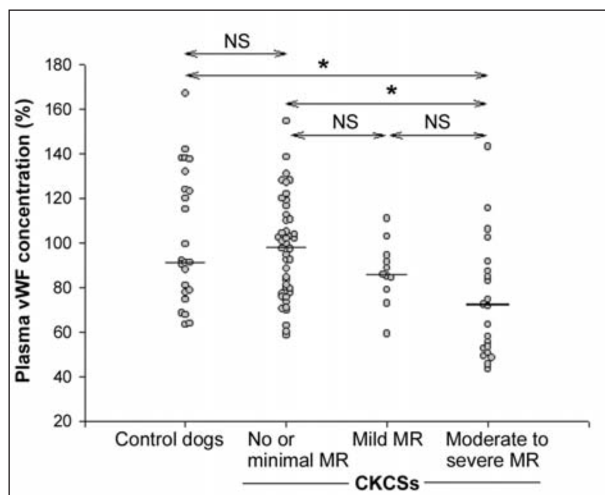

Figure 1—Plasma concentration of von Willebrand factor (vWF) in 24 control dogs and 76 untreated Cavalier King Charles Spaniels (CKCSs) with different degrees of clinically inapparent mitral regurgitation (MR). Dogs with mild and moderate to severe MR had regurgitant jets occupying 15% to 50% and > 50% of the left atrial area, respectively. Double-headed arrows indicate comparisons between groups. NS = Between groups, mean values of vWF concentration were not significantly different. *Between groups, mean values of vWF concentration were significantly ($P < 0.001$) different. Horizontal bars indicate median value for each group.

Effects of disease-related variables and other factors on plasma vWF and fibrinogen concentrations—A significant inverse relationship was found between plasma vWF concentration (dependent variable) and each of the following variables: LVEDD, MVP severity, weight, and regurgitant jet size. When plasma fibrinogen concentration was the dependent variable, the LVEDD and LA:Ao ratio were significant disease-related predictors (**Table 2**; **Figure 2**). The relationships of age, sex, or platelet count with plasma vWF and fibrinogen concentration did not reach significance in any of the analyses. Plasma concentrations of vWF or fibrinogen in the 53 CKCSs with platelet counts ≥ 100,000 platelets/μL did not differ from the concentrations in the 23 CKCSs with platelet counts < 100,000 platelets/μL. The significant predictors generally accounted for only a minor part of the variability in plasma concentrations of vWF and fibrinogen.

Relationship between plasma vWF concentration and closure time in the CKCSs and control dogs—In

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Table 2—Assessment of the influence of disease-related and other variables on plasma vWF and fibrinogen concentrations in 76 untreated CKCSs with clinically inapparent MR as a result of myxomatous mitral valve disease by use of multiple regression analyses.

Dependent variable	Disease-related variable assessed	Significant predictor*	R^2 value	P value
vWF concentration†	LVEDD‡	LVEDD	0.13	0.001
	MVP severity	MVP severity	0.17	< 0.001
		Weight	0.08	0.006
	Jet size	Jet size	0.21	< 0.001
	LA:Ao ratio	Weight	0.17	< 0.001
Fibrinogen concentration§	LVEDD	LVEDD	0.10	0.005
	MVP severity	NS	NA	NA
	Jet size	Weight	0.10	0.007
	LA:Ao ratio	LA:Ao ratio	0.11	0.003

The R^2 value indicates the variable-related variance.

*In each analysis, age, sex, weight, platelet count, and a disease-related variable were included as possible predictors. Only the variables for which $P < 0.01$ are reported. †All significant predictors were inversely related to plasma vWF concentration. ‡Normalized to dog size (value divided by weight [kg]$^{0.29}$). §All significant predictors were directly related to plasma fibrinogen concentration.

LVEDD = Left ventricular end-diastolic diameter. LA:Ao ratio = Left atrial-to-aortic root ratio. NS = Not significant. NA = Not applicable.

See Table 1 for remainder of key.

