ORIGINAL RESEARCH

Long-term follow-up of renal function assessing serum cystatin C in dogs with diabetes mellitus or hyperadrenocorticism

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Background: Serum cystatin C (sCysC) is used as biomarker for glomerular filtration rate (GFR). The effects of diabetes mellitus (DM) on renal function in dogs are unclear. Some renal variables have been evaluated in dogs with hyperadrenocorticism (HAC), but not sCysC.
Objectives: The purpose of this study was the validation of a particle-enhanced nephelometric immunoassay (PENIA) for measuring canine sCysC, and to assess renal function in dogs with DM or HAC.
Methods: A PENIA was analytically validated for canine sCysC by determining imprecision and linearity. In a longitudinal 6-month study, renal function of 14 DM dogs was assessed, using serum creatinine, GFR, urinary protein-to-creatinine (UPC) ratio, urinary markers, systolic blood pressure (SBP), and sCysC, and compared to 17 healthy dogs at baseline. Furthermore, sCysC was measured at initial presentation and during a 12-month follow-up in 22 HAC dogs.
Results: The sCysC intra- and inter-assay variation coefficients were < 8% and highly linear (r = .997). About 33% and 67% of DM dogs had persistent proteinuria and systemic hypertension, respectively, but there were no significant differences in GFR, UPC, and urinary markers over time, and compared with healthy dogs at initial presentation. Serum CysC decreased significantly (P < .05) over time within the DM group. It did not change significantly over time within the HAC group.
Conclusions: A PENIA measured sCysC linearly and precisely. There were no clinically relevant renal alterations over time in dogs with DM, although persistent proteinuria was observed. In dogs with HAC, sCysC measurement was not useful, although significant GFR changes occurred over time.

Introduction

Serum and urinary renal markers allowing early and site-specific detection of kidney dysfunction have gained much interest in veterinary medicine.[1] Cystatin C (CysC) is a protease inhibitor produced at a constant rate by all nucleated cells. It is freely filtered by the glomerulus and completely catabolized by the proximal tubules.[2] Serum CysC concentration is therefore mainly influenced by glomerular filtration rate (GFR) and is an established endogenous GFR marker in human medicine, with some advantages over serum creatinine.[2,3] Serum CysC was investigated infrequently in healthy dogs and dogs with chronic kidney disease.[4–6] Analytic, biologic, and clinical validation of canine sCysC are needed. Serum CysC can be measured using ELISA and particle-enhanced turbidimetric immunoassay (PETIA), or particle-enhanced nephelometric immunoassay (PENIA).[7] One recent study suggests that PENIA is a more precise method for

evaluating canine CysC than PETIA.[7] Although PENIA was used to measure sCysC in dogs, the method has yet to be validated.[8,9]

Further investigation of sCysC as a renal function marker in dogs is therefore warranted. In human medicine, the influence of extra-renal factors on sCysC, such as treatment with exogenous glucocorticoids, is still unclear.[10,11] A promotor-mediated glucocorticoid-induced production of sCysC has been suggested to explain increased serum levels of CysC in patients receiving glucocorticoids, which could lead to a misinterpretation of sCysC in such patients.[10] However, a more recent study could not demonstrate a sCysC elevation in patients with lupus nephritis receiving long-term steroid therapy.[12] As dogs with naturally occurring hyperadrenocorticism (HAC) are continuously exposed to increased concentrations of endogenous corticosteroid hormones, we hypothesized that these dogs might be an interesting model to assess the effect of glucocorticoids on sCysC levels. Hyperadrenocorticism is a common endocrine disorder in middle-aged to old dogs. Glomerular and tubular alterations have been described in canine HAC.[13–16] Therefore, dogs with HAC represent a well-defined group to assess CysC as an early marker of renal alterations. To our knowledge, there are no previous reports on sCysC measurements in dogs with HAC.

