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Weekly Biological Variation of Urine Protein Creatinine Ratio and Urine Specific Gravity in Healthy Dogs

Yanick Couture[1] | Deborah Keys[2] | Stacie Summers[1]

[1]Oregon State University, Carlson College of Veterinary Medicine, Corvallis, Oregon, USA | [2]Kaleidoscope Statistics, Athens, Georgia, USA

Correspondence: Stacie Summers (stacie.summers@oregonstate.edu)

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ABSTRACT

Background: Urine protein-creatinine ratio (UPC) and urine specific gravity (USG) are important measurements in the determination of renal proteinuria and chronic kidney disease. Biological and analytical variation estimates of these analytes to calculate the index of individuality (IoI) and a reference change value (RCV) are important to determine whether a population-based reference interval can be used to detect clinically meaningful changes and facilitate the interpretation of serial measurements.

Objective: Determine the biological variation of UPC and USG using calculations of RCV and IoI in healthy dogs.

Animals: Eleven healthy client-owned young adult dogs.

Methods: Prospective observational study. First-morning urine samples were collected by voiding once weekly for 6 consecutive weeks for batch analysis. Twenty random samples were run in duplicate. Urine protein concentration, urine creatinine concentration, and USG were measured using a colorimetric pyrogallol red molybdate complex, enzymatic Jaffe method, and manual refractometer, respectively. Restricted maximum likelihood estimations were used to determine within-individual, between-individual, and analytical coefficients of variation and calculation of RCV and IoI.

Results: All dogs were non-proteinuric at enrollment (UPC <0.2) and remained non-proteinuric on subsequent measurements. Urine protein concentration, urine creatinine concentration, UPC, and USG had intermediate individuality. The RCV was 73% for urine protein concentration, 68% for urine creatinine concentration, 31% for UPC, and 3% for USG.

Conclusion: Population-based reference intervals for UPC and USG should be interpreted cautiously for single measurements and calculated RCVs should be applied to serial measurements to identify clinically meaningful changes.

1 | Introduction

The evaluation of proteinuria using the urine protein-creatinine ratio (UPC) is crucial in the diagnosis and monitoring of renal proteinuria in dogs [1–3]. Persistent renal proteinuria serves as an important marker of renal injury, increased risk for adverse outcomes, and response to renoprotective interventions [4]. Renin-angiotensin-aldosterone system modifying agents and dietary modification are recommended in dogs with persistent renal proteinuria, and UPC is used to monitor the response to treatment [5–9].

Urine concentration as measured by urine specific gravity (USG) reflects the ability of the kidneys to retain or excrete water and is tightly regulated by homeostatic systems to maintain hydration [10]. Persistently decreased USG can indicate suboptimal

Abbreviations: BUN, blood urea nitrogen; CV_A, analytical variation; CV_G, between-individual variation; CV_I, within-individual variation; IoI, index of individuality; RCV, reference change value; SDMA, symmetric dimethylarginine; UPC, urine protein-creatinine ratio; USG, urine specific gravity.

kidney function and can be present without azotemia in dogs with early-stage chronic kidney disease [11]. However, USG varies depending on the environment, activity level, and time of day [12–15]. The potential variability of USG could make the interpretation of abnormal results a clinical challenge.

Veterinarians must be sure a dog's abnormal UPC or USG is indicative of disease by documenting persistence and excluding non-renal causes. Changes among serial measurements within an individual dog must be more than expected from analytical and biological variation to document clinical relevance. It is also important to consider the analytical and biological variation of UPC and USG when the measurement is slightly outside a population-based reference interval. Analytical variation is the variation attributed to analyzer imprecision among replicate measurements of the same specimen [16, 17]. Biological variation is the fluctuation of an analyte around a homeostatic set point within the same animal [16, 18]. To determine the expected degree of biological variation and utility of a population-based reference interval, the reference change value (RCV) and index of individuality (IoI) are calculated from coefficients of variation (CV) estimates of within-individual variability (CV_I), between-individual variability (CV_G), and analytical variability (CV_A). The RCV indicates the percent difference required between 2 serial results from an individual for the change to be considered clinically relevant rather than attributable to biological or analytical variation [16, 17]. The RCV can be applied when monitoring a laboratory result, and its relevance depends on the analyte's calculated IoI. The IoI of an analyte determines whether a population-based reference interval (IoI <0.7), subject-based reference interval calculated using the RCV (IoI >1.7), or both (IoI 0.7–1.7) should be used to interpret an analyte measurement [16]. Using biological variation parameters, clinicians can more confidently monitor a dog's renal function.

Healthy individuals are commonly used in biological variation studies because biological variation estimates often apply to dogs with chronic, stable disease [19, 20] Few studies have evaluated the biological variation of urine analytes in dogs [15, 21–23] using variable study designs, including the interval between urine collections. Two studies documented the weekly [21] and bi-weekly [23] variations in UPC in healthy dogs. One study evaluated the day-to-day variation in UPC in female colony-bred dogs with stable hereditary glomerulonephropathy [22]. We aim to determine the weekly biological variation of first-morning urine protein concentration, urine creatinine concentration, UPC, and USG in healthy client-owned adult dogs using prospective determination of IoI and RCV.

2 | Materials and Methods

2.1 | Animals

Healthy dogs were prospectively enrolled from December 2022 to March 2023 and recruited on a volunteer basis from Carlson College of Veterinary Medicine Veterinary Teaching Hospital at Oregon State University. Signed informed owner consent and clinical history questionnaires were required for study participation. The study was approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC-2022-0298). Dogs were 1–7 years old with equal numbers of neutered males and females. At enrollment, all dogs were deemed clinically healthy based on a physical examination, CBC, serum biochemistry profile, complete urinalysis, and UPC (Oregon Veterinary Diagnostic Laboratory, Corvallis, OR). The serum was shipped to an outside laboratory for measurement of symmetric dimethylarginine (SDMA) concentration (IDEXX BioAnalytics, West Sacramento, CA). A repeat urinalysis was performed at the conclusion of the sample collection period.

When CV_A:CV_I is ≤ 0.5, a minimum of 10–15 study subjects is suggested to achieve sufficient study power to allow for the calculation of biological variation components [17]. Dogs were included if they had no clinically relevant physical examination findings or abnormalities on their CBC, serum biochemistry panel, SDMA, urinalysis, and UPC, with no clinical signs of disease, including serum creatinine concentration ≤ 1.4 mg/dL, serum SDMA concentration <18 μg/dL, and USG >1.025. Included dogs were not receiving medications except for heartworm, intestinal parasite, flea, and tick preventatives. Dogs were excluded if they had a recent history of clinical illness (in the past 6 months), clinically relevant abnormal physical examination or laboratory findings, a history of positive PCR or antibody test or both for tick-borne disease (symptomatic or asymptomatic), or active urine sediment (pyuria or hematuria defined by white blood cells ≥ 6 per high power field or red blood cells ≥ 6 per high power field, respectively) on their urinalysis at the time of enrollment (Week 0) or the end of the study period (Week 6) to exclude extra-renal variables potentially affecting UPC [4, 24]. Animals with a history of vaccinations within the last 30 days or vaccinations during the study period were also excluded.

2.2 | Sample Collection and Handling

All dogs remained on their normal dietary regimen throughout the study. First-morning urine samples were collected by voiding, using provided ladles and stored in sterile cups. Samples were collected at home once weekly for 6 consecutive weeks, on the same day of the week, and at approximately the same time of day for each dog within a 2-h window. If the sample could not be immediately brought to the laboratory, caregivers were instructed to refrigerate the sample, and a 4-h grace period was allowed to minimize the effect of refrigeration before processing for freezing. Urine samples were separated into aliquots and frozen at −80°C within 8 h of collection. At the end of the collection period, all frozen urine samples were batch-submitted to a commercial laboratory (IDEXX Bioanalytics, West Sacramento, CA) for analysis of urine creatinine and urine protein concentrations using urine supernatant, and results were used to calculate the UPC. All samples were batch-run in randomized order on the same day to minimize analytical variability. Twenty random samples were run in duplicate to calculate CV_A, a minimum number suggested in published guidelines to minimize pre-analytical variables present in laboratory settings such as handling and machine calibration [16, 17]. Urine protein concentration (mg/dL) was determined using a colorimetric method with a colorimetric pyrogallol red molybdate complex. Urine creatinine concentration (mg/dL) was determined using a colorimetric enzymatic method with creatinine amidohydrolase (enzymatic Jaffe method). The UPC was calculated based on the

ratio of urine protein to urine creatinine concentration and recorded to 2 significant figures.

Serial USGs were determined 6–8 months after the sample collection period by thawing frozen whole urine aliquots for 30–60 min and mixing by hand. A handheld refractometer (Reichert Analytical Instruments), calibrated daily at the Oregon Veterinary Diagnostic Laboratory, was used to determine USG and was performed by the authors (Y.C., S.S.). Each sample was measured in succession and then shuffled and blinded for repeat analysis. Distilled water and disposable wipes were used to clean the refractometer between measurements. USG results above the upper limit of detection of the refractometer (USG > 1.050) were estimated visually.

2.3 | Statistical Analysis

All analyses were performed using Microsoft Excel (Microsoft Office 365, 2023) or SAS (Windows version 9.4, SAS Institute Inc. Cary, North Carolina USA). Data obtained in duplicates were averaged, and box and whisker plots were constructed to qualitatively describe CV_G and CV_I (GraphPad Prism version 9.5.1 for Windows, Boston, Massachusetts USA). Statistical analysis was performed to determine biological variation estimates according to previously reported guidelines [17, 25].

Conditional studentized residual diagnostic plots were used to confirm model assumptions of normality and equal variance. Data was assessed for outliers using a 3-step process by evaluating results falling outside 3 times the interquartile range (Part 1) across all dogs, (Part 2) within each dog, and (Part 3) using dog-level averages (where a single average was calculated for each endpoint and dog). For Part 1, no outliers were identified. For Part 2, a single time point for 2 dogs for urine creatinine concentration and 2 time points for 1 dog for urine protein concentration were identified as outliers. For Part 3, no outliers were identified. Outliers were retained in the analysis because the outlying values were consistent with the trend in the paired urine protein or urine creatinine concentrations, resulting in consistent UPC compared to other time points for the dog. A random effects model was fitted using restricted maximum likelihood (REML) using SAS Proc Mixed. Dog and time point (nested within the dog) were included in the model as random effects. Hence, the variation was partitioned into 3 components for each endpoint: (a) CV_G, (b) CV_I, and (c) variation between duplicates (CV_A). The CVs were calculated by dividing the square root of each respective variance estimate by the overall mean. The CV_A:CV_I ratios were calculated to determine analytical performance according to published guidelines, with a ratio ≤ 0.5 indicating desirable performance such that analytical variation is not expected to significantly affect biological variation [26, 27]. The IoI was calculated using the following inverted formula:

$$\text{IoI} = \frac{CV_G}{\sqrt{CV_I^2 + CV_A^2}}$$

The RCV was calculated using the following formula, with the Z score being 1.96 for a 2-sided interpretation:

$$\text{RCV}(\%) = Z * \sqrt{2} * \sqrt{\left(CV_I^2 + CV_A^2\right)}$$

3 | Results

Fourteen clinically healthy dogs were enrolled in the study. Three dogs ultimately were excluded: one dog was excluded because of a suspected foreign body obstruction necessitating an emergency visit; another dog was excluded because of repeatable minimally concentrated urine; and another dog was excluded because of a diagnosis of mast cell tumor near the end of the sample collection period. As such, 11 dogs ultimately were included in the final analysis. The population of included dogs consisted of 6 neutered males and 5 spayed females. The median age was 4 years (range, 1–7 years). The breeds included two golden retrievers, one English springer spaniel, and the remaining 8 dogs were mixed breed dogs.

The CV_A:CV_I ratio was < 0.5 for all analytes, indicating desirable analytical performance. The mean, variance, CV (CV_A, CV_I, CV_G), IoI, and RCV of urine protein concentration, urine creatinine concentration, UPC, and USG based on 6 consecutive weekly urine samples from the 11 dogs are shown in Table 1. The UPC and USG data from individual dogs are summarized in Figures 1 and 2, respectively. The weekly median (range) urine protein concentration, urine creatinine concentration, UPC, and USG for the 11 dogs is found in the Supporting Information. All dogs were non-proteinuric at enrollment (UPC < 0.2) and remained non-proteinuric on subsequent measurements. The majority (10/11, 90.9%) of dogs had concentrated urine at enrollment (USG > 1.030), except for one dog with a baseline USG of 1.027. Three dogs (3/11, 27.3%) had USGs < 1.030 on some serial measurements, but all of these dogs had other measurements > 1.030.

TABLE 1 | Mean, variance, and biological variation components of urine protein concentration, urine creatinine concentration, urine protein-creatinine ratio (UPC), and urine specific gravity (USG) in 11 clinically healthy dogs sampled once a week for 6 weeks.

Analyte	Mean	Variance	CV_I %	CV_G %	CV_A %	CV_A: CV_I	IoI	RCV %
Urine protein	20.18 mg/dL	25.56	26.21	25.06	3.89	0.15	0.95	73
Urine creatinine	229.70 mg/dL	3126.49	24.53	24.34	1.92	0.08	0.99	68
UPC	0.09	0.000095	10.77	11.08	3.19	0.30	0.99	31
USG	1.053	0.000078	1.20	0.84	0.08	0.07	0.70	3

Abbreviations: CV_A, analytical variation; CV_G, between-individual variation; CV_I, within-individual variation; IoI, inverted index of individuality; RCV, reference change value.

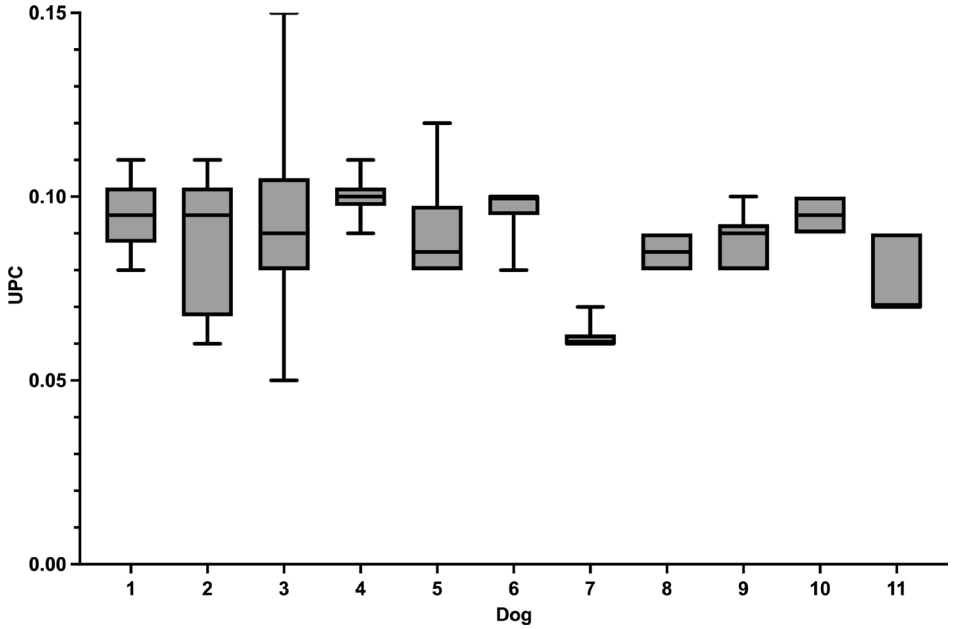

FIGURE 1 | Box and whisker plots for urine protein-creatinine ratio (UPC) in 11 clinically healthy dogs sampled once weekly for 6 weeks. Analyte concentrations are on the y-axis and dog numbers are on the x-axis. Boxes represent the interquartile range from the 25th to 75th percentile. The horizontal bar in each box represents the median value. The whiskers indicate the range.

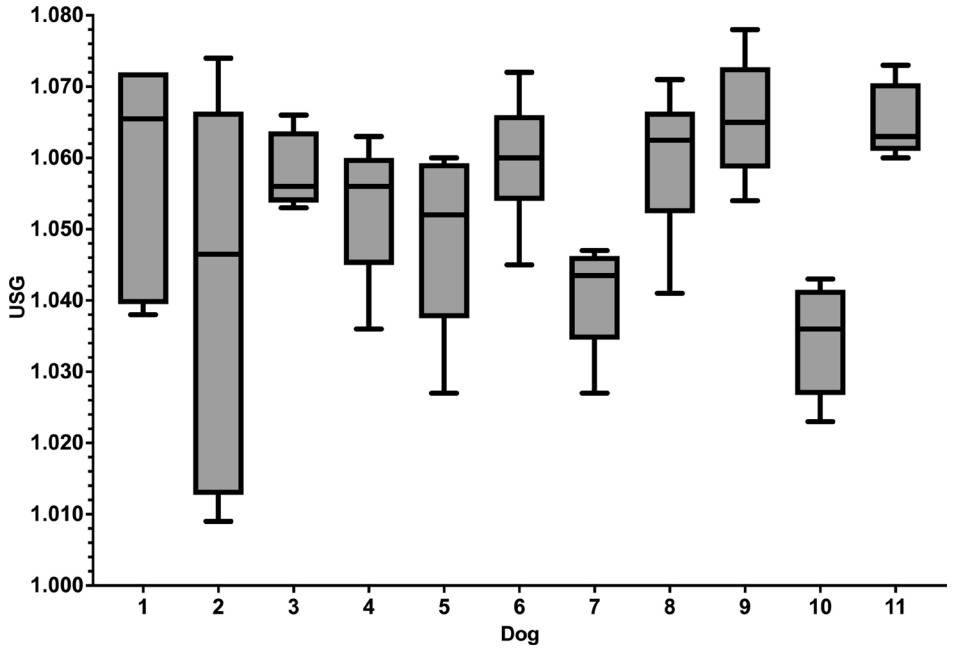

FIGURE 2 | Box and whisker plots for urine specific gravity (USG) in 11 clinically healthy dogs sampled once weekly for 6 weeks. Analyte concentrations are on the y-axis and dog numbers are on the x-axis. Boxes represent the interquartile range from the 25th to 75th percentile. The horizontal bar in each box represents the median value. The whiskers indicate the range.

The calculated IoI indicated intermediate individuality for urine protein concentration (0.95), urine creatinine concentration (0.99), UPC (0.99), and USG (0.7), with RCVs of 68%, 73%, 31%, and 3%, respectively.

4 | Discussion

Two previous studies have evaluated the biological variation of UPC in clinically healthy non-proteinuric dogs, and one of these studies included the evaluation of USG [21, 23]. Both studies had a similar methodology to our study, including voided urine collections. Similar to our study, one study [21] found intermediate individuality for UPC based on weekly urine collections. On the contrary, another study documented high individuality for UPC, which might be explained by differences in sample collection frequency because this study used urine collected biweekly and for a shorter period of time [23]. For USG, the former study found USG to have intermediate individuality similar to our study. Considering the findings of these 3 studies, including

our study, the population-based reference interval for UPC and USG could be beneficial in the assessment of these urine parameters. Applying the RCV to baseline measurements, especially when a finding is slightly outside of the population-based reference interval, can provide additional insight as to whether a clinically meaningful change has occurred rather than a change attributable to biological or analytical variability. Additionally, the repeatability of a USG measurement consistent with inadequate urine concentration (< 1.030) still should be documented, as demonstrated by certain dogs in our study that had USG < 1.030 on some serial measurements but had other measurements > 1.030.

Consensus recommendations advise repeating UPC on 3 or more occasions at least 2 or more weeks apart to confirm the persistence of proteinuria and improve the specificity of the test, given the potential for day-to-day variation [4]. Our results further assist veterinarians in interpreting the clinical relevance of changes in serial UPC measurements. Identification of a clinically relevant increase in UPC might signify the need for more careful monitoring, which in turn might lead to earlier intervention. Additionally, the calculation of a subject-based reference interval using baseline data obtained in health could help identify dogs that do not fit the definition of proteinuria (UPC > 0.5) but have a clinically relevant increase in UPC. For UPC, an increase of > 31% in healthy dogs signifies a 95% probability that this increase reflects a true change in the UPC measurement not attributable to biological variation. A > 31% increase in UPC over time could be an early marker of non-azotemic renal disease when pre- and post-renal causes of proteinuria are excluded (e.g., an increase from 0.18 to > 0.24). For USG, only a decreasing value is clinically relevant. Serial monitoring of USG, surrogate markers of glomerular filtration rate (e.g., serum creatinine and SDMA concentrations), and UPC can be used as early markers of non-azotemic chronic kidney disease in dogs [11]. A decrease in USG by > 3% in a healthy dog on repeat evaluation could signify a clinically meaningful change. Although the RCV of USG was small in our study, a 3% decrease in USG still can represent a result that can affect clinical interpretation. A 3% decrease in USG (e.g., 1.050 to < 1.019) therefore can signify a clinically relevant change and should alert the clinician. If the percent change in serial measurements for UPC or USG is higher than the RCV, the veterinarian should consider other causes for a physiological change in UPC or USG beyond primary renal disease. For example, recent exercise or hypertension could increase the UPC, and diet change could affect the USG [28–30]. Additionally, dietary protein can affect urine protein concentration, as evidenced by the benefit of protein restriction on decreasing UPC in proteinuric dogs [4].

In most clinical situations, a single test result typically is regarded as a reasonable estimate of the dog's homeostatic baseline. If possible, repeating UPC and USG measurements during health can increase the confidence of a dog's homeostatic set point. Using the CV_A and CV_I from our study, we determined that 5 urine samples for UPC and 1 urine sample for USG are required to estimate the measured values within 10% of the true homeostatic point with a probability of 95% [18]. The RCV is calculated using a CV_A that is specific to the machine and methodology used to measure the analyte and therefore veterinarians need to know the CV_A of the laboratories they use and use published species-specific CV_I (such as the data of our study) to calculate an RCV for monitoring purposes.

If biological variation components of healthy individuals can be applied to dogs with chronic stable disease as previously stated [19, 20], the RCV can be applied when monitoring dogs receiving renin-angiotensin-aldosterone system (RAAS) inhibition for the treatment of renal proteinuria, such that a decrease of > 31% in the initial UPC signifies a response to treatment that cannot be solely attributable to biological or analytical variation. However, it is plausible that biological variation estimates from a population of healthy animals are not readily applicable to animals with stable, chronic disease. Differences in CV_I between healthy people and those with disease have been documented for a few analytes, including serum creatinine and albumin concentrations, where high CV_I has been documented in disease states such as renal insufficiency [20]. For analytes that have more variation in diseased states, calculated RCVs from healthy populations might be lower than those calculated from the diseased states [20]. Furthermore, differences in severity and biological variation of proteinuria in dogs might depend on the underlying nephropathy [31, 32]. Limited information is available regarding the biological variation of blood and urine analytes in dogs with kidney disease. One study investigated the short-term (day-to-day) biological variation of UPC in dogs with stable hereditary X-linked glomerulonephropathy, but the findings from that study cannot be directly compared to our study because of differences in the timing of urine collections (daily vs. weekly) [22]. Future studies investigating weekly biological variation parameters in dogs with specific glomerulopathies might find differences in values published from healthy dogs, increasing veterinarians' confidence when monitoring response to standard treatment of proteinuria.

Weekly collections were chosen in our study to encompass the peaks and troughs of physiological fluctuation and to minimize day-to-day changes in health status [17]. Studies in humans have shown that CV_I increases as the sampling interval increases up to 4–5 days but then remains stable for up to 2 weeks when measuring blood analytes, whereas the CV_I of urinary analytes has not been similarly studied [16]. In practice, veterinarians often will monitor UPC over weeks to months depending on the clinical scenario. Veterinarians are encouraged to utilize RCVs derived from studies that closely mirror the population and sampling interval of their dogs [33].

Pre-analytical variables, such as urine collection time and method, urine storage time, and urine temperature, were carefully considered in our study, and results applied to samples collected in a similar manner in clinical practice [14, 15]. Although a cystocentesis sample eliminates bacterial and cellular contamination from the genital and lower urinary tracts, voided samples are easier to obtain, can be collected at home, and eliminate the risk of iatrogenic hemorrhage as a consequence of cystocentesis [10]. Furthermore, no significant difference has been reported in the UPC of urine obtained by voided versus cystocentesis-obtained samples in dogs [34]. The urine collected in our study was a first-morning sample because less variability in UPC is present in morning samples than in samples collected before bedtime in children, although this situation has not been thoroughly investigated in dogs

[35]. A 2-h window was allowed for flexibility in obtaining samples, and within-day variation might have played a part in some of the variations in our study. However, a single UPC has been found to be a reliable indicator of 24-h urinary protein loss [1]. Urine specific gravity in dogs has less variation when urine is sampled in the morning because of limited water access overnight [14, 15]. First-morning samples also eliminate the variable of exercise, which can transiently increase UPC in dogs [29]. Urine was collected at home in our study because higher UPC has been found in dogs that had urine collected in-hospital in some studies [36, 37].

Our study had some limitations. Owners of dogs were not specifically instructed to have water or food withheld from their dogs before first-morning urine collection, which might have resulted in variable access to water across serial time points, thus potentially affecting USG [10]. Furthermore, USG results above the upper limit of detection of the refractometer (USG > 1.050) were estimated visually, affecting the analytical precision of those measurements when compared to results below this limit. Unlike in clinical practice, urine samples were frozen at −80°C within 8 h of collection, and testing was performed 6–8 months later. Previous studies have found that the UPC did not change significantly during 3 months of storage at −20°C [38], but longer storage time could have had an impact on results. A 4-h grace period was allowed if the sample could not be immediately transported to the laboratory, with a requirement that the sample be refrigerated. Previous studies have found a limited effect of refrigeration on UPC if determined < 12 h after collection, although storage before freezing might have had an effect [39]. Additionally, urine supernatant is the preferred specimen for measurement of urine protein and creatinine concentrations to calculate UPC. In our study, whole urine was separated into aliquots and stored at −80°C until shipment and measurement of urine protein and creatinine concentrations at an outside laboratory. Urine was thawed, centrifuged at 500 g, and urine protein and creatinine concentrations were measured using urine supernatant. During thawing, cells within the sample could have lysed, releasing small molecular weight proteins into the sample. The pyrogallol red molybdate complex method used in our study to measure urine protein concentration is based on the formation of a colored complex between pyrogallol red, molybdate, and basic amino acid groups of protein molecules in the urine. Although the method used to measure urine protein concentration is most sensitive to the detection of large molecular weight proteins albumin and gamma globulins, low molecular weight intracellular proteins could have interfered with this assay, potentially increasing the apparent concentrations of urine protein. Lastly, the stability of USG in frozen urine samples has not been thoroughly investigated in veterinary medicine. One study evaluated the stability of urine osmolality in frozen samples up to 3 months in 5 healthy greyhounds [39]. Although urine osmolality and USG are not entirely interchangeable, urine osmolality strongly influences USG, allowing some degree of extrapolation of USG from urine osmolality and vice versa [15, 40]. In the previous study, urine osmolality decreased slightly from baseline after 2 weeks when samples were stored at −80°C, but the investigators concluded that this change was unlikely to be of clinical relevance [39]. As such, sample storage might have affected our USG results.

In conclusion, the biological variability detected with weekly voided urine sampling and the subsequent calculated individuality of UPC and USG were consistent with what was observed in a previous study in healthy dogs [21]. The UPC and USG had intermediate individuality, indicating that population-based reference intervals can be used cautiously for interpretation in many scenarios, but in some cases, establishing a dog's baseline and applying RCV to subsequent measurements could be useful. When collecting first-morning urine samples at similar frequencies in healthy dogs, an increase in UPC > 31% from baseline could be clinically meaningful and might signify progressive proteinuria. For USG, a decrease of 3% would signify a clinically meaningful change in a healthy dog. These findings, therefore, can help clinicians monitor and identify clinically relevant progression of proteinuria and suboptimal kidney function, allowing for earlier diagnosis and intervention.

Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Approved by the Oregon State University Institutional Animal Care and Use Committee (IACUCC; IACUC-2022-0298). Authors declare human ethics approval was not needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.