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Biological variation of biochemical urine and serum analytes in healthy dogs

Anna K. Selin[1,2] | Inger Lilliehöök[1] | Johannes Forkman[3] | Anders Larsson[4] | Lena Pelander[1] | Emma M. Strage[1]

[1]Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]AniCura Albano Animal Hospital and AniCura Gärdets Animal Clinic, Stockholm, Sweden

[3]Department of Crop Production Ecology, Swedish University of Agriculture Sciences, Uppsala, Sweden

[4]Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden

Correspondence
Anna K. Selin, Department of Clinical Sciences, Swedish University of Agricultural Sciences, Box 7054, 75007 Uppsala, Sweden.
Email: anna.selin@slu.se

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Abstract

Background: Biological variation (BV) of urinary (U) biochemical analytes has not been described in absolute terms, let alone as a ratio of the U-creatinine or fractional excretion in healthy dogs. These analytes are potential diagnostic tools for different types of kidney damage and electrolyte disorders in dogs.

Objectives: We aimed to investigate the BV of specific gravity, osmolality, creatinine, urea, protein, glucose, chloride, sodium, potassium, calcium, and phosphate in urine from healthy pet dogs.

Methods: Blood and urine samples from 13 dogs were collected once weekly for 8 weeks. Samples were analyzed in duplicate and in randomized order. For each sample, U-analyte and serum concentrations were measured, and U-analyte/U-creatinine and fractional excretion (FE) were calculated. Components of variance, estimated by restricted maximum likelihood, were used to determine within-subject variation (CV_I), between-subject variation (CV_G), and analytical variation (CV_A). Index of individuality (II) and reference change values were calculated.

Results: CV_I for all urine analytes varied between 12.6% and 35.9%, except for U-sodium, U-sodium/U-Cr, and FE-sodium, which had higher CV_Is (59.5%-60.7%). For U-protein, U-sodium, U-potassium, U-sodium/U-creatinine, FE-urea, FE-glucose, FE-sodium, FE-potassium, and FE-phosphate II were low, indicating that population-based RIs were appropriate. The remaining analytes had an intermediate II, suggesting that population-based RIs should be used with caution.

Conclusion: This study presents information on the biological variation of urinary and serum biochemical analytes from healthy dogs. These data are important for an appropriate interpretation of laboratory results.

KEYWORDS
biomarker, canine, osmolality, renal, urine specific gravity

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1 | INTRODUCTION

Urine contains a large amount of diagnostic information,[1] and research on urinary biomarkers has been growing within veterinary medicine over recent years. In human medicine, urinary biomarkers are well established as diagnostic tools. Urinary biomarkers can reveal kidney injury at an early stage and indicate which part of the nephron is injured. Three possible causes for increased concentration of urinary biomarkers are leakage through damaged glomeruli (eg, protein),[2] increased production and/or leakage from damaged tubular cells (eg, alkaline phosphatase, gamma glutamyltranspeptidase, kidney injury molecule-1, and neutrophil gelatinase-associated lipocalin concentration),[3] and decreased reabsorption due to loss of tubular epithelial cell function (eg, glucose, sodium, and cystatin C).[3–5]

A recent study on dogs with acute kidney injury (AKI) showed that the fractional excretion (FE) of different substances (electrolytes, minerals, protein, and glucose) differed among separate types of acute kidney damage, impacting prognoses.[6] Two other studies showed that the FE of phosphate (FE-P) and electrolytes (FE-Na, FE-K, FE-Cl) was higher in dogs with advanced chronic kidney disease (CKD) compared to dogs with less severe kidney disease.[7,8] Furthermore, FE-Na has been reported as both an indicator of acute kidney injury in dogs with heatstroke[9] and a prognostic indicator in dogs with AKI.[10] Urine (U) analytes are also likely relevant to other clinical situations, for example, electrolyte disorders in dogs.

Excretion of analytes into urine can be affected by several factors, such as diet, water intake, and exercise.[11] Therefore, U-analytes should preferably not be interpreted in isolation. Normalization by relating to creatinine (Cr) through a ratio (U-analyte/U-Cr) is a common way to adjust for varying urinary water content. Another way to interpret analyte excretion is by FE, which gives an estimate of the urinary excretion of a substance and creatinine compared with blood concentrations.[11]

Population-based RIs are traditionally used in veterinary practice,[12] though subject-based RIs, that is, repeated sampling of the same animal, are preferred for some analytes. Biological variation (BV) is defined as the random fluctuation of an analyte around a homeostatic setting point. This fluctuation is usually expressed as a coefficient of variation (CV), where within-individual variation (CV_I) reflects changes in the same individual over time, and between-individual variation (CV_G) shows the difference between individuals. In addition, there is analytical variation (CV_A), that is, variation attributable to the analytical method.[13,14] Population-based RIs are considered of limited value for analytes with lower CV_I than CV_G, that is, a high index of individuality (II).[13,14] In these cases, large deviations from the individual's normal values can occur without the values falling outside the population-based RI, which can lead to misinterpretation.[15] For analytes with high II (≥1.7), subject-based RIs are recommended, based on reference change value (RCV) calculated from CV_I and CV_A.[13] The RCV indicates how much the value of an analyte must differ between two samples to be considered significant.[13] For analytes with an II ≤0.7, the population-based RI is appropriate.[13] To our knowledge, only a limited number of studies have evaluated the biological variation of biochemical analytes in dog urine, and these focus on urine specific gravity (USG),[16,17] U-protein,[18,19] and gamma-glutamyl transferase (GGT).[20]

Our aim was to investigate the biological variation of USG, osmolality (U-Osmo), U-Cr, urea (U-Urea), protein (U-Prot), glucose (U-Glu), sodium (U-Na), chloride (U-Cl), potassium (U-K), calcium (U-Ca), and phosphate (U-P) in urine from healthy pet dogs, presented as U-analyte concentrations, U-analyte/U-Cr, and FE-analyte.

2 | MATERIALS AND METHODS

2.1 | Animals

2.1.1 | Biological variation

Eighteen privately owned healthy dogs were enrolled in this study. The study was approved by the Uppsala Animal research ethics committee (5.8.18-01610/2020). All owners gave informed written consent. The dogs were maintained in their home environment during the study and received their regular dry dog food. Inclusion criteria were age ≥1 year, weight ≥3 kg, and healthy status based on owner reported observations and a physical examination by the veterinarian. Dogs were excluded if they were abnormal on examination or prestudy analyses. Those analyses included hematology (hemoglobin [Hb], hematocrit, red blood cell count [RBC], white blood cell count [WBC], and platelets [PLT]), serum biochemistry profile (C-reactive protein [CRP], creatinine, albumin, protein, alanine aminotransferase [ALT], alkaline phosphatase [ALP], fructosamine, sodium, chloride, potassium, calcium, and phosphate), a fresh urine sample evaluated using a urinary dipstick (blood, glucose, ketone, and protein), and U-sediment. If positive for protein on the dipstick, a urine protein/creatinine ratio (UPC) was performed, and dogs were excluded if UPC >0.5. Exclusion criteria also included estrus, pregnancy, and medication, except for tick prevention. Finally, dogs were excluded if abnormalities were detected on the repeated weekly clinical examination, fresh urine analysis, health questionnaire, or if they showed signs of profound stress during sampling.

2.2 | Study design

2.2.1 | Biological variation

In this prospective study, blood and urine samples were collected from healthy dogs once a week for 8 consecutive weeks. Sampling was performed from April 2020 to July 2020. The owners were told

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to practice the urine collection procedure two times a week before the start of the study to acclimate their dogs to the collection procedure. A midstream morning urine sample was taken after an overnight fast (10-12 hours) at approximately the same time for each individual dog. The same type of plastic container (Uripet, WDT, CuraVet, Queensland, Australia) was used to collect urine from all participants, and a new container was used each time urine was collected. The urine sample was kept at 2-8°C until centrifugation and analysis (maximum 3 hours after collection). Weekly urine analyses included urine dipstick (Multistix 7, Siemens, Erlangen, Germany) and sediment. One aliquot of urine was mixed with hydrochloric acid (3.3 M) at a 1:20 ratio to avoid crystallization and saved together with aliquots of supernatant obtained after centrifugation at 500*g* (EBA 200, Hettich, Tuttlingen, Germany). All urine aliquots were placed at −20°C within 4 hours of urine collection and transferred to −80°C within 7 days.