Diabetic kidney disease (DKD) is a common and serious complication of diabetes mellitus (DM) in people. It leads to progressive kidney function loss and proteinuria.[17,18] Microalbuminuria is an early predictor of glomerular dysfunction in human type-I DM patients, while urinary immunoglobulin (Ig) G indicates more advanced changes in glomerular permeability[19], and urinary retinol-binding protein (uRBP) and N-acetyl-β-D-glucosaminidase (uNAG) complementarily reflect tubular dysfunction in human diabetic patients.[20,21] Dogs with DM may be at risk for DKD due to hypertension and proteinuria in up to approximately 45% and 50% of affected dogs, respectively, and due to their less strict glycemic control compared to people.[22] However, research on renal effects of DM in dogs is limited.[22–25] In such dogs, the urinary albumin to creatinine (uALB/c) ratio was increased, but tubular urinary markers, sCysC and GFR, have not been investigated.[24] In addition to the latter serum renal markers, urinary markers allow early and site-specific detection of renal dysfunction.[1]

The first objective of the current study was to validate a nephelometric canine sCysC assay. A second aim was to assess renal function in dogs with DM using routine renal markers including serum creatinine and urea concentrations, as well as systolic blood pressure (SBP), urinary protein-to-creatinine (UPC) ratio, GFR, sCysC concentrations, and select urinary renal markers in a prospective follow-up study. A third objective was to evaluate sCysC in dogs with HAC in a follow-up study.[16]

Materials and Methods

Analytic validation of the serum cystatin C (sCysC) assay

This study was performed at the Faculty of Veterinary Medicine, Ghent University. Serum CysC levels were measured using a PENIA validated for use with human samples (Behring Nephelometer [BN] ProSpec; Siemens Healthcare Diagnostics, Marburg, Germany). Polystyrene particles coated with specific antibodies to human CysC aggregate in the presence of canine CysC when mixed with canine serum pools with low, medium, and high sCysC concentrations. The intensity of light rays, scattered by the immune complexes, is measured and is proportional to the concentration of CysC in the sample.

The PENIA method was validated previously for canine sCysC by determining assay sensitivity, imprecision, and linearity.[26] The analytic sensitivity was calculated according to a protocol outlined in an earlier report.[27] In the current study, the lowest measurable canine sCysC concentration was determined based on the mean and corresponding SD of the assay diluent (blank sample). The limit of detection (LOD) was then calculated as 2 times the SD above the mean blank sample value, which was obtained from 20 replicate measurements. Assay imprecision was evaluated by measuring the intra- and inter-assay coefficients of variation (CV). Serum samples from 6 healthy, 2 DM, and 4 HAC dogs with increasing sCysC concentration ranges (0.14–0.18, 0.20–0.26, and > 0.28 mg/L) were measured in duplicates and on 3 consecutive days. The intra-assay CV was determined by dividing the SD of the parallel measurements by their mean and multiplying by 100. The inter-assay CV was determined similarly from the measurements on 3 consecutive days. The linearity was evaluated by calculating the correlation coefficient (r) between the expected and measured sCysC concentration of a sample that was serially diluted 1:4 with physiologic saline.

Animals

Dogs diagnosed with DM between 2009 and 2012 were included in the study. Diabetes mellitus was diagnosed

based on the history, physical examination, presence of concurrent fasting hyperglycemia (> 6 mmol/L), and glucosuria, and an increased serum fructosamine concentration (> 300 μmol/L) (Multigent fructosamine assay, Architect c Systems; Abbott, Wiesbaden, Germany). Prior to treatment with lente insulin (Caninsulin; Intervet International bv, Boxmeer, the Netherlands), a diagnostic work-up to detect concurrent diseases was performed in each case, based on physical examination, blood and urinalysis, and abdominal ultrasonographic examination. Exclusion criteria were the presence of other systemic diseases, such as neoplasia, cardiac disease (class B2, C, or D) classified according to the American College of Veterinary Internal Medicine (ACVIM) guidelines[28], systemic infections, and use of medications with a possible effect on renal function (eg, nonsteroidal anti-inflammatory drugs and angiotensin-converting enzyme inhibitors). Hypothyroidism was not an exclusion criterion if treated and well controlled. A low-dose dexamethasone suppresion test was performed in dogs with DM difficult to regulate, and a history, physical examination, and biochemical results compatible with HAC were documented. Dogs with both HAC and DM were excluded. Assessment of adequate glycemic control was based on control of clinical signs, owner opinion, body weight, fructosamine measurements, and serial blood glucose curves if available (8/12 dogs). Rechecks were performed after 1, 3, and 6 months, and included a CBC, serum chemistry, urinalysis (including urine culture), and SBP measurement using the Doppler ultrasonographic technique and a standardized procedure according to the ACVIM guidelines.[29] Systemic hypertension was defined as a SBP > 150 mmHg.[29] In cases with diabetic ketoacidosis (DKA), based on presence of compatible clinical signs, venous blood gas results, and presence of ketonuria, further diagnostics (eg, abdominal ultrasound, thoracic radiographs, canine pancreatic lipase immunoreactivity [cPLI] measurement) were performed to detect an underlying disorder. Affected dogs were treated with aggressive fluid and insulin treatment and supportive therapy. Samples were taken only after resolution of the DKA status, usually 10–14 days after initial presentation.

