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Urinary Cystatin C, Glucose, Urea, and Electrolytes in Dogs at Various Stages of Chronic Kidney Disease

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ABSTRACT

Background: There is limited knowledge of urine analytes in different stages of chronic kidney disease (CKD) in dogs.

Objectives: To study markers in urine and fractional excretion (FE) of markers in dogs of different stages of CKD and a healthy control group (C).

Animals: Fifty dogs in various stages of CKD and a control group of 30 healthy dogs.

Methods: In this cross-sectional observational study, dogs presenting to a referral hospital and given a diagnosis of CKD using standard methods, and healthy dogs, were included. Urinary cystatin C (uCysC), glucose (uGlu), protein (uProt), creatinine (uCr), urea (uUrea), sodium (uNa), potassium (uK), chloride (uCl), calcium (uCa), and phosphate (uP) were measured with an automated chemistry analyzer. Included analytes were normalized to uCr, FE of electrolytes and urea was calculated, and results compared among groups.

Results: Age, bodyweight, and sex were not different among groups. Urinary CysC/uCr and FE of electrolytes increased with IRIS stage. Median (IQR) for uCysC/uCr was 0.08 (0.04–0.25) 10^{-3} in dogs with CKD stage 1 and 0.03 (0.02–0.045) 10^{-3} in control dogs ($p = 0.0002$).

Conclusion and Clinical Importance: Urinary CysC might be a potential marker of early CKD, preferably as part of a panel of urinary markers. FE of electrolytes seemed to depend on the serum creatinine level in dogs with azotemic CKD.

1 | Introduction

Chronic kidney disease (CKD) in dogs is defined as the presence of functional or structural damage to one or both kidneys with a duration of more than 3 months [1]. Early diagnosis of CKD enables therapeutic management, which could slow progression to advanced stages [2–4]. Novel urinary biomarkers including low molecular weight proteins and tubular enzymes might serve as early indicators of kidney damage or dysfunction, before GFR changes occur [5–7]. These markers can also provide information regarding which compartment of the kidney is affected, and might contribute to early diagnosis of tubulointerstitial nephropathies without proteinuria [5–8].

Serum cystatin C (sCysC), a low-molecular-weight protein (13 kDa), is a cysteine protease inhibitor and a member of the super cystatin

Abbreviations: AKI, acute kidney injury; Alb, albumin; CKD, chronic kidney disease; CRP, C-reactive protein; CysC, cystatin C; FE, fractional excretion; GFR, glomerular filtration rate; Glu, glucose; IRIS, International Renal Interest Society; sCysC, serum cystatin C; uCa, urinary calcium; uCl, urinary chloride; uCr, urinary creatinine; uCysC, urinary cystatin C; uK, urinary potassium; uNa, urinary sodium; uP, urinary phosphate; UPC, urine protein: creatinine ratio; uProt, urinary protein.

family [9]. It is produced at a stable rate by all nucleated cells and cleared by glomerular filtration in both humans and dogs [10]. Filtered cystatin C is reabsorbed by a megalin-facilitated endocytosis in the proximal tubules and catabolized [11]. Consequently, proximal tubular injury or dysfunction will reduce reabsorption and degradation, which results in a larger amount of urinary cystatin C (uCysC) [12]. In experimental and spontaneous acute kidney injury (AKI) in dogs, uCysC increases before the development of azotemia [13–15], and histological evidence of nephrotoxicity correlates with uCysC in dogs with gentamicin or tenofovir induced AKI [13, 14]. There are fewer studies of uCysC in dogs with CKD [16] compared to dogs with AKI, but in one study uCysC was higher in 13 dogs with CKD compared to control dogs [16], and another study concluded that uCysC/uCr might be useful for early detection of renal injury in dogs with leishmaniosis [17].

Fractional excretion (FE) of electrolytes (FE-Na, FE-Cl, FE-K, and FE-P) is higher in dogs with advanced CKD than in dogs with less severe kidney disease [18, 19]. Knowledge about urinary CysC, glucose (uGlu), urea (uUrea), and electrolytes in different stages of CKD, as well as their potential for detecting tubular dysfunction in the early stages of CKD, is still limited. The urinary analytes included in this study can be analyzed on standard biochemistry instruments, allowing analysis in practically all large veterinary laboratories.

The primary objective of this study was to compare uCysC, uGlu, uUrea, and electrolytes normalized to urinary creatinine (uCrea), as well as FE of electrolytes and uUrea, among dogs in different International Renal Interest Society (IRIS) stages of CKD and healthy control dogs (C). A secondary objective was to evaluate the utility of these potential biomarkers for diagnosis of CKD Stage 1.

2 | Material and Methods

2.1 | Study Design

This cross-sectional observational study was performed in Sweden at the University of Agricultural Sciences (SLU), in Uppsala. The study was approved by the Uppsala Ethics committee (C340/11, C12/15, 5.2.18–13 750/2019) and all owners provided written informed consent.

Dogs with CKD, > 6 months of age, were sampled. The diagnosis of CKD (defined as structural or functional abnormalities of one or both kidneys with a duration of at least 3 months) had been made using standard methods (clinical signs, results of urine analysis, blood pressure measurements, hematological and biochemical analyses, abdominal ultrasonography, and, when relevant, renal scintigraphy). For a diagnosis of CKD stage 1, obvious ultrasonographical abnormalities (multiple cysts, irregular renal margins, or markedly reduced renal size) or persistent renal proteinuria or evidence of proximal tubular dysfunction had to be present. Exclusion criteria for the CKD group were the presence of other systemic diseases or medications (except for tick prevention, oral glycosaminoglycan supplements, and sodium pentosane polysulfate injections). If a dog was medicated with an angiotensin converting enzyme inhibitor or phosphate binder, the drug was withdrawn 1 week before inclusion and reintroduced the day after the study. Renal diets were allowed. All dogs with CKD were assigned an IRIS stage (1–4) based on stable serum creatinine (sCr) concentration.

The healthy control group (C) consisted of dogs owned by clients, students, or staff. All control dogs had undergone a thorough physical examination (i.e., urine analysis, blood pressure measurements, hematological and biochemical analyses, abdominal ultrasonography, and renal scintigraphy for GFR). Dogs in the control group were excluded if they were given any type of medication (except tick prevention and glycosaminoglycans) at the time of study inclusion. All control dogs and most of the CKD dogs were initially recruited for a previous study [20].

2.2 | Sampling and Laboratory Analyses

Blood was drawn from the cephalic vein into serum tubes. Serum analytes (C-reactive protein/CRP/, Albumin/Alb/, Protein/Prot/, Crea, Urea, Sodium/Na/, Potassium/K/, Chloride/Cl/, Calcium/Ca/, and Phosphate/P/) from dogs in the control and CKD groups were analyzed fresh at the time of the hospital visit on Architect c4000 (Abbott Diagnostics, Lake Forest, IL, US). Leftover serum samples from these dogs were frozen at −80°C, and sGlu and sCysC were batch analyzed on Architect c4000 within 9 years of sampling.

Urine samples from each dog, collected within a time frame of 4 h of serum collection, were analyzed fresh (USG, dipstick, and sediment). Another aliquot was immediately stored (−80C°). Sample collection occurred between February 2012 and September 2019. Urine was kept frozen until thawed in April 2021 for batch biochemistry analysis of urine analytes (uCysC, uGlu, uProt, uCrea, uUrea, uNa, uK, uCl, uCa, and uP) on Architect c4000. Except for uCysC and uCa, urinary methods and assay performance have previously been described [21]. In the present study, uGlu was measured down to 0.015 mmol/L; recovery after dilution (O/E%) was 105% at this level.

Urinary CysC was analyzed on Architect c4000 with immunoturbidometric reagents from Gentian Diagnostics, Moss, Norway. The method was adjusted for urine according to a previous publication [22]. Mean intra-assay CV for canine urine was 1.0% (mean concentration 0.93 mg/L) and 2.7% (mean 0.20 mg/L). Recovery (O/E%) after dilution down to 0.13 mg/L was between 75% and 106%. Urinary Ca was analyzed with the standard Architect c4000 uCa method. The intra-assay CV in canine urine was 1.2% (mean 1.27 mmol/L) and 2.0% (mean 0.23 mmol/L). Recovery (O/E%) after dilution down to 0.12 mmol/L was between 96% and 111%.

For uCysC ($n = 57/80$), uCa ($n = 2/80$), uNa ($n = 10/80$), uCl ($n = 5/80$), and uProt ($n = 2/80$) results below the measuring range (uCysC 0.1 mg/L, uCa 0.12 mmol/L, uNa 20 mmol/L, uCl 20 mmol/L, uProt 0.04 g/L) were set to half this value. For results above the measuring range, samples were reanalyzed with adjusted dilution or rerun with the serum method.

A digital refractometer (PAL-USG [DOG], Atago and Tokyo, Japan) was used for determination of USG, and osmolality was analyzed using an automatic osmometer (Automatic Micro-Osmometer Type 15, Löser Messtechnik, Berlin, Germany).

2.3 | Statistical Analyses

Statistical calculations were performed using a commercially available software program (JMP Pro 16, SAS Institute, Cary, North Carolina), and GraphPad Prism 10 (GraphPad Software, Boston, USA). Data were assessed for normality by visual inspection of graphs and by the Shapiro–Wilks test. Urinary variables were not normally distributed, and, therefore, presented using medians and interquartile ranges (IQRs).

Normalization with uCr concentration was performed for all urinary analytes. For calculation of ratios, identical units were used, and results were without units. Fractional excretion was calculated for Na, K, Cl, Ca, P, and Urea, using the following formula [23]:

$$\%\,FEX = \frac{(\text{urine concentration of X}) \times (\text{serum concentration of creatinine})}{(\text{urine concentration of creatinine}) \times (\text{serum concentration of X})} \times 100$$

Age, bodyweight (BW), sex, storage time, FE of Na, K, Cl, Ca, P, and urea, and urine analytes (uCysC, uGlu, uUrea, uNa, uK, uCl, uCa, and uP) normalized to uCr were compared among groups using the nonparametric Wilcoxon/Kruskal–Wallis test (rank sums). When significant differences were found among groups, Wilcoxon Each Pair test was used to detect differences between groups. A $p < 0.05$ was used, and Bonferroni correction of p values was performed for group comparisons. Because of the small number of dogs, IRIS stage 3 and 4 were treated as one group in all statistical analyses. The correlation between FE-electrolytes and sCr was calculated using Spearman correlation.

To evaluate the effect of storage, univariable linear regression analyses were performed with concentrations of urinary markers as dependent and storage time as independent variables. Variables with $p < 0.25$ in the univariable analyses were included in a stepwise backward multiple regression model in order to evaluate associations between the urinary marker and the independent variables age, BW, group affiliation (C, CKD 1, CKD 2, CKD 3 + 4) and storage time. Thereafter, the variable with the highest P-value was removed in each step until all remaining variables were significant. Residuals were plotted, visually inspected, and assessed for normality using Q-Q and P–P plots.

3 | Results

A total of 80 dogs were included. The breeds represented were mixed breed dogs ($n = 11$), Labrador retriever ($n = 5$), boxer ($n = 4$), golden retriever ($n = 4$), and ≤3 individuals of 37 other breeds. The median (IQR) age of all dogs was 6.2 (2.8–9.3) years and the median BW was 19.4 (11.4–25.7) kg. There were 51 females of which 13 were spayed, and 29 males of which 11 were neutered. Urine was obtained by cystocentesis in 63 dogs and by spontaneous voiding in eight. In nine dogs, information regarding urine sampling technique was missing. The CKD group included 50 dogs (16 dogs in CKD stage 1, 25 dogs in stage 2, four dogs in stage 3, and five dogs in stage 4). There were 30 control dogs (C). There were no differences in age, BW, storage time, or sex among groups (Table 1). More detailed information regarding criteria for CKD 1 diagnosis (e.g., structural parenchymal abnormalities/proteinuria), and uCysC/uCr, and uGlu/uCr for all individual dogs in CKD stage 1 are provided in Table S3.

Urinary CysC/Cr increased with IRIS stage and differed among all groups ($p < 0.002$), except between CKD 1 and CKD 2. In CKD stage 1 dogs, six out of 16 (38%) had a uCysC/uCr above the range of the control dogs. Twelve out of 25 (48%) dogs in CKD stage 2 and all (100%) dogs in CKD stage 3 + 4 had an uCysC/uCr above the range of the control dogs.

There was no difference among groups for uGlu/uCr, but uGlu/uCr was above the range of the control dogs in three of 16 dogs in CKD stage 1, four of 25 in CKD stage 2, and three of nine dogs in CKD stage 3 + 4. In six of 10 dogs with uGlu/uCr above the range of the control dogs, glucose was also detected on the dipstick. Urinary Urea/uCr and FE-Urea did not differ among groups. Results from group comparisons are presented in Figure 1 and in Tables S1 and S2.

TABLE 1 | Demographical and clinicopathological variables for included dogs.

	C ($n = 30$)	CKD 1 ($n = 16$)	CKD 2 ($n = 25$)	CKD 3 + 4 ($n = 9$)
Age (years), median (IQR)	4.9 (3–7.8)[a]	6.7 (2.8–8.6)[a]	4.7 (1.8–9.6)[a]	9.8 (6.8–11.2)[a]
Weight (kg), median (IQR)	19.8 (14.4–25.1)[a]	17.1 (7.8–27.6)[a]	20 (10.0–25.4)[a]	20 (6.8–40.7)[a]
Sex (F/FC/M/MC)	17/5/5/3[a]	7/3/3/3[a]	11/4/7/3[a]	3/1/3/2[a]
Storage time, (days), median (IQR)	2159 (2032–2260)[a]	2246 (1938–2636)[a]	2180 (1819–2618)[a]	2383 (1837–2428)[a]
uCrea (umol/L)	15 843 (9752–22 333)[a]	6015 (5028–11 006)[b]	6082 (4052–10 855)[b]	4212 (3483–6051)[b]
Osmolality (mOsmol/kg)	1330 (837–2004)[a]	719 (387–1175)[b]	480 (406–657)[b]	407 (336–481)[b]
USG	1.035 (1.023–1.047)	1.018 (1.011–1.031)	1.015 (1.011–1.019)	1.010 (1.010–1.016)
UPC, median (IQR)	0.06 (0.04–0.11)	1.37 (0.20–5.9)	0.3 (0.09–1.7)	0.97 (0.16–5.33)
sCrea (umol/L), median (IQR)	83.5 (74.8–98.3)	82.0 (65.0–89.5)	173 (144–200)	440 (310–743)

Note: Significant differences ($p < 0.005$) are noted where superscripted letters differ between groups. For USG, UPC, and sCrea comparisons among groups were not performed because these variables were used for group affiliation.
Abbreviations: C, healthy dogs; CKD, chronic kidney disease; F, female; FC, female castrated; M, male; MC, male castrated; sCrea, serum creatinine; uCrea, urine creatinine; UPC, urine protein creatinine ratio; USG, urine specific gravity.

Urinary Na/uCr, uK/uCr, uCl/uCr, uCa/uCr, and uP/uCr were not different among groups, but FE of Na, K, Cl, Ca, and P increased with IRIS stage (Figure 2). FE of Na ($r = 0.40$), K ($r = 0.61$), Cl ($r = 0.48$), Ca ($r = 0.24$), and P ($r = 0.37$) were significantly correlated with sCr. Urinary concentrations of electrolytes, as well as electrolytes normalized with uCr and FE of electrolytes, are provided in Table S2.

In the univariable linear regression analyses performed to evaluate the effect of storage, uUrea, and uP had a $p > 0.25$, and were, therefore, not included in the multiple regression model. For uCysC and uGlu, a linear association could not be established because the majority of samples had unmeasurable or low concentrations, respectively. Consequently, stepwise backward multiple regression models were run for uNa, uK, uCl, uCrea, and

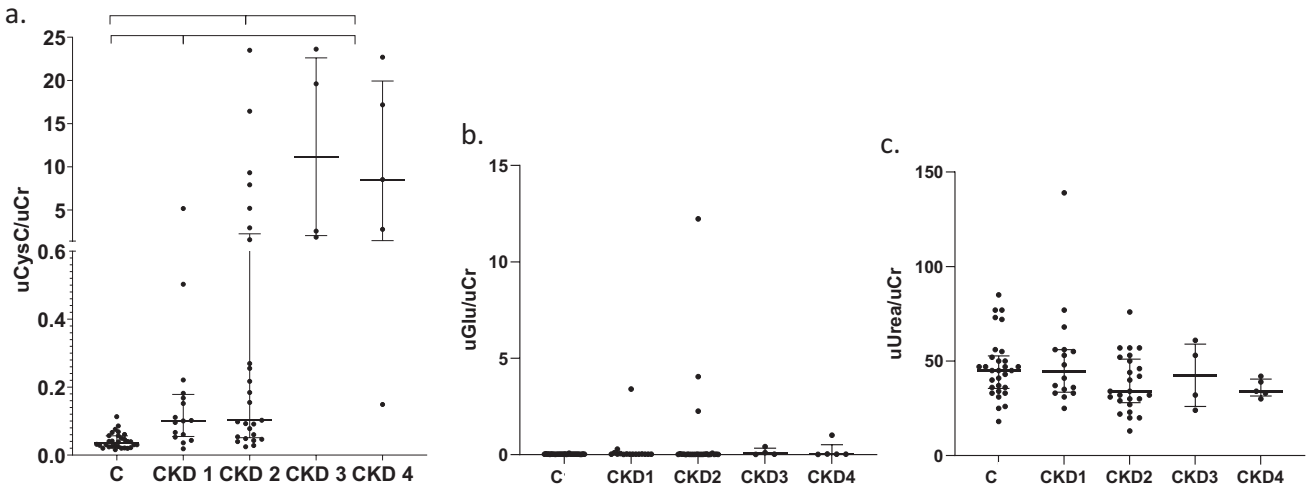

FIGURE 1 | (a–c): Urinary analyte/creatinine ratios for (a) uCysC (10^{-3}), (b) uGlu, and (c) uUrea, in control- and CKD stage 1–4 groups. Urinary CysC/uCr at low levels, showing the difference between C and CKD 1. Significant differences (<0.005) between groups are marked with bars. The groups of dogs with CKD stage 3 and 4 were combined for statistical analyses, due to their small sizes. The median and IQR is showed by the horizontal lines. C, healthy dogs; CKD 1–4, chronic kidney disease IRIS stage 1–4; uCr, urine creatinine; uCysC, urine cystatin C; uGlu, urine glucose.

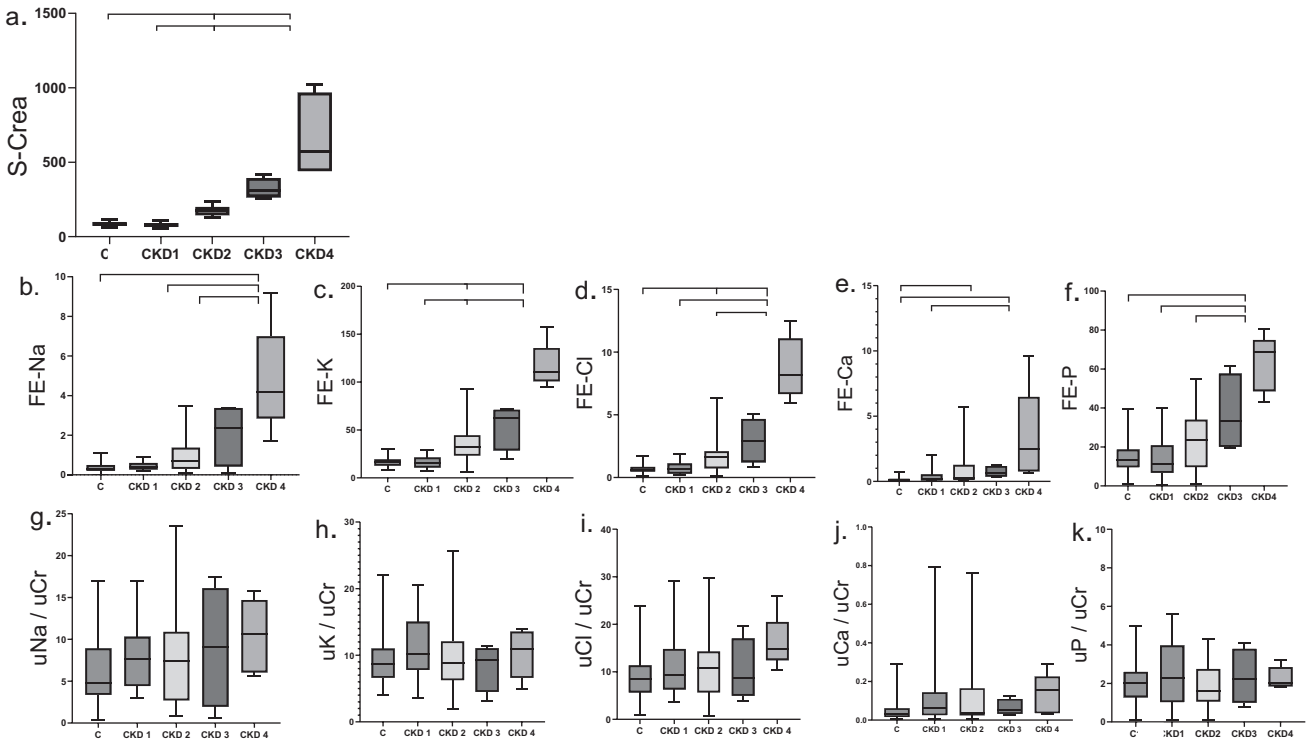

FIGURE 2 | Results of (a) serum creatinine, (b–f) fractional excretion (FE) of electrolytes, and (g–k) urinary electrolytes/uCr-ratio for all dogs. There was an association between FE-electrolytes and sCrea (sCr). The groups of dogs with CKD stage 3 and 4 were combined for statistical analyses, due to their small sizes. Significant differences ($p < 0.005$) between groups (a–k) are marked with bars. C, healthy dogs; Ca, calcium; CKD 1–4, chronic kidney disease IRIS stage 1–4; Cl, chloride; Cr, creatinine; FE, fractional excretion; K, potassium; Na, sodium; P, phosphate; u, urinary.

uCa. Storage time was not retained in the final model for any of these urinary analytes. Group affiliation was the only independent predictor of uNa, uK, and uCl. Group affiliation and age were independent predictors of uCrea. For uCa, no significant model could be obtained.

4 | Discussion

This study compared urinary CysC, Glu, urea, and electrolytes normalized to uCr, and FE of electrolytes in dogs with different stages of CKD and control dogs. Urinary CysC/uCr increased with IRIS stage, and uCysC/uCr was significantly higher in CKD stage 1 dogs than in control dogs ($p = 0.0002$). FE of Na, K, Cl, Ca, and P increased with IRIS stage.

In dogs with CKD stage 1, 38% had a uCysC/uCr above the range of the control dogs, and this number increased with IRIS stage to 100% in stage 3+4 dogs. In dogs with highly diluted urine, mildly elevated levels of uCysC might be overlooked when assessing uCysC concentration (without normalization). In such cases, the normalized value is of use for detection of increased uCysC. In the present study, many dogs in the healthy control group and CKD 1 and 2 had uCysC concentration below the measuring range (0.1 mg/L), which were set to half this value for statistical analyses. In these dogs, differences in uCysC/uCr values were solely the result of differences in uCr concentration. In order to avoid this in the future, methods with lower measuring ranges are warranted.

Sixteen of the 27 CKD dogs with increased uCysC/uCr in this study also had proteinuria (UPC > 0.5). Urinary Alb and CysC compete for the same receptors on the luminal face of the tubular cells, and a competitive inhibition of the uCysC reabsorption might occur, especially if the degree of albuminuria is severe [12, 24]. Eleven CKD dogs had an elevated uCysC/uCr without proteinuria, and in these dogs uCysC/uCr contributed new information about tubular injury. Because uCysC is stable during transport [16], easily analyzed using biochemistry analyzers, high in urine from dogs with tubular injury, and low in urine from control dogs, it might represent a clinically useful urinary biomarker.

Stage 1 CKD is diagnosed when morphological or functional abnormalities of the kidney are present and, therefore, some dogs in CKD stage 1 are suspected to have active tubular epithelial cell injury or decreased tubular function, or both, and others do not. Of particular interest are increased tubular markers in the dogs without proteinuria or azotemia, because the diagnosis of CKD 1 in nonproteinuric dogs is currently challenging, and in this scenario uCysC might be of help as a diagnostic tool.

Fractional excretion of Na, K, Cl, Ca, and P increased with IRIS stage. This is in accordance with results from two other studies that showed higher FE-Na, FE-K, FE-Cl, and FE-P in dogs with advanced CKD compared to dogs with less severe CKD [18, 19]. In contrast, the urinary concentration of all electrolytes decreased with IRIS stage. This decrease in concentration is probably caused by urine dilution because when electrolytes were normalized to uCr, this pattern was lost (Figure 2). In dogs with azotemia in the present study, sCr seemed to dominate the FE formula and thereby the calculated FE*x* (FE of analyte) results. The calculated value for FE might, therefore, reflect primarily the sCr concentration and not the actual FE of the electrolyte. One study in people investigated the relationship between FE*x* and GFR in patients with CKD and AKI. They concluded that a decrease in estimated GFR (eGFR) had a distinct impact on FE*x* and that calculated FE of electrolytes increased progressively along with the decline of eGFR in both CKD and AKI [25]. This is, to our knowledge, not evaluated prospectively in dogs.

Another important factor to consider when interpreting FE is biological variation. In a study of healthy dogs, intraindividual variation in FE for Cl, K, Ca, and P ranged from 24% to 33%, while sodium (Na) exhibited a significantly higher variation of 61% [21]. Currently, no studies have investigated whether the extent of intraindividual variation of electrolytes changes with kidney disease. However, in the present study, any influence of biological variation is likely overshadowed by the prominent role of sCr in the FE formula when applied to azotemic dogs.

A potential limitation of this study is the storage time. Urine samples from included dogs were stored at −80°C for up to 9 years, but there was no difference in storage time among groups. Long-term storage of urine samples at −80°C is common practice in human research for preservation of urine metabolites, and many urine metabolites are considered stable at −80°C [26–28]. The stability will depend on handling, storage conditions, and nature of the specific analyte [29, 30]. Urinary Crea, Urea, Na, Cl, K, Ca, and P are stable in human urine at −22°C for more than 12 years [28]. Also, uProt was studied for 2.5 years at −70°C and was stable during this time [31]. The stability of these analytes is expected to be similar in canine urine. For uGlu, no long-time stability data was found. The concentration of CysC in canine urine was studied for 3 months at −80°C and showed stability during this time [16].

In conclusion, uCysC/uCr was significantly higher in dogs with CKD stage 1 than in the control dogs and might be a potential marker of early CKD, preferably as part of a diagnostic panel of urinary markers. FE of Na, K, Cl, Ca, and P increased with IRIS stage, but sCr had a dominant impact in the formula, and it is advised to interpret calculations of FE of analytes in azotemic dogs with caution.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Approved by Uppsala Ethics committee (C340/11, C12/15, 5.2.18–13 750/2019). 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.