Blood was collected from the cephalic vein within 3 hours of urine collection by the same phlebotomist. A tourniquet and a 20-gauge needle (BD Microlance, 0.9 × 40 mm, BD Diagnostics, Oxford, United Kingdom) were used, and blood was collected directly into a 5-mL serum gel tube (BD Diagnostics). Blood samples were kept at room temperature for 30 minutes before centrifugation at 2100*g* for 5 minutes (EBA 200, Hettich, Tuttlingen, Germany). Aliquots with serum were frozen at −20°C within 2 hours of sample collection. All specimens were transferred from −20°C to −80°C within 7 days and stored until batch analysis within 5 months.

2.3 | Analytical methods

2.3.1 | Biological variation

Nine biochemical analytes (Cr, Urea, Prot, Glu, Na, Cl, K, Ca, and P) were analyzed in both urine and serum using an automated chemistry analyzer (Architect c4000, Abbott Diagnostics, Lake Forest, IL, USA) with reagents from Abbott Diagnostics intended for use in urine and serum. The analytical methods consisted of arsenazo III (Ca), phosphomolybdate (P), hexokinase/G-6-PHD (Glu), urease with GLDH (Urea), enzymatic (Cr),[21] ion-selective electrode measurement for electrolytes, the Biuret method for serum protein, and the turbidimetric method using benzethonium chloride for U-Prot.[22] The USG was analyzed using a digital refractometer (PAL-USG [DOG], Atago, Tokyo, Japan), and osmolality was analyzed using an automatic osmometer (Automatic Micro-Osmometer Type 15, Löser Messtechnik, Berlin, Germany).

For analysis, samples were divided into three groups consisting of serum samples, acidified urine for analysis of U-Ca and U-P, and supernatant urine for all other analytes. All samples from each group were batch analyzed in duplicates in randomized order. Before and after analysis of the serum samples using Architect c4000, two commercial control samples (Sero, Billingstad, Norway) were analyzed for each analyte. For urine samples, a commercial control sample (U-trol, Thermo Fisher Scientific, Waltham, MA, USA) and one canine urine control sample were analyzed before and after the run. Distilled water was used as a control for the refractometer, and Milli-Q water/purified water for the osmometer.

Standard measurement ranges (Architect c4000) for the included analytes were U-Cr 220-35 360 μmol/L, U-Urea 32-1420 mmol/L, U-Na 20-400 mmol/L, U-Cl 20-300 mmol/L, U-K 1-300 mmol/L, and U-P 3-120 mmol/L. Both urine and serum samples were analyzed according to the manufacturer's instruction, except for U-Prot, U-Glu, and U-Ca. The manufacturer's recommended lowest measurement limit for U-Prot was 0.068 g/L. Recovery upon dilution down to 0.045 g/L for U-Prot was 76%-100%. The U-Prot intra-assay coefficients of variation for two samples, mean 0.053 and 1.7 g/L, were 4.3% and 0.5%, respectively. The interassay variation for a low sample (0.071 g/L) was 6.4%, and the interassay variation for a high sample (1.7 g/L) was 0.3%. Based on repeated analysis of saline, the limit of blank was 0.0027 g/L for U-Prot (mean + 3 SD). Based on our linearity studies and CV, we accepted U-Prot concentrations down to 0.045 g/L.

For measuring lower concentrations than the standard application, adjusted applications with increased sample volume were made for U-Ca and U-Glu. The ordinary measuring range for U-Ca was 0.5-6.0 mmol/L; with the adjusted sample volume (×4), the measuring range was extended downward. Recovery upon dilution (O/E %) down to 0.04 mmol/L was 96%-101%. The U-Ca intra-assay coefficients of variation for two samples (mean 0.19 and 0.48 mmol/L) were ≤2.4%, and the interassay variation for two samples (mean 0.36 and 0.48 mmol/L) was ≤3.3%. The limit of blank for U-Ca was <0.01 mmol/L. The lowest U-Ca concentration in the BV study was 0.17 mmol/L. All samples were analyzed with the adjusted application. Four samples had U-Ca concentrations above 1.5 mmol/L, and the samples were rerun with the standard application.

For U-Glu, the ordinary measuring range was 0.06-44.00 mmol/L; with the adjusted sample volume (×5), the measuring range was extended downward. Recovery upon dilution was 95%-112% down to 0.04 mmol/L. Intra-assay coefficients of variation for two samples (mean 0.58 and 0.79 mmol/L) for U-Glu was below 1.2%, and interassay variation for two samples (mean 0.087 and 0.58 mmol/L) was below 2.5%. The limit of blank for U-Glu was 0.007 mmol/L. The lowest U-Glu concentration in the BV study was 0.18 mmol/L. All samples were analyzed with the adjusted application. For all other urine methods, recovery (O/E %) after dilution was between 92% and 105% for the range of concentrations in the study, and CV_I and CV_G were below 3.5%.

Calculation of creatinine ratios was performed by dividing the concentration of the analyte by the creatinine concentration. Fractional excretion was computed by the formula (the unit of the quota is percent):

$$FE_X = \frac{(\text{urine concentration of } X) \times (\text{serum concentration of creatinine})}{(\text{urine concentration of creatinine}) \times (\text{serum concentration of } X)} \times 100.$$

For the calculation of the ratios and FEs, the included numbers had the same unit.

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2.4 | Statistical analyses

2.4.1 | Biological variation

Calculation of biological variation was performed for urine concentrations, serum concentrations, U-analyte/U-Cr ratios, and FEs. Three levels of analyses for outliers were carried out. The Cochran test, with a significance level of $P < 0.05$, was used to detect analytical outliers in sets of duplicate results. This test was also used to detect within-subject outliers, where results from all sampling occasions for each subject were compared with each other. Finally, the Reed criterion was used to detect outliers between subjects.[15]

Variance components were estimated using restricted maximum likelihood (REML). The R software[23] was used for the analyses. For each analyte, y_{ijk} was set to denote the observation of the kth sample at the jth point of time for the ith dog, and $t(y_{ijk})$ was set to denote the transformed observation, as described next. The random-effects model

$$t(y_{ijk}) = \mu + a_i + b_{ij} + e_{ijk}$$

was fitted, where μ is the overall mean level, a_i is the effect of the ith dog, b_{ij} is the effect of the jth point of time on the ith dog, and e_{ijk} is the effect of the kth sample of the ith dog at the jth point of time. These effects were assumed to be independently and normally distributed, with an expected value 0 and variances σ_G^2 between dogs, σ_I^2 between points of time, and σ_A^2 between samples, that is, $a_i \sim N(0, \sigma_G^2)$, $b_{ij} \sim N(0, \sigma_I^2)$, and $e_{ijk} \sim N(0, \sigma_A^2)$. The model was fitted using the lmer function of the lme4 package of R,[24] providing estimates $\hat{\mu}$, $\hat{\sigma}_G^2$, $\hat{\sigma}_I^2$, and $\hat{\sigma}_A^2$.

To achieve an approximate normal distribution, observations were transformed using the power transformation, $t(y) = y^\lambda$. The parameter, λ, was computed using the powerTransform function of the car package,[25] applied after an initial fit of the model to the untransformed observations. This function uses the Box-Cox method for finding the lambda, which maximizes the likelihood of the normally distributed data. In the special case where $\lambda = 0$, the Box-Cox method applies the logarithmic transformation, $t(y) = \ln(y)$, instead of $t(y) = y^\lambda$. The significance of the power transformation was tested using the testTransform function of the car package. The transformation was only applied when significantly needed ($P < 0.05$). Conditional residuals, before and after transformation, are shown in File S1. Estimated lambdas and the P values for the tests of the transformations are provided in the Table S1.

When a transformation was not applied (U-Osmo, S-Cr, S-Ca, S-P, S-Glu, S-K, S-Cl, S-Prot, S-Na, U-Prot/U-Cr, FE-K, and FE-Prot), coefficients of variation and their 95% confidence intervals were computed by dividing the estimates of the standard deviations and their 95% confidence interval limits by the estimate of the intercept. For all other analytes, the transformation was applied, and the estimate of the intercept was back-transformed to the original scale: $m = \hat{\mu}^{1/\lambda}$. The estimated standard deviations were approximately back-transformed by multiplication with the derivative of the inverse transformation, $y = t^{1/\lambda}$, evaluated at $\hat{\mu}$:

$$y'(\hat{\mu}) = \left.\frac{dy}{dt}\right|_{t=\hat{\mu}} = \frac{1}{\lambda}\hat{\mu}^{1/\lambda - 1}.$$

Thus, $s_G = y'(\hat{\mu})\sigma_G$, $s_I = y'(\hat{\mu})\sigma_I$, and $s_A = y'(\hat{\mu})\sigma_A$. Finally, the interindividual or group coefficient of variation (CV_G), the intra-individual coefficient of variation (CV_I), and the between duplicates or analytical coefficient of variation (CV_A) were calculated as $CV_G = s_G / m$, $CV_I = s_I / m$ and $CV_A = s_A / m$, respectively. The upper and lower 95% confidence intervals for these coefficients of variation were computed in the same way, from the upper and lower 95% confidence intervals for the variance components σ_G^2, σ_I^2, and σ_A^2.