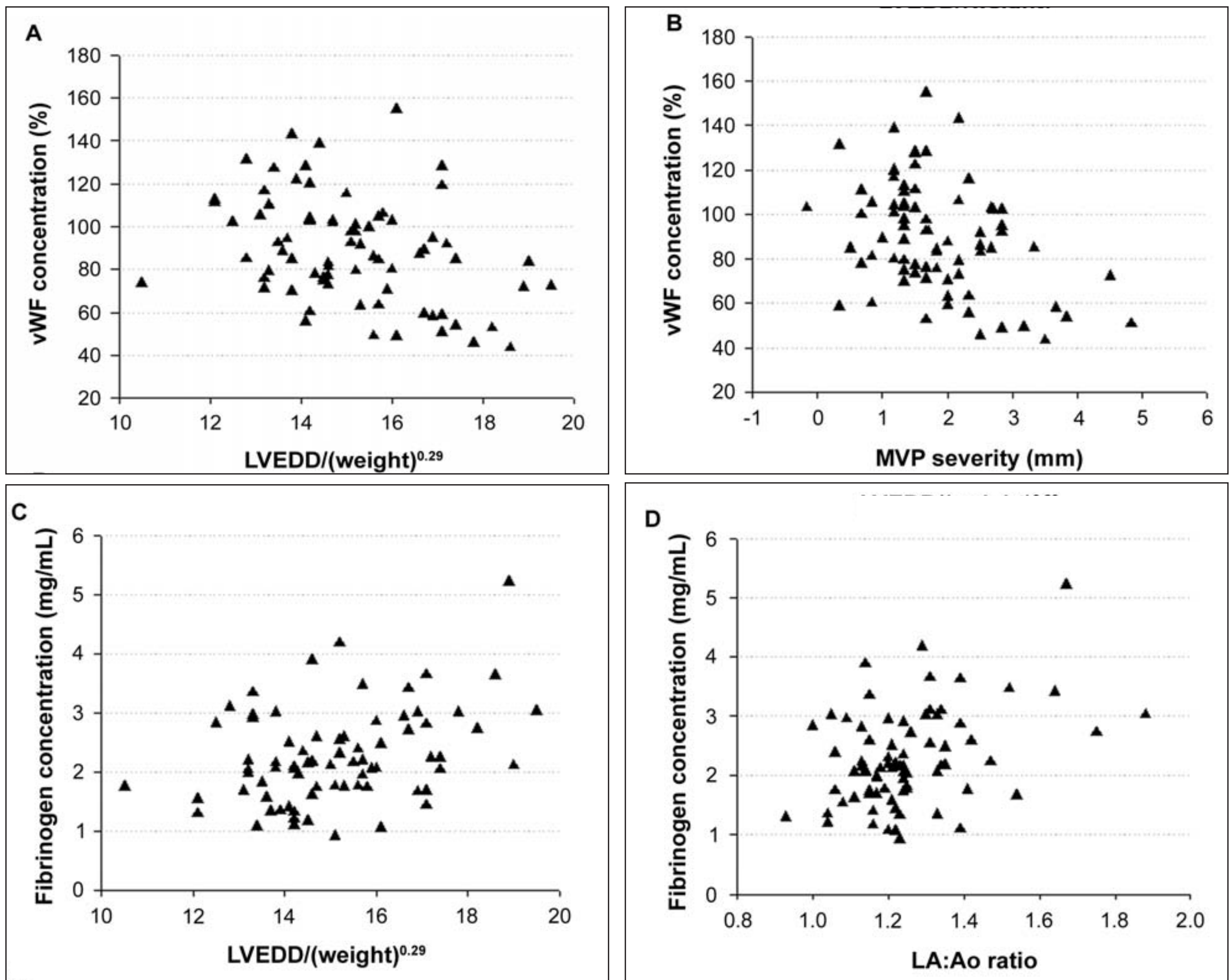

Figure 2—Relationships between echocardiographic variables and plasma vWF and fibrinogen concentrations in 76 untreated CKCSs with different degrees of clinically inapparent MR. A—Scatterplot of left ventricular end diastolic diameter (LVEDD) versus plasma vWF concentration (R^2 = 0.13; P = 0.001). B—Scatterplot of the severity of mitral valve prolapse versus plasma vWF concentration (R^2 = 0.17; $P < 0.001$). C—Scatterplot of LVEDD versus plasma fibrinogen concentration (R^2 = 0.10; P = 0.005). D—Scatterplot of left atrial-to-aortic (LA:Ao) ratio versus plasma fibrinogen concentration (R^2 = 0.11; P = 0.003). Each value of LVEDD was divided by weight (kg)$^{0.29}$ to normalize it to the size of the dog.[29]