Serum samples of dogs with HAC, previously collected[16] at time of diagnosis and during a 12-month period between 2009 and 2011, were analyzed for sCysC. Samples had been stored in aliquots at −80°C until assayed. Hyperadrenocorticism had been diagnosed based on history, physical examination, biochemical changes, and a result consistent with HAC on at least one of the screening tests: low-dose dexamethasone suppression test, urinary cortisol-to-creatinine ratio in 2 consecutive morning urine samples, or an adrenocorticotropic hormone-stimulation test. The dogs were rechecked after 3, 6, and 12 months by renal variables including serum creatinine and urea, UPC, SBP, GFR, and select urinary markers. Assessment of the response to treatment was based on adequate control of clinical signs and consistent laboratory tests.[16]

Healthy, client-owned dogs were recruited and sampled at a single time point in the period between 2009 and 2012. Dogs were considered healthy based on the absence of significant abnormalities in history, on physical examination, and routine blood and urinalysis, including CBC, serum chemistry, and urine culture.

This study was carried out in strict accordance with the recommendations in the Guide for the care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the local ethical committee (EC 2008/066) of Ghent University. All owners were informed about the study and gave their written informed consent.

Sampling methods

All dogs were fasted for at least 10 h prior to the test day and fed immediately after blood sampling. Water was provided ad libitum. Morning blood and urine samples were taken by jugular vein puncture and cystocentesis, respectively (10 mL syringe; 22 G needle; Terumo Europe N.V., Leuven, Belgium; Terumo Corporation, Laguna, Philippines, respectively). After centrifugation at 1500*g* for 3 min at room temperature (Jouan B4i; Thermo Scientific, Waltham, MA, USA), serum and plasma samples, and urine supernatant were collected and stored at −80°C until further analysis. Urinary dipstick analysis (Combur stick; Roche Diagnostics, Burgess Hill, UK), urine specific gravity (USG) (manual refractometer, Uricon; Atago, Tokyo, Japan), UPC (Iricell IQ; Instrumentation Laboratory, Zaventem, Belgium), sediment analysis, and bacterial culture (BioMerieux Media Square, Brussels, Belgium) were performed. Dogs were considered nonproteinuric when their UPC was < 0.2, borderline proteinuric when their UPC was 0.2–0.5, and proteinuric when their UPC was > 0.5, and if sediment could not explain proteinuria.[30] Presence of an active sediment (bacteriuria or > 5 RBC or WBC or epithelial cells per high power field/[40× objective] or > 1–3 casts/hpf) was suggestive for a postrenal (urinary or extra-urinary) cause of the observed proteinuria.

Urinary markers

Urinary albumin and IgG (uIgG) were determined with a canine and uRBP with a human ELISA (Immunology Consultants Laboratory, Newberg, OR, USA), and urinary NAG was determined with a colorimetric assay (B-N-Acetylglucosaminidase Assay kit; Sigma-Aldrich, St Louis, MO, USA). All assays were previously validated and the data published by our group.[1,31] The results are expressed as a ratio to the urinary creatinine concentration (uCr), which was determined with a modified Jaffé reaction.[1,30]

Plasma exogenous creatinine clearance test

Glomerular filtration rate was measured by plasma clearance of exogenous creatinine (Cl_{creat}), as previously described.[14] Briefly, a creatinine solution (40 mg/kg of an 80 mg/mL solution) was injected via a cephalic catheter. Blood was collected from the jugular vein prior to and 5, 15, 60, 120, 240, 360, and 480 min postinjection in EDTA tubes. Samples were centrifuged within 2 h and stored in aliquots of 300 μL at −20°C until assayed. Plasma creatinine concentrations were determined by an in-house validated enzymatic method (Vettest 8008; Idexx, Hoofddorp, The Netherlands). The upper limit of quantification was 1202 μmol/L. Pharmacokinetic analyses were performed using WinNonlin (WinNonlin Version 4.0.1; Scientific Consulting Inc, Apex, NC, USA). Individual plasma data were subjected to noncompartmental analysis[32] for clearance calculation. The plasma Cl_{creat} was determined by dividing the actually administered dose of exogenous creatinine by the corresponding area under the plasma concentrations vs time curve (AUC), and indexed to body weight (mL/min/kg).