The most commonly used calculation for II was published by Fraser.[15] Using the original formula, a low II corresponds to high individuality, and caution should be taken when using population-based RI. In this study, II was calculated with the inverse formula that has been recommended according to veterinary guidelines for biological variation studies. This formula may seem more logical since a high index corresponds to a high individuality (ie, results from different individuals vary greatly for the analyte in question),[13,14] and the use of RCV is recommended.

The II was calculated by the formula[13,14]

$$II = CV_G / \left(CV_I^2 + CV_A^2\right)^{1/2}.$$

For bidirectional analyses, when both decreased and increased concentrations are of interest, both lower and upper RCVs are needed. These were computed as $y^\lambda \pm z\left(2\left(\hat{\sigma}_I^2 + \hat{\sigma}_A^2\right)\right)^{1/2}$, where $z = 1.96$. When no transformation was applied, $\lambda = 1$. The limits were back-transformed to the original scale using:

$$RCV = \left(y^\lambda \pm 1.96\sqrt{2\left(\hat{\sigma}_I^2 + \hat{\sigma}_A^2\right)}\right)^{1/\lambda}.$$

This equation for RCV was obtained by substituting the confidence limits $y^\lambda \pm 1.96\sqrt{2\left(\hat{\sigma}_I^2 + \hat{\sigma}_A^2\right)}$ for t in the equation for the inverse function, $y = t^{1/\lambda}$.

3 | RESULTS

3.1 | Biological variation

Five dogs were excluded from the study because of a history of polydipsia/polyuria (n = 2), persistent proteinuria (n = 1), signs of urinary tract infection (n = 1), and estrus (n = 1). The remaining 13 dogs consisted of 3 intact females, 5 spayed females, 2 intact

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males and 3 neutered males. The dogs were 1.5-7.0 years old (median 4.0, mean 3.8). The breed distribution included Labrador retriever (n = 4), Cairn terrier (n = 1), Border collie (n = 1), Irish wolfhound (n = 1), Pointer (n = 1), Pomeranian (n = 1), Rhodesian ridgeback (n = 1), and mixed breed dogs (n = 3). The dogs weighed between 3.0 and 55.0 kg (median 24.0, mean 23.5), body condition scores were between 4.5 and 5.0 for five of the dogs, and 5.0 for the remaining eight dogs, on a scale of 1-9.[26] The dogs were sampled in a sitting position except for one, the largest dog, which was more comfortable lying on its side. All dogs except one had been treated with tick prevention medication (fluralaner [n = 6], afoxolaner [n = 4], and sarolaner [n = 2]).

Seven dogs had two sampling occasions missing, and one serum sample was excluded due to hemolysis, which led to a total of 89 urine samples and 89 blood samples. If a dog had, at one or more sample occasions, a result below the lowest measurable concentration range for an analyte, which made it impossible to estimate variances for the dog, the data for that analyte in that dog had to be excluded. This occurred for one dog which had U-P excluded, two dogs which had U-Na excluded, and five dogs which had U-Prot excluded, that is, these dogs had concentrations below the lowest measurable limit in one or more sampling occasions.

There were 11 U-analytes and 9 S-analytes (since USG and osmolality were not included for serum) analyzed on the 89 urine samples and 89 blood samples. The final number of sample analyses was 922 for U-analyte concentrations and 656 for U-analyte/U-Cr and FE-analytes. The dataset used for statistical analyses is provided in File S2.

The urine results were analyzed for outliers and for the 922 U-analyte concentrations there were 12 analytical outliers and one within-dog outlier detected and excluded (Table S2). Of the 656 U-analyte/U-Cr, 5 duplicate outliers and 6 within-dog outliers were removed. Of the 656 FE-analyte results, 4 duplicate outliers and 3 within-animal outliers were excluded (Table S2). Analytical variation is expressed as duplicate variation for U-analyte/U-Cr ratio and FE, since the difference between their replicates is based on analytical variation from analytes in a ratio/formula. There were no between-animal outliers detected.

The CV_I, CV_G, CV_A, and II for all U-analyte concentrations, U-analyte/U-Cr, and FE are presented in Tables 1-3, and Box plots showing the distribution for each dog are presented in Figures 1-3.

The CV_I for all urine analytes both as U-analyte concentration, U-analyte/U-Cr, and FE varied between 12.6% and 35.9%, except for U-Na, U-Na/U-Cr, and FE-Na which had CV_Is of 59.5%, 60.0%, and 60.7%, respectively. The CV_G for all urine analytes both as U-analyte concentrations, U-analyte/U-Cr, and FE-analyte, except U-Prot, varied between 5.3% and 46.1%, with U-Ca/U-Cr having the highest CV_G of 46.1%. Fractional excretion of glucose showed both the lowest CV_I (12.7%), and the lowest CV_G (5.3%). Results from the U-Prot REML demonstrated a relatively high analytical and within-animal variation, and this high CV_A and CV_I masked the CV_G of U-Prot, and resulted in a remarkably low CV_G value for U-Prot (0.0004%). Distribution of U-protein in all dogs is presented in Figure 1.

The CV_A for U-analyte, U-analyte/U-Cr, and FE-analyte was ≤2.1%, except for U-Prot which had a CV_A of approximately 5.0% including U-Prot/U-Cr and FE-Prot. Analytical performance goals

TABLE 1 Biological variation data for 11 canine urine biochemical analytes concentrations.

Biological variation							
Analyte	Units	N	Median (range)	CV_I % (95% CI)	CV_G % (95% CI)	CV_A % (95% CI)	II
USG		13	1.043 (1.013-1.057)	15.1 (12.9-17.8)	14.4 (8.9-22.4)	1.0 (0.9-1.2)	0.95
U-Osmo	mOsmol/kg	13	1753 (429-2448)	17.7 (15.2-21.0)	17.5 (10.9-27.2)	0.7 (0.6-0.8)	0.99
U-Cr	µmol/L	13	20 100 (7379-37 241)	22.1 (19.0-26.1)	19.0 (11.4-30.0)	1.4 (1.2-1.6)	0.86
U-Urea	mmol/L	13	1037 (242-1688)	24.2 (20.8-28.8)	20.4 (12.1-32.2)	1.7 (1.5-2.0)	0.84
U-Prot	g/L	8	0.12 (0.05-0.30)	35.9 (29.6-43.7)	<0.1 (<0.1-18.4)	5.2 (4.4-6.4)	0.00
U-Glu	mmol/L	13	0.52 (0.18-0.95)	23.1 (19.8-27.3)	19.6 (11.7-30.8)	0.9 (0.8-1.1)	0.85
U-Na	mmol/L	11	78.0 (20.0-240)	60.0 (51.0-72.0)	25.5 (0.00-47.5)	0.7 (0.6-0.8)	0.42
U-Cl	mmol/L	13	166 (24-340)	27.0 (23.2-32.0)	25.1 (15.3-32.3)	0.9 (0.8-1.0)	0.93
U-K	mmol/L	13	134 (42.4-235)	30.0 (25.8-35.4)	21.4 (11.9-34.4)	0.9 (0.7-1.0)	0.70
U-Ca	mmol/L	13	0.55 (0.17-1.56)	28.4 (24.3-33.8)	39.7 (25.7-60.7)	1.0 (0.9-1.2)	1.40
U-P	mmol/L	12	76.2 (22.1-140)	25.5 (21.8-30.4)	27.8 (17.1-43.8)	1.3 (1.1-1.5)	1.09

Abbreviations: CI, confidence interval 95%; CV_A, analytical coefficient of variation; CV_G, between-subject of coefficient of variation; CV_I, within-subject coefficient of variation; II, index of individuality based on $II = CV_G / \left(CV_I^2 + CV_A^2\right)^{1/2}$; N, number of dogs; U-Ca, urine calcium concentration; U-Cl, urine chloride concentration; U-Cr, urine creatinine concentration; U-Glu, urine glucose concentration; U-K, urine potassium concentration; U-Na, urine sodium concentration; U-Osmo, urine osmolality; U-P, urine phosphate concentration; U-Prot, urine protein concentration; USG, urine specific gravity; U-Urea, urine urea concentration.

TABLE 2 Biological variation data for eight urine U-analytes/U-Cr ratios measured in dogs.

Biological variation						
Analyte	N	Median (range)	CV_I % (95% CI)	CV_G % (95% CI)	Duplicate CV % (95% CI)	II
U-Urea/U-Cr	13	49.5 (22.8-82.8)	20.6 (17.7-24.5)	17.7 (10.6-27.9)	1.5 (1.3-1.8)	0.86
U-Prot/U-Cr	8	0.044 (0.023-0.080)	18.3 (14.9-23.0)	22.1 (12.3-38.3)	5.2 (4.3-6.4)	1.16
U-Glu/U-Cr	13	0.025 (0.017-0.062)	14.3 (12.3-17.0)	20.7 (13.5-31.5)	1.4 (1.2-1.6)	1.43
U-Na/U-Cr	11	3.88 (0.95-12.2)	59.5 (50.5-71.5)	27.0 (0.00-49.6)	1.4 (1.2-1.6)	0.45
U-Cl/U-Cr	13	8.00 (2.24-23.0)	28.6 (24.5-34.0)	33.7 (21.1-52.1)	1.3 (1.1-1.5)	1.18
U-K/U-Cr	13	6.26 (2.69-13.9)	25.8 (22.1-30.6)	23.8 (14.1-37.4)	1.1 (0.9-1.3)	0.92
U-Ca/U-Cr	13	0.028 (0.010-0.112)	35.0 (30.1-41.5)	46.1 (29.8-70.5)	1.4 (1.2-1.7)	1.31
U-P/U-Cr	12	3.51 (1.49-6.94)	24.1 (20.5-28.7)	23.4 (14.1-37.1)	1.7 (1.5-2.0)	0.97

Abbreviations: CI, confidence interval 95%; CV_G, between-subject of coefficient of variation; CV_I, within-subject coefficient of variation; Duplicate CV, duplicate coefficient of variation; II, index of individuality based on $II = CV_G / \left(CV_I^2 + CV_A^2\right)^{1/2}$; N, number of dogs; U-Ca/U-Cr, urine calcium ratio to urine creatinine; U-Cl/U-Cr, urine chloride ratio to urine creatinine; U-Glu/U-Cr, urine glucose ratio to urine creatinine; U-K/U-Cr, urine potassium ratio to urine creatinine; U-Na/U-Cr, urine sodium ratio to urine creatinine; U-P/U-Cr, urine phosphate ratio to urine creatinine; U-Prot/U-Cr, urine protein ratio to urine creatinine; U-Urea/U-Cr, urine urea ratio to urine creatinine.

TABLE 3 Biological variation data for eight FE-analytes measured in dogs.

Biological variation							
Analyte	Units	N	Median (Range)	CV_I % (95% CI)	CV_G % (95% CI)	Duplicate CV % (95% CI)	II
FE-Urea	%	13	73.1 (47.6-133.2)	15.6 (13.4-18.5)	10.1 (5.0-16.5)	2.1 (1.8-2.5)	0.64
FE-Prot	%	8	0.0006 (0.0003-0.0012)	19.6 (16.0-24.7)	17.6 (8.7-31.4)	5.4 (4.5-6.6)	0.87
FE-Glu	%	13	0.04 (0.03-0.06)	12.6 (10.9-15.0)	5.3 (0.0-9.7)	1.9 (1.7-2.2)	0.42
FE-Na	%	11	0.22 (0.05-0.83)	60.7 (51.5-73.0)	19.1 (0.00-40.1)	1.5 (1.3-1.8)	0.31
FE-Cl	%	13	0.58 (0.18-1.28)	29.6 (25.4-35.1)	24.1 (13.9-38.2)	1.5 (1.3-1.8)	0.81
FE-K	%	13	11.0 (4.7-20.0)	23.9 (20.5-28.3)	14.0 (6.0-23.4)	1.5 (1.3-1.7)	0.59
FE-Ca	%	13	0.09 (0.04-0.32)	32.6 (27.9-38.7)	37.2 (23.6-57.3)	1.7 (1.5-2.0)	1.14
FE-P	%	12	22.0 (7.8-45.9)	30.1 (25.7-36.0)	18.7 (8.5-31.5)	2.0 (1.8-2.4)	0.62

Note: The formula behind the FE calculation is = (U-concentration of X) × (plasma concentration of creatinine)/(urine concentration of creatinine) × (plasma concentration of X) × 100.

Abbreviations: CI, confidence interval 95%; CV_G, between-subject of coefficient of variation; CV_I, within-subject coefficient of variation; Duplicate CV, duplicate coefficient of variation; FE-Ca, fractional excretion of calcium; FE-Cl, fractional excretion of chloride; FE-Glu, fractional excretion of glucose; FE-K, fractional excretion of potassium; FE-Na, fractional excretion of sodium; FE-P, fractional excretion of phosphate; FE-Prot, fractional excretion of protein; FE-urea, fractional excretion of urea; II, index of individuality based on $II = CV_G / \left(CV_I^2 + CV_A^2\right)^{1/2}$; N, number of dogs.

based on biological variation for all parameters are presented in Table S3.

Nine analytes had an II of ≤0.7, indicating that they were suitable for interpretation using population-based RI. These were U-Prot, U-Na, U-K, and U-Na/U-Cr and FE-Urea, FE-Glu, FE-Na, Fe-K, and FE-P. Eighteen analytes had an intermediate II, that is, between 0.7 and 1.7. These analytes were USG, Osmo, U-Cr, U-Urea, U-Glu, U-Cl, U-Ca, U-P, and U-Urea/U-Cr, U-Prot/U-Cr, U-Glu/U-Cr U-Cl/U-Cr, U-K/U-Cr, U-Ca/U-Cr, U-P/U-Cr, FE-Prot, FE-Cl, and FE-Ca. Since the U-analytes were not normally distributed, and had to be transformed before analysis, RCV varied with different concentrations. For the biochemical urine analytes with an intermediate II, the estimated RCV at different levels is presented in Figures 4-6, and RCV for all analytes is provided in Table S4. None of the U-analytes had a result with an II ≥1.7.

Serum results were analyzed for outliers. For the 801 samples, there were two within-dog outliers. The dataset used for statistical analyses is provided in File S2. The nine serum analytes had a CV_I of 0.4%-19.4% and a CV_G of 0.5%-16.6% (Table 4). Box plots showing the distribution for each dog are presented in Figure 7 and RCV for serum analytes with II 0.7-1.7 and II ≥1.7 in Figure 8, where S-Na represented both the lowest CV_I and CV_G, S-Urea and

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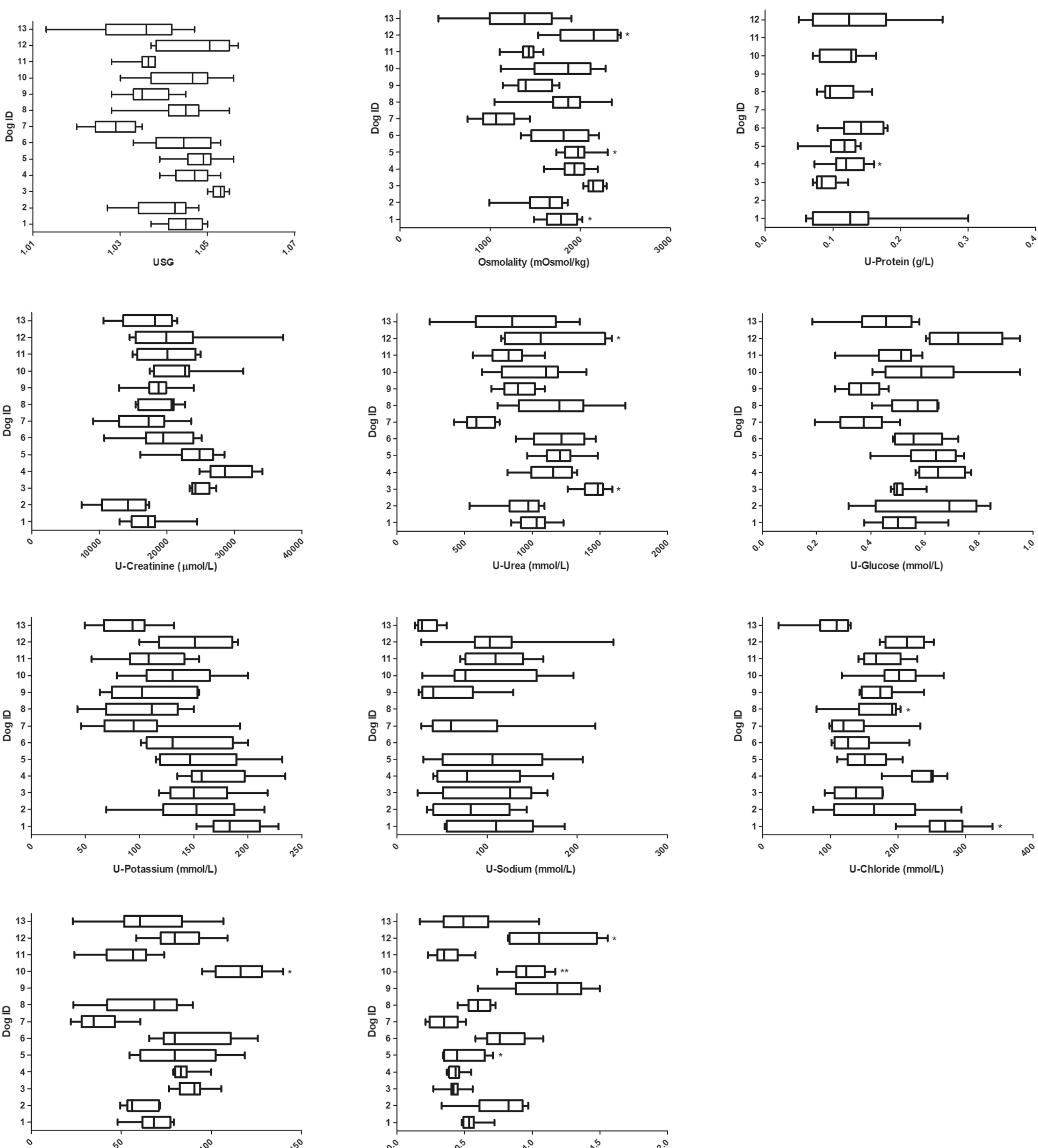

FIGURE 1 Distribution of urine analytes in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

S-Crea represented the highest CV_I and CV_G, respectively. One serum analyte, S-Na, had an II <0.7, indicating that it was suitable for interpretation using the population-based RI. Seven serum analytes, S-Urea, S-Tot Prot, S-Glu, S-Cl, S-K, S-Ca, and S-P, had an intermediate II, that is, between 0.7 and 1.7. One serum analyte, S-Cr, had an II of ≥1.7.

4 | DISCUSSION

This study presents data on biological variation for 11 urinary biochemical analytes from healthy dogs. The CV_I for all analytes both as U-analyte concentrations, U-analyte/U-Cr, and FE varied between 12.6% and 35.9%, except for U-Na, which was noticeably

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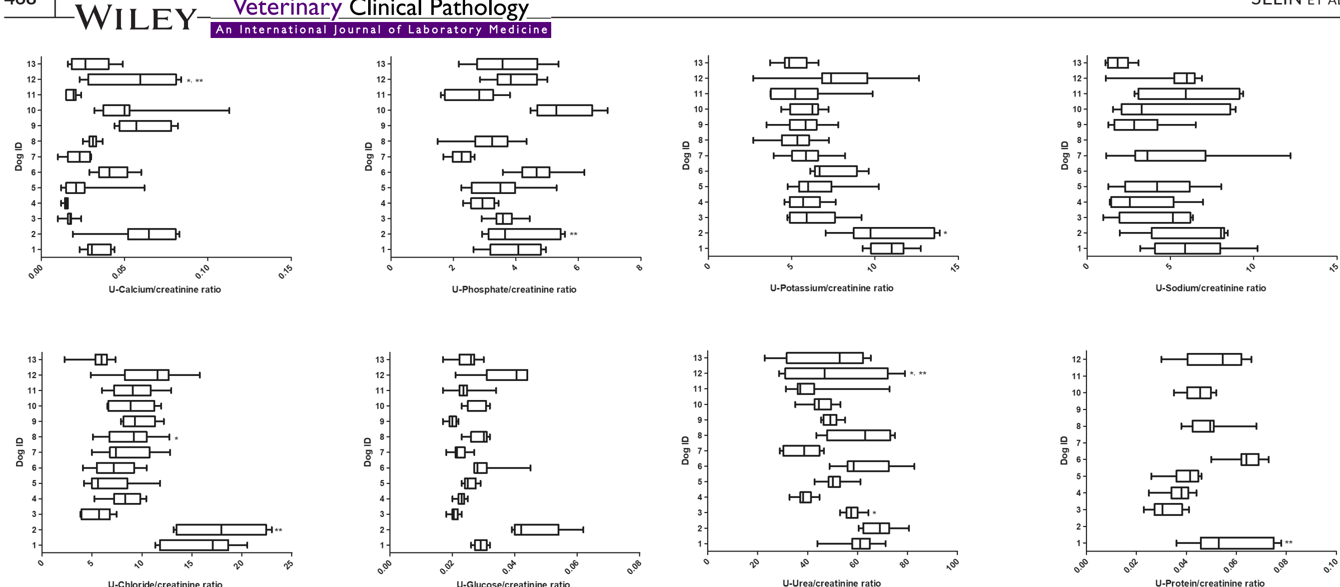

FIGURE 2 Distribution of urine analytes in relation to urinary creatinine in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

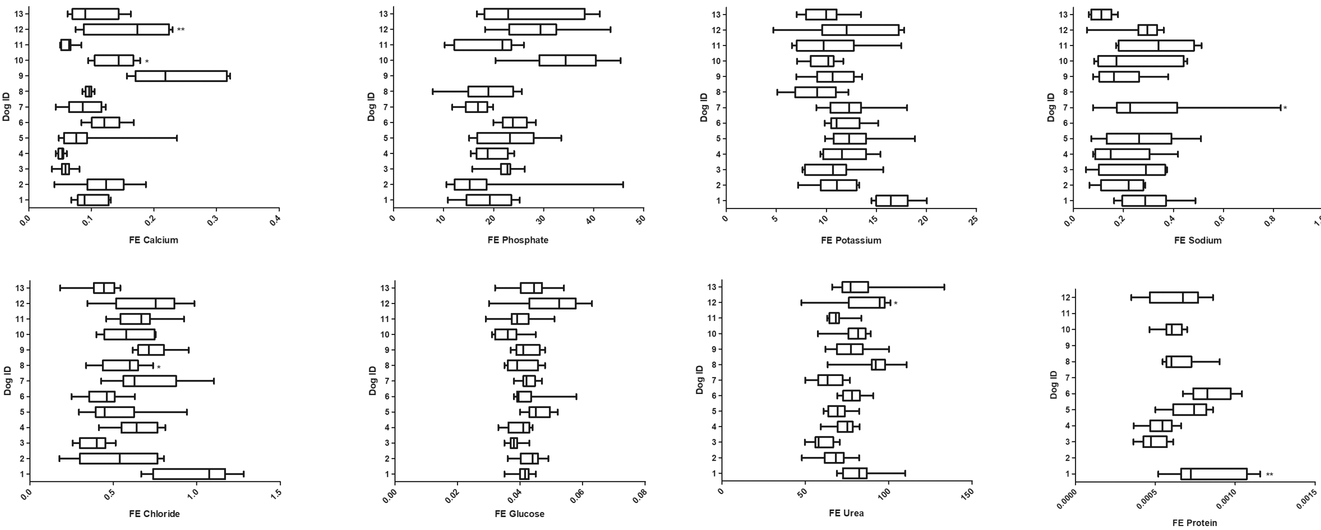

FIGURE 3 Distribution of fractional excretion (FE) of urine analytes in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

higher (60.0%). The CV_I for U-Na was approximately 60.0% regardless of whether U-Na was studied as the U-analyte concentration, as a ratio to U-Cr, or as a FE-Na. Although no earlier studies were found with dogs concerning BV of U-Na, U-Na/U-Cr, or FE-Na, one study in humans showed a CV_I for U-Na of 35.8%.[27] The high CV_I for U-Na in this study may be explained by the effects of body homeostasis and diet, even though the diet was kept the same for each individual dog throughout the study. Effects from varying water content in the urine might be another explanation, but as previously stated, the CV_I for U-Na was high even when related to U-Cr or evaluated as FE. Conversely, S-Na had the lowest CV_I and CV_G among the serum analytes. This indicates that the kidneys' regulatory focus is to keep the S-Na constant causing varying U-Na levels. The high CV_I for all forms of U-Na should be evaluated in future studies.

The kidneys treat various analytes differently, and the use of FE may be more relevant for the interpretation of some analytes than others. For example, urinary excretion of U-K and U-P is expected. On the other hand, for U-Prot, filtration is avoided, and U-Glu and small proteins that manage to pass filtration are being reabsorbed in the proximal tubuli. Therefore, the low CV_I and CV_G for FE-Glu were expected. To the authors' knowledge, there are no previous publications on the BV of FE of biochemical analytes in canine urine. There are, however, previous studies by van Vonderen et al on the variation of USG and U-Osmo in healthy dogs during 2 consecutive days,[17] and by Rudinsky et al on the

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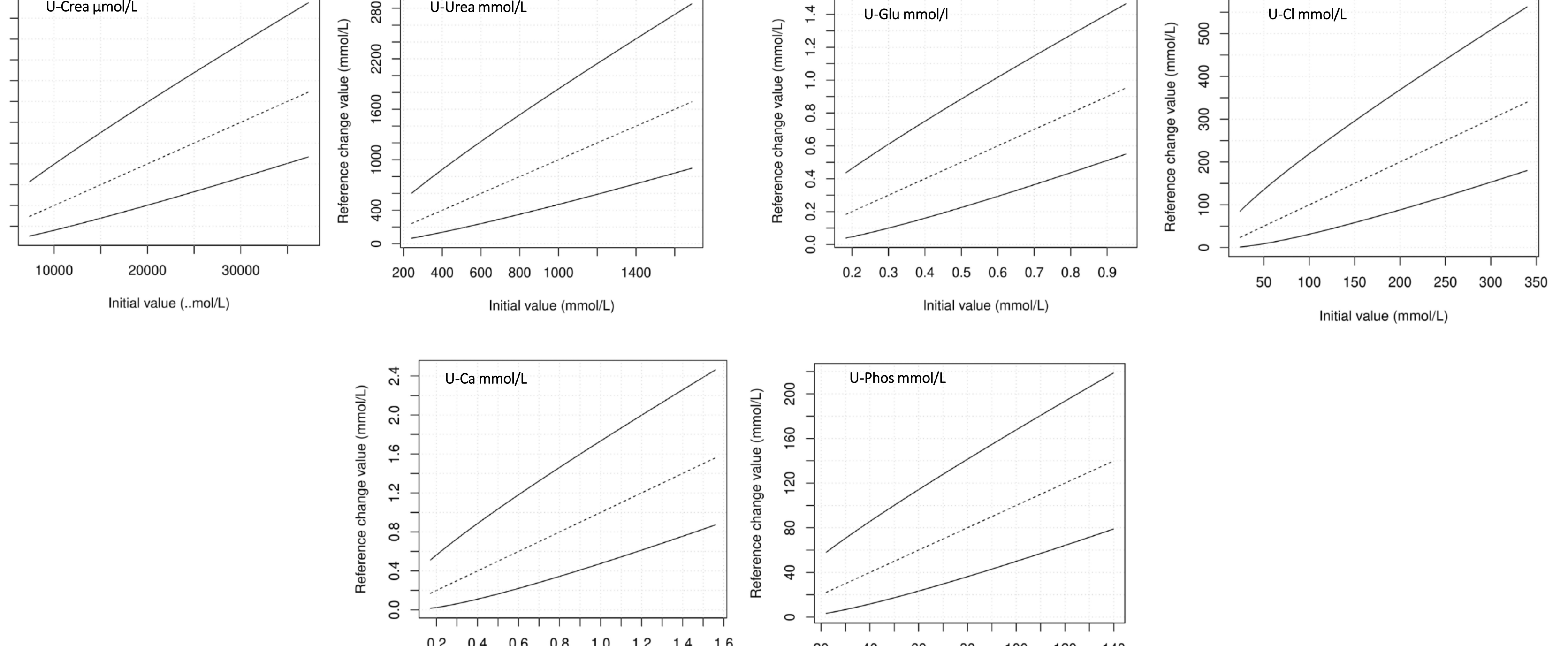

FIGURE 4 Graphs illustrating reference change value (RCV) for the six U-analyte concentrations with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

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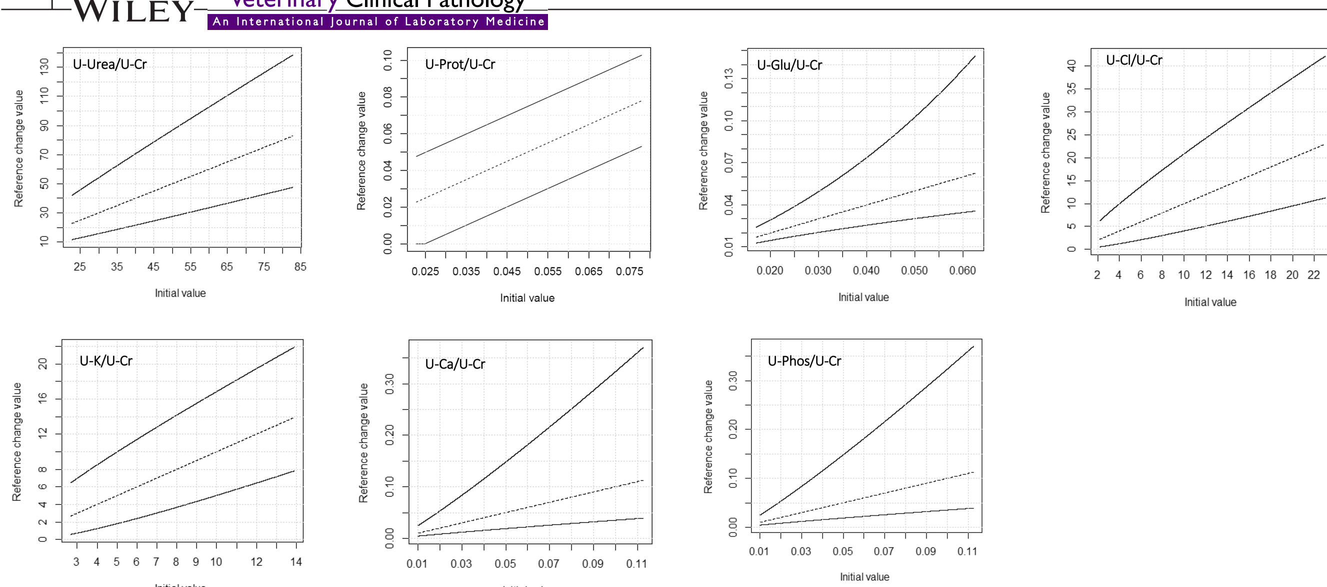

FIGURE 5 Graphs illustrating reference change value (RCV) for the seven U-analyte/U-Cr with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

variability of USG on six time points over 2 weeks.[16] These earlier articles reported a quite similar range in USG and U-Osmo values as this study. The BV of U-Prot, U-Cr, and UPC was investigated by Mårtensson et al in a study with a similar design as ours,[19] and the results are comparable with the exception of CV_G for U-Prot, which was 33.1% in Mårtensson's study and remarkably low in our (<0.1%). In our study, U-Prot in five dogs had at least one result below the measuring range, which is why these dogs had to be excluded. This is likely to cause a lower CV_G and may also have affected the CV_I. The remarkably low CV_G is also due to the high CV_A and CV_I, which masked the CV_G when using the REML method. However, since there are only eight dogs, the 95% confidence interval for CV_G was wide (<0.1%, 18.4%), as reported in Table 1.

The U-analyte concentration in this study that varied the least within (CV_I) and between dogs (CV_G) was USG. Nevertheless, it should be noted that the results were based on the morning urine sample from each dog. Ricos et al,[27] as well as Gowans and Fraser,[28] noted that 24-hour samples are more representative of biological variation than spot samples. Nonetheless, for practical feasibility and less stress for the included dogs, this study focused on morning voided urine. The U-Urea/U-Cr ratio had the lowest CV_I of all analytes. A greater variation for U-urea could perhaps have been expected. That pattern might have been different if the dogs had been on a more mixed or extreme raw food diet, or had larger variations in hydration status since urea is partially reabsorbed in the loop of Henle as a part of keeping the body's fluid volume constant. It should also be noted that in serum, S-Urea had one of the highest CV_I and CV_G. In that way, urea might be seen as opposite of sodium; that is, for urea, a greater variation is accepted in the blood resulting in a smaller variation in urine. Therefore, biological variation data improve our understanding of how the body maintains homeostasis.

Biological variation of serum parameters in pet dogs has been studied before by Ruaux et al,[29] Leissing et al,[30] Jensen et al,[31] and Pagitz et al.[32] In terms of design and results, the study by Ruaux et al is most consistent with this study. There were a few disparities seen in CV_I for S-Prot and CV_G for S-Crea, where CV_I for S-Prot in this study was 3.6%, whereas it was 15.3% in Ruaux et al's study,[29] and the CV_G for S-Crea was 16.6% in this study and 31.0% in the previous study. The reason for this discrepancy may be that our study had fewer dogs, sampled under standardized conditions, and followed for 6-8 weeks instead of 12 weeks, as in the earlier study.

The U-analytes data in this study were not normally distributed, which is commonly the case for urinary biochemical analytes.[33] For normally distributed observations, coefficients of variation and RCVs can be computed on the original scale, that is, without any need for transformation. In this case, the distance from the RCVs to the initial value is the same everywhere, regardless of the initial value. In this study, with non-normally distributed data, another strategy for computation of RCV was required for many variables, and coefficients of variation were calculated on the transformed scale and then back-transformed.[13,34] Similarly, for log-normally distributed data, there is an equation for computing RCVs.[35,36] Under the assumption of log normality, the upper RCV is a percent of the initial value, which is independent of the initial value, and the same holds for the lower RCV. There are occasions, such as for our urinary data, where biological data are neither normally distributed nor log-normally distributed. The problem with skewed urinary data was solved using the power transformation. A method was introduced for computing

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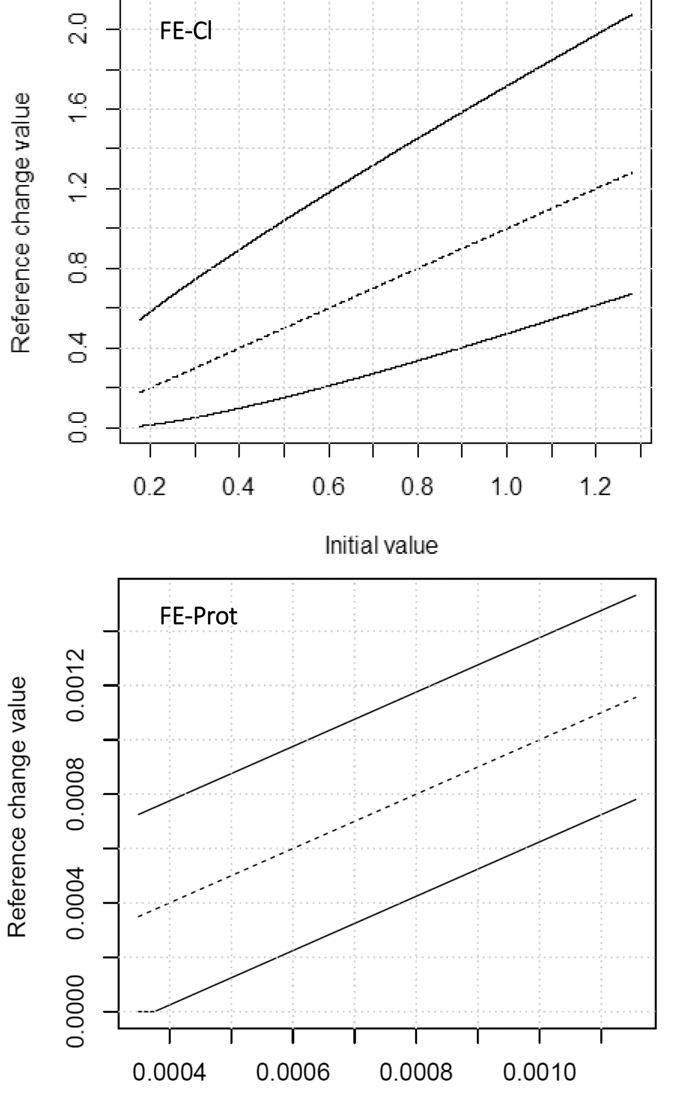

FE-Ca

Reference change value

Initial value

FIGURE 6 Graphs illustrating reference change value (RCV) for the three FE-analytes with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

TABLE 4 Biological variation of 9 biochemical serum analytes in 13 dogs sampled once a week for 8 weeks.

Biological variation							
Analyte	**Unit**	**N**	**Median (Range)**	**CV_I % (95% CI)**	**CV_G % (95%)**	**CV_A % (95% CI)**	**II**
S-Cr	µmol/L	13	81.2 (39.6-99.7)	5.4 (4.6-6.3)	16.6 (11.2-24.8)	0.9 (0.8-1.1)	3.04
S-Urea	mmol/L	13	5.4 (2.9-10.1)	19.4 (16.7-23.0)	15.6 (8.9-24.8)	1.3 (1.2-1.6)	0.80
S-Tot Prot	g/L	13	61.8 (53.5-71.1)	3.6 (3.0-4.3)	5.1 (3.3-8.0)	0.6 (0.5-0.7)	1.42
S-Glu	mmol/L	13	5.0 (3.4-6.2)	6.0 (5.1-7.1)	6.7 (4.2-10.3)	2.2 (1.9-2.6)	1.05
S-Na	mmol/L	13	145.8 (142.2-148.4)	0.4 (0.1-0.6)	0.5 (0.3-0.9)	0.7 (0.6-0.8)	0.68
S-Cl	mmol/L	13	114.8 (108.1-119.6)	1.0 (0.8-1.2)	1.3 (0.9-2.1)	0.7 (0.6-0.8)	1.09
S-K	mmol/L	13	4.5 (4.0-5.1)	3.8 (3.3-4.5)	3.7 (2.3-5.8)	0.8 (0.7-0.9)	0.96
S-Ca	mmol/L	13	2.4 (2.2-2.6)	1.9 (1.6-2.3)	2.9 (1.9-4.4)	1.0 (0.8-1.1)	1.37
S-P	mmol/L	13	1.3 (0.8-1.7)	7.5 (6.4-8.9)	8.2 (5.1-12.6)	0.7 (0.6-0.8)	1.09

Abbreviations: CI, confidence interval 95%; CV_A, analytical coefficient of variation; CV_G, between-subject of coefficient of variation; CV_I, within-subject coefficient of variation; II, index of individuality based on $II = CV_G / \left(CV_I^2 + CV_A^2\right)^{1/2}$; N, number of dogs; S-Ca, serum calcium concentration; S-Cl, serum chloride concentration; S-Cr, serum creatinine concentration; S-Glu, serum glucose concentration; S-K, serum potassium concentration; S-Na, serum sodium concentration; S-P, serum phosphate concentration; S-Prot, serum protein concentration; S-Urea, serum urea concentration.

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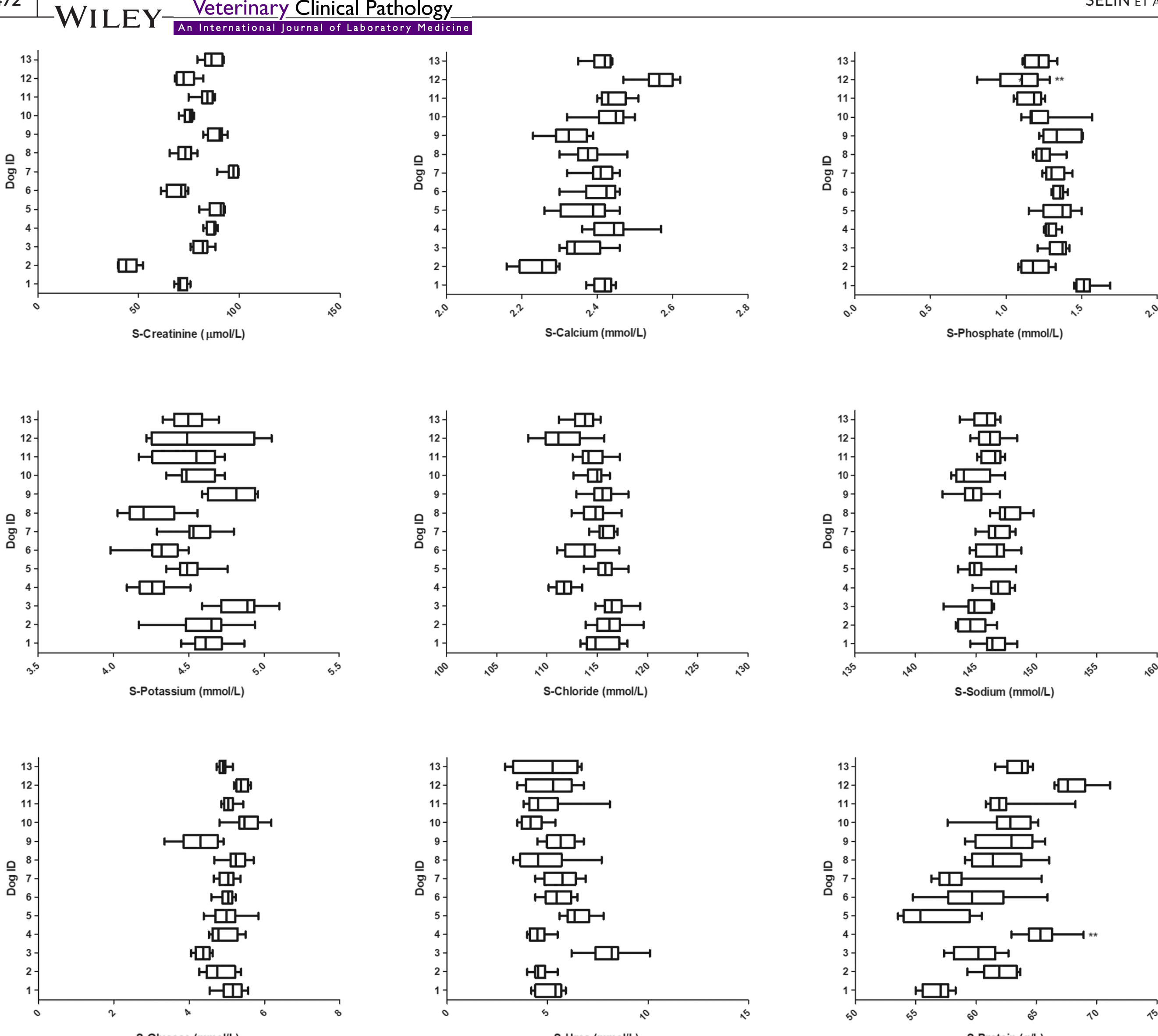

FIGURE 7 Distribution of serum analytes in 13 dogs sampled once a week for 6-8 weeks. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

the coefficients of variation and their 95% confidence intervals using the derivative of the inverse transformation evaluated for the average value. This is an application of the delta method.[37] To the authors´ knowledge, it is the first time this particular transformation has been used. Furthermore, an equation was provided for the computation of RCVs for this study. Since this equation is more complex than other equations,[32] the RCVs were presented graphically (Figures 4-6). This method has the advantage that it can be applied to any distribution of positive observations. By choosing the value of the parameter λ that maximizes the likelihood of the data, instead of restricting the choice to either $\lambda = 0$ (log-normal distribution) or $\lambda = 1$ (normal distribution), calculations should be more accurate.

Although the Box-Cox method always improves the likelihood, some plots of conditional residuals against conditional fitted values, presented in File S1, suggest the opposite. In these cases, the plots of sample quantiles against theoretical quantiles often show improved distribution. Conditional residuals are conditioned on the random effects of the model, which in our study are the dogs and the points of time. As such, the conditional residuals reflect analytical variability but not inter- and intraindividual variability. It is difficult to choose whether to transform based on the plots of conditional residuals, as these only describe the variation between samples within points of time. Instead, we tested if the transformation significantly improved the likelihood since this method considers all variance components.

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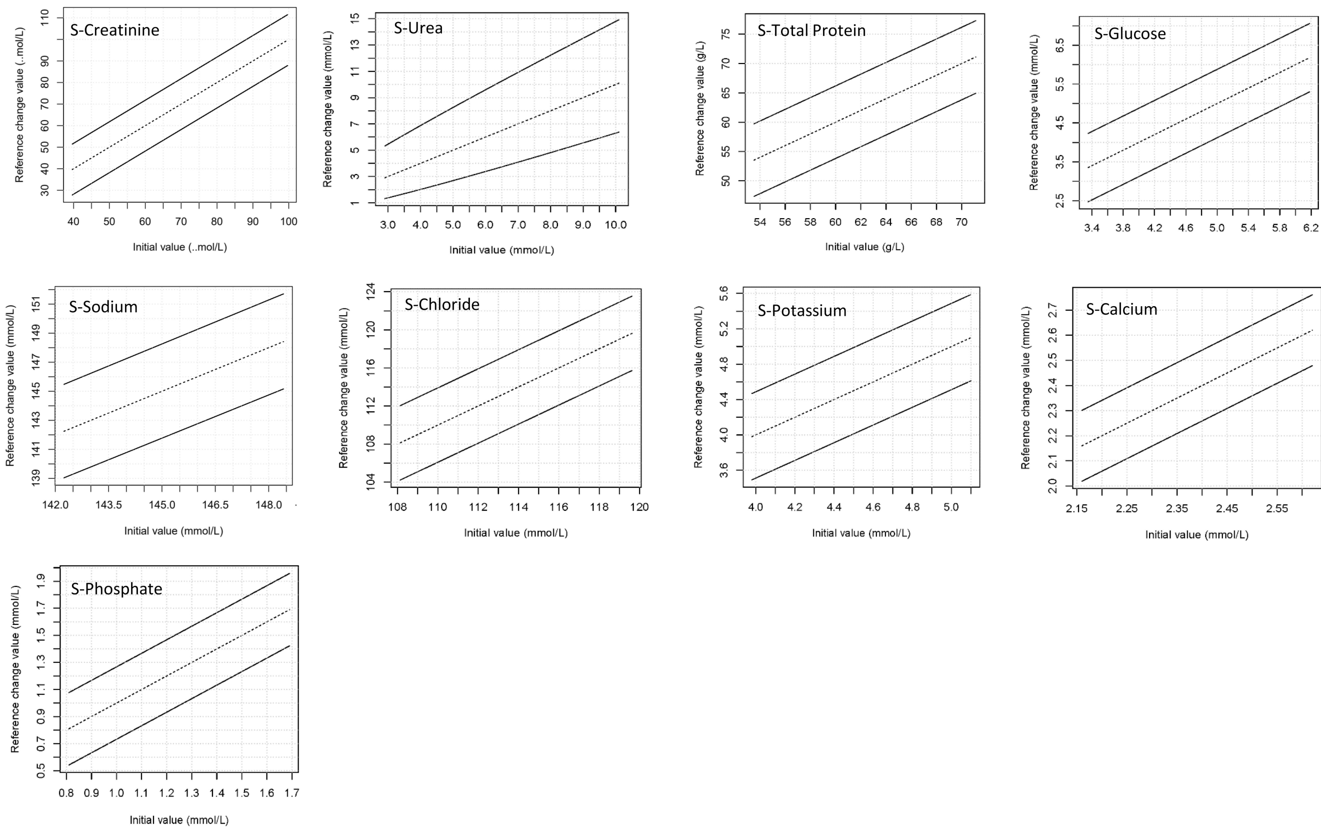

FIGURE 8 Graphs illustrating reference change value (RCV) for the eight S-analytes with index of individuality 0.7-1.7 and II ≥1.7. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

When many observations are identical, it is not possible to transform the data into a continuous normal distribution. This difficulty was experienced especially for USG, U-Na, U-Ca, S-Urea, and S-P. The results for these five analytes should be considered approximate.

There are several examples of clinical applicability of the results from this study. Included analytes are considered useful diagnostic tools in different types of kidney damage and electrolyte disorders in dogs. Data on BV are useful when interpreting results in these situations. Nonetheless, RCV usage requires a prior data point for comparison, which excludes the use of RCV in certain situations. The RCV charts (Figures 4-6) are useful when evaluating if the difference between two samples for a specific analyte has clinical significance.

In summary, this study presents information on the biological variation of urinary biochemical analytes from healthy dogs. These data are important for the correct interpretation of laboratory results.

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DISCLOSURE

The authors declare no conflicts of interest.

ORCID

Anna K. Selin https://orcid.org/0000-0002-8295-4208
Inger Lilliehöök https://orcid.org/0000-0002-9526-242X

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