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the CKCSs, a significant inverse relationship was identified between plasma vWF concentration and closure time (R^2 = 0.15; $P < 0.001$), with regurgitant jet size as a covariate (R^2 = 0.53; $P < 0.001$; **Figure 3**). No significant interaction was detected between plasma vWF concentration and regurgitant jet size. Closure time was significantly longer in the CKCSs with moderate to severe MR, compared with closure times in CKCSs with no or minimal MR, CKCSs with mild MR, and control dogs. Closure time was significantly longer in CKCSs with mild MR, compared with closure time in CKCSs with no or minimal MR (Table 1). In the control dogs, no relationship between closure time and either plasma vWF concentration or regurgitant jet size was identified (P = 0.97 and 0.88, respectively).

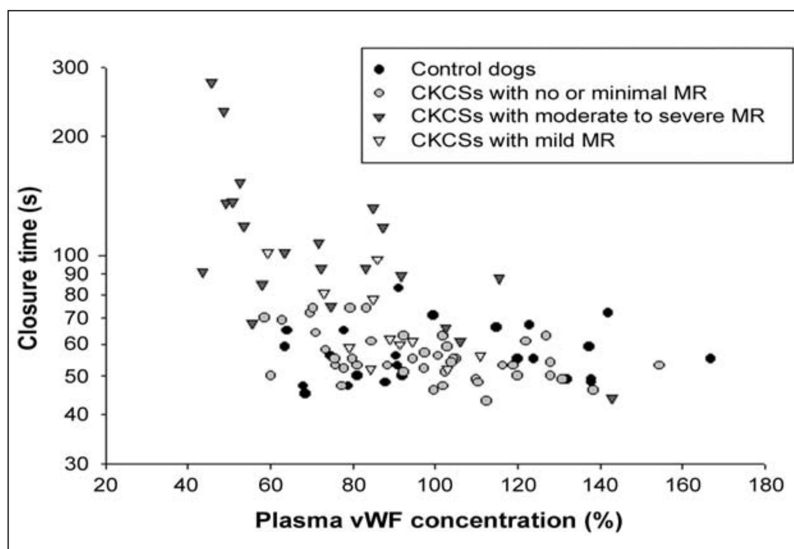

Figure 3—Scatterplot of closure times measured with a platelet function analyzer (including a combination of collagen and adenosine diphosphate as the platelet agonist) versus plasma vWF concentration in 24 control dogs and 76 untreated CKCSs with different degrees of clinically inapparent MR. Notice that the plasma concentration of vWF was inversely related to closure time in CKCSs; such a relationship was not identified in control dogs. Regurgitant jet size was a strong covariate in the statistical analysis. The intervals on the y-axis are displayed logarithmically.

Multimeric composition of vWF in CKCSs, compared with that of control dogs—Western blot analyses revealed a relative absence of high molecular weight bands in the samples obtained from CKCSs with moderate to severe MR (ie, CKCSs with regurgitant jet size of 55% to 90%) and a vWF concentration < 54%, compared with samples obtained from CKCS with no or minimal MR and control dogs with a vWF concentration of 78% to 132% or 54% to 69%. This finding indicated that CKCSs with moderate to severe MR had less **high-molecular-weight multimers (HMWMs)** than the other study dogs (**Figure 4**).