Statistical analysis

Analyses were performed with a commercial software program (Systat, version 12.00.08). The level of significance was set at 5% ($P < .05$), and ANOVA was used to test the status effect on all variables (USG, UPC, sCr, serum urea, sCysC, Cl_{creat}, SBP, urinary markers) at time point zero (T0). When a significant status effect was observed, a post hoc hypothesis test (paired *t*-tests with Bonferroni's correction) was performed. Repeated measures ANOVA was used to test the effect of time point on different variables within a group (HAC or DM group). When a statistically significant effect of time point was observed, pairwise comparisons (paired *t*-test with Bonferroni's correction) were performed between time points.

Results

Validation of sCysC PENIA

The LOD of the Behring PENIA for sCysC was 0.049 mg/L. The intra- and inter-assay CVs were satisfactory for the lower (1.74% and 2.92%), middle (2.73% and 8.65%), and high (8.46% and 4.1%) concentration serum pools, respectively. The measurement of serially diluted canine sera provided a linear series of results ($r = .997$) (Figure S1).

Dogs with DM

This study included 14 dogs with DM, with a median age of 9 years (range 4–10.8) and median body weight of 17.7 kg (range 9–59). In 2 dogs, only data obtained at presentation were collected, but 12 diabetic dogs were monitored over a 6-month period. Dogs showed symptoms for a median period of 3 weeks (one to36 weeks). The most common clinical signs were polyuria and polydipsia (14/14 dogs), weight loss (9/14), and polyphagia (7/14). Diabetic ketoacidosis was present in 7/14 dogs, accompanied by additional signs such as vomiting (6/14), anorexia (4/14), diarrhea (3/14), and lethargy (3/14). Six of the 7 dogs with DKA had pancreatitis, based on abdominal ultrasonographic examination alone, or in combination with increased cPLI (3/6 dogs, median 968 μg/L, reference interval < 200 μg/L). Eleven dogs were not treated and 2 had been treated for < 10 days prior to inclusion in the study. One dog was treated for 8 weeks, but was not well controlled and was referred due to DKA. After initial stabilization, all dogs were treated with porcine insulin zinc suspension (Caninsulin; Intervet International bv), at a starting dose of 0.5 IU/kg twice daily. Between T0 and T3, 2/14 DM dogs were euthanized because the owners declined further insulin treatment.

Dogs with HAC

The median age of the 22 dogs with HAC was 10.3 years (range 7.9–13.7 years), with a median body weight of 27.5 kg (range 8.8–56.6 kg). Dogs were symptomatic for a median time of 6 months (range one to 24 months). Pituitary-dependent hypercortisolism (PDH) was confirmed in 21 animals and adrenal-dependent hypercortisolism in one dog.[16] Choice of therapy (surgically [$n = 10$]; or medically [$n = 12$]) relied on the owners' decision; no randomization of the 2 treatment groups was performed. Three dogs had already been treated with trilostane (Vetoryl; Dechra Limited, Staffordshire, UK) for at least 6 months, but

were poorly controlled. Twelve dogs were treated with trilostane at a starting dose of 1 mg/kg bid or 2 mg/kg once daily (sid). The 10 remaining dogs in the HAC group underwent a transsphenoidal hypophysectomy and cortisone acetate replacement therapy (starting dose of 1 mg/kg bid, tapered over 4 weeks to 0.25 mg/kg bid), levothyroxine (15 μg/kg bid, Forthyron; Eurovet Animal Health, Bladel, the Netherlands), and desmopressin (0.01% solution, one drop into the conjunctival sac every 8 h; tapered and tailored to the individual patient, Desmopressin nasal spray, Prasco Laboratories, Mason, OH, USA). Nine dogs (6 surgically and 3 medically treated) with PDH were followed up for 12 months and 5 (one surgically and 4 medically treated) were followed up for 6 months.

