Evaluation of plasma activity of matrix metalloproteinase-2 and -9 in dogs with myxomatous mitral valve disease

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Objective—To investigate whether plasma activity of matrix metalloproteinase (MMP)-2 and -9 was associated with severity of myxomatous mitral valve disease (MMVD) in dogs and to assess potential associations between MMP activity and dog characteristics, echocardiographic variables, systolic arterial blood pressure (SAP), heart rate, cardiac troponin I (cTnI) concentration, and C-reactive protein concentration.

Animals—75 client-owned dogs.

Procedures—Severity of MMVD was assessed by use of echocardiography. Plasma activity of latent (pro-MMP) and active MMP-2 and -9 was analyzed via zymography. Plasma concentration of cTnI was analyzed with a high-sensitivity cTnI assay, and C-reactive protein concentration was analyzed with a canine-specific ELISA.

Results—Pro–MMP-9, active MMP-9, and pro–MMP-2 were detected, but active MMP-2 was not. No significant differences were found in MMP concentrations among the 4 MMVD severity groups. Activity of pro–MMP-9 decreased with decreases in SAP and was higher in male dogs than in female dogs. Activity of MMP-9 decreased with increases in left ventricular end-systolic dimension and with decreases in SAP and cTnI concentration. Left ventricular end-systolic dimension was the variable most strongly associated with MMP-9 activity. No associations were found between the activity of pro–MMP-2 and investigated variables.

Conclusions and Clinical Relevance—Plasma MMP-9 activity decreased with increases in the end-systolic left ventricular internal dimension and decreases in SAP. Hence, evaluation of MMP-9 activity has the potential to provide unique information about the myocardial remodeling process in dogs with MMVD. (*Am J Vet Res* 2011;72:1022–1028)

The ECM is important for cardiac performance.[1] Disruption or discontinuities within the myocardial ECM network will result in a loss of structural support, which in turn will result in changes in function.[2] Breakdown and accumulation of extracellular components are regulated by a family of zinc-dependent

Abbreviations

CKCS	Cavalier King Charles Spaniel
CRP	C-reactive protein
cTnI	Cardiac troponin I
ECM	Extracellular matrix
HR	Heart rate
IQR	Interquartile range
IVSd	End-diastolic interventricular septal dimension
IVSs	End-systolic interventricular septal dimension
LA:Ao	Left atrial-to-aortic root ratio
LVIDd	End-diastolic left ventricular internal dimension
$LVIDd_{inc}$	Increase in end-diastolic left ventricular internal dimension
LVIDs	End-systolic left ventricular internal dimension
$LVIDs_{inc}$	Increase in end-systolic left ventricular internal dimension
LVPWd	End-diastolic left ventricular posterior wall dimension
LVPWs	End-systolic left ventricular posterior wall dimension
MMP	Matrix metalloproteinase
MMVD	Myxomatous mitral valve disease
MR	Mitral valve regurgitation
SAP	Systolic arterial pressure

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proteolytic enzymes (ie, the MMPs) and their tissue inhibitors.[3] Various cell types within the myocardium, including the myocytes, can express and synthesize MMPs,[4] which are secreted into the extracellular space in a latent form (pro-MMPs). Presence of disease can stimulate activity through a number of enzymatic pathways, which results in excessive breakdown of extracellular components and thus triggers myocardial remodeling.[2,5] In the large MMP family, the gelatinases are involved in cardiac remodeling processes.[6]

Myxomatous mitral valve disease is by far the most common cardiac disorder in dogs, and the disease usually develops in middle-aged and old dogs.[7] The highest prevalence is found in small and medium-sized breeds.[8] The disease is characterized by progressive degeneration of the mitral valve, which causes MR and subsequently chronic volume overload with left atrial dilation and left ventricular eccentric hypertrophy. Affected dogs can compensate for MR for years, but eventually left-sided congestive heart failure may develop. Changes in ECM composition and structure have been reported in dogs with MR.[9,10] Medical treatment of dogs with MR has failed to attenuate left ventricular remodeling, despite improvement of cardiomyocyte function, which indicates an important role of ECM in maintaining cardiac geometry and function.[11] It has been suggested[12] that the MMPs are involved in the pathogenesis of MMVD. Increased information on the action of MMPs may increase the understanding about cardiac remodeling and possibly prompt new ideas on how to treat the actual heart disease rather than only the resulting circulatory disturbances.[13]

To our knowledge, activity of circulating MMPs has not been investigated in any species with naturally occurring MMVD of different severities. Accordingly, the objectives of the study reported here were to investigate whether plasma activity of MMP-2 and MMP-9 was associated with severity of MMVD in dogs and to investigate potential associations between MMP activity and dog characteristics, echocardiographic variables, SAP, HR, and concentrations of 2 other biomarkers (ie, cTnI and CRP).

