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Clinical Relevance of Cystatin C as a Renal Marker in Dogs with Chronic Mitral Valve Insufficiency

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Abstract : Cystatin C is a low molecular weight 13 kilodalton protein. It is known to be a more sensitive marker of glomerular filtration rate than creatinine in humans. The purpose of the present study was to demonstrate the changes of renal markers including cystatin C according to the severity of chronic mitral valve insufficiency (CMVI) and to investigate the clinical relevance of cystatin C as an early renal marker in dogs with CMVI. A retrospective study was performed to assess renal function according to International Small Animal Cardiac Health Council (ISACHC) system classification of heart failure in dogs with CMVI. Thirty seven dogs were divided into a group 1 (healthy dogs ; n = 10), a group 2 (ISACHC I ; n = 10) and a group 3 (ISACHC II-III ; n = 17). In all dogs, serum concentrations of bun (sUr), creatinine (sCr) and cystatin C (sCys-C) were measured with an automated analyzer. In dogs with CMVI, sCys-C concentrations were significantly correlated with sCr concentrations and were independent of age, BW, SBP, and sex. Renal dysfunction tended to occur more frequently as the severity of CMVI increases. In dogs with mild CMVI, only sCys-C concentrations were statistically higher than in healthy dogs. This study demonstrates the clinical relevance of sCys-C. sCys-C may be a valuable renal marker for early diagnosis of renal dysfunction in dogs with CMVI.

Key words **:** CMVI, cystatin C, dog, renal dysfunction.

Introduction

Renal dysfunction is often reported as a potential consequence of chronic heart failure (CHF) in humans and dogs (2,24,32). Although the exact pathogenesis remains unclear, decreased cardiac output associated with lower renal perfusion and hemodynamic alterations as a result of the activation of neurohormonal systems can affect renal function and decrease the glomerular filtration rate (GFR) (2,24,34). A previous study reported that 21% of human patients diagnosed with CHF had associated renal dysfunction (21). Information concerning the actual prevalence of renal dysfunction in dogs with CHF is not available in the veterinary literature. However, one study described that renal dysfunction occurred in dogs with CMVI, which is the most common cause of CHF in small-breed dogs (24).

Renal dysfunction is usually progressive and strongly associated with mortality in humans with CHF (2,7,36). In patients with CHF, it is not only fatal by itself causing renal failure but also detrimental in managing CHF resulting in hypertension, anemia, and volume overload (30). Therefore, early diagnosis and therapeutic intervention of renal dysfunction may slow the progression of renal disease and prolong the median survival time in dogs with CMVI.

The direct measurement of GFR is considered the best method for assessing renal function because it is directly proportional to functional renal mass (1,4,35). However, measuring the GFR can be challenging because the procedure is time-consuming and stressful to patients with CMVI (12,25). The sCr and sUr are renal markers that indirectly indicate the GFR (12,26). They are easily measured and have been widely used for assessing renal function to date (12,26). However, the serum concentrations of these markers do not increase until approximately 75% of the functional renal mass is lost (1,9,13,22). In addition, these markers are influenced by extrarenal factors, such as age, dietary protein, hydration status, and muscle mass (1,5,7). Therefore, different renal markers are required for an early and accurate diagnosis of renal dysfunction in dogs with CMVI.

Cystatin C is a low molecular weight 13 kilodalton protein and a member of the cystatin superfamily of cysteine protease inhibitors (1,12,35). Cystatin C has properties that make it an ideal endogenous GFR marker. It is produced at a constant rate by all nucleated cells and is freely filtered at the glomerulus (31,35). It is absorbed and catabolized in the cells of the proximal tubule, and is not secreted during tubular secretion (31,35). In addition, previous studies in humans demonstrated that sCys-C concentration was independent of diet, sex, body weight (BW), and muscle mass (1,29).

