Received: 20 April 2022 | Accepted: 14 December 2022

DOI: 10.1111/jvim.16619

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Short-term intra-individual variation of urinary biomarkers in dogs with stable chronic kidney disease

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Abstract

Background: Active-ongoing kidney damage is present in animals with stable chronic kidney disease (CKD), as reflected by biomarkers in urine. Interpretation of serial messurements of biomarkers requires knowledge of its intra-individual variation.

Aims: To evaluate the short-term intra-individual variation of urinary neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 (uNGAL, uKIM-1, respectively) in dogs with stable CKD, and to determine whether normalization to urinary creatinine (uCr) decreases variation.

Animals: Twenty-five dogs with naturally-occurring stable CKD.

Methods: Prospective, observational study. Dogs were diagnosed with CKD based on the International Renal Interest Society guidelines. Dogs were included only if the variation in serum creatinine concentration was <25% on at least 2 measurements during the 3 months preceding inclusion, and only if serum creatinine variation was <20% during the 14-day study period. Urine samples were collected on days 0, 4, 10 and 14. uNGAL and uKIM-1 were measured using ELISA.

Results: The median coefficients of variation (CV) of uNGAL and uNGAL/uCr were 42% (range, 7%-127%), and 44% (range, 8%-100%), respectively, and the CV 90th percentiles were 97% and 83%, respectively. The median CV of uKIM-1 and uKIM-1/uCr were 29% (range, 16%-91%), and 23% (range, 6%-76%), respectively, and the CV 90th percentiles were 56% and 52%, respectively.

Conclusions and Clinical Importance: Changes of >100% and >60% for uNGAL and uKIM-1, respectively, in serial measurements are higher than the normal expected variation and therefore might indicate need for further investigation for underlying causes of kidney damage.

KEYWORDS
acute kidney injury, coefficient of variation, creatinine, KIM-1, NGAL

Abbreviations: AKI, acute kidney injury; CKD, chronic kidney disease; CV, coefficient of variation; IRIS, international renal interest society; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; SDMA, symmetric dimethylarginine; uCr, urinary creatinine; uKIM-1, urinary kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin.

1 | INTRODUCTION

Chronic kidney disease (CKD) and acute kidney injury (AKI) are common in dogs, and were historically considered 2 separate entities, featuring different characteristics. CKD is an irreversible and progressive disease in which changes such as fibrosis and degeneration of the kidney parenchyma predominate, while in AKI, acute but potentially reversible damage predominates. With growing understanding of the pathophysiology governing CKD progression, it is suggested that CKD and AKI are not completely separate processes, and AKI can lead to the development of CKD and vice versa.[1,2]

Novel biomarkers of kidney injury are extensively investigated in both human and veterinary medicine. Sensitive biomarkers are extremely important because of the low sensitivity of the available conventional (functional) markers (ie, serum creatinine [sCr] and symmetric dimethylarginine) for kidney injury, especially when it is unassociated with changes in kidney function.[1,3-6] Preliminary data using these novel injury biomarkers supports the presence of active renal injury in animals with an apparently stable CKD, which might contribute to the progression of the disease.[2,7] Indeed, the degree of active damage in cats with CKD is associated with overall survival.[8] Sensitive and specific biomarkers of renal injury that could predict the progression rate would be advantageous and complimentary to the functional markers. These markers might also provide an opportunity for early diagnosis and earlier therapeutic interventions, before an irreversible decrease in kidney function occurs, and the disease progresses to the point where clinical signs become apparent and quality of life is affected.

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein synthesized in low concentrations in renal tubules, intestinal, hepatic, and pulmonary tissue, and is 1 of the most studied renal biomarkers.[9] Upregulation of NGAL synthesis occurs with acute renal tubular injury, rendering this marker as an early (ie, preceding the increase in creatinine) sensitive marker of AKI, in both human and veterinary patients.[10-15] Kidney injury molecule-1 (KIM-1) is another well-established marker of tubular injury. Its expression is low in normal kidneys,[16] but increases substantially with tubular injury, therefore KIM-1 is a potential diagnostic marker for AKI in human patients and in dogs.[16-22]

Since presence of injury markers have been documented in animals with CKD, it is possible that monitoring these injury markers will allow identification of active kidney damage in animals with CKD, and animals prone to rapid CKD progression. This will facilitate closer monitoring and timely therapeutic intervention.[2,7] Currently, interpreting the effects of therapeutic interventions in animals with CKD is challenging as functional markers are very slow to change in response to therapeutic interventions, therefore, attributing a (delayed) change of a functional marker to a specific intervention might be inaccurate. The availability of real time markers indicating active injury might facilitate evaluation of therapeutic interventions in animals with CKD. Yet, correct interpretation of the changes in the concentration of a biomarker requires knowledge of its natural fluctuations during the disease course.

The biological variation of a biomarker results from a combination of pre-analytical, analytical and post-analytical variations, caused by factors related to the patient and imprecision of the analytical process.[23,24] Patient related factors contributes to intra and inter-individual variations and include diet, activity level, muscle mass, biochemical abnormalities, and medications.[23,24] The relationship between the intra-individual and inter-individual variability of a biomarker is reflected by its index of individuality. This index determines if a population based reference range is appropriate; a biomarker with a low index of individuality, namely high variability among different individuals compared with its intra-individual variability is not well suited for population based reference range. For such biomarkers, other measures such as the subject-based reference value, critical difference or 90th percentile CV are more adequate.[23,25,26] To facilitate the interpretation of serial biomarker measurements in dogs with CKD, the degree of short-term intra-individual variation must first be established and accounted for.

We hypothesized that variation of urinary NGAL and KIM-1 (uNGAL and uKIM-1, respectively) in dogs with stable CKD will be low and comparable to the documented variability of these biomarkers in human patients. The aim of this study was to evaluate the short-term intra-individual variation of uNGAL and uKIM-1 in dogs with stable CKD. A secondary aim was to assess whether normalizing the concentration of biomarkers to urinary creatinine (uCr) decreases the daily variation.

2 | MATERIALS AND METHODS

2.1 | Animals

The study was approved by the ethical institutional committee (HUNER-2021-094). Dogs presented to the Koret School of Veterinary Medicine, The Hebrew University VTH and were diagnosed with stable CKD were prospectively enrolled. Diagnosis and staging of CKD were made according to the IRIS guidelines and staging system. Urine protein to creatinine ratio was measured in all dogs with urine dipstick of >30 mg/dL. Dogs with urine protein to creatinine ratio > 0.5 were considered proteinuric. CKD etiologies were classified as glomerulopathy, juvenile onset nephropathy, post-AKI, and unknown. Glomerulopathy was diagnosed based on the presence of persistent proteinuria (urine protein to creatinine ratio > 2 in ≥3 measurements), in the absence of pre- and post-renal causes for proteinuria. Juvenile onset nephropathy was diagnosed based on compatible clinical signs (eg, polyuria and polydipsia), ultrasonographic findings,[27] and a diagnosis of CKD before 3 years of age, and when other CKD etiologies were excluded. Etiology was regarded as "post-AKI" when CKD was diagnosed in a dog with a previous history of AKI (>3 months after the AKI diagnosis) without evidence of pre-existing CKD. If the CKD etiology was not identified, it was regarded as "unknown." Only dogs with stable CKD, defined by <25% variation in sCr in at least 2 measurements during the 3 months preceding inclusion, were considered. Dogs were finally included only if sCr variation was <20% comparing measurements on the first and last day of the 14-day study period. Dogs were excluded if they had a positive urine culture before study initiation. During the study dogs were fed a renal diet and were

managed with conventional treatment at the discretion of the attending clinician, without alterations in this treatment during the study period. Dogs presenting new clinical signs such as vomiting, diarrhea, weakness, anorexia during the study period were excluded.

2.2 | Study design and sample collection

To assess the short-term intra-individual variation of the urinary biomarkers, urine was collected 4 times over 14 days (days 0, 4, 10, and 14). sCr was measured at days 0 and 14 to assure CKD stability during the study period. Blood for sCr was collected in plain tubes with gel separators, allowed to clot in room temperature, centrifuged, and analyzed within 60 min of collection (Cobas 6000, Roche, Mannheim, Germany). Urine samples for uCr, uNGAL, and uKIM-1 were collected during the hospital visit at days 0 (cystocentesis) and 14 (void) and by the owners at days 4 and 10 (voided). Samples collected by the owners were initially frozen at −20°C and then transported to the VTH laboratory while refrigerated, and immediately stored at −80°C for up to 24 months pending analysis. In validation studies, uNGAL and uKIM-1 were stable in urine stored at −20°C for 14 days, and in long-term storage at −80°C and repeated freeze-thaw cycles did not affect their concentration.[28-30]

Before analysis, urine samples were thawed and centrifuged at 14 000 RPM for 2 min. uCr was measured at the VTH laboratory as above. uNGAL and uKIM-1 were measured in duplicates by commercial sandwich ELISA kits (BioPorto Diagnostics, Tuborg Havnevej, Denmark and ICL Inc. Portland, Oregon, USA, respectively), previously validated for canine urine, with an intra-assay coefficient of variation (CV) of <10% for both.[22,31] The intra-assay CVs in the present study were 8.3% and 4.1% for NGAL and KIM-1, respectively. All urine samples for both assays were initially diluted 1:100 using a proprietary diluent. Samples in which concentrations were above or below the detection limit were run again after diluting the sample 1: 200 or not diluted at all, respectively. All samples from a single dog were run on the same plate and on the same day by the same operator.

For uNGAL a standard curve was created using 8 dilutions (0-400 pg/mL) of canine NGAL reference standard, which were evaluated on the same plate as the experimental samples. The samples were placed in duplicates into anti-canine NGAL antibody-coated wells and then incubated for 1 hour with gentle agitation. Wells were washed to remove unbound antibody and then a secondary biotinylated antibody was added and incubated for 1 hour. Wells were washed and streptavidin-horse radish peroxidase reagent was added and incubated for 1 hour. After incubation, wells were washed and tetramethylbenzidine-based peroxidase substrate was added for 10 min while gently agitated in the dark. Finally, a dilute sulfuric acid stop solution was added, and the optical density of the solution in the well was measured at 450 nm (reference wavelength 620 nm) using a plate reader (SpectraMax Paradigm Multi-mode detection platform, Molecular Devices, Austria). The concentrations of the experimental samples were calculated from a standard curve of the optical densities.

For KIM-1 a standard curve was created using 6 dilutions (0-1000 pg/mL) of canine KIM-1 reference standard, which were evaluated on the same plate as the experimental samples. The samples were placed in duplicates into anticanine KIM-1 antibody-coated wells and then incubated for 2 hours with gentle agitation. Wells were washed to remove unbound antibody and then a secondary biotinylated antibody was added and incubated for 20 min. Wells were washed and streptavidin-horse radish peroxidase reagent was added and incubated for 20 min. After incubation, wells were washed and tetramethylbenzidine-based peroxidase substrate was added for 10 min while gently agitated in the dark. Finally a dilute sulfuric acid stop solution was added, and the optical density of the solution in the well was measured at 450 nm using a plate reader. The concentrations of the experimental samples were calculated from a standard curve of the optical densities using curve-fitting software.

2.3 | Statistical analysis

Normality of sCr concentration was assessed using the Shapiro-Wilk test. Since sCr was not normally distributed, difference in sCr between day 0 and day 14 was assessed using the Wilcoxon Signed Ranks Test. For urinary KIM-1 and NGAL analyses, the SD and mean of the 4 samples were calculated for each dog, and the CV was calculated by dividing the SD by the mean. CV were calculated for uNGAL, uKIM-1 and for their respective ratios with uCr (uNGAL/uCr and uKIM1/uCr, respectively). The 90th percentile of all CVs were also calculated. The CVs of the normalized and non-normalized markers were compared using Mann-Whitney *U*-test. Spearman's correlation test was used to investigate the correlation between urine protein to creatinine ratio and the biomarkers. All tests were 2-tailed, and in all, $P < .05$ was considered significant. Analyses were performed using a statistical software package (SPSS 22.0 for Windows, IBM Corp., Armonk, New York, USA).

3 | RESULTS

3.1 | Dogs

Twenty-five dogs with stable CKD were included in the study, of which 17 were females (13 spayed) and 8 were males (4 neutered). Median age of all dogs was 54 months (range, 8-234 months). Mixed breed dogs were the most common (13/25), followed by Labrador Retriever (3/25 dogs), Border collie (2/25 dogs) and 1 dog of the following breeds: Shih Tzu, Yorkshire Terrier, Belgian Malinois, Cocker Spaniel, Japanese Spitz, Golden Retriever, and Boxer.

CKD etiologies included glomerulopathy (2 dogs), juvenile onset nephropathy (6 dogs), and post-AKI (6 dogs with median time of 21 months [range, 3-48 months] from AKI diagnosis). CKD etiology was unknown in 11 dogs and interstitial nephritis was suspected.

When stratifying the dogs to CKD Stages, 17 dogs, 5 dogs, and 3 dogs were classified as Stage 2, 3, and 4, respectively. The median

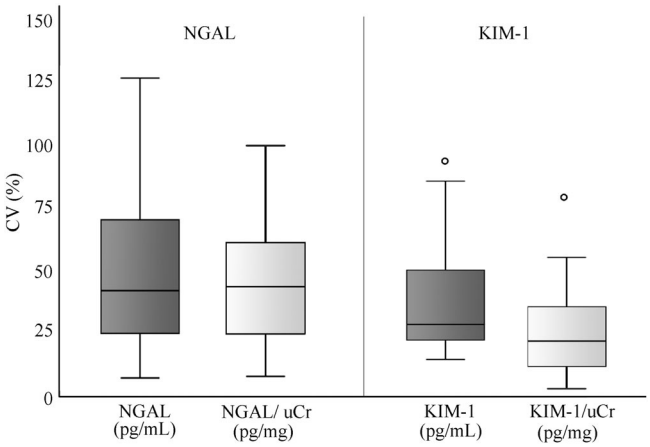

FIGURE 1 Coefficients of variation (CV) of urinary neutrophil gelatinase-associated lipocalin (NGAL) and Kidney injury molecule-1 (KIM-1) absolute concentrations (dark gray) and normalized to urinary creatinine (light gray; NGAL/uCr and KIM-1/uCr) in 96 urine samples of 25 dogs with stable chronic kidney disease. Data are presented as box and whiskers. The box represents the 2nd and 3rd quartiles, the horizontal line within the box represents the median, the whiskers represent 1.5 times the interquartile range, and the circles indicate outlying data.

sCr concentration at day 0 was 2.1 mg/dL (range, 1.4-7.3 mg/dL) and remained unchanged ($P = .12$) on day 14 (median 2.1 range, 1.3-7.6 mg/dL), with a median percent change of 7% (range, 1%-18%).

The median urine protein to creatinine ratio was 0.5 (range, 0.1-2.5); 7 dogs were proteinuric while the remaining 18 dogs were non-proteinuric (Table S1). There was no correlation between urine protein to creatinine ratio and uNGAL ($P = .85$) or uKIM-1 ($P = 1.0$).

3.2 | Variability of uNGAL and uKIM-1

Four samples, each from a different dog, were not included in the analysis because of lack of sufficient urine volume for measurement of biomarkers. The uNGAL and uKIM-1 concentrations of the individual dogs over time are presented in Figure S1. In each dog, the median biomarker's concentration was calculated for the 4 time points. Median (and range) was also calculated for the entire population, based on the medians calculated for each dog. Median uNGAL, uNGAL/uCr, uKIM-1, and uKIM-1/uCr for the entire cohort were 21 679 pg/mL (range, 23-122 760 pg/mL), 20 355 pg/mg (range, 16-202 049 pg/mg), 12 979 pg/mL (range, 2973-45 101 pg/mL), and 12 786 pg/mg (range, 3860-26 198 pg/mg), respectively.

The median CV of uNGAL was 42% (range, 7%-127%). The median CV of uNGAL/uCr was 44% (range, 8%-100%) and was not statistically ($P = .75$) different from the CV of uNGAL (Figure 1). The CV 90th percentiles for uNGAL and uNGAL/uCr were 97% and 83%, respectively.

The median CV of uKIM-1 was 29% (range, 16%-91%). The median CV of uKIM-1/uCr was 23% (range, 6%-76%) and was significantly ($P = .04$) lower compared with the uKIM-1 not normalized to uCr (Figure 1). The CV 90th percentiles for uKIM-1 and uKIM-1/uCr were 56% and 52%, respectively.

There was a positive, significant correlation between uNGAL and uKIM-1 ($r = .65$, $P < .01$); however, when normalized to uCr, there was no statistically significant correlation between uNGAL/uCr and uKIM-1/uCr ($r = .26$, $P = .21$).

4 | DISCUSSION

In this study the short-term intra-individual variations of the renal biomarkers uNGAL and uKIM-1 in dogs with stable CKD were evaluated. The results support the notion that active kidney damage is present in dogs with stable CKD, but its degree varies substantially among dogs as reflected by high inter-individual variation of urinary biomarkers. Intra-individual variation was low. Normalization of the concentration of either biomarker to uCr did not decrease the variability to a clinically important extent.

Unlike AKI, CKD is mostly considered an insidious, slowly progressive and irreversible disease, finally resulting in fibrosis and gradual loss of functional renal parenchyma over time.[32] However, recent evidence propose existence of active, ongoing injury in animals with apparently stable CKD,[1,2,7,33,34] suggesting that CKD progression might be the consequence of active damage, which could be either episodic or sustained.[2] The results of our study further support this, as dogs included in this study had stable CKD, based on both clinical assessment and serial sCr measurements; however, the concentrations of uNGAL and uKIM-1 suggest presence of varying degrees of active kidney damage. Several studies have evaluated uNGAL/uCr and uKIM-1/uCr in healthy dogs, demonstrating substantially lower concentrations of the biomarkers compared with the concentrations documented herein, further supporting the notion that active on going damage is present in dogs with CKD.[33,35-37] Recognition of active, ongoing kidney damage beyond the expected biomarker short-term variation in a particular dog will facilitate early identification of increased risk for progression and enable timely therapeutic intervention.

When using biomarkers to monitor diseases, it is important to take into account the nonspecific variation of the biomarker, resulting from pre-analytical, analytical, and post analytical variations.[37-39] When assessing urinary markers, variations might even be higher compared with serum markers, as these might result from changes in urine concentration, therefore normalization to uCr has been suggested.[23]

To minimize variation unrelated to the kidney disease, we elected to include only dogs with stable CKD, limit the study period to a relatively short time while assuring that there is no change in kidney function during the 14-day study period. We have therefore assumed that the variations documented should be considered normal variations of these biomarkers, unrelated to changes in kidney function.

The median CV for uNGAL and uKIM-1 suggests a mild-moderate degree of variability. These findings are consistent with the results of a recent study evaluating these same markers in human patients with

CKD.[23] In the latter study, the variation of both uNGAL and uKIM overtime were comparable to our results, and also not substantially different from the variation documented in healthy dogs.[40] In a study evaluating the variation of uNGAL/uCr in healthy dogs, a CV of 87.2% was documented. Although apparently higher compared to the CV in our study, one should take into consideration that uNGAL's concentration in healthy dogs is much lower, thus a small change in concentration is reflected by a relatively high variation.

In this study, the median CV was only 42% and 29% for uNGAL and uKIM-1 respectively (ie, relatively low intra-individual variation) while the variation among dogs included in the study was high (23-122 760 pg/mL for uNGAL and 2973-45 101 pg/mL for uKIM-1; ie, high inter-individual variation). In these instances, a population-based reference range for uNGAL and uKIM-1 in dogs with CKD would be insensitive to detect clinically meaningful changes, thus the difference between sequential measurements within an individual dog is probably more suitable and should be favored.[25,26] Additionally, CKD is an heterogeneous disease, which likely results in different patterns of biomarkers behavior in each patient. Therefore, the preferred approach would be to use serial urinary biomarker measurements; the first measurements would be used to establish a baseline to which subsequent measurements will be compared.

As a result of the variability observed and to avoid falsely diagnosing an increase in the degree of kidney damage, we suggest using the 90th percentile of the intra-individual CVs as the limit for the expected variation.[23] AKI is typically reflected by a substantial increase in these biomarkers even in the absence of an apparent change in kidney function (ie, AKI Grade 1),[11,15] therefore using the 90th percentile is not expected to decrease the sensitivity for an acute insult. According to our results, changes of >100% for uNGAL and >60% for uKIM-1 (normalized to uCr or not) should be considered meaningful and clinically relevant, as these exceed the expected variation. Patients in which such an increase in the degree of active damage is documented, might benefit from further diagnostics to elucidate its cause, closer monitoring, and therapeutic intervention if indicated. The CV 90th percentile (and not the median CV) was suggested as a cutoff point to decrease unnecessary diagnostics with biomarkers fluctuations, while maintaining high sensitivity to acute kidney injury. Other comparable measures such as the subject-based reference value, or critical difference could also be used for selecting a cutoff point above which an investigation should be considered.

The CVs of uNGAL/uCr and uKIM-1/uCr were smaller compared with uNGAL and uKIM-1; however, both were not substantial and the former was not statistically significant ($P = .57$ and $P = .04$, respectively). Normalizing urinary biomarkers to uCr and reporting the concentrations as a ratio is common, yet more cumbersome.[41,42] Urine concentration and tubular flow rate effect the absolute concentration of urinary biomarkers and normalization to uCr accounts, at least partially, for these effects. Normalization to uCr is based on the assumption that uCr excretion rate is constant within an individual over time and will negate changes in urine concentration. This approach is well accepted in animals with CKD when assessing the degree of proteinuria. In human medicine 24-hour urine collection is a common practice to evaluate the concentration of different urinary biomarkers and to quantify the amount of proteins (mostly albumin) in the urine; however, this is technically challenging (and often not feasible) in animals.[42] Indeed, urine protein to creatinine ratio correlates well with 24-hour collection and is being used routinely in veterinary medicine.[43] On the other hand, there is evidence to suggest that normalization to uCr might be redundant, as there is no significant difference between the absolute and normalized biomarker concentration in the current study, and even misleading where uCr excretion is not constant (eg, in AKI).[23,41,42,44] Our results suggest that normalization of uNGAL and uKIM-1 decreases the daily variation, and thus might be preferred over using the absolute biomarker concentration, as long as the patient is considered in steady state. Yet the differences in the CVs with or without normalization were not substantial (and not significant for uNGAL), especially when considering the magnitude of change expected in animals with AKI. The reason for the relatively modest decrease in CV with normalization to uCr likely relates to the fact that most dogs with CKD have isosthenuric urine and thus normalization might be less important compared to dogs with normal urine concentration ability, such as some dogs with IRIS Stage 1 CKD.

In this study, NGAL and KIM-1 were selected as representatives of tubular injury markers as these are 2 of the most common biomarkers studied in humans. Our results indicate different variation for each 1 of these biomarkers, consistent with the findings of a previous study in human patients.[23] As a result of their diagnostic and prognostic utility, renal urinary biomarkers are expected to become part of the routine diagnostic schemes for diagnosing and monitoring dogs with CKD. Thus, the intra-individual variation of each potential marker needs to be evaluated and applied separately in dogs with apparently stable CKD.

This study has several limitations: first, only 25 dogs were included of which 68% were classified as IRIS Stage 2, while dogs with Stage 1 were not included. It is possible that a larger cohort including more dogs with early as well as advanced CKD stages would have allowed recognition of an expected variability range for each CKD stage. Moreover, including dogs with Stage 1 CKD might have led to a greater difference between the CVs of the normalized and absolute biomarkers concentrations, as some of these dogs maintain urine concentrating ability. Second, the etiology of CKD might have an effect on the variability, specifically glomerular disease in which urinary biomarkers might originate from the circulation, decreasing the specificity of the biomarker to the urinary system. Third, several pre-analytical factors might have contributed to the inter and intra-individual variability. These include time and place of sampling, food intake, exercise and muscle mass, processing and storage conditions, freeze-thaw cycles as well as other pre-analytical factors that were not consistent among all samples.[24] Although all dogs were fed a commercial renal diet and received a rather uniform treatment for CKD, some of the aforementioned pre-analytical factors were not fully standardized. Fourth, the study protocol included urine collection at home by the owners and storage of the samples in a home freezer until transported our laboratory. Validation studies have shown stability of uNGAL and uKIM-1 in −20°C and during thaw-freeze cycles. Although unlikely, it is possible that mishandling

of the samples by the owners could have influenced the results. Fifth, inter-assay variability was not assessed in this study, and it is possible that variability will further increase when monitoring patients over time. Finally, we included dogs presumed to have stable CKD based on sCr measurements; however, there is no consensus regarding the definition of stable CKD, and stricter definitions might be used. Changes in kidney function during the study period were not likely because of the meticulous evaluation of the dogs' clinical status and monitoring of sCr before, at the time of inclusion and at the end of the study, yet, it has been suggested that active ongoing injury might occur without an apparent increase in sCr,[2] making it even more challenging to completely exclude any acute (although minor) kidney damage within this period of time.

In conclusion, changes greater than 100% and 60% in uNGAL and uKIM-1 concentration respectively within an individual over time should be regarded as exceeding the expected intra-individual variation of these markers, and might indicate the need for further diagnostic investigation. Normalization of the absolute urinary biomarker concentration to uCr might decrease nonspecific variability and hence should be considered.

ACKNOWLEDGMENT

No funding was received for this study.

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

Approved by Koret School of Veterinary Medicine, The Hebrew University, according to the standards for care and use of animals in research, HU-NER-2021-094.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Chen H, Maron L, Segev G. Short-term intra-individual variation of urinary biomarkers in dogs with stable chronic kidney disease. *J Vet Intern Med*. 2023;37(1):184-190. doi:10.1111/jvim.16619