

Evaluation of the Measurement of Serum Cystatin C by an Enzyme-Linked Immunosorbent Assay for Humans as a Marker of the Glomerular Filtration Rate in Dogs

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ABSTRACT. The serum cystatin C (Cys-C) concentration is a better filtration marker than plasma creatinine (Cre) concentration in humans. In veterinary medicine, a few studies have shown that the serum Cys-C concentration in dogs is also a better marker than the plasma Cre concentration. The purpose of this study is to evaluate the applicability of measuring the serum Cys-C concentration by an enzyme-linked immunosorbent assay (ELISA) as a marker of the glomerular filtration rate in dogs with various renal dysfunctions. The serum Cys-C concentration in dogs with chronic kidney disease (CKD) was significantly higher (1.23 ± 0.21 mg/L) than that in 76 control dogs (0.85 ± 0.15) ($P < 0.001$). The reference range of the serum Cys-C concentrations in samples from the 76 control dogs was 0.55–1.15 mg/l. Serum Cys-C concentration was more strongly correlated with plasma iohexol clearance ($r = -0.704$, $P < 0.001$) than plasma Cre concentration in dogs ($r = -0.598$, $P < 0.001$). In a receiver operating characteristics analysis, significant differences between the serum Cys-C and plasma Cre concentrations were found with regard to their AUC (0.949 [SE, 0.019] and 0.849 [SE, 0.029]) and diagnostic sensitivity (90.3% and 73.6%) for detecting decreased PCio ($P < 0.05$). Therefore, the measurement of serum Cys-C concentration by ELISA is more useful for the detection of early CKD than measuring the plasma Cre concentration.

KEY WORDS: chronic kidney disease, cystatin C, glomerular filtration rate.

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The glomerular filtration rate (GFR) is an adequate marker of renal function. The methods for measuring GFR in dogs and cats include plasma or urinary clearance of inulin, creatinine, and iohexol [8, 10, 14, 17, 18]. Detection of reduced GFR by such renal clearance methods provides the most useful clinical index of the degree of renal damage. However, these methods are not widely used in the clinical settings, as they are labor-intensive and technically difficult.

In veterinary clinical practice, plasma urea nitrogen (UN) and creatinine (Cre) concentrations are widely used as endogenous markers for evaluating renal function in dogs and cats because they are easy and inexpensive to perform. However, they usually increase after a severe reduction in GFR [5]. For example, the GFR level has to halve before the Cre concentration rises significantly. Factors that can alter the plasma UN concentration include high protein intake, fever, intestinal hemorrhage, and catabolic state. In dogs, the plasma Cre concentration is influenced by the ratio of body weight to muscle mass [5], and small quantities of Cre are secreted by the renal tubules in male dogs [11]. Thus, plasma UN and Cre concentrations cannot detect mildly reduced renal function.

Cystatin C (Cys-C) is a cysteine proteinase inhibitor [1]. This protein is produced by all nucleated cells, and its production rate is constant [1]. Cys-C is freely filtrated by the glomerulus and is not secreted by the tubular cells [13].

Cys-C is reabsorbed by the tubule epithelial cells and is subsequently catabolized; thus, it does not return to the circulation [13, 24]. For these reasons, the serum Cys-C concentration may be an alternative endogenous marker of the GFR. Many studies in humans have shown that the serum Cys-C concentration is a better filtration marker than the plasma Cre concentration [12, 16, 19]. In veterinary medicine, a few studies have also shown that the serum Cys-C concentration in dogs is a better marker than the plasma Cre concentration [2, 28]. In one study, the serum Cys-C concentration in dogs was not affected by age, gender, or body weight; whereas, another study reported that serum Cys-C concentrations were increased in < 1 year and > 8 years old dogs and dogs weighing > 15 kg [6]. The renal and extrarenal factors including acute renal failure, neoplasia, and other diseases that affect the serum Cys-C concentration in dogs have not been sufficiently evaluated.

In human medicine, Cys-C can be determined by immunoassay methods such as a latex or polystyrene particle-enhanced turbidimetric assay (PETIA) [16, 19], a particle-enhanced nephelometric assay (PENIA) [12], a colloidal gold assay [26], or an enzyme-linked immunosorbent assay (ELISA) [27, 29]. In previous reports, the serum Cys-C concentration in dogs was measured by polystyrene PETIA [2, 6, 15, 20, 28]. However, it is difficult to use the polystyrene PETIA method for the measurement of canine serum Cys-C concentration in Japan, because this method is unavailable in Japanese commercial laboratories. No study has evaluated serum Cys-C concentrations by methods other than the polystyrene PETIA as a GFR marker in dogs.

The purpose of this study was to evaluate the applicability of serum Cys-C concentration measured by an ELISA as

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a parameter of GFR in dogs with various renal dysfunctions, to establish a reference range for serum Cys-C concentration in healthy dogs, and to determine the renal and extrarenal factors that affect the serum Cys-C concentration.

MATERIALS AND METHODS

Animals: One hundred and seventy-four dogs were included in this study. Forty-three dogs were clinically healthy beagles that were raised as laboratory animals in the Department of Veterinary Internal Medicine at Nippon Veterinary and Life Science University (NVLU). One hundred and thirty-one dogs were client-owned dogs presented to the Nephrology Service in the Veterinary Medical Teaching Hospital at NVLU and 19 veterinary hospitals for measurement of plasma iothexol clearance (PCio). Eighty-eight dogs were diagnosed with chronic kidney disease (CKD) by detection of renal proteinuria (urinary protein: creatinine ratio > 0.5) and/or decreased PCio (< 30 ml/min/m²) (discussed below) in conformity with the common definition of CKD [22], and were classed according to CKD stage (class 1, n=37; class 2, n=26; class 3, n=24; class 4, n=1) (IRIS 2006). These dogs did not have other diseases. Nine of the 88 CKD dogs had renal proteinuria without decreased PCio. Fifty five of 88 dogs were treated with administration of angiotensin converting enzyme inhibitor (ACEI) and/or a manufactured renal diet. Ten dogs did not have CKD, but had malignant neoplastic disease (transitional cell carcinoma of the bladder [n=2], renal lymphoma [n=2], or mammary tumor [n=1]), or congestive heart failure (CHF) according to the ISACHC (International Small Animal Cardiac Health Council) class 2 (mitral valve insufficiency [n=3] or patent ductus arteriosus [n=2]). The remaining 33 dogs that did not have any diseases and 43 healthy beagle dogs that were described previously were assigned as controls.

Measurement of the serum Cys-C concentration: The serum Cys-C concentration was measured by an ELISA [27]. Blood samples were collected from the jugular vein and were clotted in blood collection tubes. All the blood samples were centrifuged at 1,500 g for 10 min, and the serum was separated and stored at -80°C until analysis. Serum samples were diluted 16-fold with 10 mmol/l phosphate buffer solution (PBS) (pH 7.4). The rabbit polyclonal anti-human Cys-C antibody (Dako, Denmark) was dissolved in 50 mmol/l tris buffered saline (TBS) (pH 8.4) to a final concentration of 8 µg/ml. A 100 µl aliquot of the solution was poured into each well of 96-well microplates (Nunc-Immuno Plate, Maxisorp, Denmark), before being immobilized overnight at 4°C. The microplates were rinsed three times with PBS containing 0.05% Tween 20, and the reaction was blocked with TBS (pH 8.0) containing 1% bovine serum albumin (BSA) at 100 µl/well. The samples (50 µl/well) were then added. The microplates were incubated for 1.5 hr at room temperature and rinsed three times with PBS containing 0.05% Tween 20. An alkaline phosphatase-labeled rabbit polyclonal anti-human Cys-C anti-

body was diluted to 1 µg/ml in TBS containing 1% BSA and added to the microplates at 100 µl/well. The mixture in the microplates was allowed to react for 1 hr at room temperature, and the microplates were then rinsed three times with PBS containing 0.05% Tween 20. The enzyme reaction test was performed, and color development was measured with a microplate colorimeter at 492/620 nm. The Cys-C concentration was calculated from the calibration curve for Cys-C standard solution (human recombinant Cys-C calibrator, DAKO), which was simultaneously measured.

Assay Validation: Firstly, the ability of the ELISA method to measure canine serum Cys-C concentrations was evaluated by comparing samples collected from 76 control dogs and 88 CKD patients.

The assay quality to measure canine serum Cys-C concentration was determined by sera collected from 5 control dogs (low concentration of serum Cys-C) and 5 dogs with CKD (high concentration of serum Cys-C). Analyses were repeated 6 times. The coefficient of variation (CV=SD/mean × 100) for each sample was calculated. The minimum limit of detection of the ELISA method for measuring serum Cys-C concentration was determined by Cys-C standard solutions (human recombinant cystatin C) containing 0.010, 0.008, 0.006, 0.004, and 0.002 mg/L.

Linearity was assessed by 4 serum control samples diluted with 10 mmol/l PBS to obtain samples containing 25, 12.5, 6.2, 3.1, 1.6 and 0.8% of the analyte.

Intra-individual variability was evaluated using 6 control beagle dogs. The serum samples were obtained every 3 hr for 24 hr after the dogs had been fasted for 12 hr.

The reference range of serum Cys-C concentration was determined using the samples taken from the 76 control dogs. The effects of age, body weight, and gender on serum Cys-C concentration were also evaluated using these samples.

The influence of dietary intake on serum Cys-C concentration was assessed in 6 clinically healthy Beagle dogs. The dogs were fed the same diet (maintenance, WALTHAM) and lived in the same conditions. Blood samples were collected before dietary intake (0 hr) at 1, 3, 6, and 10 hr after the intake.

Plasma iothexol clearance (PCio): PCio was performed for all dogs as previously described to determine GFR [8, 13, 18]. The dosage of iothexol was 90 mg of iodine/kg for non-azotemic dogs and 45 mg of iodine/kg for azotemic dogs. A half-milliliter of heparinized blood was collected from the jugular vein before iothexol injection. Iothexol was administered via the cephalic vein (time 0), and then heparinized blood was sampled again at 120, 180, and 240 min for the non-azotemic dogs and 120, 240, and 360 min for the azotemic dogs. The plasma iodine concentration was determined by cerium arsenite colorimetric methods [18].

PCio was calculated using the 1-compartment model corrected with the Broshner-Mortensen formula [7]. The area under the curve (AUC) was estimated from the slope (α) and intercept (A) of the elimination phase of the curves, as determined by linear regression analysis of the final three

plasma samples. Clearance values (Cl) were calculated as $Cl = \text{dose of iohexol} / AUC$ ($AUC = \alpha / A$). The PCio was then calculated as $PCio = 0.990778 \times Cl - 0.001218 \times Cl^2$. The clearance values (ml/min) were standardized to body surface area (BSA, ml/min/m²). BSA (m²) was calculated from body weight (BW/g) by the general formula: $BSA = K \times (BW)^\alpha / 10^4$, where K is a shape constant (10.1 for dogs), and α is the mass exponent (0.71 for dogs) [23]. In this study, a PCio of <30 ml/min/m² was considered to represent decreased GFR.

Statistics: Statistical analysis was performed using commercial computer software (Dr. SPSS for Windows [SPSS Japan Inc.]). Descriptive statistics were calculated for the assay validation and the reference range of the serum Cys-C concentration in dogs. Gender differences were assessed using the Student's-t test in 76 control dogs. Pearson's correlation coefficient was used to assess the relationship between the serum Cys-C concentration and age or body weight in the 76 control dogs. Linear regression analysis was used to assess the PCio value and the correlation between the serum Cys-C concentration and plasma Cre concentration in all dogs. Receiver operating characteristics (ROC) analysis was performed to compare the sensitivity and specificity of the serum Cys-C concentration with those of the plasma Cre concentration. The values are presented as means \pm SD. A P-value of <0.05 was considered statistically significant.

RESULTS

Assay Validation: The assay coefficient of variation were 2.78% for samples collecting from 5 control dogs (0.77 ± 0.22 mg/L), and 1.86% for samples collecting from 5 dogs with CKD (1.49 ± 0.03 mg/L). The CV values of Cys-C standard solutions containing 0.010, 0.008, 0.006, 0.004, and 0.002 mg/l were 2.5, 3.2, 3.6, 5.6, and 4.1% respectively. The minimum limit of detection of the ELISA method using human recombinant cystatin C was considered 0.006 mg/l. Dilutional linearity was not observed after serial dilution. However, an almost linear decrease in serum Cys-C concentration was observed in highly diluted samples (6.2, 3.1, 1.6 and 0.8%) (Fig. 1).

No significant intra-individual variability was found in the 6 healthy dogs. The serum Cys-C concentration remained stable over 24 hr (mean: 0.82 ± 0.03 mg/l, coefficient of variation [CV]: 3.3%).

No significant gender difference was observed in the control dogs (intact females: 0.86 ± 0.16 mg/l [n=32], intact males: 0.86 ± 0.13 mg/l [n=34], $P=0.924$). No significant correlation was found between the serum Cys-C concentration and age ($r=-0.138$, $P=0.251$); whereas, body weight was significantly correlated with the serum Cys-C concentration ($r=0.349$, $P<0.05$). The serum Cys-C concentration in 11 dogs that weighed less than 5 kg (0.68 ± 0.17 mg/l) was lower than that in 65 heavier dogs (0.88 ± 0.13 mg/l) ($P<0.05$).

No significant influence of dietary intake on the serum

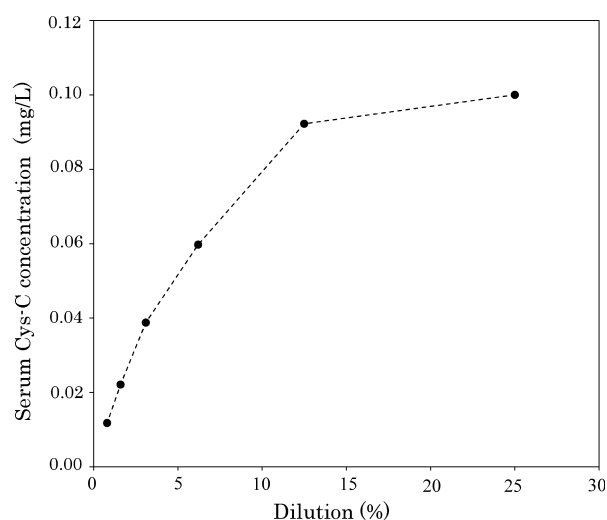


Fig. 1. Dilutional linearity for the serum Cys-C concentration in 4 control samples.

Cys-C concentration was found in 6 control beagle dogs. The serum Cys-C concentration remained stable in these dogs before dietary intake and at 1, 3, 6, and 10 hr after (0.84 ± 0.04 mg/l, CV=4.4%) (Fig. 2).

The clinical utility of the serum Cys-C concentration: In the 76 control dogs and 88 dogs with CKD, the mean Cys-C serum concentration was 0.85 ± 0.15 mg/l (range: 0.52–1.18 mg/l) (reference range: 0.55–1.15 mg/l [determined as mean \pm 2SD]) and 1.23 ± 0.21 mg/L (range: 0.62–1.58 mg/l), respectively. In 5 dogs with neoplastic disease and 5 dogs with congestive heart disease, the mean serum Cys-C concentrations were 0.93 ± 0.13 mg/l (range: 0.81–1.15 mg/l) and 0.80 ± 0.12 mg/l (range: 0.67–0.99 mg/L), respectively. There were no significant differences in the serum Cys-C concentrations between the control dogs and the dogs that were suffering from neoplastic disease or the dogs with congestive heart disease ($P=0.764$, $P=0.928$, respectively) (Fig. 3). Serum Cys-C concentration was higher than the upper reference range (1.15 mg/l) in 60 (75.9%) of 79 CKD dogs with decreased PCio value. In nine CKD dogs without decreased PCio, the serum Cys-C concentrations were within reference range. Only one of the 76 control dogs (1.3%) showed an increase in the serum Cys-C concentration (1.18 mg/l). Plasma Cre concentration was higher than the upper reference range (1.4 mg/l) in 41 dogs (51.9%) with CKD decreasing PCio value. Five of the 76 control dogs (7.0%) showed an increase in plasma Cre concentration (Fig. 3).

In all 174 dogs, the serum Cys-C concentration and plasma Cre concentration was significantly correlated with PCio ($r=-0.704$, $P<0.001$, and $r=-0.598$, $P<0.001$, respectively) (Fig. 4). A significant correlation was seen between serum Cys-C and the plasma Cre concentration ($r=0.718$, $P<0.001$).

In order to compare the diagnostic accuracy of Cys-C and Cre by ROC analysis, all 174 dogs were classified as normal

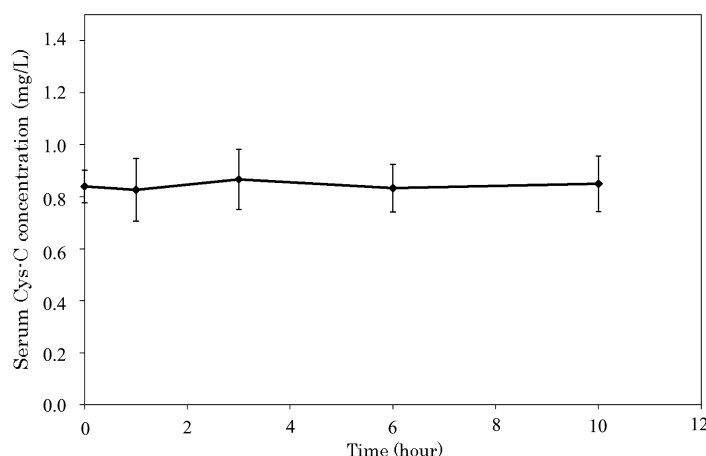


Fig. 2. Variations in the serum Cys-C concentration in 6 clinically healthy Beagles after dietary intake. Blood samples were collected before dietary intake (0 hr) and at 1, 3, 6, and 10 hr after dietary intake. No significant influence of dietary intake was found on the serum Cys-C concentration (mean: 0.84 ± 0.04 , CV: 4.4%).

PCio (> 30 ml/min/m²) or decreased PCio (< 30 ml/min/m²). The sensitivity and specificity were 90.3 and 88.2% for serum Cys-C (cutoff value=1.05 mg/l), and 73.6 and 88.2% for plasma Cre (cutoff value=1.27 mg/dl) (Fig. 5), respectively. The AUC was 0.949 (standard error [SE]=0.019) and 0.849 (SE=0.029) for serum Cys-C and plasma Cre, respectively. The serum Cys-C concentration had significantly higher sensitivity for detecting decreased GFR than Cre ($P<0.05$).

DISCUSSION

Many human studies and several veterinary studies have suggested that serum Cys-C concentration may be an alternative endogenous marker of GFR. In previous veterinary reports, a polyethylene PETIA was shown to reliably measure canine serum Cys-C [2, 6, 15, 20, 28]. Almy *et al.* [2] showed that Western blot analysis demonstrated considerable cross-reactivity of the rabbit polyclonal anti-human Cys-C antibody with canine Cys-C. In the present study, an ELISA using the same anti-human Cys-C antibody is available to measure canine serum Cys-C. However, it is not possible to assess analytical recovery by this method because no purified canine Cys-C is available. Therefore, we cannot conclusively demonstrate that the ELISA correctly measured the canine serum Cys-C concentration. Dilutional linearity was only observed in highly diluted samples in this method (6.2, 3.1, 1.6 and 0.8%). Therefore, all serum samples had to be diluted 16-fold with 10 mmol/l PBS to measure the serum Cys-C concentration.

The serum Cys-C concentration was not significantly related to age or gender in the present study, as previously reported [28]. However, a significant correlation between the serum Cys-C concentration and body weight was found ($r=0.349$, $P<0.05$). In particular, the serum Cys-C concen-

tration in dogs weighing less than 5 kg (0.68 ± 0.17 mg/l) was lower than that in 65 heavier dogs (0.88 ± 0.13 mg/l) ($P<0.05$). Braun *et al.* [6] showed that the serum Cys-C concentration was significantly lower in dogs with a body weight of < 15 kg than in those with a body weight of > 15 kg; however, they did not measure GFR. In present study, these dogs showed normal PCio values (51.52 ± 23.68 ml/min/m², range 32.9 ± 106.09 ml/min/m²). The serum Cys-C in the small sized dogs was lower without normal PCio value because the number of nucleated cells produced Cys-C could be smaller than in the large sized dogs. However, there is no reports confirming this hypothesis, so further study that involves more dogs with normal GFR is required.

The reference range of serum Cys-C concentration in the 76 control dogs ($0.55\text{--}1.15$ mg/l) was a little lower than that in humans ($0.70\text{--}1.57$ mg/l in an ELISA) [29] and those in other veterinary studies ($0.68\text{--}1.60$ mg/l [28] or $0.76\text{--}1.44$ mg/l [2]). Such differences might result from the diverse assay methods for Cys-C. Therefore, a comparison of measurements obtained via the PETIA and ELISA methods from the same canine sample is required in further studies.

In this study, the serum Cys-C concentration was more strongly correlated with PCio ($r=-0.704$, $P<0.001$) than plasma Cre concentration ($r=-0.598$, $P<0.001$). The serum Cys-C concentration was increased in about 75% of CKD dogs with decreased PCio, whereas plasma Cre concentration was within reference range in about 50% of CKD dogs (Fig. 3). Additionally, in the ROC analysis, significant differences between the serum Cys-C and plasma Cre concentrations were found in the AUC (0.949, [SE, 0.019]) and 0.849 [SE, 0.029]) and the diagnostic sensitivity (90.3% and 73.6%) for detecting decreased PCio ($P<0.05$). Numerous human studies have shown that the sensitivity of serum Cys-C for detecting reduced GFR is much higher than that of plasma Cre [3]. Although Werhner *et al.* [28] showed that

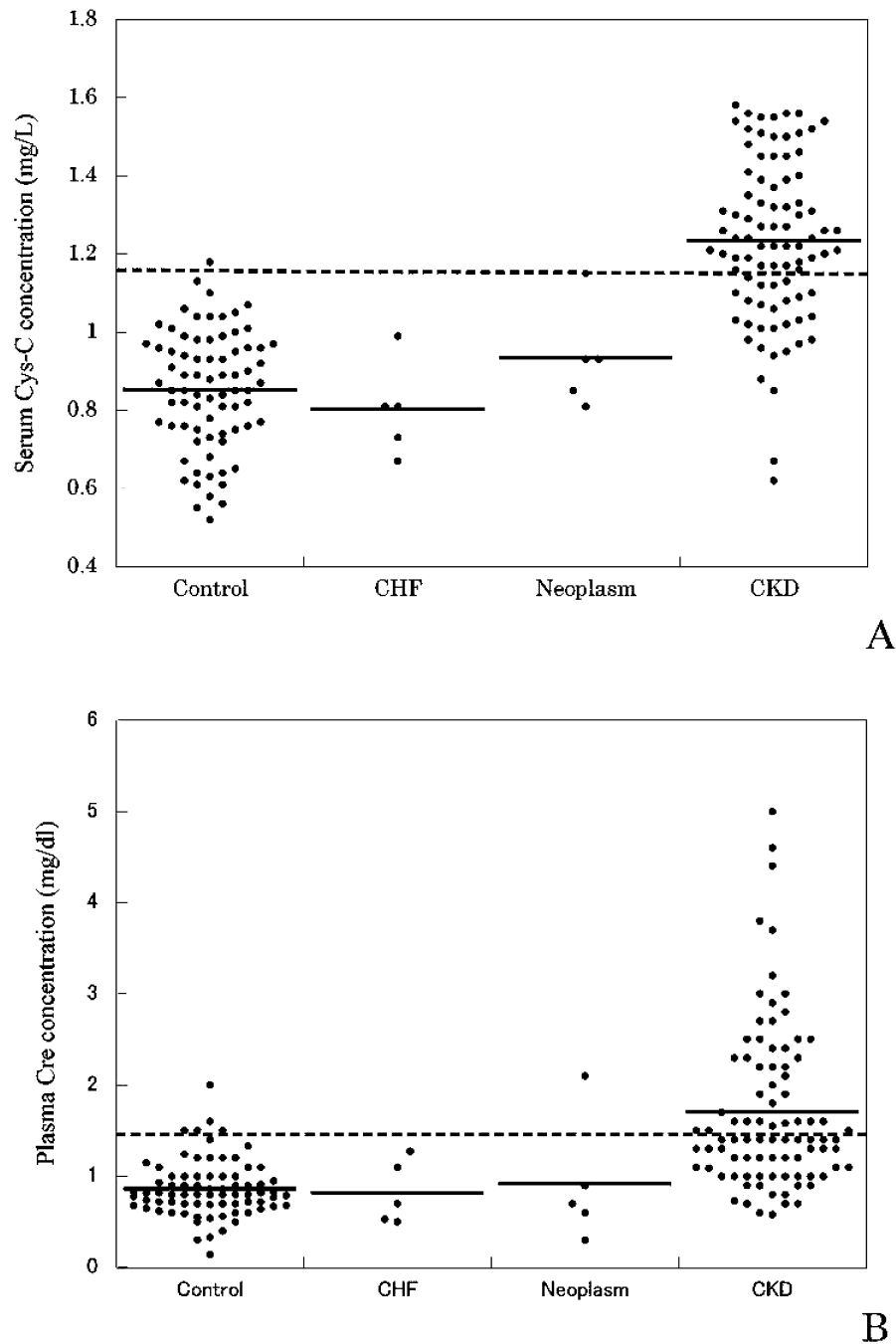


Fig. 3. Serum Cys-C (A) and plasma Cre concentrations (B) in 174 dogs with different diseases. The dotted line indicates the upper reference range for the serum Cys-C and plasma Cre concentration; CHF = congestive heart failure.

the sensitivity of serum Cys-C for detecting reduced GFR (76%) was significantly higher than that of plasma Cre (65%), the sensitivity of serum Cys-C was lower than 80%, which is the critical level for it to be considered as an adequate diagnostic test. In the present study, the sensitivity of the serum Cys-C concentration was higher than 80%. On

the basis of these results, the serum Cys-C concentration is more useful to evaluate for detecting reduced GFR than plasma Cre concentration. Furthermore, measuring the serum Cys-C concentration may be allowed to detect early chronic kidney disease.

In the present study, the serum Cys-C concentration did

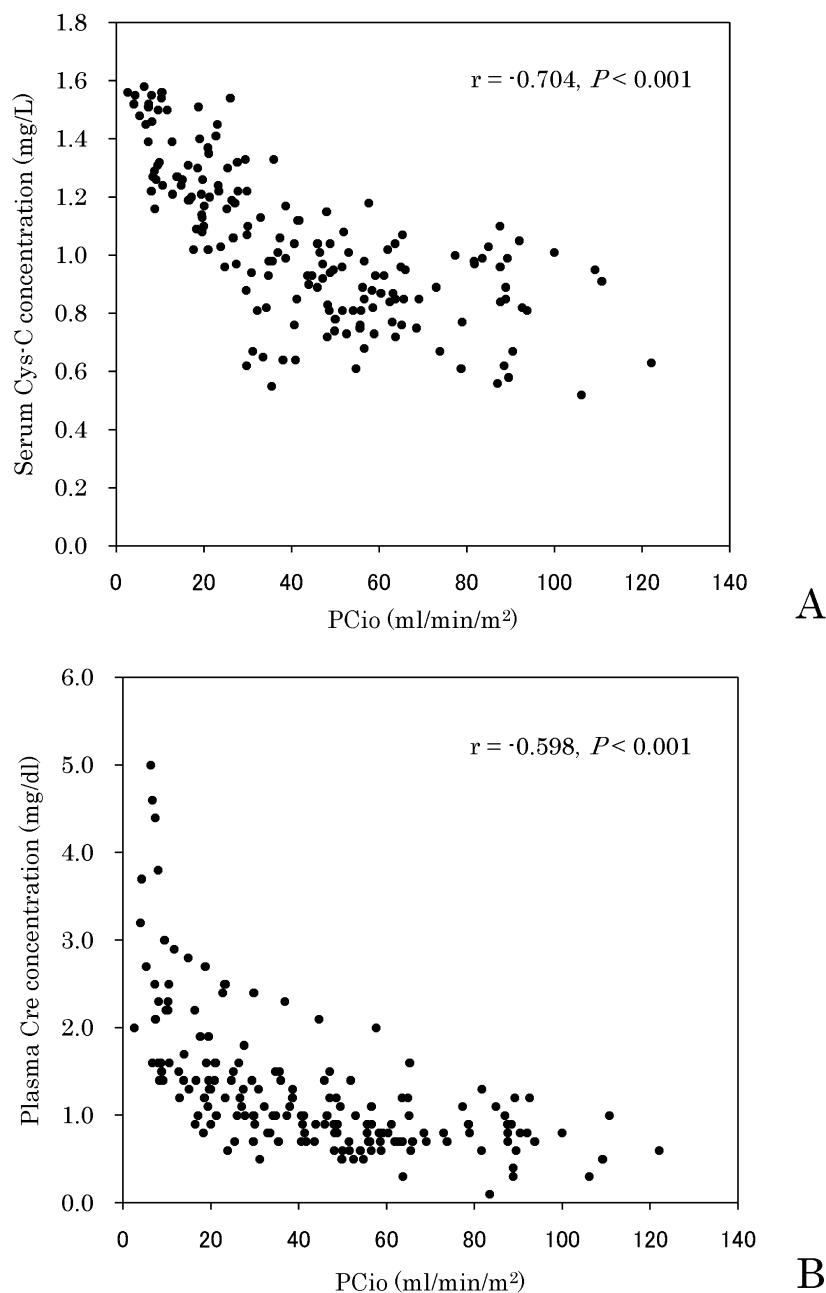


Fig. 4. The correlation between serum Cys-C (A) and plasma Cre concentrations (B) and PCio in 174 dogs with various renal dysfunctions.

not increase in dogs with neoplasms or congestive heart failure. However, the numbers of dogs with these disorders were small in our study. Although all 5 dogs with congestive heart failure were categorized as ISACHC class 2, many cases with a more severe class are needed to assess the effect of this disorder on the serum Cys-C concentration. Several human studies have shown that the serum Cys-C concentration is increased in hypothyroidism and after the administration of glucocorticoid, and is decreased in hyperthyroidism

[4, 24], although the effects of these hormones on the serum Cys-C concentration are unknown in animals. The present study also could not show the effects of these hormones, because all 174 dogs did not have the endocrine disease, or were not treated with glucocorticoid. In human studies, the serum Cys-C concentration is useful as a biomarker for early and differential diagnosis of acute kidney injury [21]. In a veterinary study, Almy *et al.* [2] only showed that a single dog with acute renal failure had a low serum Cys-C con-

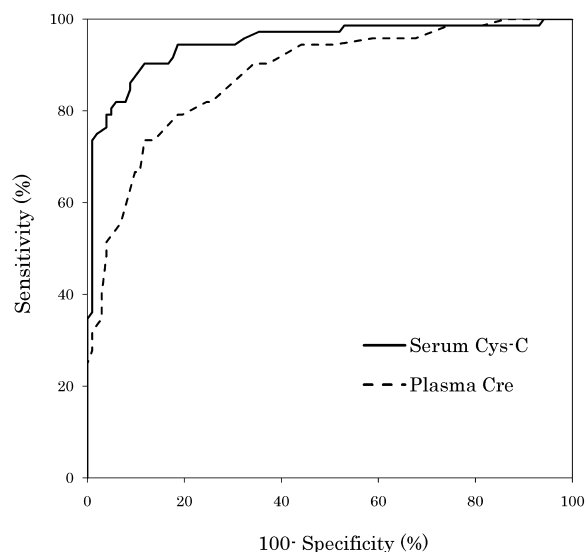


Fig. 5. ROC plots of the sensitivity and specificity of serum Cys-C and plasma Cre concentrations in 174 dogs, classified into those with normal and reduced PCio (< 30 ml/min/m²).

centration. Further study is necessary to assess the effects different types of kidney diseases or other diseases on the serum Cys-C concentration in dogs. In the present study, the effects of the treatment of CKD on the serum Cys-C concentration were not able to evaluate, because most CKD dogs undergoing treatment were previously given both ACEI and a manufactured renal diet, and these dogs demonstrated the various GFR. Further study is necessary to evaluate the effects of the ACEI or diet on the serum Cys-C concentration in dogs with the same degree of GFR. In other veterinary or human studies, the effect of any drugs or diets on that concentration has been not reported.

The amino acid sequence of canine Cys-C is still unknown. Thus, serum Cys-C might show a different response to anti-human Cys-C antibody. The availability of canine serum Cys-C for early diagnosis of chronic kidney disease might be improved by developing a measurement system using anti-canine Cys-C antibody.

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