In humans, sCys-C is suggested to be a better renal marker for GFR than sCr (15,18,23,35). According to the previous studies, sCys-C concentrations correlated more strongly with GFR measured using the creatinine clearance test than did sCr concentrations (15,35). Moreover, changes in the sCys-C concentrations were more prominent in the patients with a mild decrease in the GFR, making it a better screening test for early diagnosis of kidney disease (15,35). Despite the few

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veterinary medicine studies on cystatin C, some studies demonstrated the clinical value of cystatin C as an early renal marker in dogs (6,35).

The purpose of the present study was to evaluate the changes in renal markers including cystatin C, according to the severity of CMVI, and to investigate the clinical relevance of cystatin C as an early renal marker in dogs with CMVI.

Materials and Methods

Animals

This retrospective study was performed to assess renal function in dogs with CMVI. Thirty-seven dogs of different breeds, sexes, and ages were used in this study. All dogs had a BW below 14 kg. The severity of CMVI was evaluated according to the ISACHC classification of heart failure (Table 1) (11). Dogs were divided into 3 groups: group 1 (healthy dogs ; n = 10), group 2 (ISACHC I ; n = 10), and group 3 (ISACHC II-III ; n = 17). Dogs were defined as healthy if their history, physical examination, complete blood count, serum biochemistry, and urinalysis were unremarkable. Physical, radiographic, and echocardiographic examinations and systolic blood pressure (SBP) measurement were performed in dogs from groups 2 and 3.

Diagnostic criteria of CMVI

Physical, radiographic, and echocardiographic examinations were performed to diagnose CMVI in dogs. On auscultation, all the dogs evaluated had a systolic murmur with the maximum point of intensity near the mitral valve area. The pulmonary edema caused by CMVI was documented by pulmonary infiltrates and left atrial enlargement on thoracic radiographs, clinical signs related with CMVI, and the need for diuretics to relieve clinical signs. Echocardiographic examination was performed on an ultrasound apparatus (Xario SSA-660A, Toshiba, Japan). Mitral valve regurgitation caused by degenerative changes in the mitral valve leaflets (prolapse, thickening) were detected on color Doppler examination. The left ventricular shortening fraction had to be > 20%. Dogs with concomitant congenital or acquired heart disease, such as aortic stenosis or bacterial endocarditis, were excluded from this study.

Table 1. International Small Animal Cardiac Health Council (ISACHC) classification of heart failure

ISACHC classification		
Class I	Ia	No evidence of compensation for underlying heart disease
(asymptomatic)	Ib	Clinical signs of compensation for underlying heart disease
Class II (symptomatic)		Mild to moderate heart failure with clinical signs
Class III	IIIa	Severe heart failure - home treatment possible
(symptomatic)	IIIb	Severe heart failure - hospitalization required

Measurement of systolic blood pressure

For the measurement of systolic blood pressure (SBP), an ultrasonic Doppler flow detector (Model 812, Parks Medical Electronics Inc., USA) was used. After applying an aqueous ultrasonic transmission gel (Eco gel 99, Seung Won Medical Corp., Korea), the Doppler flow probe was placed on palmar of the thoracic limb (median artery). The occluding cuff was placed proximally to the Doppler flow probe. The pressure of the cuff was increased until the flow signal disappeared and was then gradually decreased. SBP was measured when the first audible signal was detected for more than 5 times and the average SBP was calculated.

Blood samples

3 mL of whole blood was collected into a serum separating tube (BD vacutainer®, Becton Dickinson and Co, UK). Serum was separated by centrifugation (Sigma, Sartorius AG, Germany) at 5000 rpm for 5 minutes immediately after collection. Serum samples were analyzed immediately or stored at - 20°C for a maximum of 6 weeks until analysis.

Analytical procedures

sCys-C was measured using a commercial particle-enhanced turbidimetric immunoassay (PETIA) and a cystatin C reagent set (Tina-quant Cystatin C, Roche Diagnostics GmbH, Germany) designed for human samples. The principle of the analysis involves the binding of cystatin C to rabbit anti-human cystatin C antibody coupled to polystyrene particles, resulting in the formation of agglutinates. The degree of turbidity caused by the formation of agglutinates was determined turbidimetrically for the measurement of the sCys-C concentration. All assays used for sUr, sCr, and sCys-C were performed using an automated biochemistry analyzer (Mindray BS-200 automatic biochemistry analyzer, Shenzhen Mindray Bio-Medical Electronics Co., China), according to the manufacturer's description.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (IBM Corporation, USA) program version 12.0. Data were expressed as mean ± standard deviation (SD). The Kruskal-Wallis test and the Mann-Whitney U test were used to compare the concentrations of sUr, sCr, and sCys-C between the groups. Spearman's correlation statistics were used to assess the relationships of sCys-C with other renal markers and biological factors. For all analysis, a value of less than 0.05 ($P < 0.05$) was considered statistically significant

Results

Baseline characteristics of the dogs

This retrospective study examined 37 dogs (Table 2). Of the 37 dogs, 10 dogs were clinically healthy (group 1) and 27 dogs were diagnosed with CMVI (groups 2 and 3). In groups 2 and 3, the most represented breeds were Shih tzu (n = 8), Maltese (n = 7) and Poodle (n = 4). Twelve dogs were female and 15 dogs were male. The age of the dogs with CMVI ranged between 8 and 16 years (median of 12 years), and the

Table 2. Baseline characteristics and SBP of 37 dogs in this study

	Group 1 Min-Max	Group 2 Min-Max	Group 3 Min-Max
Sex - Female (n)	5	4	8
Male (n)	5	6	9
Age (years)	2-10	9-16	8-16
BW (kg)	5.8-13.6	2.45-9.6	3-13.6
SBP (mmHg)	90-130	100-170	100-165

BW, body weight; SBP, systolic blood pressure; Min, minimum; Max, maximum.

Table 3. Spearman's correlation coefficients among sUr, sCr, and sCys-C concentrations

	Correlation between sCys-C and sUr	Correlation between sCys-C and sCr
Group 1	r = 0.037	r = 0.090
Group 2&3	r = 0.467	r = 0.593

sUr, serum urea; sCr, serum creatinine; sCys-C, serum cystatin C.

BW ranged between 2.4 and 13.6 kg (median of 5.7 kg). SBP ranged between 100 and 170 mmHg, with a mean ± SD of 127.74 ± 20.32 mmHg. The drugs for CMVI treatment were already being administered in 89% of the animals when the concentrations of sUr, sCr, and sCys-C were measured in this study.

Correlation of sCys-C with other renal markers and diverse biological factors

Correlation of sCys-C concentrations with sUr and sCr concentrations

The correlation coefficients of sCys-C concentrations with sUr and sCr concentrations are shown in Table 3. In group 1, there were no statistically significant correlations between sCys-C and sUr concentrations (r = 0.037, p = 0.920) and between sCys-C and sCr concentrations (r = 0.090, p = 0.806). On the other hand, sCys-C concentrations were positively correlated with sUr (r = 0.467, $p < 0.05$) and sCr (r = 0.593, $p < 0.01$) concentrations in groups 2 and 3, respectively.

Effects of age, sex, BW, and SBP on the concentrations of sCys-C in dogs with CMVI.

In groups 2 and 3, the Spearman's correlation coefficients between sCys-C and other factors, such as age, BW, and SBP, are shown in Fig 1. No significant correlation was found between sCys-C and age (r = 0.361, p = 0.064), between sCys-C and BW (r = 0.152, p = 0.449), and between sCys-C and SBP (r = 0.085, p = 0.672). In addition, there was no statistical difference in the sCys-C concentration between females and male dogs (p = 0.427) (Fig 2).

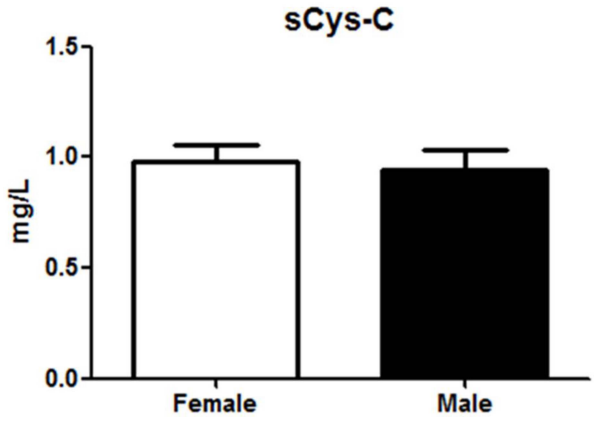

Fig 2. Box and whisker diagram for comparison of sCys-C concentration between sexes. The sCys-C concentration was not statistically different between sexes in dogs with CMVI (p = 0.427).

Table 4. Concentrations of sUr, sCr, and sCys-C, and percentage of azotemic dogs in 37 dogs. Results expressed as mean values ± SD

Groups	sUr (mg/dl)	sCr (mg/dl)	sCys-C (mg/L)
Group 1	12.15 ± 2.63	0.65 ± 0.12	0.6 9 ± 0.1
Group 2	19.85 ± 12.37	0.82 ± 0.38	0.92 ± 0.33
Group 3	48.52 ± 53.76	1.38 ± 1.18	0.98 ± 0.31
Azotemic dogs (%)*		10 %	35 %

sUr, serum urea; sCr, serum creatinine; sCys-C, serum cystatin C.
*A dog was regarded as azotemic status if concentrations of sUr or sCr exceeded 26 mg/dL and 1.3 mg/dL, respectively.

Concentrations of sUr, sCr, and sCys-C in dogs

The concentrations of sUr, sCr, and sCys-C, and the percentage of azotemic dogs are shown in Table 4. The mean concentration of sUr, sCr, and sCys-C increased with an increase in the severity of CMVI. Statistical differences in these concentrations between the three groups are illustrated in Fig 3. There were statistically significant differences in the concentrations of sUr, sCr, and sCys-C between groups 1 and

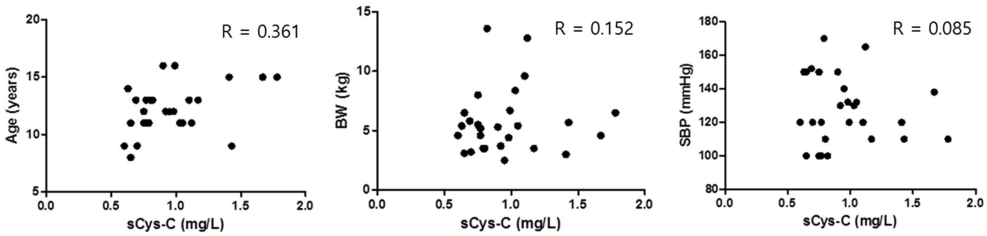

Fig 1. Scatterplots between age, BW, SBP and sCys-C in dogs with CMVI. No significant correlation was found between age, BW, SBP and sCys-C.

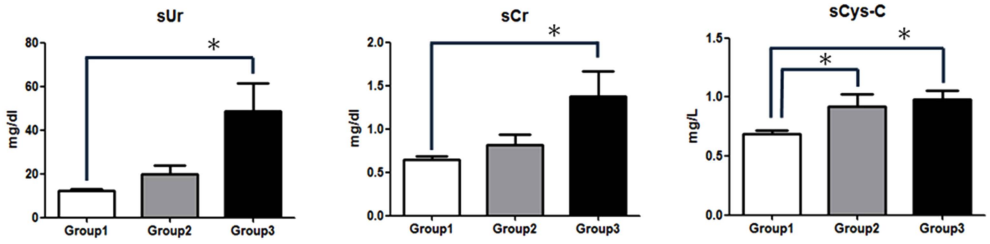

Fig 3. Box and whisker diagram for comparison of sUr, sCr, and sCys-C concentrations among groups. Between group 1 and 3, the concentrations of sUr, sCr, and sCys-C were statistically different. Between group 1 and 2, the concentration of sCys-C was statistically different. *There was a statistically significant difference between groups (P value < 0.05).

3. In group 3, these concentrations were 48.52 ± 53.76 mg/dl, 1.38 ± 1.18 mg/dl, and 0.98 ± 0.31 mg/dl, respectively. These values were significantly higher than those obtained in group 1 (12.15 ± 2.63 mg/dl, p = 0.000; 0.65 ± 0.12 mg/dl, p = 0.008; and 0.69 ± 0.1 mg/dl, p = 0.003, respectively). Between groups 2 and 3, there were no statistically significant differences in the concentrations of sUr (p = 0.057), sCr (p = 0.084), and sCys-C (p = 0.273). Similarly, there were no statistically significant differences in the concentrations of sUr (p = 0.026) and sCr (p = 0.098) between groups 1 and 2, except for sCys-C. The concentration of sCys-C in group 2 (0.92 ± 0.33 mg/dl) was significantly higher than that in group 1 (0.69 ± 0.1 mg/dl, p = 0.009). The reference ranges of sUr and sCr, in the laboratory where the analyses were performed, were 8-26 mg/dL and 0.5-1.3 mg/dL, respectively. Among the dogs diagnosed with CMVI, 10% of the dogs in group 2 and 35% of the dogs in group 3 were azotemic.

Discussion

Cardiorenal syndrome (CRS) is defined as “disorders of the heart and kidney, whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other” (2). CMVI, which results in congestive heart failure, is the most common cause of CRS in small-breed dogs (3). In this study, renal function was evaluated by measuring sCys-C using a human PETIA assay. The correlation of sCys-C with other renal markers, the effects of physiological factors on sCys-C, and the potential of sCys-C as an early renal marker were evaluated in dogs with CMVI.

To date, veterinary assays for measurement of sCys-C are not available. All previous studies on sCys-C in dogs were performed using human PETIA assays, some of which yielded meaningful results (1,12,16,35). In addition, human and animal sCys-C amino acid sequences revealed a high degree of homology, approximately between 46% and 79% (27). Therefore, we measured sCys-C in dogs using the human PETIA assay, which is considered acceptable for measuring sCys-C in dogs. Even though the stability of sCys-C has not been evaluated in dogs, human sCys-C is known to be stable for 7 days at 4°C and indefinitely at - 20°C (14). Therefore, the frozen samples, which were stored at - 20°C for 6 weeks, were regarded as acceptable for analysis in this study.

We did not perform a clearance test of exogenous substances to estimate the GFR in this study. However, previous studies have evaluated the correlation between GFR and renal markers (12). The sUr concentrations are easily influenced by various extra-renal factors, such as food intake, dehydration, and hemorrhage, and consequently are inappropriate for estimating the GFR (8,22,28). The sCr concentrations showed a significant correlation with GFR in patients with chronic kidney disease. However, no correlation was observed in patients with normal GFR (12,17). The sCys-C concentrations correlated with the GFR in the entire GFR range but the correlation was weak in patients with normal GFR (17,33). Our results indicated the lack of correlation between sCys-C and sUr concentrations and between sCys-C and sCr concentrations in healthy dogs. Measuring renal markers is clinically less useful in healthy dogs because GFR estimation by these markers is not accurate. A significant correlation was observed between sCys-C and the concentrations of other renal markers in dogs with CMVI. The correlation was stronger between sCys-C and sCr concentrations than between sCys-C and sUr concentrations. This results shows that sCyc-C can be used as a reliable renal marker in dogs with CMVI, which are considered as having renal dysfunction.

sCys-C is known to be less influenced by biological factors, whereas sCr is dependent on the dog breed, age, diet, and muscle mass (5,12,37). Human studies indicated that the sCys-C concentration was not affected by diet, muscle mass, and sex (4,10,12). In previous veterinary medic ine studies, sex and BW did not affect the sCys-C concentrations. However, the relationship between age and sCys-C concentrations was not clearly demonstrated (6,12,35). In this study, no significant correlations were found between age, BW, sex, and sCys-C concentrations in dogs with CMVI. sCys-C may be used as a renal marker regardless of the presence of other biological factors in dogs with CMVI. It is well-known that hypertension is a risk factor of renal damage, resulting in renal failure (19). Of interest, there was no correlation between sCys-C concentrations and SBP in this study. Most dogs maintained normal SBP regardless of the severity of CMVI. This may be due to antihypertensive drugs prescribed to the dogs for the management of CMVI before they were included in this study. Additional studies involving a larger number of dogs are needed to clarify the effects of these biological factors and SBP on sCys-C.

CRS is commonly encountered in clinics and renal dysfunction is prone to occur with the increase in the severity of CHF in humans and dogs (20,24). A previous study reported that 50% of the dogs with CMVI and 70% of the dogs in New York Heart Association class IV were azotemic (24). In the present study, the concentrations of renal markers, including sCys-C, tended to increase with the increase in the sever-

ity of CMVI. In addition, 10% of the dogs in group 2 and 35% of the dogs in group 3 were azotemic. Even though we could not elucidate the pathogenesis of CRS, there are some possible reasons for the azotemic status, depending on the severity of CMVI. The outcomes of CMVI, the drugs used for the management of CMVI, and other age-related underlying diseases that result in renal dysfunction may affect renal function in dogs with CMVI.

sCys-C can be a good alternative to sCr as the screening method for detecting decreases in the GFR associated with CMVI for several reasons. Several studies in humans and dogs showed that sCys-C concentrations were more strongly correlated with GFR compared to sCr concentrations (15,35). In addition, among the three renal markers, only sCys-C concentrations were statistically higher in dogs with mild CMVI, who might have early renal dysfunction, than in healthy dogs. The most important clinical finding in this study is that some dogs had elevated sCys-C concentrations with normal sCr concentrations. In 3 dogs of group 2 and 5 dogs of group 3, sCys-C concentrations were higher than the upper limit of sCys-C (0.84 mg/L) measured in healthy dogs, whereas sCr concentrations were within the reference range. It is notable that all 8 dogs had CMVI, which might affect renal dysfunction. Our results also indicated that sCys-C concentrations were elevated in all dogs with elevated sCr concentrations, suggesting that it is unnecessary to measure sCys-C when the sCr concentration is already elevated. On the basis of these results, we speculate that sCys-C can be an early renal marker for detecting renal dysfunction in dogs with CMVI.

This study has several limitations. First, the number of samples in each ISACHC group was not sufficient for statistical analysis. Two ISACHC groups were considered as a single group for statistical analysis. In addition, a reliable normal range of sCys-C measured using PETIA assay was not established owing to the lack of samples in this study. Second, GFR was not calculated because the procedure for GFR estimation is time-consuming, labor-intensive, and stressful to dogs with CMVI. Therefore, the degree of correlation between the GFR and renal markers such as sCys-C and sCr was not evaluated. Third, most dogs were diagnosed as having only CMVI. However, some dogs had other associated diseases, including hyperadrenocorticism, hypothyroidism, and pancreatitis. Even though data on the effects of other diseases on sCys-C concentration are limited in veterinary medicine studies, other diseases may impact kidney function and change the sCys-C concentration.

The present study highlighted the clinical relevance of sCys-C as a renal marker in dogs with CMVI. Based on the results, sCys-C may be a valuable renal marker for early diagnosis of renal dysfunction in dogs with CMVI. However, for clinical application, further studies are required to establish the normal range of sCys-C and clarify the effects of other diseases on sCys-C.

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