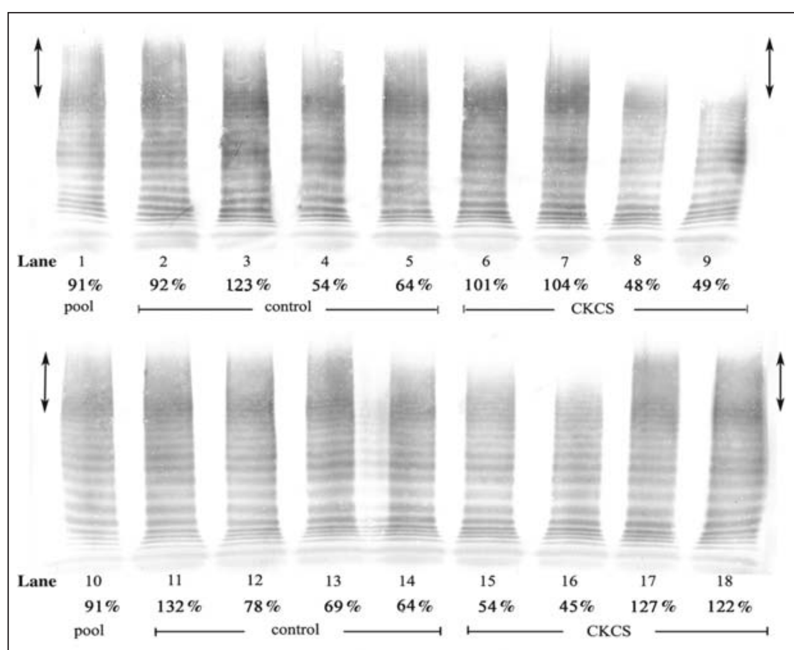

Figure 4—Images of western blots prepared from agarose gel electrophoresis of plasma vWF obtained from 8 control dogs (plasma vWF concentrations of 54% to 132%), 4 CKCSs with no or minimal MR (plasma vWF concentrations of 101% to 127%), and 4 CKCSs with moderate to severe MR (plasma vWF concentrations of 45% to 54%). Plasma vWF concentrations (%) are listed below each sample lane identifier. The direction of the electrophoresis was from top to bottom with the largest proteins at the top; double-headed arrows indicate the zone containing high-molecular-weight multimers. Notice that in CKCS with moderate to severe MR and low plasma vWF concentration, there is a low occurrence of high-molecular-weight multimers. Lanes are as follows: 1 and 10 = Pooled sample of plasma from 8 to 10 control dogs; 2 through 5 and 11 through 14 = Plasma from individual control dogs; 6, 7, 17, and 18 = CKCSs with no or minimal MR; and 8, 9, 15, and 16 = CKCSs with moderate to severe MR.

Discussion

The results of the study reported here indicated that all CKCSs with MVP had higher plasma fibrinogen concentration than that of clinically normal control dogs, and a weak direct association was identified between the plasma fibrinogen concentration and 2 disease-related variables (LVEDD and LA:Ao ratio) in the CKCSs. Furthermore, the plasma vWF concentration was lower in CKCSs with moderate to severe MR, compared with the concentrations in the CKCSs with no or minimal MR and the control dogs. Qualitative analysis of the multimeric composition of vWF indicated less HMWMs in CKCSs with MR and a plasma vWF concentration of 45% to 54%, compared with the amount in CKCSs with no MR and a plasma vWF concentration of 101% to 127% (the amount in control dogs with a plasma vWF concentration of 78% to 132%) and the amount in control dogs with a plasma vWF concentration of 54% to 69%.

In humans with cardiovascular disease, high plasma fibrinogen concentration is associated with an increased risk

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of thrombosis and is an independent marker of cardiovascular risk. Fibrinogen is also an acute phase protein; therefore, its concentration in plasma may vary greatly. Despite the variability in plasma concentration, the association between plasma fibrinogen concentration and cardiovascular disease in humans based on a single measurement is strong and consistent.[15,16] An increase in plasma fibrinogen concentration has been reported in humans with MR, compared with the concentration in unaffected individuals.[30] In our study, we detected higher plasma fibrinogen concentration in CKCSs with MR, compared with that of control dogs; furthermore, there was a relationship between plasma fibrinogen concentration and both the LVEDD and LA:Ao ratio in the CKCSs, indicating that the changes in plasma fibrinogen concentration might be associated with the mitral valve disease. However, large epidemiologic studies to investigate plasma fibrinogen concentration and cardiovascular risk in dogs, as well as breed variability studies, are needed before any broader conclusions regarding plasma fibrinogen concentration in dogs with cardiovascular disease can be made.

Measurement of plasma D-dimer concentration has been used to detect thromboembolic disease in dogs.[18,19] It appears that plasma D-dimer concentration has a high negative predictive value for thromboembolic disease in dogs as it does in humans and that only very high plasma concentrations of D-dimer have a high positive predictive value. In the present study, we did not detect any differences in plasma D-dimer concentration among the groups of dogs. Consequently, it might be concluded that there is no active thromboembolic disease in dogs with MR or that the thromboembolic disease is too mild to cause a noticeable change in plasma D-dimer concentration in those dogs.

Assessment of APTT and PT were included in the hemostatic profile in our study because of the limited availability of assays suitable for measuring markers of hypercoagulability in dogs. Thrombogenesis is a complex process involving platelets, leukocytes, endothelial cells, and fluid-phase hemostatic proteins. Because of this complexity, it has been suggested that in vitro coagulation screening tests designed to detect factor deficiencies (ie, assessment of APTT and PT) are not good predictors of an in vivo state of hypercoagulation.[7] However, in humans, a shortened APTT is associated with increased risk of acute myocardial infarction.[31] No significant difference in values of APTT was found among the groups of dogs in our study, although the APTT varied tremendously among the CKCSs. A hereditary single factor deficiency (without clinical evidence of bleeding) could be speculated to cause severe prolongation of APTT. Single factor analyses in CKCSs with prolonged APTTs would indicate whether a hereditary single coagulation factor deficiency is present in those dogs. However, other causes of prolonged APTT such as acquired coagulopathies cannot be ruled out, although this is considered unlikely because the dogs in our study had no signs of clinical disease. The clinical importance of the shorter PT detected in some CKCSs in our study, compared with PTs in the control dogs, is unknown. The finding was not consistent in all the groups of CKCSs and was not associated with any disease-related variable.

An interesting result of the study reported here was that CKCSs with moderate to severe MR had a lower plasma vWF concentration, compared with the concentrations in CKCSs with no or mild MR and control dogs. The wide overlap between the 4 groups of dogs was likely a result of the large variation in plasma vWF concentration and the fact that regurgitant jet size can be regarded only as a semiquantitative measure of the degree of MR. However, the significant relationship between plasma vWF concentration and 2 other disease-related variables (LVEDD and MVP) supports the finding of a difference in plasma vWF concentration among the groups. In our study, a strong association between plasma vWF concentration and the closure time measured by use of the platelet function analyzer was identified in CKCSs. Because of the wide range of plasma vWF concentration (64% to 167%) found in control dogs in this study, the same association might be expected in the control dogs; however, no relationship was identified between plasma vWF concentration and closure time in the control dogs. The reason for this discrepancy could be that CKCSs with MR, low plasma vWF concentrations, and long closure times also have a loss of the HMWMs of vWF and that a loss of HMWMs has a greater influence on closure time than does total plasma vWF concentration. This could explain the lack of correlation between plasma vWF concentration and closure time in the control dogs. The low plasma vWF concentration (and possible subsequent loss of HMWMs of vWF) may only partly explain the long closure time found in CKCSs with MR. Mitral regurgitation is also likely to have an independent influence on the platelets because regurgitant jet size accounted for much of the variation in closure time in the statistical analysis and because shear stress can affect platelet function directly. The shear stress-dependent platelet dysfunction is likely to affect only platelet function tests that involve conditions of high shear, such as the platelet function analyzer used in the study of this report, and this may explain why a decrease in platelet aggregation response was not detected in an earlier study[22] that evaluated the platelet aggregation response in CKCSs. We speculate that the shear stress caused by MR has an influence on platelet function indirectly by altering the vWF multimers and possibly directly by exhausting the platelets as previously suggested.[23,24]

In humans, a high plasma vWF concentration has been a consistent finding in patients with cardiovascular disease, including individuals in whom atherosclerosis is thought not to be involved in the pathophysiologic development of the cardiovascular disease (such as patients with idiopathic dilated cardiomyopathy,[32] severe mitral regurgitation,[33] and aortic valve disease[11]). Furthermore, plasma vWF concentration is regarded as an excellent marker of endothelial dysfunction in humans. However, decreased plasma vWF concentration has been detected in some patients with MVP[34-36] and congenital cardiac defects,[37] but the methods used for measuring plasma vWF concentration in those earlier studies may have been different from

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methods used at present. Therefore, we hypothesized that CKCSs with MR in our study would have a greater plasma vWF concentration, compared with the concentration in control dogs; however, a lower plasma vWF concentration was detected in CKCSs with MR. It is plausible that some form of endothelial dysfunction is present in dogs with heart disease. First, results of a study[12] in dogs indicated that metabolites of nitric oxide were present in lower amounts in CKCSs with mild, moderate, or severe MR, compared with the amounts in CKCSs with no MR and control dogs, suggesting that MR-affected dogs have endothelial dysfunction. Second, other investigations have revealed that coronary endothelial dysfunction precedes experimentally induced heart failure in dogs[38] and is present after episodes of ischemia and reperfusion in dogs.[39] Finally, endothelial dysfunction is generally associated with all forms of heart failure in humans. Therefore, it can be speculated that plasma vWF concentration might not be a good marker of endothelial dysfunction in dogs with diseases that involve substantial shear stress to the blood. Further studies are warranted to elucidate whether dogs are generally different from humans with regard to the usefulness of plasma vWF concentration as a marker of endothelial dysfunction.

Results of the evaluation of the multimeric composition of vWF in the study dogs indicated a loss of HMWMs in the 4 CKCSs with MR and low plasma vWF concentration, compared with the other CKCSs and control dogs evaluated. In humans, low plasma vWF concentration and bleeding tendency have been associated with MVP[34-36] and a decrease in the HMWMs of vWF has been reported in patients with aortic stenosis[40,41] and congenital cardiac defects.[37] It has been speculated that the decrease in HMWMs is caused by accelerated clearance of the largest multimers via accelerated platelet and vWF interactions in blood flowing through a stenotic aortic or regurgitant mitral valve.[42] However, results of studies[43,44] have indicated that high shear stress per se can alter the molecular conformation of vWF, thereby leading to an enhanced proteolytic susceptibility of vWF molecules that may account for the loss of HMWMs. We speculate that CKCSs with MR have a decrease in plasma vWF concentration and HMWMs (compared with unaffected dogs) as a result of increased turbulence and shear stress to the blood. A weakness of our study is that vWF multimer analysis was performed in only 16 selected dogs (ie, 8 control dogs and 8 CKCSs). Therefore, it is not possible to conclude whether there is a loss of HMWMs of vWF in CKCSs with MR and normal vWF concentration and whether CKCSs without MR but with low plasma vWF concentration also have a loss of HMWMs. However, if CKCSs without MR and with low plasma vWF concentration have a loss of HMWMs, long closure times (measured by the platelet function analyzer)—not normal times such as those we identified in those dogs—would be expected. In addition, because all of the 4 CKCSs with MR and low plasma vWF concentration but none of the control dogs with low plasma vWF concentration included in the vWF multimer analysis had a loss of HMWMs, these results suggest that there is a loss of HMWMs in CKCSs with MR, which results in low plasma vWF concentration.

Overall, our data indicated that the fibrinogen concentration in plasma was higher in all CKCSs, compared with that of control dogs, and was associated with values of the LVEDD and LA:Ao ratio in CKCSs. Surprisingly, a lower plasma vWF concentration was detected in CKCSs with moderate to severe MR, compared with the concentrations in the CKCSs with no or minimal MR and control dogs. The explanation for this finding is a matter of speculation, but it is suggested that there is increased destruction of the HMWMs of vWF via shear stress in the CKCSs with more severe MR, thereby reducing the total amount of plasma vWF (as indicated by the results of the quantitative ELISA). Because of this phenomenon, plasma vWF concentration may not be useful as a marker of endothelial dysfunction in canine diseases that involve considerable shear stress to the blood. The clinical importance of the changes in the hemostatic markers that were evaluated in our study, their role in the pathophysiologic progression of MMVD, and the risk of thromboembolism in dogs with MR remain to be elucidated.

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[a]ACL9000, Instrumentation Laboratory, Warrington, UK.
[b]IL Test APTT-SP, Instrumentation Laboratory, Warrington, UK.
[c]IL Test PT-Fibrinogen, Instrumentation Laboratory, Warrington, UK.
[d]IL Test D-Dimer, Instrumentation Laboratory, Warrington, UK.
[e]IL Test von Willebrand factor, Instrumentation Laboratory, Warrington, UK.
[f]Tarnow I, Kristensen AT, Olsen LH, et al. Changes in coagulation parameters in Cavalier King Charles Spaniels with mitral regurgitation, in *Proceedings.* of the 21st Annual Forum of the American College of Veterinary Internal Medicine 2003;1009.
[g]Stromatol stromatolytic agent, Mascia Brunelli Spa, Milano, Italy.
[h]PFA-100, Dade Behring Marburg Gmbh, Marburg, Germany.
[i]SeaKem, BioWhittaker Molecular Applications, Rockville, Me.
[j]Immobilon-P, Millipore, Billerica, Mass.
[k]Polyclonal rabbit anti-human von Willebrand Factor, DAKOCytomation, Glostrup, Denmark.
[l]Vivid3 echocardiograph, General Electric Medical, Milwaukee, Wis.
[m]SAS statistical software, version 8, SAS Institute, Cary, NC.

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