Materials and Methods

Animals—Client-owned dogs were prospectively recruited at the cardiology unit of the Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, between May 2007 and October 2008. Inclusion criteria were that dogs had to weigh < 15 kg and either have evidence of MMVD or be free from physical or echocardiographic evidence of cardiac disease. Dogs with congenital heart disease, other acquired cardiovascular disorders, or important organ-related or systemic diseases were not included in the study. Pregnant bitches, dogs treated with glucocorticoids, and dogs with detectable neoplasms were excluded because of possible influence on MMP results. Dogs with heart failure that required treatment to prevent clinical signs of the condition were allowed to participate in the study. Informed owner consent was obtained, and the study was approved by the Local Ethical Committee in Uppsala, Sweden.

Most of the dogs in the study were part of another study[14] conducted to evaluate cTnI and CRP concentrations in dogs with MMVD. However, the study populations differed because 6 dogs included in that other study[14] were excluded from the study reported here on the basis of small mammary gland tumors or glucocorticoid treatment.

Procedures—A database that consisted of results of various examinations and collection of a blood sample during a single visit was established for each dog. All examinations were performed on nonsedated dogs in a quiet examination room. An owner interview was conducted to provide data about age, sex, reproductive status, and medical history. A general physical examination was performed on each dog, and blood pressure was indirectly measured by use of an automated oscillometric device.[a] Dogs were minimally restrained in a standing position, and an appropriate neonatal cuff, which had a width of approximately 40% of the circumference of the tail, was applied to the base of the tail with the artery marker placed on the ventral aspect of the tail. Once reliable consecutive readings were obtained, the mean of 5 consecutive blood pressure measurements was calculated and recorded.

A blood sample was collected via jugular venipuncture into 5-mL tubes that contained EDTA. Samples were centrifuged within 30 minutes after collection. Plasma was harvested and transferred into 1.5-mL plastic cryotubes. All samples were stored at –80°C for subsequent analysis.

Echocardiography was performed last and was used to verify the diagnosis of MMVD and to exclude other primary or secondary cardiac diseases. In addition, echocardiography was used for assessment of MMVD severity. Echocardiographic examinations were all performed and evaluated by 1 experienced echocardiographer (JH). Dogs were placed in right and then left lateral recumbency on an ultrasound examination table. The echocardiographic evaluation was conducted by use of an ultrasonographic unit[b] equipped with a 5-MHz phased-array transducer and ECG monitoring.

Echocardiographic analysis—Standardized imaging planes[15] were digitally stored. Assessment of mitral valve structures was conducted from the right parasternal long-axis views and left apical 4-chamber view. The same views were used to assess the degree of MR by use of color Doppler echocardiographic mapping.

Diagnosis of MMVD was based on characteristic valvular lesions of the mitral valve apparatus (thickened and prolapsing mitral valve leaflets) and detection of MR on color Doppler echocardiography, as described elsewhere.[16,17] Regurgitation through the mitral valve was subjectively assessed as the area of the regurgitant jet relative to the area of the left atrium, as described elsewhere,[17] with the following slight modifications: regurgitation scores were recorded as none, mild (≤ 30%), moderate (> 30% to 50%), and severe (> 50%). Screening of potential regurgitations through the tricuspid, aortic, and pulmonic valves was performed routinely by use of color Doppler echocardiography. The LA:Ao was measured from the right 2-D short-axis view.[18] The M-mode measurements of the left ventricle were obtained by use of standard techniques[19] from a right parasternal short axis view. The M-mode values were used