Seventeen healthy dogs with a median age of 8.7 years (range 6.6–13.3) and median body weight of 20.5 kg (range 6–51.2 kg) were recruited. There were no age and body weight differences between the healthy group and the HAC and DM groups (Student's *t*-test, $P > .05$).

Renal variables in dogs with DM and healthy control dogs at initial presentation and follow-up

At presentation, sCr, urea, SBP, and Cl_{creat} did not significantly differ between the 2 groups (Table 1). The median SBP for each group was higher than the cutoff value of 150 mmHg[29], and hypertension was observed in 6/12 dogs with DM (50%). Urine specific gravity was significantly lower in the DM group ($P = .029$). Overt proteinuria (UPC > 0.5) was recorded in 4/14 (28%) DM dogs and 1/17 healthy dogs (5%), although UPC was not statistically different ($P = .940$). Borderline proteinuria (UPC 0.2–0.5) was noted in 4 diabetic dogs (29%) and in 5 control dogs (29%). One of the controls had overt proteinuria. None of the healthy dogs had an active sediment or positive urine culture. Serum CysC levels and renal urinary markers (uALB/uCr, uIgG/uCr, uRBP/uCr, and uNAG/uCr) at presentation did not differ significantly between groups.

Serum Cr and urea concentration, USG and UPC did not change significantly over time in dogs with DM (Table 2). One dog presented with mild azotemia at T3, but this normalized at T6. Persistent proteinuria was observed in 4/12 dogs with DM (33%). Borderline proteinuria that either persisted or even developed to overt proteinuria was found in 2 DM dogs. In 2 other DM dogs, borderline proteinuria detected at T0 was transient. At T6, 2 DM dogs suffered from *Escherichia coli* cystitis, with one of them having transient borderline proteinuria at that time point. The other dog showed persistent borderline proteinuria during complete follow-up, with even overt proteinuria at the time of diagnosis of the cystitis. At T6, 8/12 dogs with DM were hypertensive (67%). Plasma clearance of creatinine (GFR) did not change significantly over time in this group. There was a significant decrease ($P < .002$) of sCysC between T0 and T3 as well as between T0 and T6. None of the urinary markers significantly changed over time (Table 2).

Renal variables in dogs with HAC at initial presentation and follow-up

There were no significant changes in sCysC in dogs with HAC, although Cl_{creat} significantly decreased

Table 1. Median (range) of renal markers in groups of dogs with hyperadrenocorticism (HAC group), diabetes mellitus (DM group), and healthy controls at initial presentation.

Renal Variable	HAC Group ($n = 22$)	DM Group ($n = 14$)	Healthy Control Group ($n = 17$)
sCr (μmol/L)	58.7 (37–121.1)	73.8 (38.9–124.6)	70 (40.7–131.7)
Serum urea (mmol/L)	4.8 (2–19.5)	5.4 (2.3–9.3)	5.0 (3.2–7.7)
USG	1.008 (1.003–1.022)*	1.031 (1.012–1.04)*	1.039 (1.010–1.050)
UPC	1.7 (0.07–16.8)	0.3 (0.1–1.8)	0.1 (0.07–0.1)
SBP (mmHg)	156 (94–204)	154 (118–228)	159 (113–192)
Cl_{creat} (mL/min/kg)	2.4 (1.1–4.3)	3.2 (1.3–4.5)	2.5 (1.8–3.6)
sCysC (mg/L)	0.3 (0.1–0.8)	0.3 (0.2–0.6)	0.3 (0.2–0.4)
uALB/uCr (mg/g)	2879 (31.19–15717)	155.6 (6.61–2464)	14.65 (1.59–449)
uIgG/uCr (mg/g)	379 (2.31–6186)	29.26 (2.68–168.9)	3.8 (0.46–46.62)
uRBP/uCr (mg/g)	2.36 (0.21–485.6)	0.51 (0.04–34.74)	0.05 (0.00–0.54)
uNAG/uCr (U/g)	5.24 (0.00–58.3)	6.18 (2.47–26.46)	2.63 (1.11–9.05)

n indicates number of dogs; sCr, serum creatinine; USG, urine specific gravity; UPC, urinary protein-to-creatinine ratio; SBP, systolic blood pressure; Cl_{creat}, plasma clearance of exogenous creatinine; sCysC, serum cystatin C; uALB/uCr, urinary albumin/creatinine; uIgG/uCr, urinary immunglobulin G/creatinine; uRBP/uCr, urinary retinol-binding protein/creatinine; uNAG/uCr, urinary N-acetyl-β-glucosaminidase/creatinine.
*Significant difference ($P < .05$) compared to the healthy control group.

($P < .05$) both at T6 and T12 compared to initial presentation (Table 3).[16] Proteinuria was noted in 13/22 dogs (60%) with HAC at presentation.

Discussion

This first report on PENIA method validation for canine sCysC shows that the assay reliably and precisely measured canine sCysC. This assay was used to measure sCysC for the first time in dogs with DM and HAC at presentation and during follow-up.

Cross-reactivity between canine sCysC and polyclonal anti-human CysC antibodies was previously reported using Western immunoblot for PETIA[5,33], but not for PENIA. Recently, we documented cross-reactivity for both feline serum and urinary CysC with polyclonal antibodies used in the human PENIA.[26] Western blot analysis could not be performed in the current study with canine serum, as the polyclonal anti-human CysC antibodies were not commercially available. However, cross-reactivity was expected because of the degree of homology (46–79%) between the human and canine genetic structure of CysC.[34,35] Our results show that the BN 100 autoanalyzer PENIA is a valid method for measuring canine sCysC in a precise and linear manner, consistent with previous results reported for plasma CysC.[7] Nevertheless, as

Table 2. Median (range) of renal biomarkers in a group of dogs with diabetes mellitus at presentation and followed up for 6 months.

Renal Variable	T0 (n = 14)	T1 (n = 11)	T3 (n = 12)	T6 (n = 12)
sCr (μmol/L)	73.8 (38.9–124.6)	73.4 (46.0–101.7)	63.7 (36.2–130.8)	63.2 (43.3–98.1)
serum urea (mmol/L)	5.4 (2.3–9.3)	5.0 (3.2–11.3)	5.2 (2.2–12.0)	5.5 (2.7–54.0)
USG	1.031 (1.012–1.040)	1.033 (1.008–1.044)	1.023 (1.009–1.043)	1.025 (1.009–1.050)
UPC	0.3 (0.1–1.8)	0.3 (0.1–2.8)	0.5 (0.1–3.4)	0.2 (0.1–2.0)
SBP (mmHg)	154 (118–228)	157 (93–180)	168 (117–205)	159 (130–230)
Cl_{creat} (mL/min/kg)	3.2 (1.3–4.5)	–	–	3.2 (1.7–4.4)
sCysC (mg/L)	0.27 (0.20–0.56)	0.28 (0.18–0.50)	0.24 (0.17–0.38)*	0.23 (0.14–0.49)*
uALB/uCr (mg/g)	155.6 (6.6–2464)	85.27 (16.06–2543)	436.38 (10.03–5467)	124.2 (10.55–1614)
uIgG/uCr (mg/g)	29.28 (2.68–168.89)	9.22 (0.50–338.64)	25.58 (2.51–460.28)	37.15 (1.59–194.43)
uRBP/uCr (mg/g)	0.51 (0.04–34.74)	0.44 (0.18–0.50)	0.45 (0.02–1.49)	0.09 (0.00–1.55)
uNAG/uCr (U/g)	6.18 (2.47–26.46)	5.47 (1.22–16.06)	4.45 (2.07–10.94)	3.920 (0.00–12.58)

T0 indicates initial presentation; T1, time point 1 month; T3, time point 3 months; T6, time point 6 months; n, number of dogs; sCr, serum creatinine; USG, urine specific gravity; UPC, urinary protein-to-creatinine ratio; SBP, systolic blood pressure; Cl_{creat}, plasma clearance of creatinine; sCysC, serum cystatin C; uALB/uCr, urinary albumin/creatinine; uIgG/uCr, urinary immunoglobulin G/creatinine; uRBP/uCr, urinary retinol-binding protein/creatinine; uNAG/uCr, urinary N-acetyl-β-glucosaminidase/creatinine; –, indicates that the measurement was not performed at that specific time point.
*Significant change compared with initial presentation (T0).

Table 3. Median (range) of renal biomarkers in a group of dogs with hyperadrenocorticism (HAC) followed up for 12 months.

Renal Variable	T0 (n = 22)	T1 (n = 15)	T3 (n = 15)	T6 (n = 14)	T12 (n = 9)
sCysC (mg/L)	0.30 (0.14–0.84)	0.30 (0.00–0.40)	0.30 (0.21–1.21)	0.30 (0.23–0.38)	0.30 (0.27–0.38)
sCr (μmol/L)	58.7 (37.0–121.1)	65.4 (46.0–167.1)*	63.6 (44.0–209.5)*	64.1 (44.2–105.2)*	67.2 (55.7–99.9)*
Serum urea (mmol/L)	4.8 (2.0–19.5)	5.5 (3.2–16.7)	4.0 (2.3–24.8)	4.9 (2.3–6.5)	5.0 (1.3–7.2)
USG	1.008 (1.003–1.022)	1.011 (1.003–1.025)	1.012 (1.004–1.039)*	1.019 (1.004–1.039)*	1.014 (1.002–1.045)*
UPC	1.7 (0.07–16.8)	0.4 (0.1–5.0)*	0.2 (0.07–8.6)*	0.2 (0.1–4.7)*	0.3 (0.1–7.5)*
SBP (mmHg)	156 (94–204)	136 (122–219)	153 (124–207)	138 (60–220)	155 (75–174)
Cl_{creat} (mL/min/kg)	2.4 (1.1–4.3)	–	–	2.1 (1.6–2.8)*	2.1 (1.4–2.3)*
uALB/uCr (mg/g)	2879 (31.19–15717)	129.70 (2.03–5323)	64 (1.45–4483)	49.25 (3.47–5236)	140.17 (0.00–336.90)
uIgG/uCr (mg/g)	379.20 (2.31–6186)	9.18 (0.00–877)	6.43 (0.75–700.33)	6.59 (1.18–712.93)	11.93 (1.29–1365)
uRBP/uCr (mg/g)	2.36 (0.21–485.6)	0.10 (0.00–25.39)	0.12 (0.00–31.41)	0.09 (0.00–0.82)	0.10 (0.00–4.77)
uNAG/uCr (U/g)	5.24 (0.00–58.30)	0.7 (0.00–58.45)	2.09 (0.00–7.41)	3.14 (0.00–6.22)	2.57 (0.00–11.78)

T0 indicates initial presentation; T1, time point 1 month; T3, time point 3 months; T6, time point 6 months; T12, time point 12 months; n, number of dogs; sCysC, serum cystatin C; sCr, serum creatinine; USG, urine specific gravity; UPC, urinary protein-to-creatinine ratio; SBP, systolic blood pressure; Cl_{creat}, plasma clearance of creatinine; uALB/uCr, urinary albumin/creatinine; uIgG/uCr, urinary immunglobulin G/creatinine; uRBP/uCr, urinary retinol-binding protein/creatinine; uNAG/uCr, urinary N-acetyl-β-glucosaminidase/creatinine; –, indicates that the measurement was not performed at that specific time point.
*Significant change compared with initial presentation (T0).

anti-human CysC antibodies were used in this study, the values of sCysC concentration obtained are relative concentrations, similar to feline data published earlier.[27] A potential limitation of this study is the prolonged storage of serum samples (up to 3 years at −80°C) until sCysC analysis using PENIA. However, sCysC is considered a stable protein in human medicine, and freezing or freeze/thaw cycles do not affect its concentration.[36]

At presentation, median sCysC concentrations did not significantly differ between dogs with DM or HAC and healthy dogs, suggesting that GFR was similar between these groups of dogs at presentation. Over time, the routine renal variables, sCr and serum urea, did not change in the DM dogs. An impaired renal function may potentially develop in diabetic dogs, due to well-known risk factors, including systemic hypertension and proteinuria, both of which were reported in people and dogs with DM.[13,37] In the current study, 50% of the DM dogs showed systemic hypertension at T0, with no changes over time. Despite all precautions taken, we cannot exclude white coat hypertension, as 57% of the healthy controls also showed an increased SBP. Questions remain about the general occurrence of hypertension in elderly dogs. