Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:80
Faculty of Veterinary Medicine and Animal Science

Urinary biomarkers in healthy dogs and dogs with kidney disease

Anna Selin

Urinary biomarkers in healthy dogs and dogs with kidney disease

Anna Selin
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Uppsala 2025

Acta Universitatis Agriculturae Sueciae
2025: 80

Cover: Picture published with permission from creator: dreamstime.com

ISSN 1652-6880
ISBN (print version) 978-91-8124-064-1
ISBN (electronic version) 978-91-8124-110-5
https://doi.org/10.54612/a.67l6lar7q3

Swedish University of Agricultural Sciences, Department of Clinical Sciences, Uppsala, Sweden
Print: SLU Grafisk service, Uppsala 2025

Urinary biomarkers in healthy dogs and dogs with kidney disease

Abstract

Urinary analytes can act as biomarkers and indicate renal tubular injury before elevations in traditional markers of decreased glomerular filtration rate, such as serum creatinine and SDMA (symmetric dimethyl arginine), occur. Acute kidney injury (AKI) in dogs has a mortality rate of up to 50%, and early detection may allow treatment before irreversible damage. Chronic kidney disease (CKD) is often diagnosed late, but early-stage diagnosis enables interventions that may slow progression. Knowledge about urinary biomarkers in healthy dogs, dogs in various stages of CKD, and grades of AKI, is needed.

Overall aim of this thesis was to study biological variation and reference intervals of urinary analytes in healthy dogs (Study I), investigate selected urinary analytes in dogs at various stages of CKD (Study II) and in dogs with, or at risk for, AKI (Study III). Healthy privately owned dogs and dogs presented to referral veterinary hospitals in Uppsala and Stockholm were included. Studied urinary analytes were urinary cystatin C (uCysC), gamma-glutamyl transferase (uGGT), N-acetyl-β-D-glucosaminidase (uNAG), glucose, urea, and electrolytes, all measurable with standard biochemical analyzers. Urinary analytes were normalized to urinary creatinine (uCr), and fractional excretion (FE) of electrolytes calculated.

The results provide data on biological variation and reference intervals (Study I). Urinary CysC/uCr increased with IRIS stage, and uCysC/uCr and uGGT/uCr were higher in dogs with early-stage CKD compared to healthy dogs (Study II). In Study III, uCysC/uCr, uGlu/uCr, and uGGT/uCr were markedly increased in the dogs with clinically apparent AKI. These analytes were also higher in the groups with critically ill and snake envenomated dogs compared to healthy dogs, suggesting non-azotemic tubular injury. In conclusion, uCysC/uCr, uGlu/uCr, and uGGT/uCr show promise as early markers of AKI and CKD in dogs.

Keywords: AKI, biological variation, canine, CKD, cystatin C, gamma-glutamyl transferas (GGT), glucose, international renal interest society (IRIS), N-acetyl-β-D-glucosaminidase (NAG)

Authors address: Anna Selin, SLU, Department of Clinical Sciences, P.O. Box 7054, SE-750 07, Uppsala, Sweden. *E-mail:* anna.selin@slu.se

Urinbiomarkörer hos friska hundar och hundar med njursjukdom

Sammanfattning

Urinanalyter, såsom enzymer och proteiner kan fungera som biomarkörer och indikera njurskada, till exempel proximal tubuliskada. Detta sker ofta innan stegring av traditionella markörer för nedsatt glomerulär filtrationshastighet, såsom serum kreatinin och symmetric dimethyl arginine (SDMA), ses. Akut njurskada (AKI) hos hund har en mortalitet på upp till 50 %, där tidig diagnostik kan möjliggöra behandling innan irreversibel skada inträffar. Kronisk njursjukdom (CKD) upptäcks ofta i ett sent skede, men tidig diagnos kan möjliggöra åtgärder som bromsar sjukdomsprogressionen. Det behövs mer kunskap om olika biomarkörer i urinen hos friska hundar, hundar med AKI, samt hundar i olika stadier av CKD.

Syftet med denna avhandling var att undersöka biologisk variation hos inkluderade urinanalyter hos friska hundar (studie I) och att studera utvalda analyter, och dess potential som tidiga diagnostiska markörer, hos hundar i olika stadier av CKD (studie II) samt hundar med, eller med risk för att utveckla, AKI (studie III). De urinanalyter som studerades var cystatin C (uCysC), gamma-glutamyl-transferas (uGGT), N-acetyl-β-D-glukosaminidas (uNAG), glukos, urea och elektrolyter. Analyserna genomfördes med ett automatiserat biokemiinstrument, som används rutinmässigt vid veterinärkliniker. Urinanalyterna normaliserades genom att kvota med urinkoncentrationen av kreatinin (uCr), och fraktionell exkretion (FE) av elektrolyter beräknades. Studierna inkluderade friska privatägda hundar och hundar remitterade till djursjukhus i Uppsala och Stockholm.

Sammanfattningsvis bidrar resultaten med data avseende biologisk variation och referensintervall (studie I). Urin CysC/uCr, uGlu/uCr, och uGGT/uCr förefaller lovande som tidiga markörer för AKI och CKD

Nyckelord: AKI, biologisk variation, CKD, cystatin C (CysC), gamma-glutamyl transferas (GGT), N-acetyl-β-D-glucosaminidase (NAG), glukos (Glu), International Renal Interest Society (IRIS), urea

Författarens adress: Anna Selin, Institutionen för kliniska vetenskaper, Sveriges lantbruksuniversitet, SE-75007 Uppsala, Sverige. *E-post:* Anna.Selin@slu.se, Anna.Selin@anicura.se

Dedication

To my family

“If the kindest souls were rewarded with the longest lives,
dogs would outlive us all”

R. Gervais

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Selin AK, Lilliehöök I, Forkman J, Larsson A, Pelander L, Strage E (2023) Biological variation of biochemical urine and serum analytes in healthy dogs. *Veterinary Clinical Pathology*, 52 (3), pp 461-474. DOI: 10.1111/vcp.13225

II. Anna K. Selin, Inger Lilliehöök, Emma M. Strage, Anders Larsson, Lena Pelander (2025). Urinary cystatin C, glucose, urea and electrolytes in dogs of different stages of chronic kidney disease. *Journal of Veterinary Internal Medicine*, 39 (3), DOI: 10.1111/jvim.70090

III. Anna K. Selin, Inger Lilliehöök, Emma M. Strage, Anders Larsson, Lena Pelander. Longitudinal study of urinary cystatin C, gamma-glutamyl-transferase, N-acetyl-β glucosaminidase, and glucose in dogs vipera berus intoxication, critical illness and acute kidney injury. Manuscript.

The contribution of Anna Selin to the papers included in this thesis was as follows:

I. Anna Selin collected all data, performed parts of and assisted in all the laboratory analyses, and drafted the manuscript. Anna Selin performed and prepared initial parts of statistical analyses, while a statistician performed the advanced parts.

II. Anna Selin collected part of the data, performed or assisted in performing all laboratory analyses and drafted the manuscript. Anna Selin performed the statistical analyses together with co-authors and in consultation with a statistician.

III. Anna Selin collected the data, performed or assisted in performing all the laboratory analyses, performed statistical analysis in consultation with a statistician and co-authors, and drafted the manuscript.

List of tables

List of figures

Abbreviations

AKI	acute kidney injury
AKI_{APP}	apparent acute kidney injury
Alb	albumin
CKD	chronic kidney disease
CRP	C-reactive protein
CysC	cystatin C
EVB	group of dogs envenomated by Vipera Berus
FE	fractional excretion
GFR	glomerular filtration rate
Glu	glucose
IC	group of dogs in need of intensive care, without clinically apparent AKI
IRIS	International Renal Interest Society
RI	reference interval
sCysC	serum cystatin C
uCa	urinary calcium
uCl	urinary chloride

uCr	urinary creatinine
uCysC	urinary cystatin C
uGGT	urinary gamma-glutamyl transferase
uK	urinary potassium
uNa	urinary sodium
uNAG	urinary N-acetyl-β glucosaminidase
uP	urinary phosphate
UPC	urine protein:creatinine ratio
uProt	urinary protein

1. Introduction

1.1 Background

Acute kidney injury (AKI) and chronic kidney disease (CKD) are most commonly diagnosed using circulating indirect markers of decreased glomerular filtration rate (GFR) such as serum creatinine concentration or symmetric dimethyl arginine (SDMA). These serum markers do not detect early disease when the kidney pathology manifests as tubulointerstitial injury or dysfunction without global decrease of kidney function. Many of the conventional diagnostics are also not specific for renal dysfunction or damage, and two examples are pre- and post-renal influences on both GFR and proteinuria (Harris and Gill, 1981). Sensitive and specific markers that would allow early detection of tubular injury are needed.

Studies of urinary biomarkers have increased in numbers in veterinary medicine over recent years. Novel urinary biomarkers, including low molecular weight proteins and tubular enzymes, may serve as early indicators of acute or chronic kidney damage or dysfunction, before changes in GFR occur (Nabity and Hokamp, 2023). In addition, some urinary markers might determine location of injury (for example urinary IgG, CRP, and Podocin for glomerular location, urinary GGT, cystatin B and C for proximal tubules, and Tamm Horsefall protein for Loop of Henle or distal tubules injury or dysfunction) (Hokamp et al., 2018; Grauer et al., 1995). Urine is also easy and non-invasive to collect; this enables sampling at home by animal owners.

Because several factors such as diet, water intake and exercise can affect dilution of urine, urinary biomarkers are often normalized. This can be achieved either through normalization with urinary creatinine, with urine specific gravity, or with urine osmolality. Normalization with urinary creatinine is most commonly performed. Renal excretion of creatinine is however not always constant, especially when the kidneys are acutely injured (Waikar et al., 2010). Another method for assessment of renal handling of an analyte is fractional excretion (FE), were the amount of an analyte in serum concentration is compared to the amount in urine, using a specific formula (Pressler, 2013).

To make correct interpretations of urinary biomarkers in diseased dogs, it is important to have reference data of healthy dogs and knowledge about

biological variation (BV) of these analytes. Biological variation refers to the random fluctuations of an analyte around a homeostatic set point, expressed as the coefficient of variation (CV) (Fraser, 2001). It includes within-individual (CV_I) and between-individual (CV_G) variation, as well as analytical variation (CV_A), which reflects method-related variability. These components are used to calculate the reference change value (RCV), indicating whether a change is significant, and the index of individuality (II), which is used to evaluate whether population-based reference intervals or RCVs should be used for interpretation of results (Fraser, 2001; Freeman et al., 2017).

This thesis examined biological variation and reference intervals in healthy dogs for 11 urinary analytes, and studied selected analytes (urinary cystatin C, gamma-glutamyl transpeptidase, N-acetyl-β-D-glucosaminidase, glucose, urea, and electrolytes) in dogs at various stages of CKD, and dogs with AKI or at risk of AKI.

Figure 1. Bim, Kiwi, and No, three of the healthy dogs that were included in Study I (Biological variation). Photo: Anna Selin

1.1.1 The ideal biomarker

A biomarker is a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”(Califf, 2018). An important characteristic of an ideal biomarker is easy sample collection. Compared to histopathologic examination, which involves anaesthesia and invasive collection of biopsies, obtaining a urine sample is an easier and less costly option. An ideal biomarker should be stable during transport and storage, easy to analyze and results easy to interpret. It would remain unaffected by other physiological processes; however, this is seldom the case, making it essential to understand which processes that influence test results (Block, 2024; Monaghan, 2021). In veterinary medicine, several biomarkers are routinely used for diagnosing renal disease, but there is still a need for diagnostic tools for early diagnosis of kidney injury or disease. For this, urinary biomarkers might be useful.

1.2 The kidney, urine production and renal disorders

1.2.1 Physiology of healthy kidneys

The primary function of the kidneys is to eliminate unwanted substances from the body. This occurs through glomerular filtration, or by active tubular secretion into the urine, or both. Examples of unwanted substances are metabolic waste products such as potassium, phosphate and degraded drugs or toxins. In addition, the kidneys play a vital role in maintaining fluid, electrolyte, and acid-base balance (DiBartola, 2012). The kidneys also have an important endocrine function, for example erythropoietin production, and in the conversion of the inactive form of vitamin D - 25-hydroxyvitamin D, into its active form, calcitriol (DiBartola, 2012; Reece, 2015). Moreover, the juxtaglomerular cells of the kidneys, support regulation of blood pressure through the release of renin in response to reduced blood (Reece, 2015)

The functional unit of the kidney is the nephron, and the kidney contains several hundred thousands of nephrons. Every nephron is composed of a glomerulus and a tubular system (Figure 2.). The tissue between the nephrons, the renal interstitium, and associated vasa recta, provide structural support, produce the extracellular matrix, and is crucial for transport and storage of water and solutes (Lemley and Kriz, 1991).

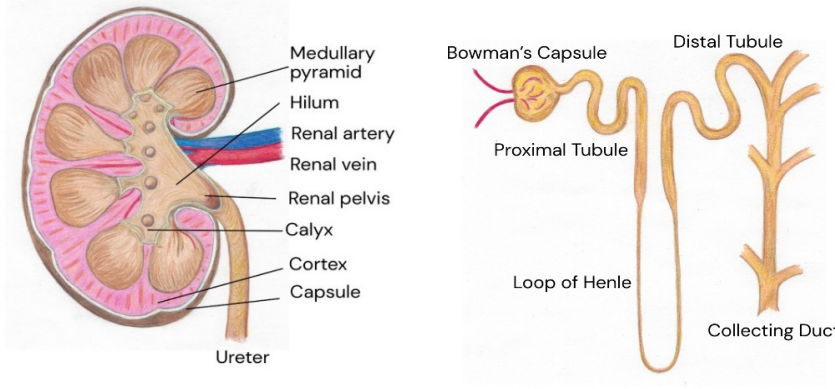


Figure 2. The anatomy of the kidney (to the left) and nephron (to the right). Illustration: Malin Vilhelmsson.

Approximately 20% of cardiac output is received by the kidneys. The blood goes via the renal arteries and into the kidneys and through the glomerulus. Glomerular plasma flow and pressure are narrowly regulated by the afferent and efferent arterioles (Deen et al., 2001). The passage into the filtrate is determined by molecular size and ionic charge. Small molecules pass freely, but with increasing size to around 60-70 kDa substances are retained with increasing efficiency. Albumin with a molecular weight of 69kDa is normally largely excluded from the filtrate. Filtrated plasma, also known as ultrafiltrate or primary urine, enters Bowman's capsule, which is the beginning of the renal tubular system. The glomerular filtration rate, GFR, represents the volume of ultrafiltrate produced by all nephrons per unit of time (DiBartola, 2012).

1.2.2 Proximal tubuli. Active transport and diffusion

Primary urine or ultrafiltrate is transformed into final urine through processes of reabsorption and secretion along the nephron. In the proximal tubuli, about 66-76% of fluid and solutes are reabsorbed into the blood. Proximal tubular cells have microvilli that increase the surface area for reabsorption and osmosis, and diffusion are the main mechanisms here, which limits energy-consuming transport (Sand, 2004; Reece, 2015). Glucose and amino acids are almost completely reabsorbed in proximal tubuli (Silbernagl, 1988). Limited amounts of albumin, and small proteins can pass through healthy glomeruli in small quantities. These are then reabsorbed by the proximal

tubular cells via megalin-mediated pinocytosis (Lazzara and Deen, 2007). Sodium reabsorption is driven by Na^+/K^+-ATPase pumps on the basolateral membrane towards the interstitial side, which eject sodium and import potassium, maintaining a low intracellular sodium concentration. Potassium diffuses out, creating a negative charge that facilitates sodium entry from the lumen. This mechanism supports efficient sodium uptake (Sand, 2004; Reece, 2015). Sodium and transport of solutes raises interstitial osmolality, drawing water from the lumen into the interstitium and then into peritubular capillaries. The loop of Henle and distal tubule create large gradients that, together with antidiuretic hormone and urea, enable water reabsorption in the collecting ducts, resulting in concentration of urine (Reece, 2015; Sand, 2004).

1.2.3 Acute kidney injury in dogs

Acute kidney injury in dogs can have many different etiologies, such as ischemia, intoxication, inflammation, infections, and congenital or familiar diseases (Thoen and Kerl, 2011). Causes of AKI can be divided into hemodynamic (pre-renal), parenchymal (intrinsic) renal injury and post-renal causes (Ettinger, 2024). The mortality of AKI in dogs is as high as 50% (Vaden et al., 1997; Segev et al., 2008; Legatti et al., 2018; Ross, 2011) but early diagnosis of AKI might improve survival by facilitating therapeutic intervention before renal parenchymal lesions become irreversible (Himmelfarb, 2008 ; Cobrin et al., 2013). There are various classification schemes of acute kidney injury available in human and veterinary medicine. All systems highlight the potential for AKI in non-azotaemic animals. A useful grading system for dogs is the IRIS-AKI grading system **(Table 1).**

Table 1. International Renal Interest Society grading of AKI in dogs.

AKI GRADE	SERUM CREATININE	CLINICAL DESCRIPTION
GRADE I	<140 µmol/l	Nonazotemic AKI a. Documented b. Progressive, nonazotemic increase in blood creatinine c. Measured oliguri
GRADE II	141-220 µmol/l	Mild AKI: a. Documented d. Progressive azotemic c. Measured oliguri
GRADE III	221-439 µmol/l	Moderate AKI
GRADE IV	440-880 µmol/l	Moderate to severe AKI
GRADE V	>880 µmol/l	Severe AKI

Table 2. International Renal Interest Society staging of CKD in dogs.

CKD STAGE	SERUM CREATININE SDMA	COMMENTS
STAGE I	<125 µmol/l <1.4 mg/dl	Normal blood creatinine, normal or mild increase SDMA, some other abnormality present such as inadequate urinary concentration ability, proteinuri, or renal imaging findings)
STAGE II	125-250 µmol/l 1.4-2.8 mg/dl	Normal or mildly increased creatinine, and SDMA. Clinical signs usually mild or absent.
STAGE III	251-440 µmol/l 2.9-5.0 mg/dl	Moderate renal azotemia.
STAGE IV	>440 >5.0 mg/dl	Increasing risk of systemic clinical signs and uremic crises

1.2.4 Chronic kidney disease in dogs

Chronic kidney disease is often regarded a result of repeated, smaller insults to the kidney (Nenov et al., 2000), or a result of genetic pathologies. However, one major injury may also lead to development of CKD (Venkatachalam et al., 2010). Consequently, AKI may result in CKD, but dogs with stable CKD may also experience an acute decrease in kidney function (acute on chronic kidney disease [ACKD]) (Dunaevich et al., 2020). The pathogenesis, clinical presentation and laboratory abnormalities of ACKD may resemble those of AKI, which sometimes makes differentiation between AKI and ACKD challenging (Dunaevich et al., 2020). A close connection between AKI and CKD has been proposed in both human and veterinary medicine. In that it has been suggested that mechanisms of pathogenesis may be shared between the two. If this is true, CKD may be thought of as a slow (or of variable pace) progressing, ongoing, kidney injury (Cowgill et al., 2023). The current definition of CKD in dogs is "presence of functional or structural damage to one or both kidneys with a duration of more than 3 months"(Polzin, 2011). Staging of CKD can be performed through the IRIS CKD staging schedule (**Table 2**).

A method for early diagnosis of CKD would be of value for the work up of dogs that are presented with clinical signs such as chronic unexplained polyuria and polydipsia (PU/PD), or recurrent infection of the urinary tract. In both scenarios, early CKD is a plausible differential diagnosis that often is difficult to diagnose or rule out. In addition, dog breeds with inherited renal diseases without overt proteinuria, such as renal dysplasia, will also benefit from a method for early diagnosis (Polzin, 2011).

1.3 Urine as a diagnostic fluid

1.3.1 Urine collection and handling

The timing and method of urine collection can significantly affect urinalysis (Callens and Bartges, 2015; Piech and Wycislo, 2019). Samples may be obtained at home by free catch or in a clinic by free catch, cystocentesis, or catheterization. Stress during hospital visits can alter results, one study found higher urine protein-to-creatinine (UPC) ratios in hospital samples than in those collected at home (Duffy et al., 2015; Citron et al., 2020). Spontaneously voided urine samples taken at home risk contamination from

environmental debris, and random containers might harbor constituents from previous use that can affect urine analyses. Urine collection in the clinic means rapid access to the laboratory and, when performed by cystocentesis, also avoids bacterial contamination from genital secretions or skin. However, urine sampling in the hospital may, besides stress, be affected by dilution from intravenous fluids, or mild hematuria caused by cystocentesis. In conclusion, knowledge about how different handling, storage conditions before analysis, and eventual transport to external laboratories can affect urine analytes is essential for both clinical practice and research.

Figure 3. Disposable Uripet, used for urine collection.

1.3.2 Urinalysis

Urinalysis can offer valuable insight into kidney function, including the ability to concentrate urine, retain essential substances, and detect signs of infection, endocrine disease (cortisol, catecholamines) (Stockham, 2025), as well as tumors in the urinary tract (Grassinger et al., 2019). Urine specific gravity (USG) measures the ratio of the weight of a urine sample to an equal volume of distilled water at a specific temperature, reflecting urine concentration. It is measured with a refractometer on fresh, well-mixed urine and sometimes confused with density, although specific gravity is a dimensionless ratio and density is mass per volume (for example kg/m^3). Glucosuria or proteinuria can slightly raise USG; a glucose concentration of 56 mmol/l or protein concentration of 10 g/L adds up to 0.005 to USG (Stockham, 2025). Glucosuria may also cause osmotic diuresis affecting urine dilution. Urine specific gravity is a key parameter when evaluating patients with suspected kidney injury or disease. In a predictive model of CKD in dogs, USG was one of 6 parameters and, among other results, a USG over 1.030 was associated with a reduced risk for development of CKD in the dogs in that study (Kokkinos et al., 2022).

Urine reagent test strips are the primary method for chemical urine analysis. Most strips are designed for humans but suitable for veterinary use, with exceptions for nitrite, specific gravity, and leukocyte pads. Urine reagent test strips reliably detect glucose at concentrations above 2.8 mmol/L (50 mg/dL) (Zeugswetter and Schwendenwein, 2020). False results may arise from ascorbic acid, cleaning agents, or improper test strip storage. Pigmented urine, certain drugs (e.g., captopril, cysteine), delayed testing, or improper storage can also affect results (Abebayehu, 2023).

Urine reagent test strips detect protein ≥300 mg/L, mainly albumin, and are less sensitive to globulins and other proteins. Results depend on urine concentration: small, detected amounts in dilute urine are more significant than if detected in concentrated urine, and negative dipstick results in dilute urine do not exclude proteinuria. Heavily pigmented or discolored urine may cause false positives (Duncan and Prasse, 1976). Positive dipstick results, especially in urine with low USG, should be confirmed with uProt/uCr (UPC).(Zatelli et al., 2010; Lees et al., 2005) Electrophoresis can be performed in the diagnostics of confirmed proteinuria, and be of help to identify glomerular- or tubular damage (Hokamp et al., 2018).

Microscopic urine sediment analysis provides valuable information on urinary tract disease and should be performed no later than 30 minutes after urine collection in order to detect casts.(Piech and Wycislo, 2019) Casts form in renal tubules from Tamm-Horsfall protein secreted by tubular epithelial cells and may incorporate intact cells or debris. They are classified as hyaline, granular, waxy, epithelial, erythrocyte, or leukocyte casts. Normally, only occasional hyaline or granular casts are observed per low-power field (100×). Increased numbers, or casts containing cells, often indicate tubular damage or inflammation, while transient increases, especially of hyaline casts, can occur after blood pressure changes or physical exertion (Piech and Wycislo, 2019). Erythrocytes and leukocytes in urine are normally few (0–2 per high-power field, 400×), with higher counts indicating hematuria or pyuria; however, sample collection methods can affect results (Smee et al., 2013).

1.4 Normalization and fractional excretion

1.4.1 Normalization

Standard practice for reporting urinary biomarkers is to present analytes either as absolute concentrations or normalized to urinary creatinine. Both approaches have their considerations. In patients with kidney disease, or polyuria from non-kidney origin, increases in analyte concentration may be missed because of urine dilution (Keen et al., 2022; Zatelli et al., 2010). Although, there are disadvantages with normalization to uCr. Normalizing single spot urinary biomarkers to creatinine in chronic kidney disease (CKD) may be appropriate if the patient is stable at a given stage. However, this is questioned in AKI because urinary creatinine excretion can fluctuate as the acutely injured kidney attempts to restore homeostasis (Waikar et al., 2010).

Urine specific gravity (USG) offers an alternative normalization method (Newman et al., 2000), and osmolality is a third option (Balar et al., 2023). Osmolality measures the total solute concentration in urine (e.g., sodium, chloride, glucose, urea) using an osmometer. Compared to USG, osmolality is more precise presenting the solute concentration of a solution, while USG provides an approximate value of the urine solute concentration (Stockham, 2025). Careful consideration of the biomarker's properties and the clinical scenario is crucial when choosing a normalization method. Further research is needed to clarify the advantages and limitations of each approach and to establish optimal strategies for different biomarkers and clinical situations.

1.4.2 Fractional excretion (FE)

Fractional excretion (FE) is the percentage of a filtered solute excreted in urine. Precise determination requires a complete 24-hour urine collection, but FE is more commonly estimated from a single spot urine sample using the following equation:

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

Non–protein-bound electrolytes are freely filtered at the glomerulus and reabsorbed by the tubules. Solutes such as amino acids and glucose are reabsorbed almost completely (>99%), yielding an FE <1 % in health. Sodium and potassium are about two-thirds reabsorbed in the proximal tubule and loop of Henle, with the remainder excreted or reclaimed under hormonal control (e.g., aldosterone). Fractional excretion values are influenced by numerous factors such as age, breed, diet, food intake, exercise, ultrafiltration rate, solute and volume status, renal function, and drug administration (Lefebvre et al., 2008). Spot-sample FE provides only an approximate reflection of 24-hour electrolyte excretion because creatinine is an imperfect GFR marker and urine electrolyte concentrations fluctuate with diet and circadian rhythm (Lefebvre et al., 2008). There are no clearly defined reference intervals of FE of electrolytes in dogs, but there are reported values for healthy control dogs (Ettinger, 2024). Fractional excretion of electrolytes is rarely used clinically in veterinary medicine because of a marked inter- and intra- patient variability (Ettinger, 2024). When quantification of FE of electrolytes is being considered, it has been suggested that a standardized diet be fed for a minimum of 1 week and that the dog has been normally hydrated for several days before samples for analyses are taken (Pressler, 2013).

Fractional excretion of most electrolytes increases as GFR decreases which severely limits the value of this test in clinical patients with renal dysfunction. Fractional excretions are relevant to many clinical situations, especially in electrolyte disorders and AKI in dogs (Brown et al., 2015; Troia et al., 2018).

1.5 Renal Biomarkers

1.5.1 Traditional markers in serum and urine.

Serum markers, that is indirect GFR markers

Serum creatinine, produced from muscle creatine and creatine phosphate, enters the blood at a steady rate and is eliminated almost entirely by glomerular filtration with negligible tubular handling, making it a useful GFR marker. Serum creatinine has shown an exponential relationship to GFR (Finco et al., 1995). However, like all indirect GFR measures, it is influenced by pre- and post-renal factors (Braun et al., 2003). Because

produced from muscle creatine, muscle mass also affect creatinine concentrations, and age and breed (e.g. Greyhounds) (Braun et al., 2003).

Urea, is produced from ammonia by the liver, filtered through the glomerulus but can be reabsorbed within tubules as a response for the body to correct dehydration or hypovolemia. Serum levels of urea can also an affect from high dietary protein or gastrointestinal bleeding (Prause and Grauer, 1998).

Symmetric dimethylarginine (SDMA), released during protein metabolism and cleared by glomerular filtration, performs similarly to creatinine but is unaffected by muscle mass, though still influenced by pre- and post-renal factors (Pelander et al., 2019; Hokamp and Nabity, 2016).

Serum cystatin C, a 13 kDa proteinase inhibitor produced at a constant rate, is freely filtered and then reabsorbed and degraded in the proximal tubule (Ghys et al., 2014). In human medicine it is a routine renal marker and often superior to creatinine, but levels can be altered by inflammation, diabetes, obesity, age, sex, muscle mass, and thyroid disease (Knight et al., 2004). In dogs, studies have not shown consistent superiority over creatinine or SDMA, and age-related increases have been reported (Ghys et al., 2014; Pelander et al., 2019).

Traditional urinary markers

The urinary biomarkers commonly used in veterinary medicine are UPC and to a lesser extent urinary albumin. Proteinuria can be pre-renal (for example from circulating immunoglobulins, such as Bence Jones proteinuria in multiple myeloma), renal, and post renal (urinary tract infections, inflammation, and hemorrhage) (Harley and Langston, 2012). Hypertension can be both a cause to proteinuria and a complication of kidney damage or disease. Also, strenuous exercise, stress, pyrexia and biological variation can affect urine protein concentration (Couture et al., 2025; Grauer, 2011). Renal proteinuria arises from impaired glomerular filtration selectivity or reduced tubular resorption of filtered proteins. Albumin is the predominant urinary protein in dogs but globulins, and other proteins can also be components of the total UPC. It is important to identify and treat proteinuria, since ongoing proteinuria is associated with tubuli toxicity and damage (from endoplasmic reticulum overload), progression and mortality, with greater proteinuria linked to higher risk (Jacob et al., 2005).

1.5.1 Selected urinary biomarkers of renal disease

Some urinary biomarkers can indicate kidney injury which otherwise might have been difficult to detect. Increased urinary biomarker can result from: 1, Leakage through damaged glomeruli (e.g., albumin, CRP, IgG). 2, Increased release from injured tubular epithelial cells (e.g., ALP, clusterin, GGT, NAG, NGAL, KIM-1). 3, Reduced reabsorption due to tubular dysfunction (e.g., cystatin C, glucose, sodium, urea, albumin). 4, Inflammation and fibrosis (TGF-β1, KIM-1) (Cobrin et al., 2013; Nabity and Hokamp, 2023). **Figure 4** shows an overview of urinary biomarkers. Many of these markers have mainly been used in research studies with methods that are not available in clinical settings.

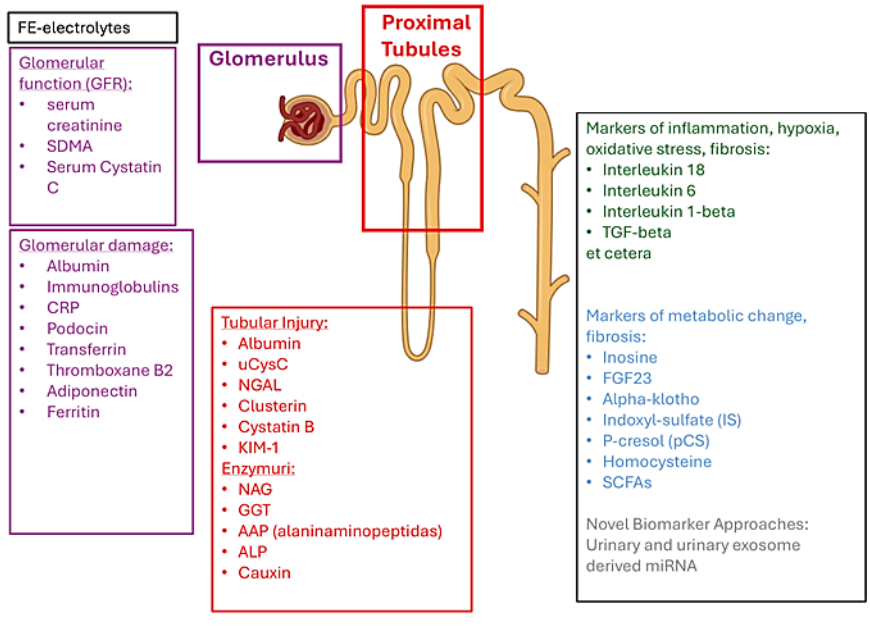


Figure 4. Biomarkers for renal disease. Created by the author with Biorender.com. (Reference; Nabity and Hokamp, Vet Clin North Am Small Anim Pract 2023, Ettinger 9th ed.)

1.5.2 Urinary cystatin C

Cystatin C is a low molecule weight protein produced by most cells in the body and freely filtered in glomerulus (Grubb, 2000). Filtered uCysC is reabsorbed by a megalin-facilitated endocytosis in the proximal tubules and catabolized (Kaseda et al., 2007). Consequently, injury or dysfunction of

proximal tubular will reduce reabsorption and degradation, which causes high concentration of CysC in urine (Conti et al., 2006; Herget-Rosenthal et al., 2007). Megalin is essential for the normal tubular uptake of uCysC. However, megalin deficiency has been found as a genetic defect, studied in mice and applied to people (Fisher and Howie, 2006), this is to the knowledge of the author not studied in dogs. Extended studies on rats, within human medicine concluded that an increase in urinary cystatin C excretion after ischemia/reperfusion injury is associated with decreased tubular uptake but not with reduced megalin expression (Jensen et al., 2017).

Urinary CysC/uCr was significantly higher in dogs with CKD compared with healthy dogs and dogs with non-renal disease. There was a pronounced increase in the CKD dogs with no overlap between the healthy dogs and the CKD dogs (Monti et al., 2012). In experimentally induced AKI by tubulitoxic substances such as gentamicin and tenofovir, uCysC increased before development of azotemia and histological evidence of nephrotoxicity correlated with uCysC (Sasaki et al., 2014; Gu et al., 2018). In a study of dogs with different stages of leishmaniosis uCysC had high sensitivity and specificity, indicating that uCysC/uCr might be useful for early detection of kidney injury (Ruiz et al., 2023). Thielemans et al. 1994 showed in an experimental nephrotoxic study on rats that the massive albuminuria increased low-molecular-weight proteins, such as CysC in urine and suggested that the cause likely is competition for a common transport mechanism (Thielemans et al., 1994). Quantification of CysC by particle-enhanced nephelometric immunoassay (PENIA) on biochemistry instrument performed well with high precision and good linearity in both human (Herget-Rosenthal et al., 2004), and canine urine samples (Monti et al., 2012).

1.5.3 Urinary enzymes (uGGT and uNAG)

Tubular epithelial cells produce many enzymes of which GGT, ALP, NAG, and several others have been used as diagnostic biomarkers for tubular damage for many years (Clemo, 1998). Since most of these enzymes are medium – to high molecular weight proteins (>70-80 kDa), enzyme activity identified in urine is most likely of renal origin (Clemo, 1998), even if severe damage of glomerular barrier might allow large plasma proteins to leak into the ultrafiltrate (Ilchyshyn et al., 2019).

Urinary GGT is present in the proximal tubule brush border. Elevated uGGT and/or uGGT/uCr have been described in dogs with induced nephrotoxic by gentamicin (Greco et al., 1985; Rivers et al., 1996), in clinical diseases such as leishmaniasis (Ibba et al., 2016), dogs envenomated by Vipera berus (Harjen et al., 2021), and in bitches with secondary kidney injury caused by pyometra (Heiene et al., 2001; De Schepper et al., 1989). In experimental studies GGT elevation preceded clinically significant abnormalities in UPC, USG, and serum creatinine (Greco et al., 1985; Rivers et al., 1996), however overlap between groups was often apparent. Tubular damage was confirmed by histopathology in the study of bitches with pyometra (Heiene et al., 2001).

Another enzyme released in renal tubular injury is NAG, which is a lysosomal enzyme. As for uGGT, uNAG shows an early response after drug-induced nephrotoxicosis (Sun et al., 2019) and in clinical cases with babesiosis (Kules et al., 2018). However, in another study of dogs treated with tenofovir uNAG was not elevated (Gu et al., 2018). Dogs with severe CKD, including depletion of tubular cells and enzyme stores, are not expected to have elevated urinary enzymes (Grauer, 2005).

It is possible to analyse uGGT using the method for serum GGT in a regular biochemistry instrument (Ilchyshyn et al., 2019), even if the method might need to be adjusted to gain an optimal measuring range. Also, uNAG can also be measured with regular biochemistry instruments, however the short stability of reagents and calibrators reduce the usability for clinical purposes.

1.5.4 Urinary glucose

Glucosuria is most often detected in hyperglycemic dogs, for example in dogs with diabetes mellitus, where the renal threshold (10-12 mmol/L) or tubular maximum for glucose reabsorption has been exceeded. Glucosuria in an euglycemic dog is indicative of renal tubular dysfunction. The cause can be an isolated tubuli injury or a complex tubular disorder with increased excretion of other molecules, such as in Fanconi´s syndrome, which mainly affect Basenji dogs (Bovee et al., 1978). Glucosuria caused by acquired proximal tubular dysfunction has been described in dogs eating jerky treats (Thompson et al., 2013), lead toxicity (King, 2016), and copper-associated hepatitis in labradors (Langlois et al., 2013). Glucosuria have also been reported in Norwegian Elkhounds with familial renal glucosuria (Heiene et

al., 2010), and dogs treated with sodium glucose cotransporter (SGLT) inhibitors (Ueta et al., 2006). Glucosuria was more common in dogs with leptospiral AKI (59%) than in dogs with AKI of other causes (18%) (Zamagni et al., 2020). Among non-selected Leptospira cases 18% were positive for glucose on dipstick (Tangeman and Littman, 2013). One study has described uGlu as the most sensitive laboratory marker to discriminate AKI from CKD in dogs (Gerber et al., 2004). Dipstick works well to detect glucosuria in clinical practice. Exact concentration can be analysed using an adapted application for urinary glucose with biochemistry instruments (Zeugswetter and Schwendenwein, 2020).

1.5.5 Urinary electrolytes and urea

Electrolytes (uNa, uCl, uK, uCa, uP) are excreted or reabsorbed in kidney tubuli in response to the body's requirements, so urinary electrolytes are commonly evaluated as fractional excretion. Extensive dysfunction of the tubular epithelial cells in dogs with kidney disease leads to increased loss of electrolytes in the urine. In dogs with advanced chronic kidney disease FE-P, FE-Na, FE-K, FE-Cl were higher compared to dogs with less severe kidney disease (Martorelli et al., 2017; Buranakarl et al., 2007). Studies in people with acute kidney injury have shown that FE-Na is lower in reversible AKI compared to persistent kidney injury (Lima and Macedo, 2018). Intact proximal renal tubules reabsorb Na and FE-Na <1% was found in patients with pre-renal azotemic oliguric patients, while AKI patients with FE-Na > 3% had poor prognosis (Espinel et al., 1979). However, this concept was not found in patients with septic AKI (Saha et al., 1987; Bagshaw et al., 2013). Increased FE-Na has also been shown to be an indicator of AKI in dogs with heat stroke (Segev et al., 2015), and decreasing fractional clearance of Na was a positive prognostic indicator in dogs with AKI (Brown et al., 2015). Fractional excretion of electrolytes differed between different types of AKI in dogs and had importance for the prognosis (Troia et al., 2018).

Urea is produced in the liver from breakdown products of proteins and amino acids. It is freely filtered in glomerulus and excreted in the urine. The kidneys regulate the amount of urea that is reabsorbed, which is important for the water balance. FE-urea has been described to be a useful tool to differentiate transient from persistent AKI in people (Lima and Macedo, 2018), while FE-urea was not different between intrinsic and volume-responsive AKI and control dogs (Troia et al., 2018).

1.5.6 Method validation

New diagnostic methods need to be validated before use to prove that the performance is acceptable. Precision is evaluated through repeated analysis of samples with different concentrations from the species of interest. Level of acceptable imprecision should be based on the clinical use of the variable (Harr et al., 2013). In research, it is often preferable to run all samples together in one batch to avoid effect of method variation over time (between assay variation) (Yu and Yang, 2017). The limit of quantification (LOQ) can be defined as the lowest measured concentration of an analyte with acceptable level of accuracy and precision (Armbruster and Pry, 2008). Linearity upon dilution is done to evaluate if test results are proportional to the analyte concentration within the measuring range (Chavan, 2022). Accuracy of an assay is preferably studied by analysis of standards or samples with known concentration but purified canine standards or calibrators are rarely available. When immunological assays produced for human samples are used for canine samples the accuracy is usually unknown, and results may vary considerably between assays (Davis et al., 2021).

1.6 Biological Variation and reference intervals

A laboratory result is not influenced only by the presence or absence of disease in an individual, but also by biological variation, which refers to variability in analytes concentration or activity around a homeostatic set point (Fraser, 2001). Variability is a result of innate physiological factors, and may or may not show daily, monthly or seasonal rhythms (Fraser, 2001). In addition to BV and analytical variation, clinical laboratory results can be affected by preanalytical factors, such as preparation, sampling techniques, handling of specimen, and analytical variation in the laboratory analyzer (Freeman et al., 2017; Flatland et al., 2020).

Biological variation is usually expressed as coefficient of variation (CV) (Fraser, 2001). The variation can be described as within-individual variation (CV_I) which reflects changes in the same individual overtime, and between-individual variation (CV_G), the difference among individuals in a group. In addition, there is analytical variation (CV_A), representing variation due to the analytical method (Freeman et al., 2017; Flatland et al., 2020). The parameters of biological variation are estimated by repeated sampling of the same individuals, under controlled conditions, and results obtained by

analysis of variance. It is important to understand both biological and analytical variation to interpret clinicopathological data. This is increasingly relevant when veterinarians interpret serial data from the same animal, or when the results are close to the reference limits or clinical decision thresholds (Flatland et al., 2020).

Population-based reference intervals (RIs) are traditionally used in veterinary practice (Friedrichs et al., 2012), usually reported as comprising 95% of the healthy population. International recommendations declare nonparametric determination from at least 120 reference individuals as the preferred method, however recruiting this number of animals is not always achieved in veterinary medicine, and acceptable alternative methods include transference and validation from previously established RIs (Geffre et al., 2009). Population-based reference intervals are helpful for interpreting results from many laboratory analytes, but there are situations where RIs has limitations. For example, if analytes have low within individual variation (CV_I) and a high between individual variation (CV_G), the results may be abnormal for the individual even though they are still within the reference interval (Freeman et al., 2017).

To evaluate if population-based RIs are appropriate to use, index of individuality (II) can be calculated. The most commonly used calculation for II was published by Fraser (Fraser, 2001). Using the original formula, a low II corresponds to high individuality, and caution should be taken when using population-based RI (Fraser, 2001). However, an inverse formula has been recommended according to veterinary guidelines for biological variation studies (Freeman et al., 2017) and was used in this thesis. This formula is calculated as $II=CV_G/(CV_I+CV_A)$ and may seem more logical since a high index corresponds to a high individuality (Freeman et al., 2017; Flatland et al., 2020). For analytes with high II ($\geq$1.7), populations-based RI are inappropriate (Freeman et al., 2017), and instead reference change value (RCV) calculated from CV_I and CV_A is recommended ($RCV = 2^{0.5}*Z*(CV_A^2 + CV_I^2)^{0.5}$) (Freeman et al., 2017). The RCV indicates how much an analyte must differ between two samples to be considered significant (Freeman et al., 2017). For analytes with an II $\leq$0.7 the population-based RI are considered to be appropriate to use, and for intermediate II (II= 0.7-1.7) population-based RI should be used with caution (Freeman et al., 2017).

2. Aims

Overall aim of this thesis was to investigate biological variation of selected urinary analytes in healthy dogs, and to study if these potential urinary biomarkers can provide diagnostic information in dogs with chronic kidney disease and acute kidney injury.

The specific aims of this thesis were to

- Investigate biological variation and establish population-based RIs for 11 urinary and 9 serum analytes in healthy dogs.
- Compare uCysC, uGGT, uGlu, uUrea, and electrolytes, normalized to creatinine and FE for electrolytes and urea, among dogs in different IRIS stages of CKD and healthy control dogs.
- Investigate the value of these urinary analytes for diagnosis of CKD Stage 1.
- Study selected urinary analytes (uCysC, uGGT, uNAG, and uGlu normalized to uCr) in dogs with AKI or at risk for AKI, and follow them over time.

3. Materials and methods

A brief description of materials and methods of included studies is presented here. A more detailed description of each study is provided in the respective papers.

3.1 Animals

3.1.1 Recruitment, and inclusion criteria (Study I,II,III)

In studies I-III, dogs were prospectively recruited. In Study I, recruitment was carried out through seminars and e-mails. Dogs for Study II and III were recruited when presented to the University Animal Hospital (SLU) in Uppsala, or AniCura Animal Hospital in Stockholm, and given a diagnosis of CKD (Study II), or hospitalized because of Vipera berus intoxication, or admitted for intensive care with or without clinically apparent AKI (Study III). Inclusion criteria were >6 months of age (>1 year for Study I), weight ≥3 kg, and of any breed and sex. All studies were approved by the Uppsala Animal Ethics Committee, and all owners provided written consent.

3.1.2 Exclusion criteria (Study I,II,III)

Exclusion criteria for dogs in Study I and Study II were presence of other systemic disease, and medications (except for tick prevention). If a dog in the CKD group 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. For dogs in Study III, envenomated by vipera berus (EVB), admitted for intensive care without clinically apparent AKI (IC), and dogs with clinically apparent AKI (AKI_{APP}), the exclusion criterion was previously known CKD. Specific exclusion criteria for dogs in the biological variation study (Study I), were pregnancy, heat, abnormalities on physical- and laboratory examination, or owner reported abnormalities.

3.1.3 Diagnostic criterias (Study II,III)

The diagnosis of AKI or CKD was made based on clinical signs and standard methods, i.e physical examination, hematological and biochemical analyses,

results of urine analysis, blood pressure measurements, abdominal ultrasonography, and when relevant, renal scintigraphy. All CKD dogs were assigned an IRIS stage (1–4), based on stable serum creatinine (sCr) concentration. For a diagnosis of CKD stage 1, obvious ultrasonographical abnormalities (multiple cysts, irregular renal margins, or markedly reduced renal size), persistent renal proteinuria, or evidence of proximal tubular dysfunction had to be present.

3.1.4 Healthy control dogs (Study II and III), and dogs included for population-based reference intervals (Study I)

The healthy control groups (C) in Study II and III consisted of 30 privately-owned dogs each. All control dogs were deemed healthy by their owners and underwent thorough physical examination, and urine analysis, as well as hematological and serum biochemical analyses. In Study II, blood pressure measurements, abdominal ultrasonography, and renal scintigraphy for GFR, were also performed in control dogs. Healthy control dogs in Study III were examined and had blood- and urine samples collected once (day 1). To generate population-based RIs, healthy control dogs from study I, II, and two student master theses (Mårtensson, 2017; Damm, 2020) were combined.

3.2 Study design and collection of samples

3.2.1 Biological variation

To study BV, blood and urine samples from 13 healthy dogs collected once a week for 8 weeks were included (paper I). In the investigation of biological variation, it is essential to standardize all aspects of the study -including examination protocols, sampling procedures, environmental conditions, and laboratory equipment to minimize confounding variables and ensure that performance variability does not influence the outcome. The same type of plastic container (Uripet, WDT, CuraVet, Queensland, Australia) was used for collection of spontaneously voided urine. A new container was used each time urine was collected.

Every dog had a scheduled collecting day and included dogs were distributed over the week, with a maximum of 4 dogs a day. A midstream morning urine sample was taken by the owner, after an overnight fast (10-12 hours) at approximately the same time for each individual dog. The urine

sample was kept at 2-8°C until centrifugation and analysis (maximum 3 hours after collection) with urine dipstick (Multistix 7, Siemens, Erlangen, Germany) and sediment analysis. One aliquot of urine was acidified by hydrochloric acid (3.3 M) at a 1:20 ratio to avoid crystallization during freezing and saved together with aliquots of supernatant obtained after centrifugation at 500*g* (EBA 200, Hettich, Tuttlingen, Germany). Fasting blood samples, withdrawn from the cephalic vein within 3 hours from urine sample, 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. Urine aliquots were placed at −20°C within 4 hours of urine collection. All specimens were transferred from −20°C to −80°C within 7 days and stored until batch analysis within 5 months from collection.

3.2.2 Population-based reference intervals

Blood samples was drawn from the cephalic vein ±3 h from urine sampling. The samples were kept at -22°C for maximum 20 days and at -80°C for maximum eight years before analysis. All urine analytes in this study have been reported stable in human urine at -22°C for >12years (Remer, 2014), with the exception of protein and glucose. Previous studies describe uProt to be stable for at least one month in -20°C (Banfi et al., 2002) and 2.5 years at -70°C (Parekh et al., 2007). These studies were performed on human urine, and stability of these analytes are expected to be similar in canine urine. A small stability study was performed where uProt was analyzed in 11 canine urinary samples at sampling and after 5-9 years storage at -80°C. There was no significant difference between results using the non-parametric Wilcoxon signed rank test (p=0.76). For uGlu no long-time stability data was found and hence population-based RI was not calculated for that analyte.

3.2.3 Study II (CKD) and III (AKI)

In Study II, urinary biomarkers were investigated in dogs at different stages of CKD, and healthy control dogs. Serum analytes (C-reactive protein [CRP], Albumin [Alb] Protein [Prot], Crea, Urea, Sodium [Na], Potassium [K], Cloride [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

analysed later on Architect c4000. Urine samples from each dog, collected within a time frame of 4 hours of serum collection, were analyzed fresh (USG, dipstick, and sediment). Other aliquots were immediately stored (-80C°), for batch analysis within 9 years of sampling.

In Study III, all dogs underwent a physical examination at each time point of sample collection, and blood and urine samples were collected on day 1 when they received an intravenous catheter. On day 2, blood was drawn from the IV catheter, in the hospital. Additional urine samples from day 7 and 14 (+/- 2 days), were collected at the hospital recheck, or at home by the owner and sent by post to the laboratory. Hematological and biochemical analyses (CRP, crea, urea, ALT, ALP, bile acid, fructosamine, glucose, albumin, total protein, cholesterol, total calcium, phosphate, sodium, potassium, chloride) were analyzed immediately, as part of the diagnostic work-up. Serum for analysis of CysC, cholesterol, and sGGT from day 1, as well as all serum for all analyses from day 2 (CRP, creatinine, and CysC), were stored at -80°C until batch analysis. Urine samples were collected within a time frame of 1-6 hours of serum collection on both day 1 and 2. Urine analyses (dip stix, urine specific gravity (USG), and sediment examination) were performed on the day of inclusion. Urine was cultured when there was an active sediment (> 3 WBC per hpf, >3 RBC per hpf) or when urinary tract infection was on the differential diagnosis list. Aliquots of urine supernatant (centrifuged in 2100xg for 5 min) were stored in -80°C until batch analysis 8-13 months after collection.

3.3 Laboratory analyses

In Study I and Study II, as well as for generation of population-based RIs, Cr, Urea, Prot, Glu, Na, Cl, K, Ca, and P, were analyzed in urine and serum using an automated chemistry analyzer (Architect c4000, Abbott Diagnostics, Lake Forest, IL, USA). The reagents were from Abbott Diagnostics, intended for use in urine and serum, and analyzed according to the manufacturer's instruction, except for uProt, uGlu and uCa. For uProt, a lower measurement limit than specified by the producer was used and validated. To extend the lower measuring range, uGlu and uCa were analysed with slightly adjusted methods where a larger sample volume was used. In Study II, serum CysC was analyzed with an immunoturbidometric method on Architect c4000 with reagents from Gentian Diagnostics, Moss, Norway.

The method described by Noraddin et al (2012) was used for uCysC. The methods were deemed acceptable based on precision, limit of quantification and recovery upon dilution. For analysis in Study I and Study II, samples were divided into three groups consisting of serum samples, acidified urine for analysis of uCa and uP, and supernatant urine for all other analytes. All samples from each group were batch analyzed in duplicates in randomized order. Two commercial control samples were analyzed before and after batch analysis for each serum analyte. For urine analytes, one commercial control sample and one canine urine control sample were analyzed.

In Study III, serum and urine samples were analyzed with a different automated chemistry analyzer, Beckman Coulter DxC, because the university laboratory shifted chemistry analyzer during the PhD project period. Standard methods and reagents from Beckman Coulter were used, except for uCysC and uNAG. Urinary CysC was analyzed with the same immunoturbidometric reagent (Gentian Diagnostics, Moss, Norway) as in Study II. To avoid problems with antigen overload each urine sample was analysed both with the standard method for serum samples and with a modified application (high sensitive CysC, hsCysC), with diluted calibrators and increased sample volume to extend measuring range down to 0.03 mg/L. The application was developed in cooperation with Beckman Coulter. Results above 0.6 mg/L, from the standard method, were used without modifications. For samples with uCysC concentration below 0.6 mg/L with the standard method, results from the high sensitivity methods were reported. Urinary NAG was analysed using a colorimetric method (Diazyme Laboratories, Poway, CA, USA) with the biochemistry instrument (Beckman Coulter).

In all studies, a digital refractometer (PAL-USG (DOG), Atago 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). Calculation of creatinine ratios was performed by dividing the concentration of the analyte by the creatinine concentration. Fractional excretion (%) was computed by the formula below.

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

For calculation of ratios and FEs, the numbers included had the same unit, except for uGGT/uCr (U/g), and uNAG/uCr (U/g).

In Study I-III, results below the measuring range were set to half this value. For results above the measuring range, samples were reanalyzed with adjusted dilution. For calculation of population-based RIs, uProt results below the measuring range of 0.045 g/l, were given a value of 0.044. In addition, uNa and uCl concentrations below 20 mmol/l, were given a value of 19 and was used for the RI´s calculations. From 70 to 109 dogs, depending on analyte, were used for calculation of RIs.

3.4 Statistical analysis

In Study I, calculation of biological variation was performed for urine concentration, serum concentration, uAnalyte/uCr ratio, and FE of all analytes. 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, Reed´s criterion was used to detect outliers between subjects (Fraser, 2001). Variance components were estimated using restricted maximum likelihood (REML). The R software (The statistical software R.R core Team R Foundation for Statistical Computing) was used for the analyses. Urinary analytes that were not normally distributed were box-cox transformed to achieve an approximate normal distribution. Before calculating RCV these analytes were back transformed to the original scale. Since data were transformed, a new formula for RCV was established. Calculations of population-based reference intervals with 90% confidence intervals were performed by bootstrap estimation utilizing the software RefVal 4.0, Department of Clinical Chemistry R, N-0027 Oslo, Norway (Solberg, 1995; Solberg, 2004). The program is using Dixon´s test to detect outliers.

For Study II and III, 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). Age,

bodyweight (BW), sex, storage time (II), and urinary analytes 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. A comparison of urinary markers, UPC and sCr between survivors and non-survivors in the AKI_{APP} group was performed. A significant level of $p < 0.05$ was applied, and Bonferroni correction of p values was used for multiple comparisons among all groups.

4. Results

4.1 Biological variation (Study I)

In Study I, the CV_I for all urinary analytes, both as concentrations, normalized to uCr and FE-analyte, varied between 12.6% and 35.9%, except for uNa, uNa/uCr and FE-Na, which were noticeably higher, around 60%. The between individual variation (CV_G) for all urinary analytes, except for uProt, varied between 5.3% and 46.1%, (uProt < 0.1%). The highest CV_G was seen for uCa/uCr (46.1%). Fractional excretion of glucose showed both the lowest CV_I (12.7%), and the lowest CV_G (5.3%). **Figure 5** shows uNa/uCr and uGlu/uCr, representing low and intermediary high II.

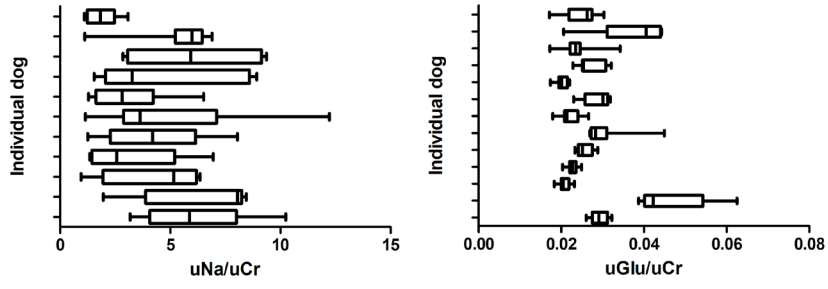


Figure 5. Distribution of urinary sodium and glucose normalized to urinary creatinine, measured in healthy dogs sampled once a week for 8 weeks, representing low (uNa/uCr) index of individuality to the left, and intermediary high (uGlu/uCr) index of individuality to the right. Boxplots show median and IQR, and whiskers indicate minimum and maximum values.

Results for uAnalytes/uCr and FE-analytes for all dogs in study I are shown in **Table 3**. The CV_A, i.e. the variation attributable to the analytical method was ≤2.1% for all analytes except for uProt, UPC, and FE-Prot, which had a CV_A of approximately 5.0%. A desirable CV_A is recommended to be maximum of half of the CV_I (Freeman et al., 2017), which was the case for all analytes.

Table 3. Biological variation of 8 urinary analytes normalized to urinary creatinine, and FE-analyte, measured in 13 healthy dogs for 8 weeks.

Biological variation	N	Median (Range)	CV_I %	CV_G %	Duplicate CV_A %	II
uAnalyte /uCr						
uUrea/uCr	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
uProt/uCr	8	0.044 (0.023-0.080)	18.2 (14.8-22.9)	22.1 (12.3-38.3)	5.3 (4.4-6.5)	1.16
uGlu/uCr	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
uNa/uCr	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
uCl/uCr	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
uK/uCr	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
uCa/uCr	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
uP/uCr	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
FE-analyte						
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)	0.0006 (0.0003-0.0012)	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

Abbreviations*:* N, number of dogs; Cr, creatinine; CV_I ,within-subject coefficient of variation; CV_G,between-subject of coefficient of variation; CV_A, Duplicate coefficient of variation; II, index of individuality based on II = $CV_G/(CV_I^2 + CV_A^2)^{1/2}$; u, urinary

Index of individuality for uProt, uNa, uK, uNa/uCr, FE-urea, FE-glu, FE-Na, FE-K, and FE-P were low (<0.7) indicating that population based RIs are appropriate. The remaining 18 urine analytes (USG, Osmo, UPC, uCr, uUrea, uGlu, uCl, uCa, uP, uUrea/uCr, uGlu/uCr, uCl/uCr, uK/uCr, uCa/uCr, uP/uCr, FE-Prot, FE-Cl and FE-Ca) had intermediate II (0.7-1.7), suggesting that population based RIs should be used with caution. In serum, II for creatinine was 3.04 indicating that population-based RI is not appropriate. Only one serum analyte, sNa, had low II (0.68), suggesting that population-based RI was appropriate. All other serum analytes (sUrea, sProt, sGlu, sCl, sK, sCa, sP) had intermediate II. Individual distribution of sK and sCr in the 13 dogs are shown in **Figure 6.**

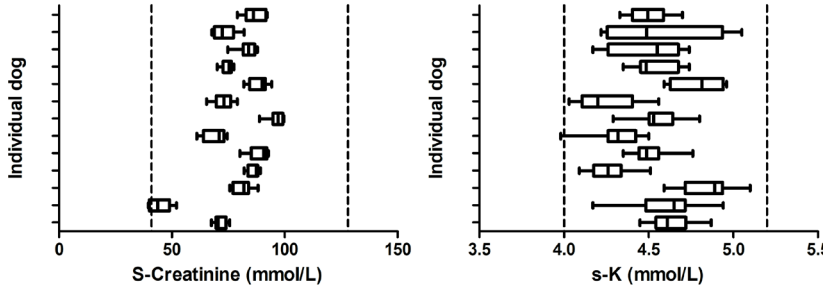


Figure 6. Distribution of serum creatinine and serum potassium (sK), in 13 healthy dogs, sampled once a week. Serum creatinine to the left, showing a high index of individuality, and sK to the right, showing an intermediate to low index of individuality. Dotted lines represent population-based reference intervals. Boxplots show median and IQR, and whiskers indicate minimum and maximum values.

Because uAnalytes were not normally distributed and had to be transformed before analysis, RCV varied with different concentrations. For biochemical uAnalyte/uCr and FE-analyte, with an intermediate II, the estimated RCV at different levels of uAnalyte/uCr and FE-analyte, respectively, is presented as RCV charts in **Figure 7 and 8.**

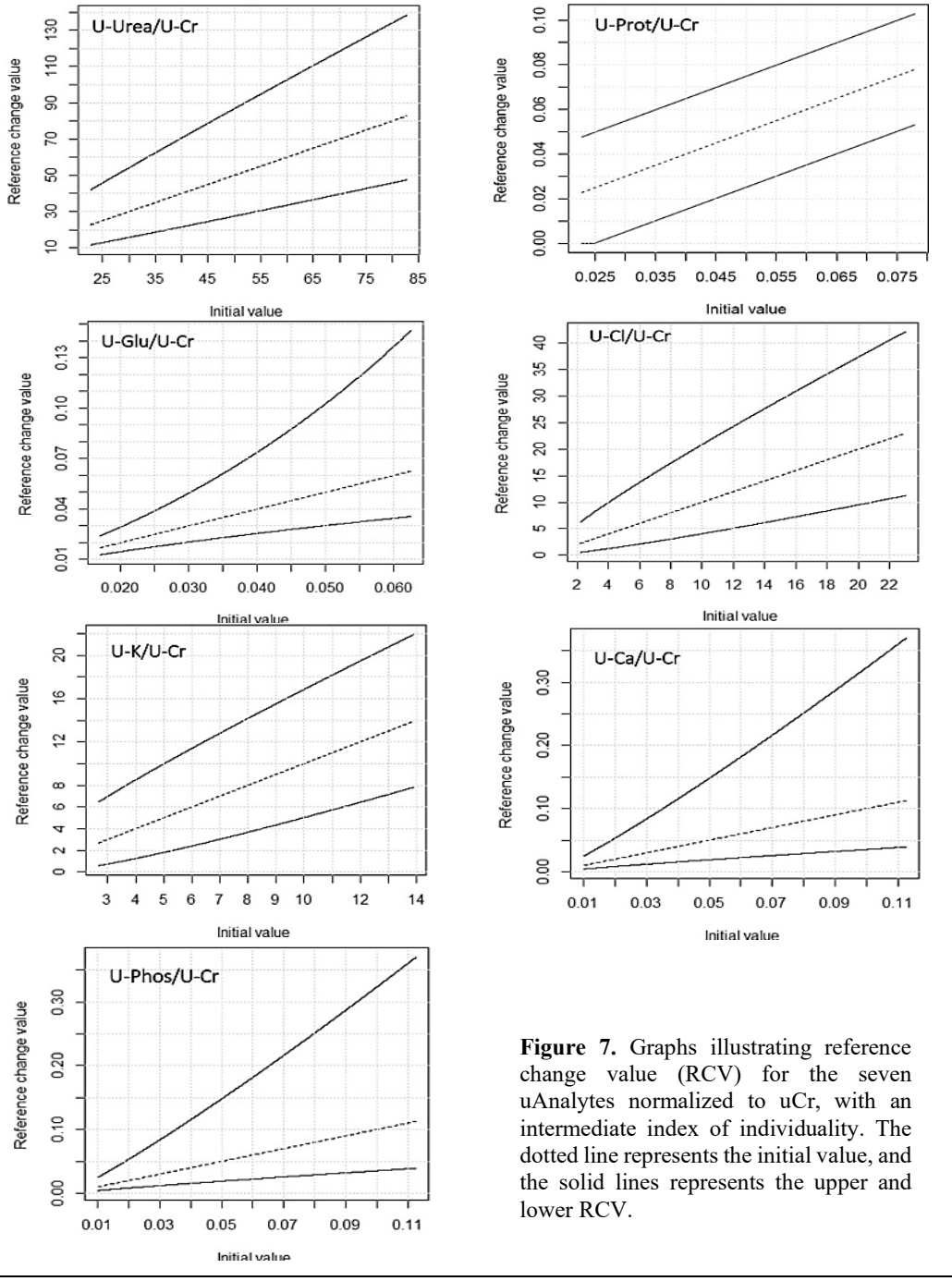
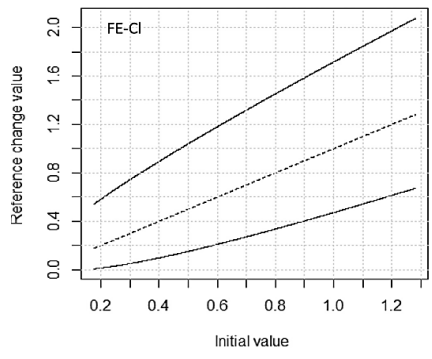
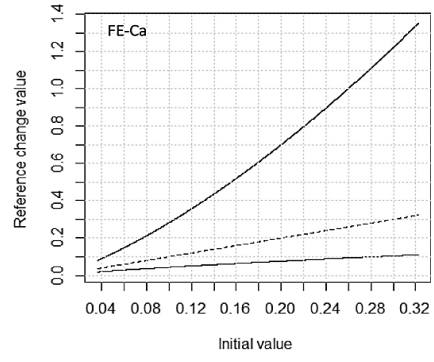


Figure 7. Graphs illustrating reference change value (RCV) for the seven uAnalytes normalized to uCr, with an intermediate index of individuality. The dotted line represents the initial value, and the solid lines represents the upper and lower RCV.





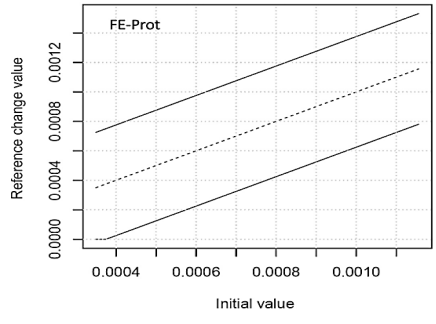


Figure 8. Graphs illustrating reference change value, RCV, (y-axis) for the three FE-analytes with an intermediate index of individuality. The dotted line represents the initial value, and the solid lines represents the upper and lower RCV.

4.2 Population-based reference intervals (Study I)

Dogs of 20 different breeds, 1-11 years of age and of any sex or neutering status, were included to calculate population-based RIs. There were 70 to 109 dogs, depending on analyte, used to calculate RIs for the different analytes. Three results were detected as outliers and excluded from the RI calculations (2 for uProt, and 1 for uProt/uCr). Eleven dogs had uProt below the measuring range of 0.045 g/l, 10 dogs had uNa below 20 mmol/l and 2 dogs had uCl below 20 mmol/l. For the calculations of uAnalyte/uCr and FE-analyte 0.044 g/l was used for uProt, and 19 mmol/l for uNa and uCl. The population-based RIs are presented in **Table 4.**

Table 4. Reference intervals for 8 urinary analytes, presented as normalized to urinary creatinine, and FE-analyte.

Analyte	N	RI lower limit (90% CI)	RI upper limit (90% CI)
uAnalyte/uCr			
uUrea/uCr	97	23.6 (19.6-27.6)	111 (75.9-145)
uProt/uCr	109	<0.021 (N/A)	0.380 (0.233-0.527)
uGlu/uCr	57	N/A	N/A
uNa/uCr	97	<0.715 (N/A)	15.9 (14.1-17.7)
uCl/uCr	97	1.66 (0.337-2.99)	23.3 (20.3-26.3)
uP/uCr	97	3.20 (2.50-3.90)	18.3 (14.5-22.1)
uCa/uCr	70	0,008 (0.004-0.012)	0.248 (0.169-0.327)
uP/uCr	97	0.53 (0.11-0.94)	5.71 (5.25-6.17)
FE-analyt			
FE-Urea	71	40.7 (36.0-45.4)	97.6 (84.8-110.4)
FE-Prot	71	<0.001 (N/A)	0.010 (0.004-0.015)
FE-Glu	57	N/A	N/A
FE-Na	71	<0.04 (N/A)	0.88 (0.60-1.16)
FE-Cl	71	0.096 (0.01-0.206)	1.37 (0.97-1.77)
FE-K	71	5.27 (4.41-6.11)	29.8 (23.8-35.9)
FE-Ca	70	0.030 (0.015-0.044)	0.704 (0.490-0.919)
FE- P	71	3.13 (0.21-6.47)	39.8 (33.9-45.8)

No RIs were calculated for uGlu/uCr and FE-Glu because of too few available values.

4.3 Urinary biomarkers in dogs at various stages of CKD and grades of AKI (Study II+III)

4.3.1 Demographical and pathophysiological data

In Study II, 30 healthy dogs and 50 dogs in various stages of CKD were included. Stage 3 and 4 dogs were combined to one group for statistical analyses. Study III included a total of 97 dogs (30 healthy control dogs, 28 dogs envenomated by Vipera Berus, and 39 dogs admitted for intensive care for a variety of reasons. Fourteen of the 39 dogs admitted for intensive care were diagnosed with clinically apparent AKI and assigned to a separate group (AKI_{app}). Thus, 25 dogs constituted the intensive care group. There were no differences in age, sex, or BW among the 4 groups of dogs in Study II or III. Clinicopathological data and results (from Day 1) for included dogs in Study II and III, are presented in **Table 5.**

Table 5. Clinicopathological data and results (median and IQR) for included dogs on Day 1 (Study II and III). Significant differences (p<0.008) are noted where superscripted letters differ among groups.

STUDY II	C	CKD1	CKD2	CKD3+4	STUDY III	C	EVB	IC	AKI_{APP}
Number dogs	30	16	25	9	Number of dogs	30	28	25	14
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)	USG	1.046 (1.033-1.051)	1.028 (1.017-1.02)	1.020 (1.013-1.036)	1.014 (1.013-1.017)
Osmo	1330 (837-2004)	719 (387-1175)	480 (406-657)	407 (336-481)	Osmo	1832 (1298-2158)	1005 (633-1523)	683 (493-1203)	454 (380-529)
UPC	0.06 (0.04-0.11)	1.37 (0.20-5.9)	0.3 (0.09-1.7)	0.97 (0.16-5.33)	UPC	0.1 (0.09-0.14)	0.22 (0.16-0.44)	0.32 (0.16-1.2)	6.0 (0.65-11.3)
uCrea	15843 (9752-22333)	6015 (5028-11006)	6082 (4052-10855)	4212 (3483-6051)	uCrea	18962 (10485-25784)	11712 (6381-18104)	7574 (3580-14334)	4492 (3710-9280)
$uCysC/uCrx10^{-3}$	0.03 (0.02-0.045)[a]	0.08 (0.04-0.25)[b]	0.082 (0.04-4.2)[b]	13.4 (3.5-34.0)[c]	$uCys/uCr\ x10^{-3}$	0.018 (0.014-0.025)[a]	0.05 (0.03-0.1)[b]	0.06 (0.03-0.4)[b]	4.5 (0.13-30.0)[c]
uGGT/uCr, U/g	14.6 (4.0-20.4)[a]	42.6 (11.2-83.8)[b]	14.0 (4.9-29.0)[ab]	16.0 (7.0-27.0)[ab]	uGGT/uCr, U/g	7.4 (2.6-13.2)[a]	27.0 (16.2-44.0)[b]	17.4 (8.7-41.9)[b]	35.6 (4.1-62.7)[b]
uGlu/uCr	0.023 (0.021-0.03)[a]	0.027 (0.025-0.05)[a]	0.02 (0.015-0-03)[a]	0.025 (0.02-0.04)[a]	uGlu/uCr	0.03 (0.02-0.04)[a]	0.04 (0.03-0.06)[b]	0.05 (0.03-0.09)[b]	0.12 (0.05-0.5)[b]
uNAG/uCr, U/g	-	-	-	-	uNAG/uCr, U/g	1.7 (0.7-3.1)[a]	2.3 (0.7-4.5)[a]	3.0 (1.1-8.1)[a]	15.2 (5.9-26.8)[b]
sCrea	83.5 (74.8-98.3)	82.0 (65.0-89.5)	173 (144-200)	440 (310-743)	sCrea	78.7 (66.5-87.1)	77.8 (62.0-88.6)	67.8 (60.1-78.2)	321.9 (194.1-488.6)
sCysC	0.31 (0.26-0.41)	0.43 (0.39-0.89)	0.93 (0.52-1.27)	2.77 (1.50-3.69)	sCysC	0.50 (0.38-0.73)	0.49 (0.34-0.59)	0.42 (0.33-0.65)	2.0 (1.1-3.5)
sCRP	7.0 (5.0-7.0)	7.0 (6.5-7.0)	7.0 (7.0-9.5)	9.0 (7.0-30)	sCRP	4.4 (3.6-6.7)	6.7 (5.1-46.2)	79.0 (19.8-217.8)	60.4 (15.1-109.0)

Abbreviations: AKI_{APP}, apparent AKI, CKD, chronic kidney disease; C, healthy dogs; EVB, envenomated by vipera berus; IC, admitted for intensive care. Regarding further abbreviations, see page 19. For USG, Osmo, UPC, uCrea, sCrea and sCysC comparisons among groups were not performed because these variables were used for group affiliation.

4.3.2 Urinary analytes in dogs in various stages of CKD

In Study II, uCysC, uGGT, uGlu, uUrea, and electrolytes normalized to uCr, as well as FE of electrolytes and urea, were compared among dogs in various stages of CKD, and a healthy control group. Results from group comparisons of uCysC/uCr, uGGT/uCr, uGlu/uCr and uUrea/uCr, are presented in **Figure 9**. Urinary CysC/uCr increased with IRIS stage. Urinary CysC/uCr and uGGT/uCr were significantly higher in dogs with CKD stage 1 compared to control dogs (p=0.0002 and 0.002, respectively).

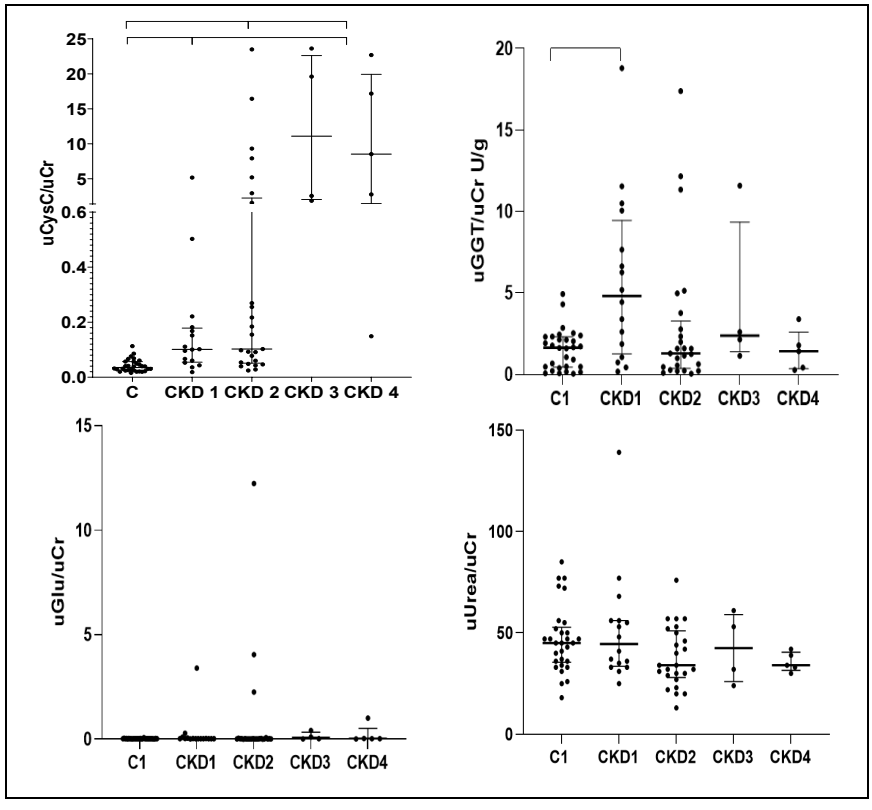


Figure 9. Results from group comparisons of uCysC/uCr (10^{-3}) (broken axis plots), uGGT/uCr (U/g), uGlu/uCr and uUrea/uCr in healthy control dogs and dogs in different stages of CKD. Significant differences (p<0.008) between groups are marked with bars. The groups of dogs with CKD 3 and 4 were combined for statistical analyses, due to their small sizes. The median and IQR is showed by horizontal lines. Abbreviations; C, healthy dogs; CKD, chronic kidney disease; uCysC, urine cystatin C; uCr, urine creatinine; uGGT, urine gamma glutamyl transferas (U/g); uGlu, urine glucose.

The urinary concentration of all electrolytes decreased with IRIS stage of CKD. This decrease in concentration was interpreted as caused by urine dilution, because when electrolytes were normalized to uCr, this pattern was lost. Fractional excretion of Na, K, Cl, Ca, and P increased with IRIS stage but there was no difference between the healthy control group and CKD1. **Figure 10** shows urinary concentration of normalized (uCr), FE of electrolytes, and serum creatinine.

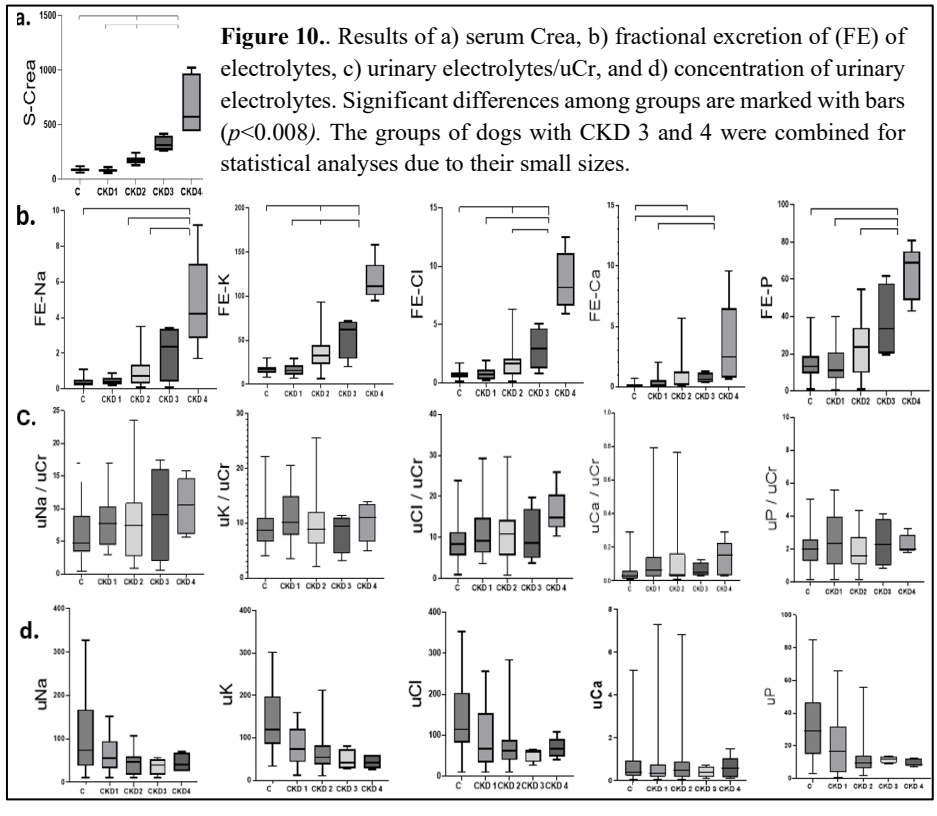


Figure 10.. Results of a) serum Crea, b) fractional excretion of (FE) of electrolytes, c) urinary electrolytes/uCr, and d) concentration of urinary electrolytes. Significant differences among groups are marked with bars (p<0.008*).* The groups of dogs with CKD 3 and 4 were combined for statistical analyses due to their small sizes.

Abbreviations; C, healthy dogs; Ca, calcium; CKD 1-4, CKD chronic kidney disease IRIS stage 1-4; Cl, chloride; Cr, creatinine; K, potassium; Na, sodium; P, phosphate; u, urinary

4.3.3 Urinary biomarkers in dogs at risk for AKI and with clinically apparent AKI (Study III).

In Study III, on day 1, uCysC/uCr, uGGT/uCr and uGlu/uCr were significantly higher in all groups of hospitalized dogs than in the healthy group. This also applied to day 2 for uCysC/uCr and uGlu/uCr. Urinary NAG/uCr was higher in the AKI_{APP} group than in all other groups, but there was no difference for uNAG/uCr between the healthy group and the EVB and IC groups. All urinary biomarkers normalized to creatinine for each group on day 1 and 2 are shown in **Figure 11.**

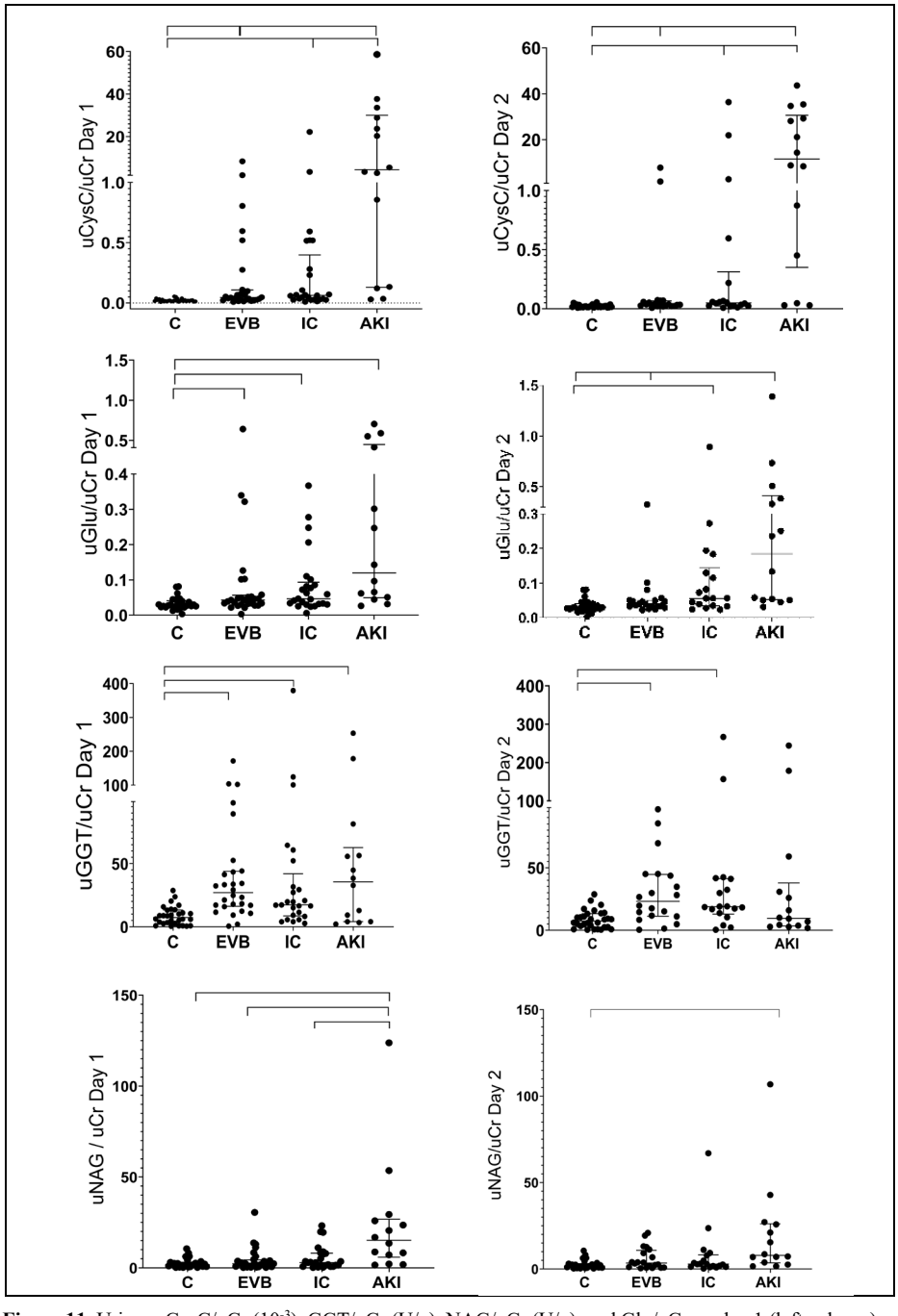


Figure 11. Urinary CysC/uCr (10^{-3}), GGT/uCr (U/g), NAG/uCr (U/g), and Glu/uCr on day 1 (left column) and day 2 (right column) in healthy dogs (C), dogs envenomated by vipera berus (EVB), dogs admitted for intensive care for a variety of reasons (IC), and dogs with clinically apparent AKI (AKI), in Study III. Significant differences between groups are marked (p<0.008) with bars. Broken axis plots.

4.3.4 Urinary analytes over time (Study III)

The urinary biomarkers, normalized to creatinine, decreased over two weeks post hospitalization in all groups except $AKI_{APP.}$ **(Figure 12).** The number of samples available from the AKI_{APP} group were 10 at day 7 and 5 at day 14. Corresponding numbers for the IC group were 8 at day 7, and 4 at day 14, and for the EVB group 12 at day 7, and 5 at day 14.

Seven of 14 dogs in the AKI_{APP} group survived > 6months post inclusion, and 7 dogs died or were euthanized during the first 2 weeks (n=4) or within 2 months (n=3) post hospitalization. **Figure 13** shows how uCysC/uCr was significantly higher from day 1 in the group that survived compared to non-survivors, (this was also true for sCysC but not for UPC, sCrea or any other uAnalyte). Non-survivors had a significantly higher uCysC/uCr on day 1 and 2 than survivors ($p < 0.002$). Urinary CysC/uCr remained high or increased during the 14 days post hospitalization in the 7 dogs that died, while the 7 dogs that survived had uCysC that decreased **(Figure 14).**

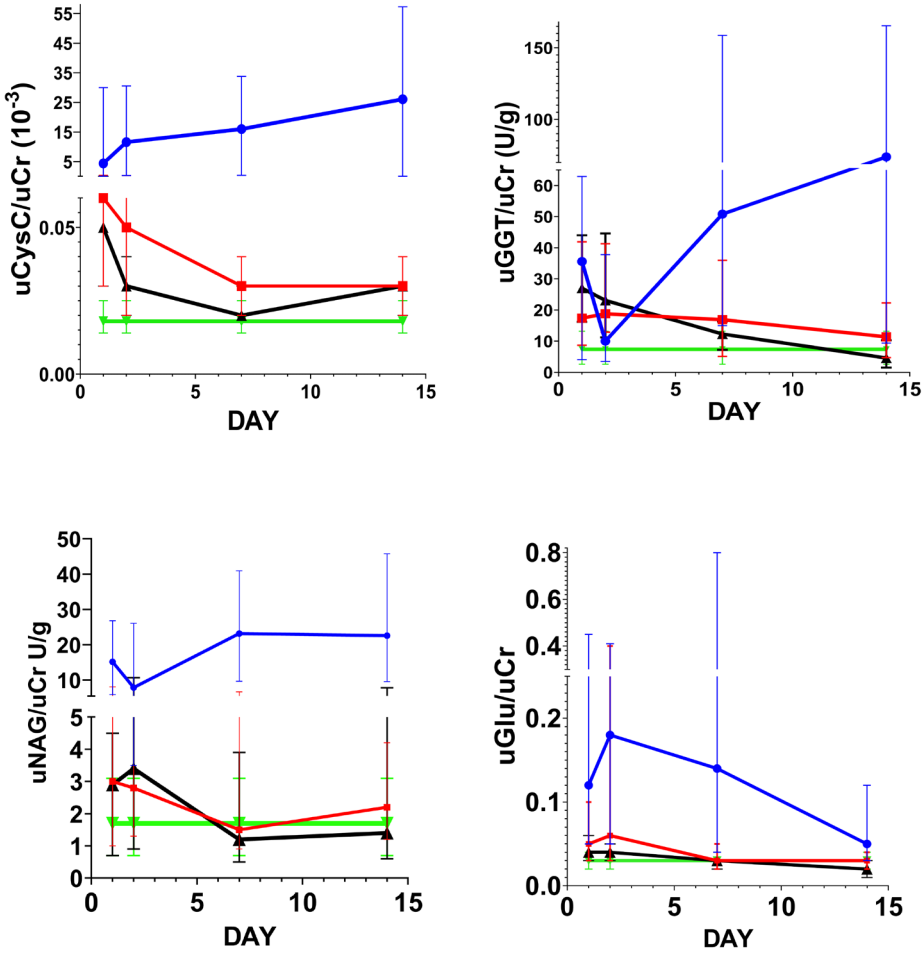


Figure 12. Urinary CysC/uCr (x10-3), uGGT/uCr (U/g), uNAG/uCr (U/g) and uGlu/uCr for dogs with AKI (blue line), dogs suffering from envenomation by Vipera berus (EVB) (black line), dogs that needed intensive care for a variety of reasons (IC) (red line), and healthy control dogs (C) (green line), sampled at day 1, 2, 7 and 14 (C only sampled day 1).

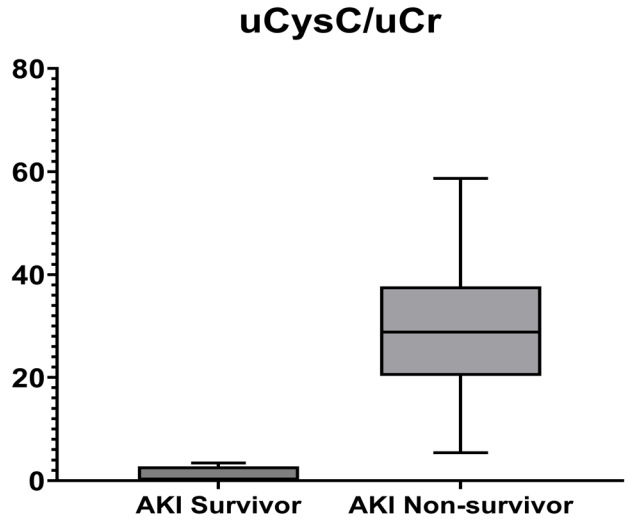


Figure 13. Urinary CysC/uCr in the group of dogs with clinically apparent AKI that did not survive (n=7) was significantly higher Day 1 compared to survivors (n=7), (p=0.002).

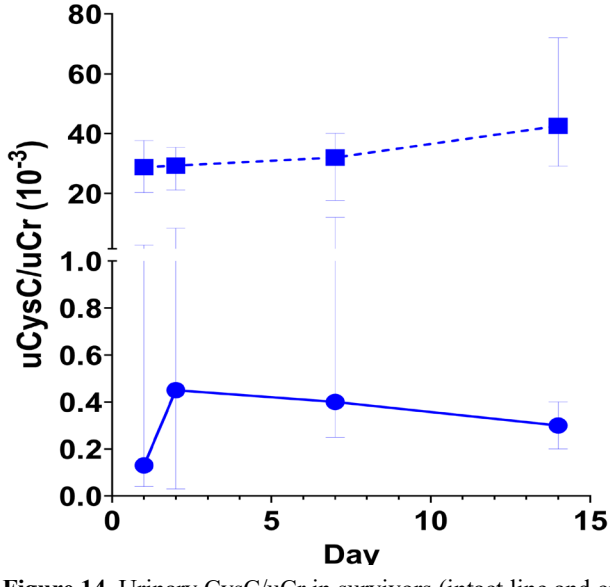


Figure 14. Urinary CysC/uCr in survivors (intact line and circles), and non-survivors (dotted line and squares) in the group of dogs with clinically apparent AKI, over time. Urinary CysC/uCr was significantly higher on Day 1 and remained high or elevated for non-survivors.

5. Discussion

5.1 Biological variation

5.1.1 Reference change value and reference intervals

In the urine and serum samples analyzed for the BV study (Study I) most analytes had an intermediate II, indicating that populations-based RI should be used with caution. In such situations RCV may be more suitable, but to use this approach a priory data point is needed for comparison, and this excludes the use of RCV in many situations. Reference change value is most often given as a percentage that represent the amount of change needed in a result, to be considered clinically relevant (Flatland et al., 2020). In this study, data was not normally distributed and RCV was dependent on concentrations. To aid in interpretation, RCV-charts were generated, where RCV could be addressed for every specific concentration. In the healthy dogs of this study, values of commonly used markers were low and normal, wherefore RCVs for diseased dogs could not be estimated from these charts. It would have been of interest to determine RCVs for commonly used markers in dogs diagnosed with CKD for comparison. Because only a single time-point results were available for each dog with CKD in study II, BV and RCVs were not calculated. However, given that CKD dogs are commonly stable within their respective stages, it is likely feasible to establish meaningful RCV values from them. One study has reported RCVs for UPC in dogs with CKD and summarized that at low UPC values (near 0.5), a minimum change in the UPC of 80% is required to demonstrate a significant difference (P>0.5) in serial values. Whereas at high UPC values (near 12), a minimum change of 35% is necessary (Nabity et al., 2007). Establishing RCVs in dogs with AKI is more challenging as the acutely injured kidney is actively striving to regain homeostasis, and rapid fluctuations of the disease process are likely to exceed effects of normal biological variation. In current veterinary practice, the use of RCV in interpretation of serum and urine analytes is virtually nonexistent, and it is to be considered whether it should be implemented in clinical settings and used more frequently.

Fractional excretion (FE) of electrolytes is rarely used in veterinary practice and described to have high intra-individual (CV_I) and inter-individual (CV_G) variability (Ettinger, 2024). However, it is probably

unfortunate to generalize this assumption to all electrolytes. In study I, FE-Ca show low CV_I and CV_G, whereas FE-Na show high CV_I and CV_G. Nevertheless, FE-Na also has a low II, which means it is suitable for interpretation using population based reference intervals. This thesis provides canine reference intervals for concentration, creatinine ratio and FE of urinary electrolytes.

5.1.2 Stratified reference interval

Of all investigated biomarkers only serum creatinine had a high II, indicating that population-based RI are inappropriate. There is limited information about biological variation of various urine- and serum analytes in dogs, but there are a few previously published articles with sCrea according to published guidelines. Two previously published articles were supportive of our data (Ruaux et al., 2012; Kopke et al., 2018), and found high II, whereas other studies demonstrated intermediate II (Pagitz et al., 2007; Jensen and Aaes, 1993; Leissing et al., 1985). The latter three studies with intermediate II only sampled one breed (beagle), and the discrepancy among studies is most probably due to sampled breeds. The high II for creatinine is likely due to different muscle mass, for example greyhounds have higher serum creatinine concentration than other breeds (Feeman et al., 2003). One way to reduce II is to stratify reference intervals. In human medicine, reference intervals are often stratified on gender and age. For creatinine in dogs one suitable stratification would be on muscle mass. In a clinical setting, information on breed and body weight might be more practical. The result from study I contributed to the development of bodyweight stratified reference intervals for serum creatinine concentration at the veterinary clinical pathology laboratory, SLU, and these were also implemented within AniCura Animal Hospitals in Stockholm.

5.2 Normalization

Normalization is important when assessing urinary biomarkers to account for urine dilution. In dogs with highly diluted urine, mildly elevated biomarker levels might otherwise be missed. A problem arises when biomarker concentrations fall below the measurable range. In Study II, many healthy controls and dogs in CKD stages 1–2 had uCysC below the detection limit (0.1 mg/L), which was set to half this value for data analysis. In these cases,

differences in uCysC/uCr ratios were solely due to uCr variation. To address this, more sensitive methods were adapted for Study III, enabling lower measuring ranges, and fewer dogs with analyte concentrations under the measuring range. Regardless of whether the biomarker was normalized to uCr or expressed as a concentration, results were similar for uCysC, uGGT, and uGlu, in study II and III (data not shown).

Normalization with creatinine is based on the assumption that creatinine excretion is constant, which is not always the case in patients with acute kidney injury (AKI), when renal homeostasis is disrupted (Waikar et al., 2010). In this context, alternative normalization approaches, such as using urine specific gravity (USG) or urine osmolality, may be preferred. In Study III, 3 of 14 dogs in the AKI group had results that deviated when normalized to uCrea compared to when normalized to USG or osmolality (data not shown). The interpretation was that these fluctuations were due to impaired uCrea excretion, rather than uCrea adjusting for dilution. However, normalization with USG or osmolality is less commonly used in veterinary practice, and may be unfamiliar to clinicians to interpret. The most important consideration is probably to be aware of the fact that changes in uCrea in a nonstable AKI patient may not always represent dilution. Instead, these changes may reflect a decrease in urinary creatinine excretion, rather than true changes in the biomarker (Waikar et al., 2010).

5.3 Urinary analytes as biomarkers

5.3.1 Urinary analytes in healthy dogs (study I-III)

Urinary CysC, uGGT, uNAG, glucose, urea, and urinary electrolytes (as concentration, normalized to uCr, and FE of electrolytes and urea) in healthy dogs in Studies I-III were in alignment with reported values and ranges for healthy dogs in other studies, where data were available (Nivy et al., 2017; Lippi et al., 2018; Perondi et al., 2019; Nivy et al., 2021). Although caution is warranted when comparing results across studies, as methodological differences may influence values, consistent results and similar cut-off values in multiple studies of healthy dogs is reassuring and of value because each study contains a relatively low number of dogs.

5.3.2 Urinary cystatin C

In study II (CKD), uCysC/uCr was higher in dogs with CKD stage 1 compared to healthy dogs and increased with IRIS stage. In addition, uCysC/uCr was significantly higher in dogs envenomated by vipera berus, in intensive care, and with apparent AKI groups compared with the healthy control group, in study III. The vipera berus envenomated and intensive care groups represent populations at risk for acute tubular injury. These findings, together with other studies of experimental and acquired kidney damage (Sasaki et al., 2014; Gu et al., 2018; Ruiz et al., 2023), suggest that uCysC/uCr may serve as a promising urinary biomarker of tubular injury or dysfunction in early stages of CKD, or grades of AKI.

Urinary albumin and cystatin C compete for the same receptors on the luminal surface of tubular cells, and competitive inhibition of uCysC reabsorption may occur, particularly if albuminuria is severe (Thielemans et al., 1994). In Study II, sixteen of the 27 CKD dogs with increased uCysC/uCr also had proteinuria (UPC > 0.5). Eleven CKD dogs had elevated uCysC/uCr without proteinuria, and in these dogs uCysC/uCr provided new information. In study III, an elevated UPC in urine samples often coexisted with an elevated uCysc/uCr. In total, 43 of 67 hospitalized dogs had an uCysC/uCr above the limit of the healthy dogs. For UPC the corresponding number was 25 of 67 (that is all dogs with an elevated UPC also had an elevated uCysC/uCr, but 18 dogs had elevated uCysC/uCr without an increased UPC). Cystatin C itself is a protein and thereby contributes to the total UPC. Only eight of 67 hospitalized dogs had glomerular range proteinuria (UPC >3) and therefore the influence of UPC receptor competition is likely not to have affected results in most dogs. Urinary CysC probably bring complementary information to UPC, since the latter may result from prerenal, renal, or postrenal causes.

Cystatin C is freely filtered in glomerulus and marked elevations in serum CysC is theoretically a possible reason for increased uCysC. The renal threshold for sCysC in dogs is currently unknown. In Study II, 6 of 16 dogs in CKD stage I had elevated uCysC/Cr with a serum CysC within the reference interval. In Study III, out of the 43 dogs that had an increased uCysC/uCr only 15 had an increased serum CysC concentration, (that is >0.7 mg/l sCysC), and 11 of these 15 dogs were in the AKI_{APP} group where azotemia was present. This indicates that the cause of uCysC/uCr increase in most dogs was not due to increased serum concentrations of CysC.

In conclusion, uCysC is shown to be low in healthy dogs, high in urine from dogs with tubular injury, stable during transport,(Sasaki et al., 2014) and easily measured with routine biochemistry analyzers, making it a promising urinary biomarker for clinical diagnostic use. A clinical application of this is that uCysC is currently one of the available urine tests at the veterinary clinical pathology laboratory, SLU, as a complementary tool in the diagnostic evaluation of patients with suspected non-azotemic CKD or acute tubular injury.

In addition, uCysC may be of prognostic value, because initial levels of uCysC/uCr were higher and remained elevated or increased in the AKI dogs that did not survive during the first two weeks post-hospitalization while it decreased in survivors. A similar pattern was observed for serum cystatin C, but not for serum creatinine or UPC. These findings suggest that uCysC/uCr may have potential as a prognostic marker in AKI, and further studies, designed for evaluation of the prognostic value of uCysC, are warranted.

5.3.3 Urinary enzymes and glucose

In Study II, uGGT/uCr was significantly higher in CKD stage 1 compared to healthy dogs, and in Study III uGGT/uCr was higher on day 1 in EVB, IC and AKI_{APP} groups, compared to healthy dogs. Elevations in uGGT/uCr, as reported in previous studies (Lippi et al., 2018; Rivers et al., 1996), are generally interpreted as indicators of acute tubular injury. Because uGGT has approximately the same size as albumin, a combination of elevated serum GGT and glomerular leakage could potentially lead to an elevated uGGT/uCr. Although, elevated serum GGT was observed in only two dogs in Study III.

A reference interval for uGGT/uCr of 5–28 U/g was established in a study of 42 healthy dogs (Ilchyshyn et al., 2019). This is in agreement with the results from the healthy dogs in study II and III. In Study II the range of uGGT/uCr in the healthy dogs was 0.6–43.6, with 28 of 30 dogs having values <25 U/g, and in study III (AKI), uGGT/uCr ranged from 0.5–28.7 U/g. A previous study in dogs with AKI (Lippi et al., 2018) proposed a cut-off of 54.3 U/g to achieve optimal specificity (89.1%) and sensitivity (85.7%) for detection acute tubular injury. Using a lower cut-off, closer to 30 U/g, would increase sensitivity but at the expense of reduced specificity, as some healthy dogs have values in this range. In summary, the cut-off

proposed by Lippi et al. makes sense although an alternative might be to consider a “grey zone” between 28.5 and 50 U/g.

Urinary GGT/uCr is stable up to three days at 20°C and 5 days at 4°C (Ilchyshyn et al., 2019), of greater concern is perhaps published data that indicate variability in measurement after two months at –80 °C (Ilchyshyn et al., 2019), which is why we did not include uGGT in the final publication of study II. However, all samples in study II were treated the same, and any long-term effect is likely to have effected results in all groups of dogs similarly. Additional long-term stability studies are needed for uGGT. In clinical settings uGGT is most often analyzed in fresh samples. Our findings together with result from previous studies suggest that uGGT/uCr is a potential urinary biomarker of early tubular injury in CKD and AKI.

Urinary NAG/uCr was elevated in 57% of the AKI dogs, 20% of the ICU dogs and 14% of the EBV-dogs on day 1. The AKI group had significantly higher uNAG/uCr than the other three groups, while no differences between the other groups were detected. In summary, uNAG/uCr did not demonstrate convincing utility as an early biomarker of AKI in study III, however, its potential should not be dismissed because it was not demonstrated in this study. Earlier studies have suggested that uNAG can be used in routine toxicity testing due to its sensitivity and specificity for detection of renal tissue injury (Sun et al., 2019; Heiene et al., 2001; Kules et al., 2018).

In Study II, 6 of the 10 dogs that had a uGlu/uCr above the highest value of the control dogs, also had glucose detected on the dipstick. Corresponding numbers for study III was only 2 of 23 dogs, for the rest of the dogs uGlu/uCr brought new information. In Study III, uGlu/uCr was higher in the EVB, IC, and AKI_{APP} groups, compared to the healthy group. In dogs with CKD (Study II) there was no significant difference in uGlu/Cr among groups. No dog in Study II or III were hyperglycemic. Urinary Glu/uCr is probably more valuable as a marker of acute tubular injury or dysfunction than for identification of CKD stage 1.

5.3.4 Electrolytes and fractional excretion

In Study I, the CV_I for uNa was ~60%, regardless if expressed as concentration, normalized to creatinine, or FE. A study in humans reported a CV_I of 35.8% (Ricos et al., 1994), but to the author´s best knowledge no previous data is available for dogs. The high variability in dogs may reflect influences of diet even though food was not changed for any of the dogs

throughout the study. Urine dilution could also affect uNa concentration, however, the CV_I remained high even when corrected for uCr or expressed as FE. In contrast, sNa demonstrated the lowest CV_I and CV_G, indicating that renal regulation prioritizes stable sNa, leading to a variable uNa. Urinary urea/uCr exhibited the lowest CV_I, although greater variability could be expected with more diverse diets or hydration states, as urea reabsorption in the loop of Henle contributes to fluid regulation. Notably, sUrea was one of the analytes with the highest CV_I and CV_G. Thus, urea may be viewed as the opposite of sodium: wider fluctuations are tolerated in blood, resulting in less variation in urine. These biological variation data reflect the mechanisms by which the body maintains homeostasis.

Fractional excretion (FE) is a calculation used to assess the fraction of a plasma constituent filtered in the glomerulus that remains in the urine (Lefebvre et al., 2008). Fractional excretion is mainly used for substances such as electrolytes and urea that are freely filtered through glomerulus and reabsorbed based on the need of the metabolite in the body.

Buranakarl et al. 2007 showed that FE-Na, FE-K and FE-Cl were associated with the degree of renal azotemia (Buranakarl et al., 2007), and FE-P has been reported to be higher in dogs with severe CKD compared to dogs with less severe CKD (Martorelli et al., 2017). The fractional excretion of Na, K, Cl, Ca, and P in the dogs with CKD in Study II also increased with IRIS stage and serum creatine concentration, while the concentration of the urinary electrolytes decreased. The decrease was probably caused by urine dilution because when electrolytes were normalized to uCr, this pattern disappeared. The increases in FE-electrolytes closely followed the elevation of serum creatinine. Serum creatinine seemed to dominate the FE-formula for the azotemic CKD dogs causing FE elevation for all electrolytes. In a study of 314 people with kidney diseases the calculated FE of electrolytes increased progressively along with the decline of estimated GFR regardless of CKD or AKI (YU, 2023). Fractional excretion should probably be used with caution in dogs with marked azotemia.

To the author´s knowledge, there are no previous publications on BV of FE of biochemical analytes in canine urine, and for FE-electrolytes clearly defined reference intervals have been missing (Ettinger, 2024).

5.3.5 A panel of biomarkers

Urinary CysC/uCr and uGGT/uCr were higher in dogs with CKD stage 1 compared to healthy dogs (Study I) and both markers together with uGlu/uCr were also higher in dogs with suspected acute tubular injury compared to healthy dogs (Study III). This suggests their potential as urinary biomarkers of tubular injury or dysfunction in dogs, to be of use before azotemia appear and can be diagnosed with currently used clinical diagnostic methods. For each marker there are advantages and disadvantages to take under consideration, although the fact that the urinary analytes were elevated concurrently strengthens the diagnosis of tubular injury. Ideally, these (and possibly other) urinary biomarkers should be used together as part of a diagnostic panel.

5.4 Methodological aspects

The biochemistry instrument at the university hospital laboratory was replaced at a point in time between the performance of study II and study III. This could perhaps have been viewed as a limitation for comparing results across studies. However, it was an advantage because with the new equipment the need for methodological improvements, identified in Studies I and II, could be addressed and solved. Methods detecting lower values of uProt were warranted, and could be established for study III, detecting down to 0.03 mg/L. Also, in healthy dogs uCysC is extremely low (often <0.1 mg/L), whereas dogs with severe tubular damage can have urine concentrations more than 300 times higher. This wide range presents a major methodological challenge. One Norwegian study investigated several urinary biomarkers, including uCysC/uCr in dogs envenomated by Vipera berus, and reported no significant differences for uCysC/uCr compared to healthy controls at any time point (Harjen et al., 2021). Different assays measure uCysC differently; bead-based methods report much lower values than validated ELISA (enzyme-linked immunosorbent assay) or PETIA (particle-enhanced turbidimetric immunoassay), likely due to poor cross-reactivity (Davis et al., 2021). In study III cystatin C was analysed with PETIA, and to capture both low and very high levels, urine samples were analysed with both the standard serum method and with high-sensitivity settings. The standard method is sufficient for most clinically relevant elevations but may be limited in detecting small amounts of uCysC in dilute

urine. To accurately detect both very low and very high concentrations, samples in study III were analyzed using both the standard serum method and a high-sensitivity application.

5.5 Limitations

A limitation of study II is the prolonged storage of urine samples. Samples from the included dogs in study II were stored at −80 °C for up to nine years; however, storage time did not differ between groups. Long-term storage of urinary samples at −80 °C is common within human research for the preservation of urinary metabolites, and many metabolites are considered stable under these conditions (Petrucci G, 2024; Beauval et al., 2022; Remer, 2014). Stability depends on sample handling, storage conditions, and the specific analyte (Stevens et al., 2019; Peakman and Elliott, 2008). Urinary creatinine, urea, Na, Cl, K, Ca, and P have been shown to remain stable in human urine at −22 °C for more than 12 years (Remer, 2014). Similarly, urinary protein was stable in a study of 2.5 years, when stored at −70 °C (Parekh et al 2007). The author could only find one stability study on uGGT in dogs, followed for two months in -80°, where levels of uGGT were stable to begin with but fluctuated around 5% at the end of the study (Ilchyshyn et al., 2019). These findings are similar to those reported in human samples (Bollick et al., 2018). For uGlu, no long-time stability study was found but urinary glucose is generally considered to maintain high stability under appropriate storage conditions. The concentration of CysC in canine urine was studied for 3 months at −80°C and showed stability during this time (Monti P et al 2012). It is worth noting that when it is stated that it was studied for three months, this does not imply that the value declined during this period, but rather that the study was concluded at that time. An alternative to long term storage in to analyze fresh, but then problems with inter-assay variation or method changes may arise.

The lack of a golden standard represents another limitation in research on urinary markers of tubular damage and dysfunction. A definitive diagnosis of tubular injury would have required renal biopsies, but this was not feasible in this study. However, the association between histopathological evidence of tubular damage and elevated uCysC/uCr, uGGT/uCr, uNAG/uCr, respectively uGlu/uCr have been showed preciously in dogs (Sasaki et al., 2014; Sun et al., 2019; Gu et al., 2018). Also, tubular dysfunction might not

be evident on histological examination of renal tissue as illustrated in a study on canine Fanconi syndrome (Thompson et al., 2013), making confirmation of its presence even more difficult. This lack of a golden standard creates a problem when the diagnostic accuracy of markers of tubular injury and dysfunction is evaluated. The use of multiple markers probably increases diagnostic accuracy and should be considered, especially in clinical situations.

6. Conclusion and clinical applications

- This thesis provides information on biological variation and reference intervals of urinary and serum biochemical analytes in healthy dogs. This data is important for appropriate interpretation of laboratory results, relevant in clinical situations such as investigation of electrolyte disorders in dogs.

- In dogs with CKD stage I, urinary CysC/uCr and uGGT/uCr was higher than in the healthy dogs and were therefore considered potential urinary biomarkers of renal tubular injury. These may be potentially useful in clinical situations when early stage kidney disease is on the differential list (for example in polyuria and polydipsia investigations, pre anesthetic profiles, and suspicion of congenital tubulointerstitial kidney disease).

- Fractional excretion of electrolytes increased with IRIS stage in dogs with CKD, but serum creatinine had a dominant impact on the FE-formula, therefore it is probably advised to interpret calculations of FE in azotemic dogs with caution.

- Urinary CysC/uCr, uGGT/uCr, and uGlu/uCr were markedly increased in dogs with clinically apparent AKI and also increased significantly in a portion of critically ill and snake envenomated dogs, compared to healthy dogs. These urinary analytes have potential as urinary biomarkers for detection of renal tubular injury or dysfunction.

- Among dogs with clinically apparent AKI, non-survivors had significantly higher uCys/uCr on day 1 and 2 than survivors. Furthermore, uCys/uCr remained high or increased in the dogs that died or were euthanized, and decreased in survivors over two weeks post hospitalization. This indicates that uCysC/uCr could be of value as a prognostic marker.

7. Future perspectives

The results of this thesis raise new questions to be answered in future research.

- The high index of individuality for serum creatinine makes the use of RCV indicated (Study I), but with stratification it is likely that population-based reference intervals will be more appropriate. Previous studies have shown that muscle mass can influence serum creatinine levels; however, the impact of different variables on urinary creatinine, for example body weight, remains to be explored in future.

- In veterinary medicine, urinary analytes are routinely normalized by urinary creatinine. Other options include normalization with USG or urine osmolality. Further research is needed to clarify the advantages and limitations of each approach and to establish optimal strategies for different biomarkers and different clinical situations.

- Among dogs with clinically apparent AKI, non-survivors had significantly higher uCysC/uCr during the first 48 hours than survivors. Furthermore, uCysC/uCr remained high or increased in the dogs that died or were euthanized-, and decreased in survivors over two weeks post hospitalization. This indicates that uCysC/uCr could potentially be of value as a prognostic marker and further investigations of this aspect are needed.

- The specificity of uCysC as a marker of tubular injury needs to be further evaluated. A study investigating the possible influence of hematuria and pyuria on uCysC levels is planned.

- Long-term storage at –80 °C is an established way to save biological material, and many analytes have been shown to remain stable over extended periods. However, some urinary analytes have primarily been studied over shorter durations such as 3-6 months, and some are primarily studied within human medicine, thus further long-term stability studies for dogs are warranted.

References

Abebayehu A (2023) Urine test strip analysis, concentration range and its interpretations of the parameters. *GSC Biological and Pharmaceutical Sciences* 22(2): 001-003.

Armbruster DA and Pry T (2008) Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 29 Suppl 1(Suppl 1): S49-52.

Bagshaw SM, Bennett M, Devarajan P, et al. (2013) Urine biochemistry in septic and non-septic acute kidney injury: a prospective observational study. *J Crit Care* 28(4): 371-378.

Balar S, Beke N, Patki D, et al. (2023) The Correlation of Urine Protein/osmolality and Protein/creatinine Ratio as Predictor of 24-hour Urinary Protein Excretion. *J Assoc Physicians India* 71(5): 11-12.

Banfi G, Bauer K, Brand W, et al. (2002) World health organization. WHO/DIL/LAB/99.1Rev2: 48.

Beauval N, Leroyer A, Hisbergues M, et al. (2022) Stability of trace element concentrations in frozen urine - Effect on different elements of more than 10 years at - 80 degrees C. *J Trace Elem Med Biol* 74: 127080.

Block G (2024) Evidence-based veterinary medicine-potential, practice, and pitfalls. *J Vet Intern Med* 38(6): 3261-3271.

Bollick YS, de Carvalho JAM, Tatsch E, et al. (2018) Reference limits of the urinary gamma-glutamyltransferase in a healthy population and effects of short-term storage on the enzyme activity. *Clin Chim Acta* 482: 46-49.

Bovee KC, Joyce T, Reynolds R, et al. (1978) The fanconi syndrome in Basenji dogs: a new model for renal transport defects. *Science* 201(4361): 1129-1131.

Braun JP, Lefebvre HP and Watson AD (2003) Creatinine in the dog: a review. *Vet Clin Pathol* 32(4): 162-179.

Brown N, Segev G, Francey T, et al. (2015) Glomerular filtration rate, urine production, and fractional clearance of electrolytes in acute kidney injury in dogs and their association with survival. *J Vet Intern Med* 29(1): 28-34.

Buranakarl C, Ankanaporn K, Thammacharoen S, et al. (2007) Relationships between degree of azotaemia and blood pressure, urinary protein:creatinine ratio and fractional excretion of electrolytes in dogs with renal azotaemia. *Vet Res Commun* 31(3): 245-257.

Califf RM (2018) Biomarker definitions and their applications. *Exp Biol Med (Maywood)* 243(3): 213-221.

Callens AJ and Bartges JW (2015) Urinalysis. *Vet Clin North Am Small Anim Pract* 45(4): 621-637.

Chavan D (2022) Analytical method validation: A brief review. *World Journal of Advanced Research and Reviews (WJARR)* 16(02), 389–402.

Citron LE, Weinstein NM, Littman MP, et al. (2020) Urine cortisol-creatinine and protein-creatinine ratios in urine samples from healthy dogs collected at home and in hospital. *J Vet Intern Med* 34(2): 777-782.

Clemo FA (1998) Urinary enzyme evaluation of nephrotoxicity in the dog. *Toxicol Pathol* 26(1): 29-32.
Cobrin AR, Blois SL, Kruth SA, et al. (2013) Biomarkers in the assessment of acute and chronic kidney diseases in the dog and cat. *J Small Anim Pract* 54(12): 647-655.
Conti M, Moutereau S, Zater M, et al. (2006) Urinary cystatin C as a specific marker of tubular dysfunction. *Clin Chem Lab Med* 44(3): 288-291.
Couture Y, Keys D and Summers S (2025) Weekly Biological Variation of Urine Protein Creatinine Ratio and Urine Specific Gravity in Healthy Dogs. *J Vet Intern Med* 39(2): e70052.
Cowgill LD, Segev G, Vaden S, et al. (2023) Differentiation of stable kidney function versus progressive dysfunction in dogs. *J Vet Intern Med* 37(6): 2241-2250.
Damm E (2020) Concentration of chemical metabolites in urine of healthy dogs.Veterinary program (Master thesis, Advanced level A2E) Swedish University of Agriculture Sciences. https://stud.epsilon.slu.se/15873/ 2020.
Davis J, Rossi G, Miller DW, et al. (2021) Ability of different assay platforms to measure renal biomarker concentrations during ischaemia-reperfusion acute kidney injury in dogs. *Res Vet Sci* 135: 547-554.
De Schepper J, De Cock I and Capiau E (1989) Urinary gamma-glutamyl transferase and the degree of renal dysfunction in 75 bitches with pyometra. *Res Vet Sci* 46(3): 396-400.
Deen WM, Lazzara MJ and Myers BD (2001) Structural determinants of glomerular permeability. *Am J Physiol Renal Physiol* 281(4): F579-596.
DiBartola S (2012) *Fluid, Electrolyte, and Acid-Base Disorders in Small Animal Practice.* Elsevier Applied renal physiology Chp 2; 26-43.
Duffy ME, Specht A and Hill RC (2015) Comparison between Urine Protein: Creatinine Ratios of Samples Obtained from Dogs in Home and Hospital Settings. *J Vet Intern Med* 29(4): 1029-1035.
Dunaevich A, Chen H, Musseri D, et al. (2020) Acute on chronic kidney disease in dogs: Etiology, clinical and clinicopathologic findings, prognostic markers, and survival. *J Vet Intern Med* 34(6): 2507-2515.
Duncan JR and Prasse KW (1976) Clinical examination of the urine. *Vet Clin North Am* 6(4): 647-661.
Espinel CH, Mendez-Picon G, Currier C, et al. (1979) FE Na effective in early diagnosis of acute rejection after kidney transplantation. *Proc Clin Dial Transplant Forum* 9: 256-259.
Ettinger SJ, Cote, Etienne, Feldman Edward, (2024) *Veterinary Internal Medicine.* St. Louis, Missouri: Elsevier. Renal Diseases, Chp 299. page; 2053-2070.
Feeman WE, 3rd, Couto CG and Gray TL (2003) Serum creatinine concentrations in retired racing Greyhounds. *Vet Clin Pathol* 32(1): 40-42.
Finco DR, Brown SA, Vaden SL, et al. (1995) Relationship between plasma creatinine concentration and glomerular filtration rate in dogs. *J Vet Pharmacol Ther* 18(6): 418-421.

Fisher CE and Howie SE (2006) The role of megalin (LRP-2/Gp330) during development. *Dev Biol* 296(2): 279-297.

Flatland B, Baral RM and Freeman KP (2020) Current and emerging concepts in biological and analytical variation applied in clinical practice. *J Vet Intern Med* 34(6): 2691-2700.

Fraser CG (2001) *Biological variation: From principles to practice.* AACC Press.

Freeman KP, Baral RM, Dhand NK, et al. (2017) Recommendations for designing and conducting veterinary clinical pathology biologic variation studies. *Vet Clin Pathol* 46(2): 211-220.

Friedrichs KR, Harr KE, Freeman KP, et al. (2012) ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol* 41(4): 441-453.

Geffre A, Friedrichs K, Harr K, et al. (2009) Reference values: a review. *Vet Clin Pathol* 38(3): 288-298.

Gerber B, Glaus TM, Unterer S, et al. (2004) [Evaluation of parameters for the differentiation of acute from chronic renal failure in the dog]. *Schweiz Arch Tierheilkd* 146(8): 365-373.

Ghys L, Paepe D, Smets P, et al. (2014) Cystatin C: a new renal marker and its potential use in small animal medicine. *J Vet Intern Med* 28(4): 1152-1164.

Grassinger JM, Merz S, Aupperle-Lellbach H, et al. (2019) Correlation of BRAF Variant V595E, Breed, Histological Grade and Cyclooxygenase-2 Expression in Canine Transitional Cell Carcinomas. *Vet Sci* 6(1).

Grauer GF (2005) Early detection of renal damage and disease in dogs and cats. *Vet Clin North Am Small Anim Pract* 35(3): 581-596.

Grauer GF (2011) Proteinuria: measurement and interpretation. *Top Companion Anim Med* 26(3): 121-127.

Grauer GF, Greco DS, Behrend EN, et al. (1995) Estimation of quantitative enzymuria in dogs with gentamicin-induced nephrotoxicosis using urine enzyme/creatinine ratios from spot urine samples. *J Vet Intern Med* 9(5): 324-327.

Greco DS, Turnwald GH, Adams R, et al. (1985) Urinary gamma-glutamyl transpeptidase activity in dogs with gentamicin-induced nephrotoxicity. *Am J Vet Res* 46(11): 2332-2335.

Grubb AO (2000) Cystatin C--properties and use as diagnostic marker. *Adv Clin Chem* 35: 63-99.

Gu YZ, Vlasakova K, Troth SP, et al. (2018) Performance Assessment of New Urinary Translational Safety Biomarkers of Drug-induced Renal Tubular Injury in Tenofovir-treated Cynomolgus Monkeys and Beagle Dogs. *Toxicol Pathol* 46(5): 553-563.

Harjen HJ, Nicolaysen TV, Negard T, et al. (2021) Serial serum creatinine, SDMA and urinary acute kidney injury biomarker measurements in dogs envenomated by the European adder (Vipera berus). *BMC Vet Res* 17(1): 154.

Harley L and Langston C (2012) Proteinuria in dogs and cats. *Can Vet J* 53(6): 631-638.

Harr KE, Flatland B, Nabity M, et al. (2013) ASVCP guidelines: allowable total error guidelines for biochemistry. *Vet Clin Pathol* 42(4): 424-436.

Harris RH and Gill JM (1981) Changes in glomerular filtration rate during complete ureteral obstruction in rats. *Kidney Int* 19(4): 603-608.

Heiene R, Bjorndal H and Indrebo A (2010) Glucosuria in Norwegian elkhounds and other breeds during dog shows. *Vet Rec* 166(15): 459-462.

Heiene R, Moe L and Molmen G (2001) Calculation of urinary enzyme excretion, with renal structure and function in dogs with pyometra. *Res Vet Sci* 70(2): 129-137.

Herget-Rosenthal S, Feldkamp T, Volbracht L, et al. (2004) Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. *Ann Clin Biochem* 41(Pt 2): 111-118.

Herget-Rosenthal S, van Wijk JA, Brocker-Preuss M, et al. (2007) Increased urinary cystatin C reflects structural and functional renal tubular impairment independent of glomerular filtration rate. *Clin Biochem* 40(13-14): 946-951.

Himmelfarb MJ, Bruce Molitoris, et al. (2008) Evaluation and initial management of acute kidney injury. *Clin J Am Soc Nephrol.* Jul;3(4):962-7.

Hokamp JA, Leidy SA, Gaynanova I, et al. (2018) Correlation of electrophoretic urine protein banding patterns with severity of renal damage in dogs with proteinuric chronic kidney disease. *Vet Clin Pathol* 47(3): 425-434.

Hokamp JA and Nabity MB (2016) Renal biomarkers in domestic species. *Vet Clin Pathol* 45(1): 28-56.

Ibba F, Mangiagalli G and Paltrinieri S (2016) Urinary gamma-glutamyl transferase (GGT) as a marker of tubular proteinuria in dogs with canine leishmaniasis, using sodium dodecylsulphate (SDS) electrophoresis as a reference method. *Vet J* 210: 89-91.

Ilchyshyn NP, Villiers E and Monti P (2019) Validation of a spectrophotometric method for GGT measurement in canine urine and determination of the urine GGT-to-creatinine ratio reference interval and biological variation in 41 healthy dogs. *J Vet Diagn Invest* 31(1): 33-39.

Jacob F, Polzin DJ, Osborne CA, et al. (2005) Evaluation of the association between initial proteinuria and morbidity rate or death in dogs with naturally occurring chronic renal failure. *J Am Vet Med Assoc* 226(3): 393-400.

Jensen AL and Aaes H (1993) Critical differences of clinical chemical parameters in blood from dogs. *Res Vet Sci* 54(1): 10-14.

Jensen D, Kierulf-Lassen C, Kristensen MLV, et al. (2017) Megalin dependent urinary cystatin C excretion in ischemic kidney injury in rats. *PLoS One* 12(6): e0178796.

Kaseda R, Iino N, Hosojima M, et al. (2007) Megalin-mediated endocytosis of cystatin C in proximal tubule cells. *Biochem Biophys Res Commun* 357(4): 1130-1134.
Keen B, Cawley A, Reedy B, et al. (2022) Metabolomics in clinical and forensic toxicology, sports anti-doping and veterinary residues. *Drug Test Anal* 14(5): 794-807.
King JB (2016) Proximal tubular nephropathy in two dogs diagnosed with lead toxicity. *Aust Vet J* 94(8): 280-284.
Knight EL, Verhave JC, Spiegelman D, et al. (2004) Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. *Kidney Int* 65(4): 1416-1421.
Kokkinos Y, Morrison J, Bradley R, et al. (2022) An early prediction model for canine chronic kidney disease based on routine clinical laboratory tests. *Sci Rep* 12(1): 14489.
Kopke MA, Burchell RK, Ruaux CG, et al. (2018) Variability of Symmetric Dimethylarginine in Apparently Healthy Dogs. *J Vet Intern Med* 32(2): 736-742.
Kules J, Bilic P, Beer Ljubic B, et al. (2018) Glomerular and tubular kidney damage markers in canine babesiosis caused by Babesia canis. *Ticks Tick Borne Dis* 9(6): 1508-1517.
Langlois DK, Smedley RC, Schall WD, et al. (2013) Acquired proximal renal tubular dysfunction in 9 Labrador Retrievers with copper-associated hepatitis (2006-2012). *J Vet Intern Med* 27(3): 491-499.
Lazzara MJ and Deen WM (2007) Model of albumin reabsorption in the proximal tubule. *Am J Physiol Renal Physiol* 292(1): F430-439.
Lees GE, Brown SA, Elliott J, et al. (2005) Assessment and management of proteinuria in dogs and cats: 2004 ACVIM Forum Consensus Statement (small animal). *J Vet Intern Med* 19(3): 377-385.
Lefebvre HP, Dossin O, Trumel C, et al. (2008) Fractional excretion tests: a critical review of methods and applications in domestic animals. *Vet Clin Pathol* 37(1): 4-20.
Legatti SAM, El Dib R, Legatti E, et al. (2018) Acute kidney injury in cats and dogs: A proportional meta-analysis of case series studies. *PLoS One* 13(1): e0190772.
Leissing N, Izzo R and Sargent H (1985) Variance estimates and individuality ratios of 25 serum constituents in beagles. *Clin Chem* 31(1): 83-86.
Lemley KV and Kriz W (1991) Anatomy of the renal interstitium. *Kidney Int* 39(3): 370-381.
Lima C and Macedo E (2018) Urinary Biochemistry in the Diagnosis of Acute Kidney Injury. *Dis Markers* 2018: 4907024.
Lippi I, Perondi F, Meucci V, et al. (2018) Clinical utility of urine kidney injury molecule-1 (KIM-1) and gamma-glutamyl transferase (GGT) in the diagnosis of canine acute kidney injury. *Vet Res Commun* 42(2): 95-100.

Martorelli CR, Kogika MM, Chacar FC, et al. (2017) Urinary Fractional Excretion of Phosphorus in Dogs with Spontaneous Chronic Kidney Disease. *Vet Sci* 4(4).

Monaghan (2021) Foundational Statistical Principles in Medical Research: Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value. *Medicina*.

Monti P, Benchekroun G, Berlato D, et al. (2012) Initial evaluation of canine urinary cystatin C as a marker of renal tubular function. *J Small Anim Pract* 53(5): 254-259.

Mårtensson F (2017) Biological variation of urine protein and urine creatinine in healthy dogs.Veterinary program (Master thesis, Advanced level A2E) Swedish University of Agriculture Sciences. http://stud.epsilon.slu.se 2017:36.

Nabity M and Hokamp J (2023) Urinary Biomarkers of Kidney Disease in Dogs and Cats. *Vet Clin North Am Small Anim Pract* 53(1): 53-71.

Nabity MB, Boggess MM, Kashtan CE, et al. (2007) Day-to-Day variation of the urine protein: creatinine ratio in female dogs with stable glomerular proteinuria caused by X-linked hereditary nephropathy. *J Vet Intern Med* 21(3): 425-430.

Nenov VD, Taal MW, Sakharova OV, et al. (2000) Multi-hit nature of chronic renal disease. *Curr Opin Nephrol Hypertens* 9(2): 85-97.

Newman DJ, Pugia MJ, Lott JA, et al. (2000) Urinary protein and albumin excretion corrected by creatinine and specific gravity. *Clin Chim Acta* 294(1-2): 139-155.

Nivy R, Avital Y, Aroch I, et al. (2017) Utility of urinary alkaline phosphatase and gamma-glutamyl transpeptidase in diagnosing acute kidney injury in dogs. *Vet J* 220: 43-47.

Nivy R, Chaim N, Hanael E, et al. (2021) Prospective evaluation of 5 urinary biomarkers as predictors of acute kidney injury in nonazotemic, hospitalized dogs. *J Vet Intern Med* 35(6): 2812-2820.

Pagitz M, Frommlet F and Schwendenwein I (2007) Evaluation of biological variance of cystatin C in comparison with other endogenous markers of glomerular filtration rate in healthy dogs. *J Vet Intern Med* 21(5): 936-942.

Parekh RS, Kao WH, Meoni LA, et al. (2007) Reliability of urinary albumin, total protein, and creatinine assays after prolonged storage. *Clin J Am Soc Nephrol* 2(6): 1156-1162.

Peakman TC and Elliott P (2008) The UK Biobank sample handling and storage validation studies. *Int J Epidemiol* 37 Suppl 1: i2-6.

Pelander L, Haggstrom J, Larsson A, et al. (2019) Comparison of the diagnostic value of symmetric dimethylarginine, cystatin C, and creatinine for detection of decreased glomerular filtration rate in dogs. *J Vet Intern Med* 33(2): 630-639.

Perondi F, Lippi I, Ceccherini G, et al. (2019) Evaluation of urinary gamma-glutamyl transferase and serum creatinine in non-azotaemic hospitalised dogs. *Vet Rec* 185(2): 52.

Petrucci G HD, Langley R et al. (2024) Effect of very long term storage and multiple freeze nd thaw cycles 11-dhydrotromoboxane-B2 and 8-isoprostaglandin, levels in human urine samples. *Sci.Rep* 2024;14; 5546.

Piech TL and Wycislo KL (2019) Importance of Urinalysis. *Vet Clin North Am Small Anim Pract* 49(2): 233-245.

Polzin DJ (2011) Chronic kidney disease in small animals. *Vet Clin North Am Small Anim Pract* 41(1): 15-30.

Prause LC and Grauer GF (1998) Association of gastrointestinal hemorrhage with increased blood urea nitrogen and BUN/creatinine ratio in dogs: a literature review and retrospective study. *Vet Clin Pathol* 27(4): 107-111.

Pressler BM (2013) Clinical approach to advanced renal function testing in dogs and cats. *Vet Clin North Am Small Anim Pract* 43(6): 1193-1208, v.

Reece (2015) *Dukes Physiology of Domestic Animals.* Pondicherry, India: Wiley Blackwell, Section III, Chp: "The kidneys and Urinary system"p.157-173.

Remer MB, Shi (2014) Long-term urine biobanking: Storage stability of clinical chemical parameters under moderate freezing conditions without use of preservatives. *Clinical Biochemistry* 47(18): 307-311.

Ricos C, Jimenez CV, Hernandez A, et al. (1994) Biological variation in urine samples used for analyte measurements. *Clin Chem* 40(3): 472-477.

Rivers BJ, Walter PA, O'Brien TD, et al. (1996) Evaluation of urine gamma-glutamyl transpeptidase-to-creatinine ratio as a diagnostic tool in an experimental model of aminoglycoside-induced acute renal failure in the dog. *J Am Anim Hosp Assoc* 32(4): 323-336.

Ross L (2011) Acute kidney injury in dogs and cats. *Vet Clin North Am Small Anim Pract* 41(1): 1-14.

Ruaux CG, Carney PC, Suchodolski JS, et al. (2012) Estimates of biological variation in routinely measured biochemical analytes in clinically healthy dogs. *Vet Clin Pathol* 41(4): 541-547.

Ruiz P, Duran A, Duque FJ, et al. (2023) Urinary cystatin C and N-acetyl-beta-D-glucosaminidase (NAG) as early biomarkers for renal disease in dogs with leishmaniosis. *Vet Parasitol* 318: 109930.

Saha H, Mustonen J, Helin H, et al. (1987) Limited value of the fractional excretion of sodium test in the diagnosis of acute renal failure. *Nephrol Dial Transplant* 2(2): 79-82.

Sand HESV (2004) *Människan fysiologi.* Liber, Njurar och urinvägar, page; 450-478.

Sasaki A, Sasaki Y, Iwama R, et al. (2014) Comparison of renal biomarkers with glomerular filtration rate in susceptibility to the detection of gentamicin-induced acute kidney injury in dogs. *J Comp Pathol* 151(2-3): 264-270.

Segev G, Daminet S, Meyer E, et al. (2015) Characterization of kidney damage using several renal biomarkers in dogs with naturally occurring heatstroke. *Vet J* 206(2): 231-235.

Segev G, Kass PH, Francey T, et al. (2008) A novel clinical scoring system for outcome prediction in dogs with acute kidney injury managed by hemodialysis. *J Vet Intern Med* 22(2): 301-308.

Silbernagl S (1988) The renal handling of amino acids and oligopeptides. *Physiol Rev* 68(3): 911-1007.

Smee N, Loyd K and Grauer GF (2013) UTIs in small animal patients: part 2: diagnosis, treatment, and complications. *J Am Anim Hosp Assoc* 49(2): 83-94.

Solberg HE (1995) RefVal: a program implementing the recommendations of the International Federation of Clinical Chemistry on the statistical treatment of reference values. *Comput Methods Programs Biomed* 48(3): 247-256.

Solberg HE (2004) The IFCC recommendation on estimation of reference intervals. The RefVal program. *Clin Chem Lab Med* 42(7): 710-714.

Stevens VL, Hoover E, Wang Y, et al. (2019) Pre-Analytical Factors that Affect Metabolite Stability in Human Urine, Plasma, and Serum: A Review. *Metabolites* 9(8).

Stockham (2025) *Fundamentals of Veterinary Clinical Pathology.* Wiley.Chapter 8, Urinary system, page; 576-583. Chapter 18, Adrenal page; 1075-1078.

Sun B, Zhou X, Qu Z, et al. (2019) Urinary biomarker evaluation for early detection of gentamycin-induced acute kidney injury. *Toxicol Lett* 300: 73-80.

Tangeman LE and Littman MP (2013) Clinicopathologic and atypical features of naturally occurring leptospirosis in dogs: 51 cases (2000-2010). *J Am Vet Med Assoc* 243(9): 1316-1322.

Thielemans N, Lauwerys R and Bernard A (1994) Competition between albumin and low-molecular-weight proteins for renal tubular uptake in experimental nephropathies. *Nephron* 66(4): 453-458.

Thoen ME and Kerl ME (2011) Characterization of acute kidney injury in hospitalized dogs and evaluation of a veterinary acute kidney injury staging system. *J Vet Emerg Crit Care (San Antonio)* 21(6): 648-657.

Thompson MF, Fleeman LM, Kessell AE, et al. (2013) Acquired proximal renal tubulopathy in dogs exposed to a common dried chicken treat: retrospective study of 108 cases (2007-2009). *Aust Vet J* 91(9): 368-373.

Troia R, Gruarin M, Grisetti C, et al. (2018) Fractional excretion of electrolytes in volume-responsive and intrinsic acute kidney injury in dogs: Diagnostic and prognostic implications. *J Vet Intern Med* 32(4): 1372-1382.

Ueta K, Yoneda H, Oku A, et al. (2006) Reduction of renal transport maximum for glucose by inhibition of NA(+)-glucose cotransporter suppresses blood glucose elevation in dogs. *Biol Pharm Bull* 29(1): 114-118.

Vaden SL, Levine J and Breitschwerdt EB (1997) A retrospective case-control of acute renal failure in 99 dogs. *J Vet Intern Med* 11(2): 58-64.

Venkatachalam MA, Griffin KA, Lan R, et al. (2010) Acute kidney injury: a springboard for progression in chronic kidney disease. *Am J Physiol Renal Physiol* 298(5): F1078-1094.

Waikar SS, Sabbisetti VS and Bonventre JV (2010) Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. *Kidney Int* 78(5): 486-494.

YU B, Jiang, L, Li, Z, (2023) Renal Fractional excretion of urea and Uric Acid have a diagnostic value to disitnguish acute kidney injury from chronic disease *Kidney International Reports* 8(WCN23-0173): 42-43.

Yu B and Yang H (2017) Evaluation of Different Estimation Methods for Accuracy and Precision in Biological Assay Validation. *PDA J Pharm Sci Technol* 71(4): 297-305.

Zamagni S, Troia R, Zaccheroni F, et al. (2020) Comparison of clinicopathological patterns of renal tubular damage in dogs with acute kidney injury caused by leptospirosis and other aetiologies. *Vet J* 266: 105573.

Zatelli A, Paltrinieri S, Nizi F, et al. (2010) Evaluation of a urine dipstick test for confirmation or exclusion of proteinuria in dogs. *Am J Vet Res* 71(2): 235-240.

Zeugswetter FK and Schwendenwein I (2020) Basal glucose excretion in dogs: The impact of feeding, obesity, sex, and age. *Vet Clin Pathol* 49(3): 428-435.

Populärvetenskaplig sammanfattning

Akut njursvikt och kronisk njursjukdom utgör vanliga och i många fall allvarliga sjukdomar hos hund. Tidig upptäckt kan möjliggöra behandling innan sjukdomstillstånden gått för långt. Akuta skador som drabbar njurarna hittas oftast inte med hjälp av blodprovanalys förrän skadorna är så stora att de orsakat svikt i funktionen hos hela organet.

Ämnen (analyter) i urin, till exempel proteiner, kan fungera som biomarkörer, då deras mängd ökar i urinen vid skada. För korrekt tolkning av laboratorieresultat är kunskap om analyternas biologiska variation nödvändig. Syftena med denna avhandling var att beskriva biologisk variation och referensintervall för flera urinanalyter hos friska hundar, samt att undersöka om ämnen som cystatin C, glukos, gamma-glutamyl-transferas (GGT), och elektrolyter ökar i urinen hos hundar i olika stadier av kronisk njursjukdom och hos hundar med risk för, eller konstaterad, akut njurskada. Analyserna utfördes med metoder som redan är tillgängliga på många djursjukhus.

I studie I togs upprepade blod- och urinprover från friska hundar för att beräkna biologisk variation för flera urinanalyter både som koncentration, efter kvotning med urin kreatinin för att justera för utspädning av urinen, samt fraktionell exkretion vilket är en beräkning för hur stor mängd av ett ämne som utsöndras i urinen i förhållande till dess mängd i blodet.

Hos hundar med kronisk njursjukdom i studie II sågs förhöjda urinnivåer av cystatin C samt GGT och man såg även att cystatin C ökade med stadie av njursjukdom. Hos hundarna med risk för eller konstaterad akut njurskada i studie III sågs högre urinnivåer av cystatin C, GGT och glukos jämfört med hos friska hundar. Hos hundar med bekräftad akut njurskada som inte överlevde var urin-cystatin C högre jämfört med hos de hundar som överlevde, detta indikerar att cystatin C kanske har värde som prognostisk markör för överlevnad hos hundar med akut njursvikt.

Sammanfattningsvis presenterar denna avhandling information om biologisk variation hos friska hundar och referensintervall för flera urinbiomarkörer. Cystatin C, glukos och GGT i urinen, kvotade med urin kreatinin, utgör lovande tidiga indikatorer för akut och kronisk njursjukdom.

Popular science summary

Acute kidney injury and chronic kidney disease are common in dogs. Early detection can enable treatment before irreversible injury occurs. Injury to the kidneys is often not detected using routine diagnostic methods until the magnitude of organ function is large enough to decrease the filtration rate. Substances (analytes) in urine, such as proteins, can function as biomarkers, as they are increased in urine in the event of damage or impaired function.

The aims of this thesis were to describe normal biological variation and reference intervals for several urinary biomarkers in healthy dogs, and to investigate whether urinary biomarkers such as cystatin C, glucose, GGT and electrolytes increase in dogs at different stages of chronic kidney disease and dogs at risk for, and confirmed, acute kidney injury. The analyses were performed with the type of automated biochemistry instruments that are available in many animal hospitals.

In study I, repeated blood and urine samples were taken from healthy dogs to calculate biological variation for several urine analytes both as concentration, normalized to urine creatinine to adjust for urine concentration, and fractional excretion, which is a calculation of how much of a substance is excreted in the urine in relation to its amount in the blood.

In dogs with early chronic kidney disease in study II, elevated urinary levels of cystatin C and GGT were seen, and cystatin C levels increased with stage of kidney disease. In dogs at risk for or with confirmed acute kidney injury in study III, higher urinary levels of cystatin C, GGT and glucose were seen compared to the control group of healthy dogs. In dogs with confirmed acute kidney injury that did not survive, urinary cystatin C was and remained elevated compared to in dogs that survived, indicating that cystatin C may serve as a prognostic marker.

In conclusion, this thesis presents information on biological variation in healthy dogs and reference intervals for several urinary biomarkers, which is important for correct interpretation of laboratory results. Urinary cystatin C, glucose and GGT normalized to urine creatinine are promising as early markers of acute and chronic kidney disease.

Acknowledgements

The present thesis was performed at the Department of Clinical Sciences at the Swedish University of Agriculture Sciences (SLU) in Uppsala. The work included in this thesis was made possible by generous support from the **Greater Stockholm Veterinary Care Foundation-** in collaboration with the Regional Animal Hospital AniCura Albano, **AGRIA Research Foundation, Marianne Thedes Foundation, Thure F and Karin Forsberg Foundation,** and **SveLand Research Foundation.**

This thesis would not have been possible without help from colleagues, friends and my family. I would like to thank you all, and not least thanks to all participating dogs and their owners for contributing to the research.

I would especially like to express my genuine gratitude to:

My main supervisor Docent **Emma Strage,** for all the knowledge you share about biological variation, clinical pathology, method validation, academic writing and the structure behind scientific work, and also for your great sense of humor.

My former main supervisor, thereafter co-supervisor professor **Inger Lilliehöök**, your expertise and dedication for laboratory work made these three studies possible, you never give up although laboratory days turned into nights, you always answer questions -vacation or not, and I am so grateful for how you review text with your alert eye for every detail**.**

My co-supervisor DVM **Lena Pelander,** for your pedagogical way of teaching everything from how to find ones way in JMP to short and clean writing, and the understanding of renal physiology and clinical management. Your kind mentorship kept me inspired the whole way.

My co-supervisor professor **Anders Larsson**. Thank you for the energy, knowledge, questions and ideas you brought to all supervisor meetings, and for always answer any questions with returning mail.

The head of department **Bodil Ström Holst** for bringing stability and joy at work, and thank you for "checking in" with some encouraging words. Professor **Jens Häggström**. Thank you for being an excellent supervisor "back in the days of veterinary school", that planted a seed of interest for research in me. Also thank you for words to cheer up at the coffee corner.

The clinical pathologists at the university hospital laboratory, **Anna Hillström**, **Luca Ecimovic**, **Harold Tvedten**, **Helena Pettersson** and **Ulrika Falkenö,** for sharing knowledge and coffee brakes. In addition, thank you to everyone in the team at the laboratory at UDS, your kind help has been invaluable. Special thanks to **Maria Lind**, **Mansour Naghibi**, and **Isabell Alnehem** for excellent laboratory work during the long days of batch analysis.

The helpful team at the KV laboratory; **Anna Svensson, Haleh Yazdan-Panh, Gabriella Hallbrink Ågrena,** and **Yongzhi Guo**, for good advice and help with laboratory questions and tasks, and for keeping the osmometer working with me.

Johannes Forkman for bringing excellent statistical help and solutions, as a co-writer in Study I. **Claudia Brömssen** for advice and encouragement within the statistics of Study II and III.

Ylva Sjunnesson –director of postgraduate studies, thank you so much for your help, support and kindness. **Susanne Pettersson** and **everyone at the KV administration** – you are making everything work, thank you so much. **Malin Persson** at the university library, your support with "Kappa-Mallen" was so filled with humor and a genuine desire to really help, you are one in a million, thank you!

Greatest thanks to my fellow PhD colleagues at SLU. **Ninni Rothlin Zachrisson,** this would not have been the same without you, you see "the silver lining" in everything, and I am thankful and happy to have you as a friend for life**. Johanna Holmberg**, you are so filled with knowledge, kindness and humor that just minutes with you can light up ones day. **Karin Kriström**, for all the laughter, the understanding and surviving together when "we run out of gas". **Aleksandar Cojkic** and **Elin Lindell**, for being my friends here and my super weekend-colleagues at Albano. **Karolina Engdahl** for lending your dog to Study I, and your kind support. **Malin Nilsson** for telling me to "submit that article", and **Momo Yu-Wen Kuo** for sweets and friendship. **Anja Pederson** and **Ida Sjöberg** for supporting chats, and many more.

My colleagues and friends at AniCura Albano Hospital, you are my second family, and you know it. Thank you to everyone. Special thanks to the laboratory-, ward- and ICU- team at Albano. **Anna Tidholm**, thank you for

all the energy and inspiration, you have paved the way for research in a clinical setting at Albano. **Erica Wiss** for all knowledge and excellent chieftainship. Thank you to **Anna Bodegård**, **Anna Jidhage**, **Alicia Lange**, **Charlotte Jeeves, Caroline Harlos** and **Lena Scott** for extra support. **Erica Brandeker** and **Sara Jensen** at AniCura Bagarmossen, and fellow colleagues at **AniCura Aros clinic** in Västerås. **Sandra Friberg** for all help with the healthy dogs.

Among the dog owners extra thanks **to Eva Giertz** and **Julia Giertz,** and **Emelie Mimmi Schneider** for helping out with many of the healthy dogs.

All my friends in life. Special thanks to **Annica Å**, **Anna F** (I know you are with me), **Efva E**, **Eva H**, **Helena B**, **Jessica N,** and **Åsa E**, for reminding me about everything on the outside, your friendship and humor means the world to me.

My family. **Mom** and **dad**, thank you for bringing love, security and support.

Daniel, you lift me up. Many parts of this thesis are written at your kitchen table. You challenge and inspire me. Thank you for your engagement, I love discussing things with you. I love you.

Oliver, for also being a night owl, I enjoy our conversations. **Paulina** because you have my back and I have yours. **Bianca** for all the light and joy you are bringing.

Olle och **Petter,** thank you for everyday life, I am so proud of you, I love you always.

I

Received: 22 November 2021 | Revised: 21 October 2022 | Accepted: 27 November 2022

DOI: 10.1111/vcp.13225

Veterinary Clinical Pathology
An International Journal of Laboratory Medicine
WILEY

ORIGINAL ARTICLE

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

Funding information
Marianne Thedes Foundation., Grant/Award Number: 2019; The Greater Stockholm Veterinary Hospital Foundation

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

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

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.

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 intraindividual 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

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

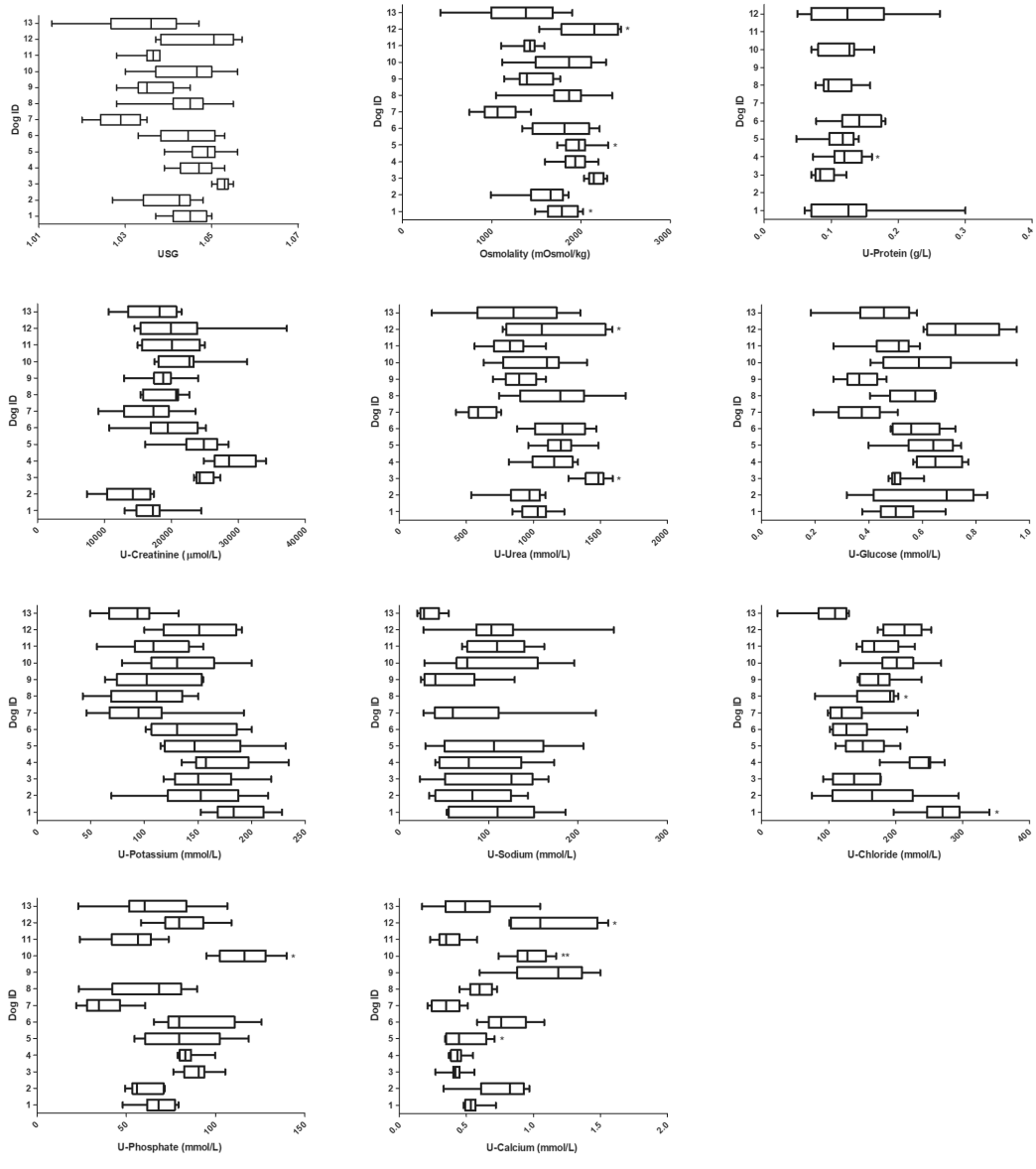


FIGURE 1 Distribution of urine analytes in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

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

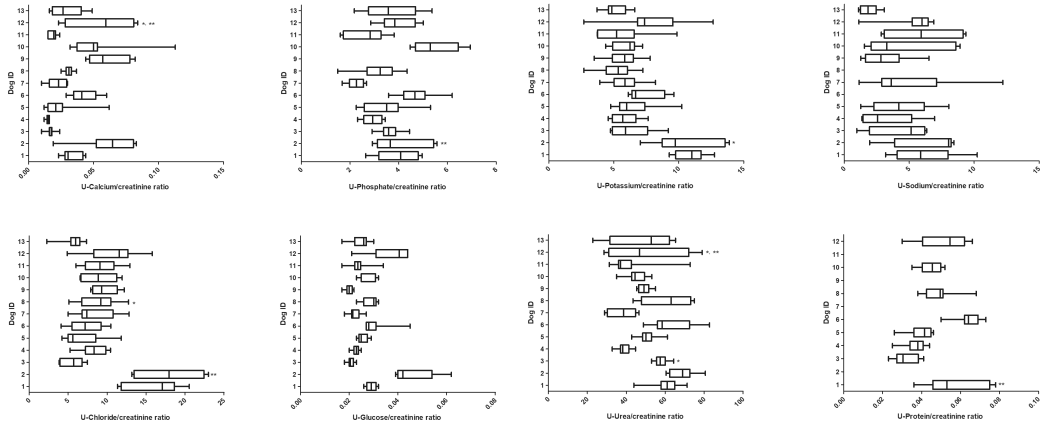


FIGURE 2 Distribution of urine analytes in relation to urinary creatinine in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

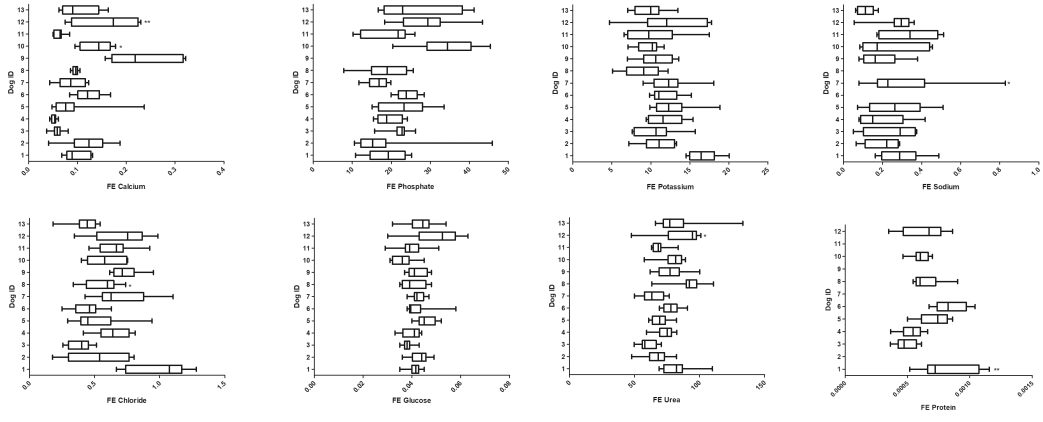


FIGURE 3 Distribution of fractional excretion (FE) of urine analytes in 8-13 dogs sampled once a week for 6-8 weeks. *Analytical outlier excluded. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

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

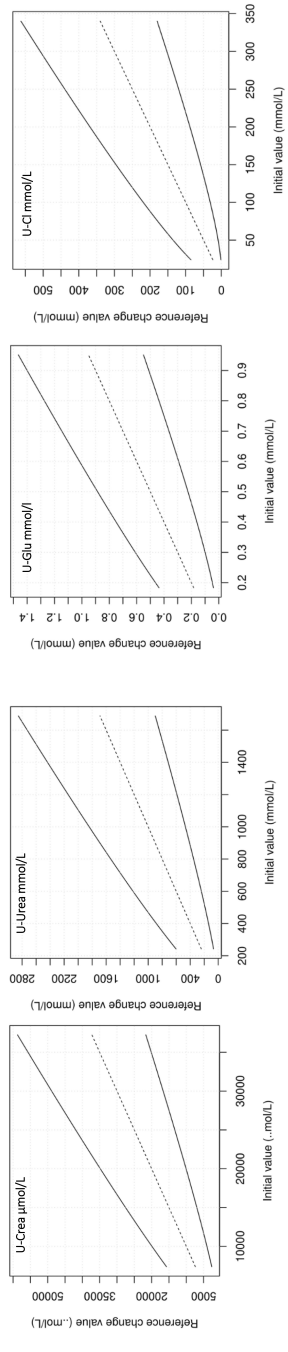


FIGURE 4 Graphs illustrating reference change value (RCV) for the six U-analyte concentrations with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

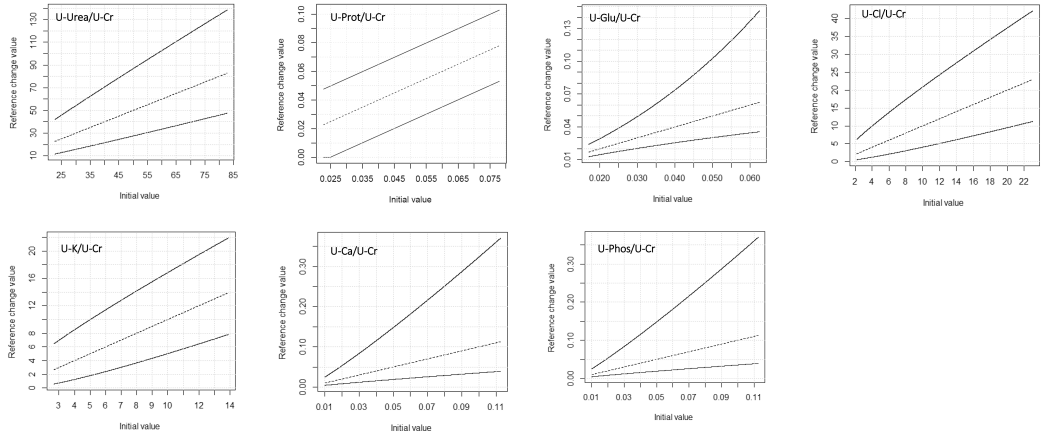


FIGURE 5 Graphs illustrating reference change value (RCV) for the seven U-analyte/U-Cr with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

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

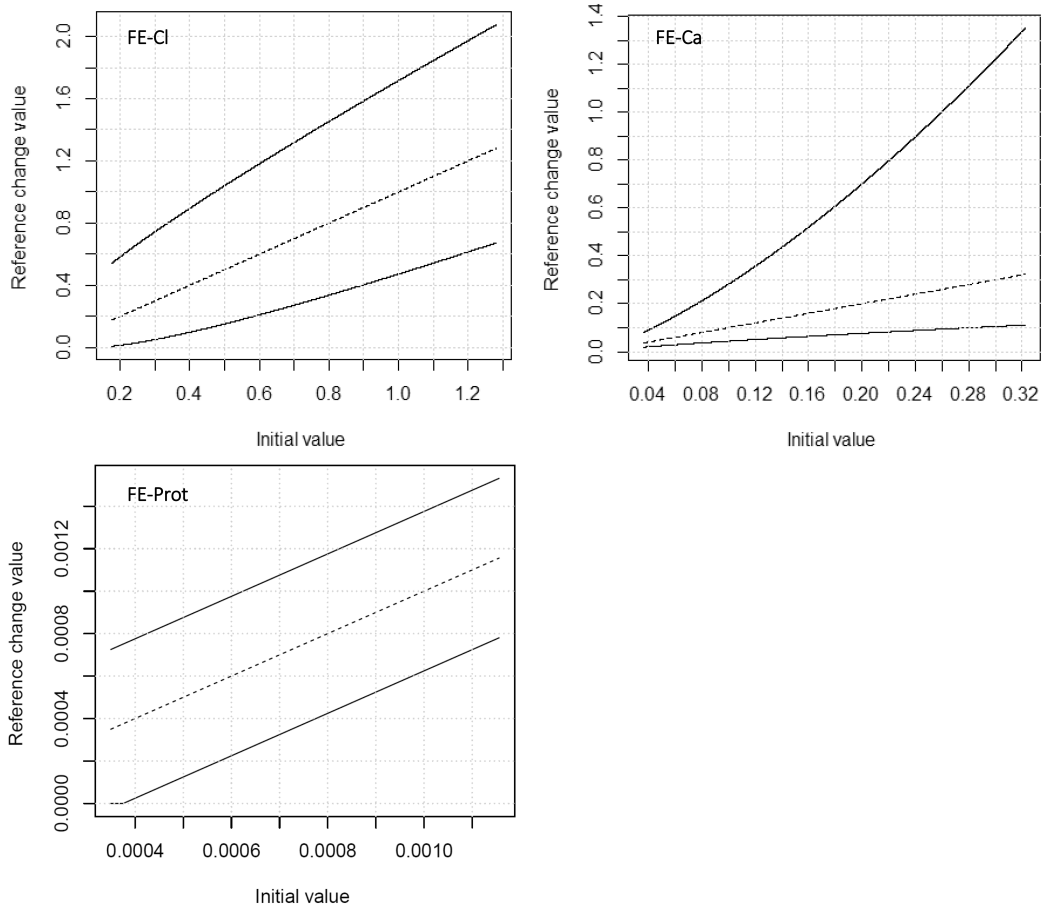


FIGURE 6 Graphs illustrating reference change value (RCV) for the three FE-analytes with an intermediate index of individuality. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

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.

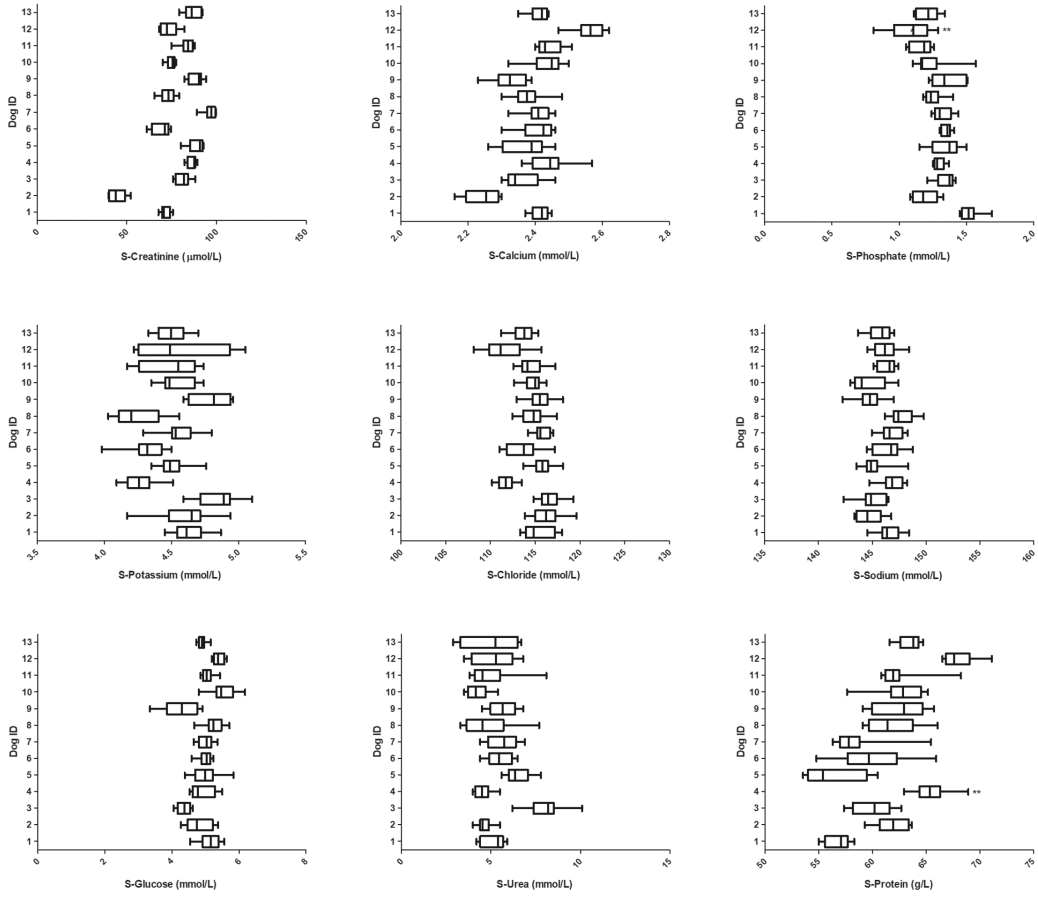


FIGURE 7 Distribution of serum analytes in 13 dogs sampled once a week for 6-8 weeks. **Within-dog outlier excluded. Whiskers indicate minimum and maximum.

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.

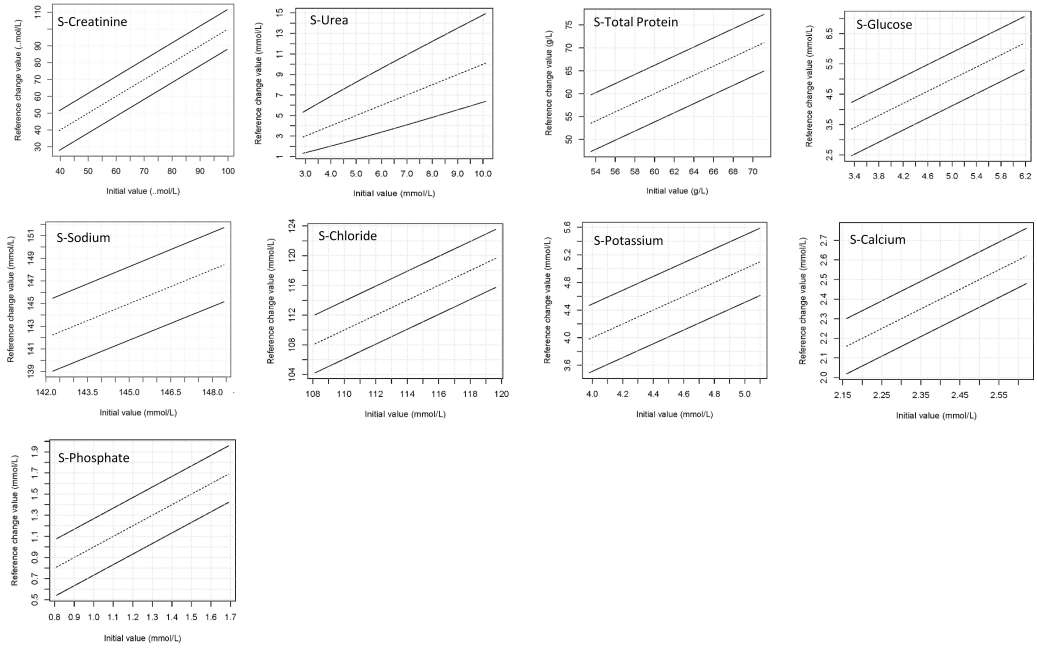


FIGURE 8 Graphs illustrating reference change value (RCV) for the eight S-analytes with index of individuality 0.7-1.7 and II ≥1.7. The dotted line represents the initial value and the solid line represents the upper and lower RCV.

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.

ACKNOWLEDGMENTS

The project was funded by The Greater Stockholm Veterinary Hospital Foundation and Marianne Thedes Foundation. We thank the staff at the Clinical Pathology Laboratory, University Animal Hospital, Uppsala, Sweden, for technical assistance. We also thank the dog owners for participating.

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

REFERENCES

1. Delanghe JR, Speeckaert MM. Preanalytics in urinalysis. *Clin Biochem*. 2016;49(18):1346-1350. doi:10.1016/j.clinbiochem.2016.10.016
2. D'Amico G, Bazzi C. Pathophysiology of proteinuria. *Kidney Int*. 2003;63(3):809-825. doi:10.1046/j.1523-1755.2003.00840.x
3. Hokamp JA, Nabity MB. Renal biomarkers in domestic species. *Vet Clin Pathol*. 2016;45(1):28-56. doi:10.1111/vcp.12333
4. Thompson MF, Fleeman LM, Kessell AE, Steenhard LA, Foster SF. Acquired proximal renal tubulopathy in dogs exposed to a common dried chicken treat: retrospective study of 108 cases (2007-2009). *Aust Vet J*. 2013;91(9):368-373. doi:10.1111/avj.12100
5. Zamagni S, Troia R, Zaccheroni F, et al. Comparison of clinicopathological patterns of renal tubular damage in dogs with acute kidney injury caused by leptospirosis and other aetiologies. *Vet J*. 2020;266:105573. doi:10.1016/j.tvjl.2020.105573
6. Troia R, Gruarin M, Grisetti C, et al. Fractional excretion of electrolytes in volume-responsive and intrinsic acute kidney injury in dogs: diagnostic and prognostic implications. *J Vet Intern Med*. 2018;32(4):1372-1382. doi:10.1111/jvim.15146
7. Martorelli CR, Kogika MM, Chacar FC, et al. Urinary fractional excretion of phosphorus in dogs with spontaneous chronic kidney disease. *Vet Sci*. 2017;4(4):67. doi:10.3390/vetsci4040067
8. Buranakarl C, Ankanaporn K, Thammacharoen S, et al. Relationships between degree of azotaemia and blood pressure, urinary protein:creatinine ratio and fractional excretion of electrolytes in

dogs with renal azotaemia. *Vet Res Commun*. 2007;31(3):245-257. doi:10.1007/s11259-006-3413-2
9. Segev G, Daminet S, Meyer E, et al. Characterization of kidney damage using several renal biomarkers in dogs with naturally occurring heatstroke. *Vet J*. 2015;206(2):231-235. doi:10.1016/j.tvjl.2015.07.004
10. Brown N, Segev G, Francey T, Kass P, Cowgill LD. Glomerular filtration rate, urine production, and fractional clearance of electrolytes in acute kidney injury in dogs and their association with survival. *J Vet Intern Med*. 2015;29(1):28-34. doi:10.1111/jvim.12518
11. Pressler BM. Clinical approach to advanced renal function testing in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2013;43(6):1193-1208. doi:10.1016/j.cvsm.2013.07.011
12. Friedrichs KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 2012;41(4):441-453. doi:10.1111/vcp.12006
13. Freeman KP, Baral RM, Dhand NK, Nielsen SS, Jensen AL. Recommendations for designing and conducting veterinary clinical pathology biologic variation studies. *Vet Clin Pathol*. 2017;46(2):211-220. doi:10.1111/vcp.12475
14. Flatland B, Baral RM, Freeman KP. Current and emerging concepts in biological and analytical variation applied in clinical practice. *J Vet Intern Med*. 2020;34(6):2691-2700. doi:10.1111/jvim.15929
15. Fraser CG. *Biological Variation: From Principles to Practice*. AACC Press; 2001:91-116.
16. Rudinsky A, Cortright C, Purcell S, et al. Variability of first morning urine specific gravity in 103 healthy dogs. *J Vet Intern Med*. 2019;33(5):2133-2137. doi:10.1111/jvim.15592
17. van Vonderen IK, Kooistra HS, Rijnberk A. Intra- and interindividual variation in urine osmolality and urine specific gravity in healthy pet dogs of various ages. *J Vet Intern Med*. 1997;11(1):30-35. doi:10.1111/j.1939-1676.1997.tb00070.x
18. Nabity MB, Boggess MM, Kashtan CE, Lees GE. Day-to-day variation of the urine protein: creatinine ratio in female dogs with stable glomerular proteinuria caused by X-linked hereditary nephropathy. Article; proceedings paper. *J Vet Intern Med*. 2007;21(3):425-430. doi:10.1892/0891-6640(2007)21[425:Dvotup]2.0.Co;2
19. Mårtensson F. Biological variation of urine protein and urine creatinine in healthy dogs. Veterinary program (Master thesis, Advanced level A2E) Swedish University of Agriculture Sciences 2017:36. http://stud.epsilon.slu.se
20. Ilchyshyn NP, Villiers E, Monti P. Validation of a spectrophotometric method for GGT measurement in canine urine and determination of the urine GGT-to-creatinine ratio reference interval and biological variation in 41 healthy dogs. *J Vet Diagn Invest*. 2019;31(1):33-39. doi:10.1177/1040638718812927
21. Kume T, Saglam B, Ergon C, Sisman AR. Evaluation and comparison of Abbott Jaffe and enzymatic creatinine methods: could the old method meet the new requirements? *J Clin Lab Anal*. 2018;32(1):e22168. doi:10.1002/jcla.22168
22. Riond B, Steffen F, Schmied O, Hofmann-Lehmann R, Lutz H. Total protein measurement in canine cerebrospinal fluid: agreement between a turbidimetric assay and 2 dye-binding methods and determination of reference intervals using an indirect a posteriori method. *Vet Clin Pathol*. 2014;43(1):78-88. doi:10.1111/vcp.12107
23. The statistical software R. *R.Core Team*. R Foundation for Statistical Computing; 2020. Accessed October 25, 2021. http://www.r-project.org/index.html
24. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J Stat Softw*. 2015;67:1-48. doi:10.18637/jss.v067.i01
25. Fox J, Weisberg S. *Fitting Linear Models. An R Companion to Applied Regression*. 3rd ed. Sage; 2019:149-228.
26. Laflamme D. Development and validation of a body condition score system for dogs. *Canine Pract*. 1997;22:10-15.
27. Ricos C, Jimenez CV, Hernandez A, et al. Biological variation in urine samples used for analyte measurements. *Clin Chem*. 1994;40(3):472-477.
28. Gowans EM, Fraser CG. Despite correlation, random spot and 24-h urine specimens are not interchangeable. *Clin Chem*. 1987;33(6):1080-1081.
29. Ruaux CG, Carney PC, Suchodolski JS, Steiner JM. Estimates of biological variation in routinely measured biochemical analytes in clinically healthy dogs. *Vet Clin Pathol*. 2012;41(4):541-547. doi:10.1111/j.1939-165x.2012.00473.x
30. Leissing N, Izzo R, Sargent H. Variance estimates and individuality ratios of 25 serum constituents in beagles. *Clin Chem*. 1985;31:83-86.
31. Jensen AL, Aaes H. Critical differences of clinical chemical parameters in blood from dogs. *Res Vet Sci*. 1993;54:10-14.
32. Pagitz M, Frommlet F, Schwendenwein I. Evaluation of biological variance of cystatin C in comparison with other endogenous markers of glomerular filtration rate in healthy dogs. *J Vet Intern Med*. 2007;21:936-942.
33. Choi SW. Life is lognormal! What to do when your data does not follow a normal distribution. *Anaesthesia*. 2016;71:1363-1366.
34. Fokkema MR, Herrmann Z, Muskiet FAJ, Moecks J. Reference change values for brain natriuretic peptides revisited. *Clin Chem*. 2006;52:1602-1603.
35. Roraas T, Stove B, Petersen PH, Sandberg S. Biological variation: the effect of different distributions on estimated within-person variation and reference change values. *Clin Chem*. 2016;62(5):725-736. doi:10.1373/clinchem.2015.252296
36. Strage EM, Ley CJ, Forkman J, et al. Homeostasis model assessment, serum insulin and their relation to body fat in cats. *BMC Vet Res*. 2021;17(1):34. doi:10.1186/s12917-020-02729-1
37. Casella G, Berger RL. *Statistical Inference*. Vol 5. 2nd ed. Duxbury Press; 2015:207-267.

SUPPORTING INFORMATION

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

How to cite this article: Selin AK, Lilliehöök I, Forkman J, Larsson A, Pelander L, Strage EM. Biological variation of biochemical urine and serum analytes in healthy dogs. *Vet Clin Pathol*. 2023;52:461-474. doi:10.1111/vcp.13225

Supporting information Paper I

Supplementary Table 2 is included

Supplementary Table 3 is included

Supporting File 1 and 2, and supporting Table 1 and 4 can be opened by the following :

Filename	Description
vcp13225-sup-0001-FileS1.pdf PDF document, 797.1 KB	**File S1**
vcp13225-sup-0002-FileS2.xlsx Excel 2007 spreadsheet , 79.7 KB	**File S2**
vcp13225-sup-0003-TableS1.pdf PDF document, 417.3 KB	**Table S1**
vcp13225-sup-0004-TableS2.pdf PDF document, 226.3 KB	**Table S2**
vcp13225-sup-0005-TableS3.pdf PDF document, 431.9 KB	**Table S3**
vcp13225-sup-0006-TableS4.pdf PDF document, 390 KB	**Table S4**

Supporting Table 2 Outlier variances identified as outliers in a study of biological variation in urine from 8-13 dogs, depending on analyte, sampled once a week for 8 weeks. For the 922 U-analyte concentrations there were 12 analytical outliers and one within-dog outliers detected and excluded. Of the 656 U-analyte/U-Cr five duplicate outliers and six within-dog outliers were removed, and of the 656 FE-analyte results four duplicate outliers and three within-animal outlier were excluded.

U-analyte	Analytical variance outlier	Within-subject variance outlier
U-Osmo	Dog 1 (3), Dog 5 (7), Dog 12 (3)	
U-Urea	Dog 3 (4), Dog 12 (2)	
U-Prot	Dog 4 (5)	
U-Cl	Dog 1 (3), Dog 8 (5)	
U-Ca	Dog 5 (7), Dog 12 (2 and 6)	Dog 10 (4)
U-P	Dog 10 (4)	
U-analyte	**Analytical variance outlier**	**Within-subject variance outlier**
U-Urea/U-Cr	Dog 3 (4), Dog 12 (2)	Dog 12 (6)
U-Prot/U-Cr		Dog 1 (3)
U-Cl/U-Cr	Dog 8 (5)	Dog 2 (5), Dog 2 (8)
U-K/U-Cr	Dog 2 (3)	
U-Ca/U-Cr	Dog 12 (6)	Dog 12 (2)
U-P/U-Cr		Dog 2 (7)
U-analyte	**Analytical variance outlier**	**Within-subject variance outlier**
FE-Urea	Dog 12 (2)	
FE-Prot		Dog 1 (3)
FE-Na	Dog 7 (6)	
FE-Cl	Dog 8 (5)	
FE-Ca	Dog 10 (4)	Dog 12 (2 and 6)

*U-Osmo= urine osmolality, U-Urea=urine urea concentration, U-Prot=urine protein concentration, U-Cl=urine chloride concentration, U-Ca=urine calcium concentration, U-P=urine phosphate concentration. U-Urea/U-Cr=urine urea ratio to urine creatinine, U-Prot/U-Cr=urine protein ratio to urine creatinine, U-Cl/U-Cr=urine chloride ratio to urine creatinine, U-K/U-Cr= urine potassium ratio to urine creatinine, U-Ca/U-Cr=urine calcium ratio to urine creatinine, U-P/U-Cr=urine phosphate ratio to urine creatinine. FE-urea=fractional excretion of urea, FE-Prot=fractional excretion of protein, FE-Na= fractional excretion of sodium, FE-Cl=fractional excretion of chloride, FE-Ca=fractional excretion of calcium.

Supporting Table 3.

Parameter	CV_G	CV_I	CV_{min}	CV_{des}	CV_{opt}	B_{max}	TE_a
USG	14,4	15,1	11,3	7,6	3,8	5,2	17,7
U-Osmo	17,5	17,7	13,3	8,9	4,4	6,2	20,8
U-Cr	19,0	22,1	16,6	11,1	5,5	7,3	25,5
U-Urea	20,4	24,2	18,2	12,1	6,1	7,9	27,9
U-Prot	<0,1	35,9	26,9	18,0	9,0		
U-Glu	19,6	23,1	17,3	11,6	5,8	7,6	26,6
U-Na	25,5	60,0	45,0	30,0	15,0	16,3	65,8
U-Cl	25,1	27,0	20,3	13,5	6,8	9,2	31,5
U-K	21,4	30,0	22,5	15,0	7,5	9,2	34,0
U-Ca	39,7	28,4	21,3	14,2	7,1	12,2	35,6
U-P	27,8	25,5	19,1	12,8	6,4	9,4	30,5
U-Urea/U-Cr	17,7	20,6	15,5	10,3	5,2	6,8	23,8
U-Prot/U-Cr	22,1	18,3	13,7	9,2	4,6	7,2	22,3
U-Glu/U-Cr	20,7	14,3	10,7	7,2	3,6	6,3	18,1
U-Na/U-Cr	27,0	59,5	44,6	29,8	14,9	16,3	65,4
U-Cl/U-Cr	33,7	28,6	21,5	14,3	7,2	11,1	34,6
U-K/U-Cr	23,8	25,8	19,4	12,9	6,5	8,8	30,1
U-Ca/U-Cr	46,1	35,0	26,3	17,5	8,8	14,5	43,3
U-P/U-Cr	23,4	24,1	18,1	12,1	6,0	8,4	28,3
FE-Urea	10,1	15,6	11,7	7,8	3,9	4,6	17,5
FE-Prot	17,6	19,6	14,7	9,8	4,9	6,6	22,8
FE-Glu	5,3	12,6	9,5	6,3	3,2	3,4	13,8
FE-Na	19,1	60,7	45,5	30,4	15,2	15,9	66,0
FE-Cl	24,1	29,6	22,2	14,8	7,4	9,5	34,0
FE-K	14,0	23,9	17,9	12,0	6,0	6,9	26,6
FE-Ca	37,2	32,6	24,5	16,3	8,2	12,4	39,3
FE-P	18,7	30,1	22,6	15,1	7,5	8,9	33,7
S-Cr	16,6	5,4	4,1	2,7	1,4	4,4	8,8
S-Urea	15,6	19,4	14,6	9,7	4,9	6,2	22,2
S-Tot Prot	5,1	3,6	2,7	1,8	0,9	1,6	4,5
S-Glu	6,7	6,0	4,5	3,0	1,5	2,2	7,2
S-Na	0,5	0,4	0,3	0,2	0,1	0,2	0,5
S-Cl	1,3	1,0	0,8	0,5	0,3	0,4	1,2
S-K	3,7	3,8	2,9	1,9	1,0	1,3	4,5
S-Ca	2,9	1,9	1,4	1,0	0,5	0,9	2,4
S-P	8,2	7,5	5,6	3,8	1,9	2,8	9,0

CV_G indicates between-dog coefficient of variation; CV_I, within-dog coefficient of variation; CV_{min}, minimally acceptable analytical coefficient of variation based on $CV_A<0.75CV_I$; CV_{des}, desirable analytical coefficient of variation based on $CV_A<0.5CV_I$; CV_{opt}, optimal analytical coefficient of variation based on $CV_A<0.25CV_I$; TE_a, allowable total error based on $CV_A< 1.65\ (0.50CV_I) + 0.250\ (CV_I^2 + CV_G^2)^{1/2}$; B_{max}, Bias based on $CV_A< 0.250\ (CV_I^2 + CV_G^2)^{1/2}$

II

Journal of Veterinary Internal Medicine

Journal of Veterinary Internal Medicine Open Access ACVIM American College of Veterinary Internal Medicine

STANDARD ARTICLE OPEN ACCESS

Small Animal Internal Medicine Nephrology/Urology

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

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

[1]Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden | [2]AniCura Albano, Animal Hospital, Stockholm, Sweden | [3]Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden

Correspondence: Anna K. Selin (anna.selin@slu.se)

Received: 23 June 2024 | **Revised:** 19 March 2025 | **Accepted:** 31 March 2025

Funding: This work was supported by the Greater Stockholm Veterinary Hospital Foundation, Agria Djurförsäkringar Research Fund, and Sveland Research Fund.

Keywords: biomarker | canine | CKD | proximal tubules | renal | veterinary

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, 5.2.18–13750/2019) 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

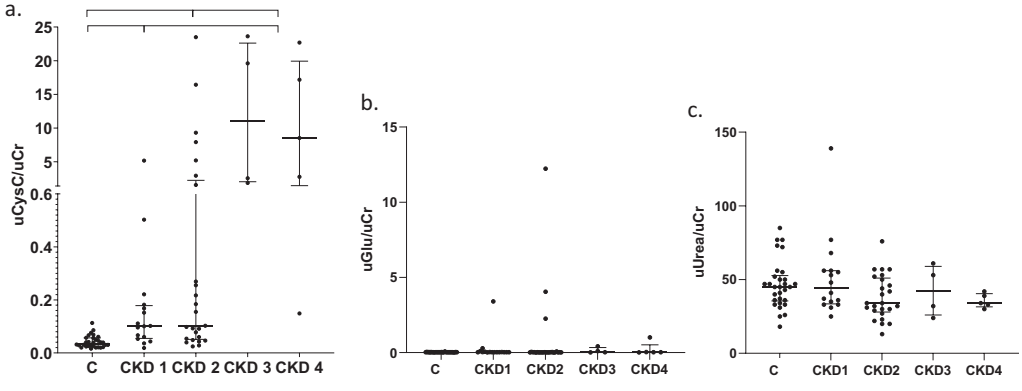


FIGURE 1 | (a–c): Urinary analyte/creatinine ratios for (a) uCysC (10^{-3}), (b) uGlu, and (c) uUrea, in control- and CKD stage 1–4 groups. Urinary CysC/uCr at low levels, showing the difference between C and CKD 1. Significant differences (< 0.005) between groups are marked with bars. The groups of 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.

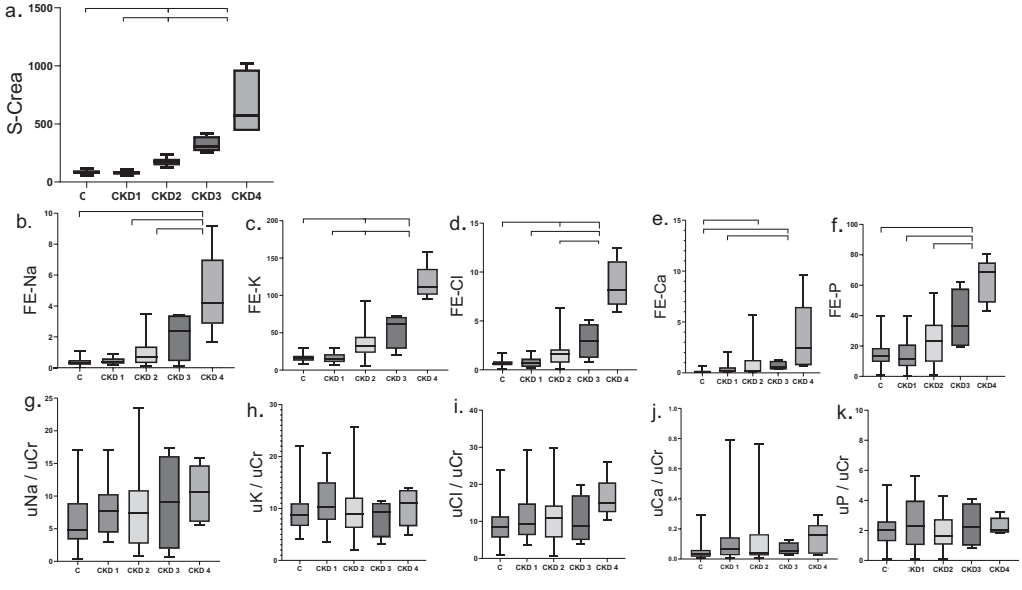


FIGURE 2 | Results of (a) serum creatinine, (b–f) fractional excretion (FE) of electrolytes, and (g–k) urinary electrolytes/uCr-ratio for all dogs. There was an association between FE-electrolytes and sCrea (sCr). The groups of dogs with CKD stage 3 and 4 were combined for statistical analyses, due to 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.

Acknowledgments

The authors thank The Greater Stockholm Veterinary Hospital Foundation, Agria Djurförsäkringar Research Fund, and Sveland Research Fund, for funding the present study. We thank the owners of included dogs for participating. We also thank the staff at the Clinical Pathology Laboratory, University Animal Hospital, Uppsala, Sweden, for technical assistance.

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.

References

1. D. J. Polzin, "Chronic Kidney Disease in Small Animals," *Veterinary Clinics of North America. Small Animal Practice* 41, no. 1 (2011): 15–30.

2. F. Jacob, D. J. Polzin, C. A. Osborne, et al., "Clinical Evaluation of Dietary Modification for Treatment of Spontaneous Chronic Renal Failure in Dogs," *Journal of the American Veterinary Medical Association* 220, no. 8 (2002): 1163–1170.

3. G. F. Grauer, "Early Detection of Renal Damage and Disease in Dogs and Cats," *Veterinary Clinics of North America. Small Animal Practice* 35, no. 3 (2005): 581–596.

4. D. G. O'Neill, J. Elliott, D. B. Church, P. D. McGreevy, P. C. Thomson, and D. C. Brodbelt, "Chronic Kidney Disease in Dogs in UK Veterinary Practices: Prevalence, Risk Factors, and Survival," *Journal of Veterinary Internal Medicine* 27, no. 4 (2013): 814–821.

5. A. R. Cobrin, S. L. Blois, S. A. Kruth, A. C. Abrams-Ogg, and C. Dewey, "Biomarkers in the Assessment of Acute and Chronic Kidney Diseases in the Dog and Cat," *Journal of Small Animal Practice* 54, no. 12 (2013): 647–655.

6. J. De Loor, S. Daminet, P. Smets, B. Maddens, and E. Meyer, "Urinary Biomarkers for Acute Kidney Injury in Dogs," *Journal of Veterinary Internal Medicine* 27, no. 5 (2013): 998–1010.

7. J. A. Hokamp and M. B. Nabity, "Renal Biomarkers in Domestic Species," *Veterinary Clinical Pathology* 45, no. 1 (2016): 28–56.

8. M. Nabity and J. Hokamp, "Urinary Biomarkers of Kidney Disease in Dogs and Cats," *Veterinary Clinics of North America. Small Animal Practice* 53, no. 1 (2023): 53–71.

9. K. Jung and M. Jung, "Cystatin C: A Promising Marker of Glomerular Filtration Rate to Replace Creatinine," *Nephron* 70, no. 3 (1995): 370–371.

10. F. S. Almy, M. M. Christopher, D. P. King, and S. A. Brown, "Evaluation of Cystatin C as an Endogenous Marker of Glomerular Filtration Rate in Dogs," *Journal of Veterinary Internal Medicine* 16, no. 1 (2002): 45–51.

11. R. Kaseda, N. Iino, M. Hosojima, et al., "Megalin-Mediated Endocytosis of Cystatin C in Proximal Tubule Cells," *Biochemical and Biophysical Research Communications* 357, no. 4 (2007): 1130–1134.

12. M. Conti, S. Moutereau, M. Zater, et al., "Urinary Cystatin C as a Specific Marker of Tubular Dysfunction," *Clinical Chemistry and Laboratory Medicine* 44, no. 3 (2006): 288–291.

13. A. Sasaki, Y. Sasaki, R. Iwama, et al., "Comparison of Renal Biomarkers With Glomerular Filtration Rate in Susceptibility to the Detection of Gentamicin-Induced Acute Kidney Injury in Dogs," *Journal of Comparative Pathology* 151, no. 2–3 (2014): 264–270.

14. Y. Z. Gu, K. Vlasakova, S. P. Troth, et al., "Performance Assessment of New Urinary Translational Safety Biomarkers of Drug-Induced Renal Tubular Injury in Tenofovir-Treated Cynomolgus Monkeys and Beagle Dogs," *Toxicologic Pathology* 46, no. 5 (2018): 553–563.

15. B. Sun, X. Zhou, Z. Qu, et al., "Urinary Biomarker Evaluation for Early Detection of Gentamycin-Induced Acute Kidney Injury," *Toxicology Letters* 300 (2019): 73–80.

16. P. Monti, G. Benchekroun, D. Berlato, and J. Archer, "Initial Evaluation of Canine Urinary Cystatin C as a Marker of Renal Tubular Function," *Journal of Small Animal Practice* 53, no. 5 (2012): 254–259.

17. P. Ruiz, A. Duran, F. J. Duque, et al., "Urinary Cystatin C and N-Acetyl-Beta-D-Glucosaminidase (NAG) as Early Biomarkers for Renal Disease in Dogs With Leishmaniosis," *Veterinary Parasitology* 318 (2023): 109930.

18. C. R. Martorelli, M. M. Kogika, F. C. Chacar, et al., "Urinary Fractional Excretion of Phosphorus in Dogs With Spontaneous Chronic Kidney Disease," *Veterinary Sciences* 4, no. 4 (2017): 67, https://doi.org/10.3390/vetsci4040067.

19. C. Buranakarl, K. Ankanaporn, S. Thammacharoen, et al., "Relationships Between Degree of Azotaemia and Blood Pressure, Urinary Protein:Creatinine Ratio and Fractional Excretion of Electrolytes in Dogs With Renal Azotaemia," *Veterinary Research Communications* 31, no. 3 (2007): 245–257.

20. L. Pelander, J. Haggstrom, A. Larsson, et al., "Comparison of the Diagnostic Value of Symmetric Dimethylarginine, Cystatin C, and Creatinine for Detection of Decreased Glomerular Filtration Rate in Dogs," *Journal of Veterinary Internal Medicine* 33, no. 2 (2019): 630–639.

21. A. K. Selin, I. Lilliehook, J. Forkman, A. Larsson, L. Pelander, and E. M. Strage, "Biological Variation of Biochemical Urine and Serum Analytes in Healthy Dogs," *Veterinary Clinical Pathology* 52, no. 3 (2023): 461–474.

22. F. H. Noraddin, M. Flodin, A. Fredricsson, A. Sohrabian, and A. Larsson, "Measurement of Urinary Cystatin C With a Particle-Enhanced Turbidimetric Immunoassay on Architect ci8200," *Journal of Clinical Laboratory Analysis* 26, no. 5 (2012): 358–364.

23. B. M. Pressler, "Clinical Approach to Advanced Renal Function Testing in Dogs and Cats," *Veterinary Clinics of North America. Small Animal Practice* 43, no. 6 (2013): 1193–1208.

24. N. Thielemans, R. Lauwerys, and A. Bernard, "Competition Between Albumin and Low-Molecular-Weight Proteins for Renal Tubular Uptake in Experimental Nephropathies," *Nephron* 66, no. 4 (1994): 453–458.

25. B. Yu, L. Jiang, Z. Li, et al., "Renal Fractional Excretion of Urea and Uric Acid Have a Diagnostic Value to Disitnguish Acute Kidney Injury From Chronic Disease," *Kidney International Reports* 8, no. WCN23-0173 (2023): S42–S43.

26. G. Petrucci, D. Hatem, R. Langley, et al., "Effect of Very Long-Term Storage and Multiple Freeze and Thaw Cycles Om 11-Dehydro-Thromoboxane -B2 and 8-Isoprostaglandin F2a, Levels in Human Urine," *Scientific Reports* 14, no. 1 (2024): 5546.

27. N. Beauval, A. Leroyer, M. Hisbergues, et al., "Stability of Trace Element Concentrations in Frozen Urine - Effect on Different Elements of More Than 10 Years at −80 Degrees C," *Journal of Trace Elements in Medicine and Biology* 74 (2022): 127080.

28. T. Remer, G. Montenegro-Bethancourt, and L. Shi, "Long-Term Urine Biobanking: Storage Stability of Clinical Chemical Parameters Under Moderate Freezing Conditions Without Use of Preservatives," *Clinical Biochemistry* 47, no. 18 (2014): 307–311.

29. V. L. Stevens, E. Hoover, Y. Wang, and K. A. Zanetti, "Pre-Analytical Factors That Affect Metabolite Stability in Human Urine, Plasma, and Serum: A Review," *Metabolites* 9, no. 8 (2019): 156, https://doi.org/10.3390/metabo9080156.

30. T. C. Peakman and P. Elliott, "The UK Biobank Sample Handling and Storage Validation Studies," *International Journal of Epidemiology* 37, no. Suppl 1 (2008): i2–i6.

31. R. S. Parekh, W. H. Kao, L. A. Meoni, et al., "Reliability of Urinary Albumin, Total Protein, and Creatinine Assays After Prolonged Storage: The Family Investigation of Nephropathy and Diabetes," *Clinical Journal of the American Society of Nephrology* 2, no. 6 (2007): 1156–1162.

Supporting Information

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

Supplementary Table 1. Urinary analytes (Cystatine, Glucose, Urea) normalized to urinary creatinine for dogs in CKD 1-4, and healthy control dogs. .

	C	CKD 1	CKD 2	CKD 3+4
uCysC/uCr, median (IQR), (10^{-3})	0.03 (0.02-0.045)[a]	0.08 (0.04-0.25)[b]	0.1 (0.04-4.2)[b]	13.4 (3.5-34.0)[c]
uGlu/uCr, median (IQR)	0.02 (0.02-0.03)[a]	0.03 (0.03-0.05)[a]	0.02 (0.02-0.03)[a]	0.03 (0.019-0.26)[a]
uUrea/uCr, median (IQR)	45.0 (35.5-52.8)[a]	44.5 (33.5-56.0)[a]	34 (28-51)[a]	34.0 (31.0-47.5)[a]

Significant differences (P <.005) are noted where superscripted letters differ between groups. Abbreviations: C, healthy dogs; CKD, chronic kidney disease; uCysC, urine cystatin C; uCr, urine creatinine; uGlu, urine glucose.

Supplementary Table 2 Urinary electrolytes as concentrations, normalized to creatinine, and fractional excretion, in dogs in different stages of CKD and healthy control group.

	C	CKD 1	CKD 2	CKD 3+4
uNa mmol/l	73.2 (37.4-166.8)[a]	54.6 (32.3-94.7)[a]	47.3 (17.1-59.5)[a]	39.5 (25.1-61.6)[a]
uK mmol/l	119.5 (85.0-198.3)[a]	73.5 (43.5-120.8)[b]	54.0 (37.0-82.5)[b]	41.0 (29.0-59.0)[b]
uCl mmol/l	114.5 (82.0-203.5)[a]	66.5 (33.5-154.8)[ab]	63.0 (40.0-88.0)[b]	62.0 (48.0-70.5)[b]
uCa mmol/l	0.42 (0.25-0.96)[a]	0.34 (0.19-0.78)[a]	0.51 (0.17-0.9)[a]	0.42 (0.16-0.7)[a]
uP mmol/l	29.0 (14.8-46.4)[a]	16.6 (3.6-31.8)[ab]	9.3 (6.1-13.9)[b]	11.1 (8.3-12.9)[b]
uNa/uCr	4.8 (3.4-8.9)[a]	7.7 (4.4-10.3)[a]	7.4 (2.7-10.9)[a]	10.6 (5.8-14.7)[a]
uK/uCr	8.7 (6.6-11.0)[a]	10.2 (7.8-15.0)[a]	8.9 (6.3-12.1)[a]	10.0 (6.6-12.3)[a]
uCl/uCr	8.5 (5.7-11.4)[a]	9.3 (6.3-14.8)[a]	10.8 (5.7-14.3)[a]	14.5 (8.7-17.4)[a]
uCa/uCr	0.03 (0.02-0.06)[a]	0.07 (0.03-0.14)[a]	0.04 (0.02-0.16)[a]	0.06 (0.04-0.16)[a]
uP/uCr	2.0 (1.3-2.6)[a]	2.3 (1.0-4.0)[a]	1.6 (1.0-2.8)[a]	2 (1.7-3.0)[a]
FE-Na, median (IQR)	0.3 (0.2-0.5)[a]	0.4 (0.28-0.6)[a]	0.6 (0.1-3.5)[a]	3.4 (1.6-4.5)[b]
FE-K, median (IQR)	16.5 (12.8-19.3)[a]	15.5 (10.8-21.3)[a]	32.5 (23.3-44.5)[b]	95 (62-112)[c]
FE-Cl, median (IQR)	0.6 (0.48-0.85)[a]	0.7 (0.3-1.3)[ab]	1.7 (0.7-2.1)[b]	5.9 (3.0-9.0)[c]
FE-Ca, median (IQR)	0.1 (0.06-0.2)[a]	0.17 (0.07-0.55)[ab]	0.24 (0.11-1.3)[bc]	0.88 (0.55-2.92)[c]
FE-P, median (IQR)	13.2 (9.5-18.7)[a]	11.1 (6.7-20.9)[a]	23.7 (9.6-34.0)[a]	54.3 (32.3-69)[b]
FE-Urea	66 (55.1-73.5)[a]	68 (47.0-78.5)[a]	56.5 (49.3-64.8)[a]	55 (44-67.5)[a]

Significant differences (P <.005) are noted where superscripted letters differ between groups. Abbreviations: CKD 1-4, chronic kidney disease IRIS 1-4; C: healthy dogs, u, urinary; Na, sodium; Cl, cloride; K, potassium; Ca, calcium; P, phosphate; Cr, creatinine; FE, fractional excretion.

Supplementary Table 3. Criteria for diagnosis, UPC and selected urinary markers, in the 16 individual dogs with stage 1 CKD.

Dog	CKD 1 criteria	GFR Total (left/right) ml/L/min	UPC	uCysC/uCr x 10^{-3}	uCysC mg/L	uGlu/uCr	uCr mmol/L	sCr umol/L
1	Structural (PKD)	38.5 (22.3/16.4)	0.06	0.08	<0.1	0.024	5.5	110
2	Structural (irregular renal outline, reduced renal size)	26.8 (1.5/20.6)	0.07	0.03	<0.1	0.021	12.9	104
3	Structural (irregular renal outline)	-	0.10	***0.12***	<0.1	0.026	3.7	88
4	Renal glucosuria	48.9 (16.4/22.5)	0.18	***0.29***	***0.17***	***3.4***	5.1	60
5	Structural (PKD)	48.0 (24.0/24.0)	0.21	0.05	<0.1	0.027	8.3	86
6	Structural (PKD)	47.2 (25.4/21.7)	0.23	0.08	<0.1	0.017	5.5	90
7	Structural (PKD)	35.0 (17.4/17.6)	0.49	0.04	<0.1	0.022	10.5	83
8	PRP	24.6 (7.29/13.0)	***0.98***	***0.13***	<0.1	0.033	3.3	100
9	PRP	53.9 (25.4/28.5)	***1.64***	0.03	<0.1	0.027	15.4	58
10	PRP	39.0 (18.0-21.0)	***2.03***	0.05	<0.1	***0.097***	9.2	64
11	PRP	39.0 (15.5/23.2)	***3.32***	0.08	<0.1	0.036	5.8	77
12	PRP	86.7 (35.0/49.0)	***3.77***	***0.40***	<0.1	0.053	1.1	68
13	PRP	59.7 (35.7/31.3)	***6.48***	0.01	<0.1	0.026	29.9	84
14	PRP	47.8 (18.2/29.6)	***7.54***	0.09	<0.1	0.027	5.0	81
15	PRP	46.7(25.0/21.7)	***14.57***	***0.35***	***0.45***	0.034	11.2	61
16	PRP	-	***18.55***	***8.30***	***5.82***	***0.29***	6.2	78

The table is sorted based on UPC. Results above range of healthy dogs are indicated by bold font. *Structural, renal parenchymal abnormalities detected by ultrasound; PKD, polycystic kidney disease; PRP, persistent renal proteinuria.

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:80

Early diagnosis of acute kidney injury and chronic kidney disease in dogs is challenging, but important for early interventions. This thesis investigates diagnostic performance of selected urinary analytes that are measurable with biochemistry analysers commonly available at veterinary hospitals. Results provide data on biological variation and reference intervals in healthy dogs. Urinary cystatin C and gamma-glutamyl transferase were identified as promising early indicators of tubular injury or dysfunction in dogs with both acute and chronic kidney disorders.

Anna Selin underwent her postgraduate education at the Department of Clinical Sciences, Swedish University of Agriculture (SLU), Uppsala, Sweden. Her undergraduate degree in veterinary medicine was obtained at the Faculty of Veterinary Medicine, SLU, Uppsala, Sweden.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).
SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

ISSN 1652-6880
ISBN (print version) 978-91-8124-064-1
ISBN (electronic version) 978-91-8124-110-5