to measure IVSd, LVIDd, LVPWd, IVSs, LVIDs, LVPWs, and fractional shortening. Values for the increases in IVSd LVIDd, LVPWd, IVSs, LVIDs, and LVPWs were calculated as percentages as follows: ([observed dimension – expected normal dimension]/expected normal dimension) × 100. Expected normal dimensions were calculated as described elsewhere[20] for the following variables: IVSd = (body weight$^{0.241}$ × 0.41), LVIDd = (body weight$^{0.294}$ × 1.53), LVPWd = (body weight$^{0.232}$ × 0.42), IVSs = (body weight$^{0.24}$ × 0.58), LVIDs = (body weight$^{0.315}$ × 0.95), and LVPWs = (body weight$^{0.222}$ × 0.64). The value for h:R, where h is the LVPWd and R is the left ventricular radius (which was calculated as LVIDd/2), was used as an indicator of left ventricular eccentric hypertrophy.[21]

Estimation of MMVD severity was determined on the basis of the obtained LA:Ao and size of the MR jet. Dogs were classified as follows: healthy (LA:Ao < 1.5 and no MR jet), mild (LA:Ao ≤ 1.5 and MR jet < 30%), moderate (LA:Ao > 1.5 and < 1.8 and MR jet < 50%), and severe (LA:Ao ≥ 1.8 and MR jet > 50%).

Measurement of MMP activity—Plasma MMP activity was analyzed by use of gelatin zymography, as described elsewhere.[22] Blood samples were diluted (1:20) with loading buffer and incubated at 20°C for 2 hours. Each lane of an 11% SDS-polyacrylamide gel[c] was loaded with 20 µL of a 1:1 mixture of sample and loading buffer. As a positive control sample, each gel was loaded with 3 µL of a diluted (1:800) MMP-9 and -2 standard suspension[d] and a molecular weight standard.[e] Zymography was performed at 4°C; resulting zymograms were soaked for 1 hour in 2.5% Triton X-100 and incubated in developing buffer (50mM Tris, 0.02% Coomassie brilliant blue 250, and 5mM $CaCl_2$)[c] at 37°C for 17 hours. Gels were then stained[f] and washed, and gelatinolytic activity was evident as clear bands against a blue background. For quantification of gelatin degradation, gels were scanned, background gray values were subtracted, and densitometric results were measured by use of the area mode of the image analysis system.[g] The densitometric result for each band was assessed by comparison with the pro–MMP-2 (positive-control standard) band on each gel; hence, the MMP values described the order of gelatinolytic activity among samples. All zymograms were analyzed for pro–MMP-2 and -9 as well as for active forms of MMP-2 and -9.

Measurement of cTnI concentrations—Plasma concentrations of cTnI were analyzed by use of a high-sensitivity cTnI assay[h] conducted in accordance with the manufacturer's instructions. The troponin amino acid sequence is highly conserved across species, which allows assays validated for use in humans to be used for canine samples.[23,24] In addition, an in-house validation was performed by a trained laboratory technician, and the tested dilutional parallelism of canine plasma confirmed that there was linearity within the assay system. The lower limit of detection for the assay used in the study reported here was 0.001 ng/mL, and the intra-assay coefficient of variation was 4.0% at low concentrations (mean ± SD, 0.49 ± 0.02 ng/mL) and 3.7% at high concentrations (33 ± 1.2 ng/mL).

Measurement of CRP concentrations—Plasma concentrations of CRP were analyzed by use of a validated[25] commercially available canine CRP ELISA assay,[i] which was conducted in accordance with the manufacturer's instructions. An in-house validation confirmed that there was linearity for dilutional parallelism within the assay system. The intra-assay coefficient of variation for the assay used in the study reported here was 7.2% at low concentrations (4.3 ± 0.48 µg/mL) and 7.0% at high concentrations (22 ± 1.2 µg/mL).

Statistical analysis—A commercially available software program[j] was used for all statistical analyses. Data were reported as medians and IQRs. A value of $P < 0.05$ was considered significant for all analyses.

