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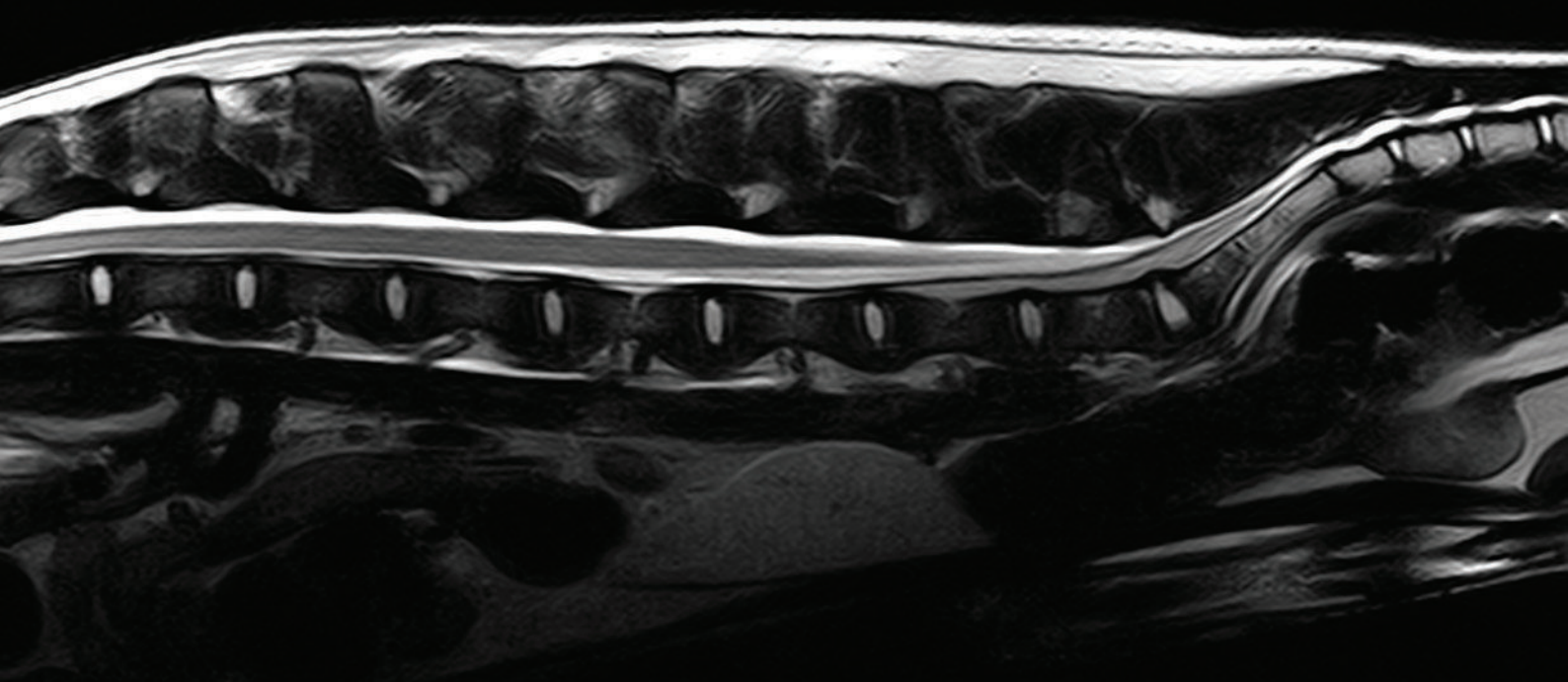

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STANDARD ARTICLE

# Variability of serum concentrations of cystatin C and urinary retinol-binding protein, neutrophil gelatinase-associated lipocalin, immunoglobulin G, and C-reactive protein in dogs

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**Background:** Markers of kidney dysfunction and damage have potential to detect chronic kidney disease (CKD) in early stages. However, data on long-term variation of these markers in healthy dogs is lacking and is crucial for the interpretation of results.

**Hypothesis/Objectives:** To determine temporal variations of serum cystatin C (sCysC) and urinary retinol-binding protein (uRBP), neutrophil gelatinase-associated lipocalin (uNGAL), immunoglobulin G (uIgG), and C-reactive protein (uCRP) in healthy dogs.

**Animals:** Eight clinically healthy adult Beagles were evaluated.

**Methods:** Longitudinal observational study. Serum cystatin C was determined by particle-enhanced nephelometric immunoassay. Urinary retinol-binding protein, uNGAL, uIgG and uCRP were determined by ELISA and concentrations were indexed to urinary creatinine. Within- and between-dog variance components (VC) and within-dog coefficients of variation (CV) were determined from blood and urine collected at eight time points over 1.5 years.

**Results:** Urinary C-reactive protein (uCRP) concentrations were consistently below the detection limit (5.28 ng/mL). Mean ± within-dog standard deviation for sCysC, uRBP/c, uNGAL/c and uIgG/c was 0.15 ± 0.01 mg/L, 0.09 ± 0.03 mg/g, 2.32 ± 2.03 μg/g and 12.47 ± 10.98 mg/g, respectively. Within-dog CV for sCysC, uRBP/c, uNGAL/c and uIgG/c was 8.1%, 33.7%, 87.2% and 88.1%, respectively.

**Conclusions and clinical importance:** Serum cystatin C, uRBP/c, uNGAL/c and uIgG/c exhibit a wide range of long-term within-dog variability. Researchers and veterinarians might need to take this into account when interpreting their results. To assess their diagnostic and predictive ability, future studies need to establish reference ranges for healthy dogs and dogs with CKD.

KEYWORDS

Canine, glomerular, tubular, marker, variation

## 1 | INTRODUCTION

Kidneys have a great compensatory ability when affected by insults that could compromise their function.[1] As a consequence, conventional markers of renal disease, such as serum creatinine, often lead to late diagnosis of chronic kidney disease (CKD).[2] This makes it difficult and challenging for veterinarians to detect kidney disease at an early stage when proper treatment might slow CKD progression and improve longevity and quality of life.[3] Furthermore, while measurement of

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**Abbreviations:** /c, creatinine ratio; BCS, body condition score; BW, body weight; CKD, chronic kidney disease; CRP, C-reactive protein; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; IgG, immunoglobulin G; LOD, limit of detection; LOQ, limit of quantification; MW, molecular weight; NGAL, neutrophil gelatinase-associated lipocalin; PENIA, particle-enhanced nephelometric immunoassay; RBP, retinol-binding protein; sCysC, serum cystatin C; SE, standard error; u, urinary; UPC, urinary protein:creatinine ratio; USG, urine specific gravity; VC, variance component

glomerular filtration rate (GFR) is the gold standard to determine kidney function, the clinical application in veterinary medicine is cumbersome as multiple sampling is often required and filtration markers or specialized equipment to analyze the markers are not commercially available.[4] Therefore, there is a search for more sensitive markers of kidney disease and indirect markers of GFR in veterinary medicine.

Combined use of sensitive indirect markers of GFR and site-specific (i.e., glomerular and tubular) markers of renal disease, allowing for earlier detection of CKD, could potentially offer veterinarians a powerful alternative, particularly in the context of routine screening or serial monitoring of individuals. Serum symmetric dimethylarginine is a more sensitive and specific indirect GFR marker when compared to creatinine.[5,6] However, there are other markers that have potential to detect early renal dysfunction. Serum cystatin C (sCysC), for example, is a low molecular weight (MW) protein that meets many of the requirements for an ideal endogenous GFR marker.[7] Serum cystatin C is a better marker of GFR than serum creatinine and has good diagnostic accuracy in predicting CKD in humans.[8,9] In dogs, sCysC has a comparable or better sensitivity to detect a decrease in GFR compared to serum creatinine.[7] However, it lacks specificity.[7] To our knowledge, there are no studies yet comparing sCysC directly to serum symmetric dimethylarginine in dogs.

Several site-specific urinary markers have also been investigated in recent years. C-reactive protein (CRP) and immunoglobulin G (IgG) are both high MW proteins associated with glomerular damage when detected in urine.[10] Retinol-binding protein (RBP) and neutrophil gelatinase-associated lipocalin (NGAL) are both low MW proteins that reflect tubular damage.[10] These markers increase in dogs with CKD and some correlate to glomerular lesions, tubulointerstitial lesions, or both of differing magnitudes.[10–13]

Despite the potential of these markers, their use is still limited to research purposes. Generally accepted reference ranges and cut-off values do not exist yet due to lack of standardized methods of analysis and large-scale studies. Moreover, information on long-term variation of both healthy and diseased dogs is lacking for sCysC and urinary (u) RBP, NGAL, IgG and CRP. Knowing the stability or variability of each marker's concentration in function of time is needed to determine how it should be interpreted when compared to established reference ranges. Therefore, the aim of this study was to determine the temporal variation of markers of kidney disease sCysC, uRBP, uNGAL, uIgG and uCRP in healthy dogs during a period of 1.5 years.

## 2 | MATERIALS AND METHODS

This longitudinal study of 1.5 years was approved by the Local Animal Ethics Committee (Faculties of Veterinary Medicine and Bioscience Engineering, Ghent University, Belgium) and performed in accordance with European and national regulations for the care and use of animals (EC2015/92).

### 2.1 | Animals

Eight healthy lean adult Beagles (three intact and one spayed female, two intact and two neutered males) were included in the study. Dogs were considered healthy if no clinically relevant abnormalities were found on their medical history, physical examination, complete blood count, serum biochemistry (serum creatinine < 1.4 mg/dL based on International Renal Interest Society guidelines for staging CKD), abdominal ultrasonography, and urinalysis on urine collected by ultrasound-guided cystocentesis (urine sediment, dipstick test, specific gravity (USG), including protein-to-creatinine ratio (UPC), and bacterial culture). At the start of the study dogs were between 2.7 and 8.3 years (mean ± standard deviation, 4.7 ± 1.7 years) and had an ideal body weight (BW) (11.58 ± 1.64 kg) and an ideal body condition score (BCS) of four based on a 9-point scale.[14]

### 2.2 | Procedures

After adapting to the study's diet for four weeks, measurements were made at week 0, 12, 24, 36, 47, 56, 68 and 83 of the study. All dogs received the same dry commercial adult maintenance diet (Veterinary™ HPM Adult Large and Medium, Virbac, Carros, France) during the entire study. The amount was adjusted weekly to maintain an ideal BW and BCS (4 - 5/9), both of which were assessed weekly. Water was provided ad libitum. Blood and urine samples were collected at the eight time points. Dogs were fasted for at least 12 hours prior to samples collections. Blood samples (5 mL) were collected from the jugular vein (21G needle). Complete blood count and serum biochemistry were repeated at weeks 24, 47, 56 and 83.[15] Serum was acquired by centrifuging blood collected in a serum tube within two hours of collection for 5 minutes at 2000 × g at 21°C. Serum was stored at -80°C in aliquots of 300 μL until analysis. Urinalysis (dipstick analysis, USG, UPC, sediment analysis, and bacterial culture) were performed on an aliquot of morning urine (5 mL) collected by ultrasound-guided cystocentesis (22G needle) at all eight time points.[15] An aliquot of 5 mL urine was centrifuged for 3 minutes at 447 × g within 30 minutes of collection. The supernatant was aliquoted (200 μL) and stored at -80°C until analysis.

### 2.3 | Assays

Serum cystatin C was measured with particle-enhanced nephelometric immunoassay (PENIA) previously validated for dogs.[15,16] Samples for sCysC were analyzed in four batches. The limit of detection (LOD) of PENIA for sCysC was 0.05 mg/L. uRBP concentrations were analyzed with a commercially available human ELISA kit (Immunology Consultants Laboratory, Portland, OR, USA). uNGAL (BioPorto Diagnostics, Hellerup, Denmark), uIgG (Immunology Consultants Laboratory, Portland, OR, USA) and uCRP (Immunology Consultants Laboratory, Portland, OR, USA) concentrations were determined with commercial canine-specific ELISA kits. uRBP, uNGAL, uIgG and uCRP assays were previously validated for use with canine urine.[12,17] All immunoassays were performed in two batches and were used according to manufacturer's instructions and performed as previously described.[12,17–19] The LOD and limit of quantification (LOQ) were determined in previous studies.[17,19] The LOD and LOQ of the uRBP, uNGAL, uIgG and uCRP assays were 14.11 ng/mL and 18.93 ng/mL, 5.35 pg/mL and 9.60 pg/mL, 19.69 ng/mL and 29.72 ng/mL and 5.28 ng/mL and 7.76 ng/mL, respectively. The concentration of each

urinary marker was expressed as a ratio to urinary creatinine (/c) to account for variations in urine concentration.[18] Urinary creatinine was determined by the modified kinetic Jaffé method.[17]

## 2.4 | Statistical Analysis

Statistical analysis was performed with R (version 3.3.2; Rstudio version 1.0.143). For the immunoassays, if the obtained concentrations of samples that were minimally diluted (1:2) were below the LOD or between the LOD and LOQ, the median between 0 and LOD or the median between LOD and LOQ were used as a value for statistics, respectively. When samples were not minimally diluted, a missing value was assigned for concentrations that fell below the LOD or between the LOD and LOQ. A random effects model using restricted maximum likelihood (lme4 package) was used to estimate the variance components (VC).[20] Two VCs were estimated: $v_1$ representing the variation between repeated measurements on the dog and $v_2$ represents the extra variation when considering observations of different dogs. The VCs were used to determine 95% reference intervals for repeated observations in the same dog and for repeated observations in different dogs. The coefficient of variation (CV), defined as the ratio of the standard deviation over the mean, was determined for the within-dog repeated observations.

# 3 | RESULTS

During the study, percentage BW changes varied between -3.9% and 14.8% compared to week 0. BCS, however, remained within the ideal range (BCS 4 – 5/9), as each unit increase is associated with a 10-15% change in weight on a 9-point scale.[21] One of the dogs had persistent mild proteinuria (UPC 0.62 ± 0.14) without azotemia from week 12. In another dog euthanized after week 56 because of multicentric lymphoma, only the first 6 of the 8 time points were included for statistical analysis. All dogs had a serum creatinine concentrations less than 1.4 mg/dL (0.62 ± 0.07 mg/dl), USG of 1.038 (median; range, 1.008-1.052), negative urine bacterial culture and unremarkable urinalysis except for microscopic hematuria (> 27 red blood cells/μL urine) in some of the samples. Microscopic hematuria was present in one sample at week 0, seven samples at week 24, one sample at week 36, two samples at week 47, and four samples at week 56. uCRP consistently had concentrations below the LOD of the assay, and was therefore not included in the statistical analysis.

**TABLE 1** Estimated variance components, $v_1$ for variation from repeated measurements on the same dog and $v_2$ for extra variation from measurements from different dogs, of sCysC, uRBP/c, uNGAL/c and uIgG/c in healthy beagles (n = 8) measured over 1.5 years

| Variable | $v_1$ | $v_2$ |
|---|---|---|
| sCysC (mg/L) | $1.42 \times 10^{-4}$ | $2.78 \times 10^{-4}$ |
| uRBP/c (mg/g) | $9.25 \times 10^{-4}$ | $1.04 \times 10^{-3}$ |
| uNGAL/c (μg/g) | 4.11 | 3.96 |
| uIgG/c (mg/g) | 120.6 | 242.9 |

sCysC, serum cystatin C; uRBP/c, urinary RBP-to-creatinine ratio; uNGAL/c, urinary NGAL-to-creatinine ratio; uIgG/c, urinary IgG-to-creatinine ratio.

**TABLE 2** Mean ± within-dog SD and within- and between-dog 95% reference intervals and CV for within-dog repeated measures of sCysC, uRBP/c, uNGAL/c and uIgG/c in healthy beagles (n = 8) measured over 1.5 years

| Variables | Mean ± SD | Within-dog 95% reference interval | Between-dog 95% reference interval | CV |
|---|---|---|---|---|
| sCysC (mg/L) | 0.15 ± 0.01 | 0.12 - 0.17 | 0.11 - 0.19 | 8.1% |
| uRBP/c (mg/g) | 0.09 ± 0.03 | 0.03 - 0.15 | 0.00 - 0.18 | 33.7% |
| uNGAL/c (μg/g) | 2.32 ± 2.03 | 0.00 - 6.3 | 0.00 - 7.89 | 87.2% |
| uIgG/c (mg/g) | 12.47 ± 10.98 | 0.00 - 33.99 | 0.00 - 49.83 | 88.1% |

SD, within-dog standard deviation; CV, coefficient of variation; sCysC, serum cystatin C; uRBP/c, uRBP-to-creatinine ratio; uNGAL/c, uNGAL-to-creatinine ratio; uIgG/c, uIgG-to-creatinine ratio.

Table 1 summarizes the estimated VCs for sCysC, uRBP/c, uIgG/c and uNGAL/c. Variation from repeated measurements on the same dog over 1.5 years was smaller than variation from measurements made on different dogs for sCysC, uIgG/c and uRBP/c. Of all markers examined, sCysC had the lowest within-dog CV (8.1%), followed by uRBP/c (33.7%), while uIgG/c and uNGAL/c had the highest within-dog CVs (88.1% and 87.2%, respectively) (Table 2).

# 4 | DISCUSSION

This longitudinal study investigates markers of kidney disease in dogs over a period of 1.5 years to determine both within- and between-dog variability for each marker in a highly controlled group of dogs, i.e., same breed, kept under the same conditions and fed the same diet. Moreover, the study and sample analyses were performed in a standardized manner to reduce further variation. Except for uNGAL/c, the variation arising from repeated measurements within the same dog was smaller than the variation from measurements made in different dogs for sCysC, uRBP/c and uIgG/c. Among all markers studied, sCysC had the smallest within-dog CV, suggesting that the change in concentrations obtained from serial measurements in healthy dogs is limited. In addition, low intra-individual variability allows the dog to be its own reference in the detection of early changes in concentration after serial measurements (i.e., trending), as in the case of serum creatinine.[10] Compared to sCysC, uRBP/c, uNGAL/c and uIgG/c exhibit a high degree of within-dog variability. This means that the obtained values from sequential samples from one healthy dog can differ substantially. As such, interpretation of a single sample for these markers should be done with caution, particularly if there is overlap between the concentration of healthy dogs and dogs with CKD.

The biological variance of sCysC was investigated a decade ago in healthy dogs.[22] Even with a longer study duration in the current study (83 weeks vs. 24 weeks); a longer time interval between measurements (median 12 weeks vs. 2 weeks); the use of a single breed instead of two; and the use of PENIA instead of particle-enhanced turbidimetric immunoassay to analyze sCysC, within-dog variability of sCysC appears to be quite low in dogs (8.1% vs. 12.3%). Currently, there is still no standardized method of analyzing sCysC and no canine-specific assay available. Therefore, it remains difficult to

compare different studies and to establish generally applicable reference intervals that reflect exact sCysC concentrations.[7]

Long-term variability of uCRP, uRBP, uNGAL and uIgG has not been previously assessed in dogs or in other companion animal species. Even in humans, data are scarce. Thus, this study is the first to demonstrate high variability over time of markers of kidney disease uRBP, uNGAL and uIgG. Yet, of the urinary markers evaluated, uRBP/c seems least prone to within-dog variability. RBP, a 21 kDa plasma protein, is freely filtered through the glomerulus in the unbound form, and in healthy animals mostly reabsorbed and catabolized in the proximal tubules.[23] The presence of RBP in urine occurs when tubules are injured, when abnormal amounts of proteins compete for reabsorption, or both.[10] An undetectable to a low uRPB/c of <0.15 mg/g is expected in healthy dogs.[10] The overall mean ± within-dog SD from the current study (0.09 ± 0.01 mg/g) corroborates this finding and only one dog had a concentration below the LOD. Day-to-day CV of 9.2 to 10.5% occurs in humans.[24] The within-dog CV of the current study is much higher, possibly as a result of the longitudinal nature of the study. Nevertheless, the variation is still moderate compared to that of uIgG/c and uNGAL/c. Therefore, uRBP/c might still be potentially useful as urinary markers reflect lesions and physiology occurring in the kidneys more directly and might be a more sensitive indicator of injury than their systemic counterparts.[25,26] Since there are contradicting studies on whether uRBP/c can detect renal dysfunction at an early stage,[12,27,28] additional studies are needed to determine the usefulness of this marker in the diagnosis of early CKD.

Both uNGAL/c and uIgG/c demonstrated high within-dog CVs. The high variability in normal dogs implies that to be a good clinical marker (i.e., to allow an accurate discrimination of health status), the difference between healthy and diseased dogs (e.g., CKD) needs to be bigger relative to markers with a small CV. NGAL is similar to RBP as a low MW protein marker of kidney disease but can also be synthesized by damaged tubular epithelial cells.[10] uNGAL/c is <6 μg/g in healthy dogs, which corroborates with the current study's overall mean.[10] Only one dog had an uNGAL concentration below the LOD. Within-dog CV of this longitudinal study also corroborates with high day-to-day biological variation of uNGAL/c in humans (CV ranging from 75% to 101%).[29–31] Variation from samples collected repeatedly within the same day is higher than between days.[32] The reason for this high variation is currently unknown. It should be highlighted that urinary levels in healthy dogs were 1000-fold lower than for the other two markers, uRBP and uIgG. To that extent, uNGAL/c might still have potential to detect early CKD, provided that the rise in concentration is high enough despite the high variation in healthy dogs. However, its ability to diagnose early CKD is still requires investigation as during early stages of canine X-linked hereditary nephropathy, when GFR is decreasing from >3.5 to 1.5-2.5 mL/min/kg, uNGAL/c increases 2-fold and then plateaus as GFR continues to decrease.[12]

Immunoglobulin G is a high MW protein (150 kDa) that plays a part in the humoral immune system.[23] uIgG/c is increased in dogs with CKD and is positively correlated to glomerular lesions.[12,13] Moreover, it is increased in early stages of CKD related to X-linked hereditary nephropathy in dogs.[12] The overall mean of uIgG/c from the current study was slightly higher than the observed maximum of 10 mg/g from healthy dogs in other studies.[10] Factors that affect uIgG concentrations in healthy dogs are still undetermined. Moreover, biological variation of uIgG/c in either humans or in other companion animal species than dogs has not been investigated. One possible explanation for the variation is that uIgG/c is significantly correlated to UPC.[12] In our study, a dog with mild proteinuria after the start of the study but was otherwise healthy had higher but relatively stable uIgG/c compared to other dogs. Despite the proteinuria, we chose to keep the dog in the study. Other than an increase in UPC between week 0 and week 12 (i.e., from 0.25 to 0.72), it fluctuated between 0.42 and 0.80 in the current study. According to Nabity and colleagues, for UPC values near 0.5, UPC must change by at least 80% before a change can be considered significant and warrant further investigation.[33] For UPC values starting at <0.2 or borderline proteinuria, as with the dog in our study, guidelines have not been proposed nor investigated. Moreover, this study was based on dogs with glomerular proteinuria caused by X-linked hereditary nephropathy, and whether this guideline can be applied to other glomerular diseases is still unknown.[33] Although this dog's proteinuria could be age-related as this dog was eight years at the start of the study, data linking proteinuria to aging in dogs is limited.[34] Future studies need to determine the age effect, as well as other factors on uIgG/c.

In accordance with the majority of other studies of markers of kidney disease in dogs that included a healthy control group, dogs in our study also had uCRP concentrations below the detection limit.[11,17,18,35–39] CRP is a major positive acute phase protein in dogs with a large MW (110 to 144 kDa) and therefore is unable to pass through an intact glomerular barrier, which probably was the case in the healthy dogs in our study.[10] Further investigation into uCRP as a marker to detect the presence of glomerular injury is still warranted.

Although hematuria could potentially interfere with accurate determination of urinary analyte concentrations, its influence on uRBP/c, uIgG/c and uCRP/c seems to be limited.[35,40] Furthermore, in the current study, hematuria was microscopic, most likely due to contamination from the cystocentesis, and only present in a small portion of the samples. Hence, the effect of hematuria on our results was negligible.

Because the dogs used in our study were from the same breed, kept in the same environment and were fed the same diet, the results of the current study cannot be applied to the general population of dogs. Therefore, studies in a large mix-breed population are required. In such studies, the contribution of breed, sexual status, and age to the variation of biomarkers can also be assessed as to determine whether separate reference ranges are necessary. Furthermore, it would be interesting to determine, especially for the biomarkers with low within-dog variation, the change in concentration needed before it can be considered as indicative of disease.

In conclusion, markers of kidney disease sCysC, uRBP/c, uNGAL/c and uIgG/c show a wide range of intra-individual variation in healthy dogs, which might affect their interpretation. sCysC had the lowest variation, while the other markers exhibited large variation. Clinically, if there is only a slight difference in concentration between healthy dogs and dogs with CKD, a value indicative of disease will likely be difficult to detect with the latter three markers, while the former might still be able to discriminate between dogs with or without renal damage. In other words, to be of added diagnostic value, the

difference between healthy dogs and dogs with CKD would have to be considerable for uRBP/c, uNGAL/c and uIgG/c. While this study provides important information on the long-term variability of markers of kidney disease in healthy dogs, future studies have to assess whether there is an overlap of concentrations between healthy dogs and dogs with CKD.

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## CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Local Animal Ethics Committee (Faculties of Veterinary Medicine and Bioscience Engineering, Ghent University, Belgium) and performed in accordance with European and national regulations for the care and use of animals (EC2015/92).

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