The nonparametric Kruskal-Wallis test was used to investigate overall associations between MMP activity and the 4 MMVD severity groups. Univariable and multivariable regression analyses were used to evaluate associations between the MMPs and dog characteristics (age, sex, breed [CKCS {yes or no}], sexually intact [yes or no], and body weight), HR attained from the echocardiogram, SAP, echocardiographic measurements (LA:Ao, $LVIDd_{inc}$, $LVIDs_{inc}$, and h:R ratio), and cTnI and CRP concentrations. Because of the high colinearity between LA:Ao and $LVIDd_{inc}$[26] only $LVIDd_{inc}$ was included in the multivariable regression model. Whether a female dog had been ovariohysterectomized was only included in the univariable regression analysis. Logarithmic transformation was performed for the regression analyses to achieve a normal distribution for skewed variables (activities of pro–MMP-9, active MMP-9, and pro–MMP-2 and concentrations of cTnI and CRP). In the multivariable regression model, analyses were performed in a backward stepwise manner,[27] starting with all variables included in the model and then removing the variable with the highest *P* value until all of the remaining variables had a value of $P < 0.05$. All variables were assessed only as main effects; no interaction terms were considered in the model. The adjusted R^2 is defined as the percentage of the total sum of squares that can be explained by the regression, and it also considers the *df* for variables added to the model.

Results

A total of 75 dogs (44 females and 31 males) with a median age of 8.1 years (IQR, 6.2 to 10.7 years) and a median body weight of 9.5 kg (IQR, 8.4 to 11.0 kg) was included in the study. Eleven dogs had unremarkable results for echocardiography and were considered healthy, 37 had mild MMVD, 11 had moderate MMVD, and 16 had severe MMVD. Two dogs classified with moderate MMVD and 9 dogs classified with severe MMVD were receiving cardiac medications at the time of enrollment in the study. The most prevalent breed was CKCS (n = 63), followed by Dachshund (10) and then by Miniature Schnauzer (1) and Shih Tzu (1).

Zymography of the plasma samples revealed gelatinase expression at the same region as for the positive control samples. Bands corresponding to the positive control pro–MMP-2, pro–MMP-9, and active MMP-9 were detected and were considered to represent canine pro–MMP-2, pro–MMP-9, and active MMP-9.[22]

Pro–MMP-2 and pro–MMP-9 were detected in all dogs, and active MMP-9 was detected in 64 (85.3%) dogs. Active MMP-2 was not detected in the study population.

Groupwise comparisons—No significant differences were detected among the various MMPs and the 4 MMVD severity groups.

Univariable regression analyses—Activity of pro–MMP-9 decreased with decreases in SAP (R^2 = 0.10; P = 0.004) and were significantly (P = 0.21) higher in male dogs than in female dogs. Activity of active MMP-9 decreased with increases in $LVIDs_{inc}$ (R^2 = 0.11; P = 0.004) and with decreases in cTnI concentrations (R^2 = 0.09; P = 0.009), SAP (R^2 = 0.07; P = 0.019), and age (R^2 = 0.07; P = 0.019). Total MMP-9 activity decreased with decreases in SAP (R^2 = 0.15; $P < 0.001$) and with increases in $LVIDs_{inc}$ (R^2 = 0.098; P = 0.004). No associations were detected between pro–MMP-2 activity and the investigated variables.

Multivariable regression analyses—Inclusion of pro–MMP-9 activity as a dependent variable and baseline characteristics (age, sex, CKCS [yes or no], and body weight), HR, SAP, $LVIDs_{inc}$, $LVIDd_{inc}$, h:R, and cTnI and CRP concentrations as independent variables revealed a major effect of SAP and sex. Systolic arterial pressure was the variable with the highest model R^2. The final model had an adjusted R^2 value of 0.14. Of the 11 variables included in the initial multivariable regression model with activity of active MMP-9 as a dependent variable, $LVIDs_{inc}$, SAP, and cTnI concentration were significantly associated with MMP-9 activity (**Figure 1**), and $LVIDs_{inc}$ was the variable with the highest model R^2. The final model had an adjusted R^2 value of 0.29.

Of the 11 variables included in the initial multivariable regression model with total MMP-9 activity as a dependent variable, SAP, $LVIDs_{inc}$, and $LVIDd_{inc}$ were significantly associated with total MMP-9 activity, and SAP was the variable with the highest model R^2. The

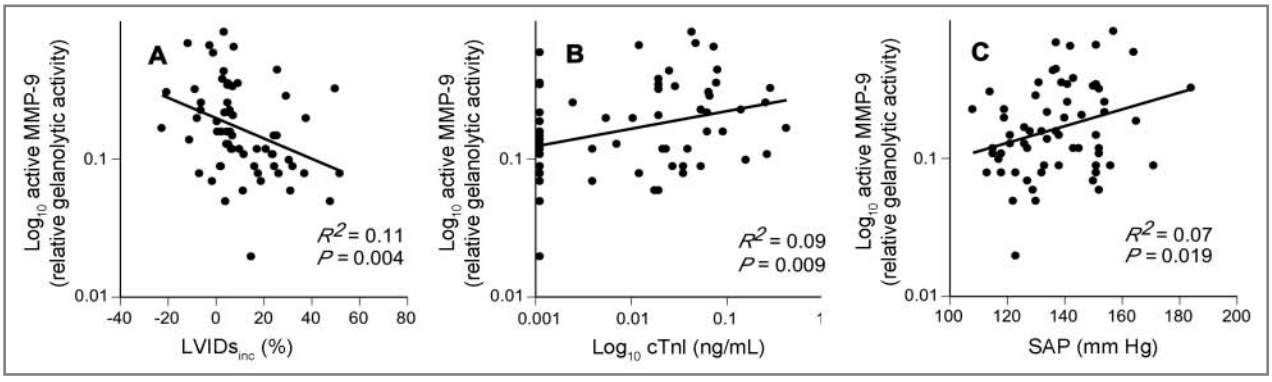

Figure 1—Associations between the log_{10} activity of active MMP-9 and the $LVIDs_{inc}$ (A), log_{10} cTnI concentration (B), and SAP (C) in 75 dogs with various severities of MMVD. These 3 variables were significant in the final multivariable model. Notice the line of best fit for the data and the corresponding R^2 values.

Table 1—Dog characteristics, HR, SAP, echocardiographic data, cTnI concentration, and CRP concentration in dogs in various stages of MMVD.*

Variable	Healthy	Mild	Moderate	Severe
No. of dogs	11	37	11	16
Sex (female/male)	10/1	24/13	6/5	4/12
Sexually intact (female/male)	9/1	17/11	3/5	2/8
CKCS (yes/no)	8/3	33/4	10/1	12/4
Age (y)	4.5 (3.7 to 7.5)[a]	7.2 (6.1 to 10)[b]	10.0 (7.6 to 11.0)[b,c]	11.0 (8.5 to 12.0)[c]
Weight (kg)	8.8 (8.2 to 10.0)[a,b]	9.2 (8.1 to 10.4)[a]	11.0 (8.8 to 11.0)[a,b]	11.0 (9.6 to 13.0)[b]
HR (beat/min)	121 (112 to 155)[a,b]	107 (99 to 131)[a]	134 (87 to 139)[a,b]	143 (121 to 163)[b]
SAP (mm Hg)	134 (123 to 151)	137(125 to 148)	151 (136 to 154)	131 (117 to 141)
LA:Ao	1.2 (1.1 to 1.2)[a]	1.2 (1.1 to 1.2)[a]	1.6 (1.5 to 1.7)[b]	2.0 (1.9 to 2.3)[c]
$IVSd_{inc}$ (%)	–10.0 (–15.0 to –5.9)[a]	–1.6 (–10.6 to 4.4)[a]	–5.7 (–9.0 to –1.0)[a]	–3.3 (–12.0 to 4.2)[a]
$LVIDd_{inc}$ (%)	–3.8 (–11.0 to 4.6)[a]	5.1 (0 to 13)[b]	27.0 (17.0 to 35.0)[c]	42.0 (32.0 to 56.0)[d]
$LVPWd_{inc}$ (%)	–2.3 (–7.8 to –2.0)[a]	1.2 (–5.2 to 6.5)[a]	–3.8 (–6.3 to 1.5)[a]	–2.1 (–11.0 to 4.4)[a]
$IVSs_{inc}$ (%)	–9.7 (–14.0 to –0.1)[a]	–1.8 (–8.6 to 5.5)[a,b]	6.7 (–6.8 to 13.5)[b,c]	14.0 (3.3 to 21.0)[c]
$LVIDs_{inc}$ (%)	4.0 (–2.5 to 6.9)[a]	5.9 (2.0 to 18.0)[a]	6.5 (3.4 to 31.0)[a,b]	25.0 (7.2 to 45.0)[b]
$LVPWs_{inc}$ (%)	–14.0 (–20.0 to –9.1)[a]	–5.7 (–9.6 to 1.5)[b]	11.0 (0.3 to 13.0)[c]	14.0 (4.0 to 21.0)[c]
h:R†	0.5 (0.4 to 0.5)[a]	0.5 (0.4 to 0.5)[a]	0.4 (0.3 to 0.4)[b]	0.3 (0.3 to 0.3)[b]
Fractional shortening (%)	29 (27 to 34)[a]	32 (29 to 37)[a]	41 (34 to 45)[b]	44 (40 to 47)[b,c]
cTnI concentration (ng/mL)	0.001 (0.001 to 0.004)[a]	0.003 (0.001 to 0.021)[a,b]	0.014 (0.008 to 0.024)[b]	0.04 (0.0241 to 0.072)[c]
CRP concentration (ng/mL)	0.90 (0.60 to 1.80)[a]	1.00 (0.60 to 1.50)[a]	0.90 (0.40 to 1.40)[a]	1.20 (0.70 to 2.95)[a]

Most values are reported as median (IQR).

*Estimation of MMVD severity was based on the obtained LA:Ao ratio and size of the MR jet. Dogs were classified as follows: healthy (LA:Ao $<$ 1.5 and no MR jet), mild (LA:Ao $\leq$ 1.5 and MR jet $<$ 30%), moderate (LA:Ao $>$ 1.5 and $<$ 1.8 and MR jet $<$ 50%), or severe (LA:Ao $\geq$ 1.8 and MR jet $>$ 50%). †For h:R, h is the LVPWd and R is left ventricular radius, which was obtained by dividing LVIDd by 2.

$IVSd_{inc}$ = Increase in IVSd. $IVSs_{inc}$ = Increase in IVSs. $LVPWd_{inc}$ = Increase in LVPWd. $LVPWs_{inc}$ = Increase in LVPWs.

[a–c]Within each row, values with different superscript letters differ significantly (P = 0.008).

(Adapted from Ljungvall I, Höglund K, Tidholm A, et al. Cardiac troponin I is associated with severity of myxomatous mitral valve disease, age, and C-reactive protein in dogs. *J Vet Intern Med* 2010;24:153–159. Reprinted with permission.)

final model had an adjusted R^2 value of 0.27. No associations were detected between pro–MMP-2 activity and the investigated variables (**Table 1**).

Discussion

To our knowledge, the study reported here was the first that was conducted to investigate circulatory activity of MMPs for patients of any species with differing severities of MMVD. Activities of pro–MMP-9, active MMP-9, and pro–MMP-2 were detected in the dogs of this study. Activity of MMP-9 decreased with increases in $LVIDs_{inc}$ and decreases in SAP; hence, this biomarker has the potential to provide unique information about the myocardial remodeling process in dogs with MMVD.

No overall significant differences were detected among the various MMPs and the 4 MMVD severity groups. It has been suggested that different cardiac diseases can lead to different activation patterns of MMPs.[28,29] Circulating activity of MMP-2 and -9 reportedly increases in human patients with acute coronary syndromes,[30,31] and animals with experimentally induced heart failure have time-dependent increases in myocardial MMP activity.[32,33] However, progression and duration of cardiac remodeling processes in acute and experimentally induced cardiac diseases differ fundamentally from those in naturally occurring chronic cardiac diseases. Myxomatous mitral valve disease is a slowly progressive disease, and changes in MMP activity could possibly be intermittently upregulated or downregulated during disease progression, which could create a unique MMP profile in a specific dog at a given time. This could possibly explain the lack of significant differences among the MMVD severity groups.

Furthermore, the MMVD severity classification system used in the present study was based on variables that primarily reflected left-sided cardiac dilation. Because changes in MMP-9 activity primarily reflect systolic dysfunction, such a classification system may not have completely reflected the pathophysiologic process because it does not account for systolic function. Activity of active MMP-9 decreased with worsening systolic function, which was indicated by increases in $LVIDs_{inc}$ and decreases in SAP. The association between MMP-9 activity and systolic dysfunction was confirmed by use of univariable and multivariable regression analyses. In dogs with MMVD, systolic function decreases during disease progression[34] as a consequence of chronic volume overload. Because of the low resistance to ventricular emptying into the left atrium, dogs with severe MR attributable to MMVD might eject > 75% of the total stroke volume into the left atrium,[35] and it might ultimately be difficult to completely compensate for the decreased forward stroke volume.[36] Hence, decreased forward stroke volume and systolic function contribute to a decrease in SAP in dogs with more severe MMVD.[k] However, all dogs in the present study had SAP within, or close to, the reference ranges, which indicated that LV forward function did not decrease dramatically and that an acceptable SAP can be maintained even in the severe stage of the disease. The SAP did not differ significantly between dogs in the different MMVD severity groups, although dogs with more-severe disease tended to have a lower SAP than did dogs with less-severe disease. Such a tendency has been described in dogs with MMVD.[k] Because MMP-9 activity was downregulated with increases in $LVIDs_{inc}$ and decreases in SAP, a classification system that involved the use of systolic variables possibly could have identified changes for the groupwise comparison.

Changes in myocardial structure with loss of the fine collagen weave surrounding the myocytes may develop because of selective induction of MMPs.[2] Evaluation of experimentally induced heart failure in animals has revealed time-dependent increases in myocardial MMP activity accompanied by alterations in cardiac ECM structure and progressive cardiac dysfunction.[32,33,37] Breakdown of structural cardiac ECM may trigger alterations in myocyte size and geometry[38] and lead to cardiac dilation in early stages of heart failure.[28,39] During disease progression, there are hemodynamic changes that can contribute to further changes in cardiac structure.[28,37] Sustained volume overload may stabilize or downregulate MMP activity, as has been described in experimentally induced cardiac failure in dogs.[28,37] Evaluation of myocardial biopsy specimens from human patients with mitral valve disease has revealed that downregulation of MMP-9 is associated with increasing amounts of fibrosis.[29] Downregulation of MMP-9 may protect against progressive uncontrolled dilation by reducing ECM breakdown and enhancing development of fibrosis.[29] However, the possibility cannot be excluded that lower MMP-9 activity is a consequence of previous changes in the structural cardiac ECM mass. Myocardial stiffness caused by generalized interstitial fibrotic changes and fibrosis may impair myocardial function and cause both systolic and diastolic dysfunction during heart failure.[40,41]

Potential associations between MMP activity and cTnI concentration were investigated, and activity of active MMP-9 was found to decrease with decreases in cTnI concentrations. Changes in MMP activity primarily reflect changes in the ECM, whereas changes in cTnI concentrations primarily reflect myocyte damage. In another study[14] conducted by our research group, we reported that plasma cTnI concentrations increase with increases in MMVD severity. Because activity of active MMP-9 was found to decrease with increases in systolic dysfunction, an inverse relation between activity of active MMP-9 and cTnI concentrations could be expected. However, the cTnI concentration was associated with variables that reflected left-sided cardiac dilation and was not significantly associated with SAP and $LVIDs_{inc}$; hence, MMP-9 and cTnI probably reflected different cardiac remodeling processes. This suggests that remodeling in a chronic cardiac disease is highly complex, with different processes occurring simultaneously and most likely with different degrees of contribution during progression.

Activity of active MMP-9 was significantly lower in younger dogs in the univariable model; however, this association was not significant in the multivariable regression model. Furthermore, male dogs had higher activity of pro–MMP-9 than did female dogs, which is in agreement with results of a report[42] on MMP-9 activity in human patients with left ventricular remodeling.

It has been suggested[43,44] that estrogen reduces the tissue effects and plasma activity of MMP-9. Activity of pro–MMP-9 did not differ significantly between ovariohysterectomized and sexually intact female dogs in our study population. However, because of the low number of ovariohysterectomized dogs in the various MMVD severity groups, no conclusions can be drawn from the present study on the potential impact of estrogen and other female sex hormones on MMP-9 activity.

Detectable activity of pro–MMP-2 was found in all dogs but was not associated with MMVD severity or other investigated variables. Activity of active MMP-2 was not detected in the study population. Type, degree, and duration of extracellular stimuli in different cardiac diseases likely affect the MMP profile within the failing myocardium.[2] The proteolytic activity of MMP-2 may play a minor role in MMVD in dogs.[12,45] Possibly, MMP-2 is not upregulated until dogs are in end-stage heart failure,[37] and inclusion of more dogs with end-stage MMVD in the present study may have influenced the MMP-2 results.

A limitation of the present study was the lack of histologic evaluation of cardiac tissues as well as the lack of measurement of tissue protease activity. Hence, local changes in the MMP system at the tissue level could not be assessed accurately. However, it is difficult to obtain cardiac tissue from a large group of client-owned dogs with differing severities of naturally occurring MMVD. Relating measurements of plasma MMP-2 and -9 activities to clinical and echocardiographic measurements is based on the premise that the heart is an important source of circulating MMP-2 and -9. To minimize influence from other disease processes in the study population, dogs with other important organ-related or systemic diseases were not included in the present study. In addition, CRP concentrations in the study population were within reported[25,46] normal variations for healthy dogs. Therefore, dogs with clinically important systemic inflammation were likely not represented in the study population. Activity of MMP was investigated at only a single time point for each dog, and a future prospective longitudinal study may increase information about the role of MMPs in dogs with MMVD. Furthermore, blood samples were stored at –80°C before analysis, and degradation of plasma protein may have affected the measured MMP activity.

A possible influence of cardiac medication on MMP activity could not be properly evaluated in the study reported here because few dogs were receiving cardiac medications at the time of enrollment and because of variations in the combinations of cardiac medications administered to these dogs. In clinical situations, it is extremely difficult to avoid stressing animals, and dogs react differently to clinical situations (including stress), which may have influenced SAP measurements in some dogs. A high proportion of CKCSs develop MMVD, and dogs of this breed predominated in the study population. Because few dogs of other breeds were in the study population, potential breed differences should be investigated more thoroughly in future studies. In the present study, no interaction terms were considered in the statistical analysis. Because of the comparably low R^2 in the regression analyses, the number of variables included in the multivariable regression analysis had to be limited to avoid oversaturation of the models. Accordingly, we cannot exclude effects of interactions among the main variables.

The balance between MMPs and tissue inhibitors appears to be important in the physiologic and pathological remodeling of ECM. Although information in another report[6] supports an important role of MMP-2 and -9 in cardiac remodeling processes, an important future investigation should be conducted to define the specific MMPs and tissue inhibitors that are expressed within the failing myocardium. Several human ELISAs for measurement of concentrations of various MMPs and tissue inhibitors are currently available. However, we have had a lack of success for properly validating human ELISAs for use on canine blood samples, probably because of a lack of species homology.

We concluded that for the study reported here, MMP-9 activity decreased with increases in $LVIDs_{inc}$ and decreases in SAP; hence, measurement of MMP-9 activity may add information when the cardiac remodeling process in MMVD is evaluated. Moreover, MMP-9 activity was associated with age, sex, and cTnI concentration. On the basis of these results, MMP-2 may play a minor role in the progression of MMVD. Additional studies on the involvement of MMPs in the cardiac remodeling process in dogs with MMVD are warranted. The significant associations found between MMP-9 activity and some of the investigated variables were rather weak, and on the basis of analysis of these results, circulatory activity of MMP-2 and -9 cannot be regarded as valuable diagnostic biomarkers by practitioners. Improved understanding of the MMPs and their regulation may provide new insights and strategies for management of dogs with MMVD.

a. Oscillometric Krutech VET420A, Jorgen Kruuse A/S, Marslev, Denmark.
b. GE Vivid 3 ultrasound unit, General Electric Co, Stockholm, Sweden.
c. Sigma-Aldrich, St Louis, Mo.
d. MMP9/2, Chemicon, Temecula, Calif.
e. Precision Plus Kaleidoscope, Bio-Rad Laboratories, Richmond, Calif.
f. Page Blue, Fermentas, Burlington, ON, Canada.
g. AlphaeaseFC, Alphainnotech, Santa Clara, Calif.
h. Access Systems AccuTnI assay, Beckman Coulter Inc, Fullerton, Calif.
i. Tridelta Phase Range CRP–canine assay, Tridelta Development Ltd, Maynooth, County Kildare, Ireland.
j. JMP, version 7.0.2, SAS Institute Inc, Cary, NC.
k. Moonarmart W. *Studies on the natural history and progression of acquired mitral insufficiency in the dog*. PhD Thesis, Department of Veterinary Clinical Sciences, Royal Veterinary College, University of London, London, 2008.

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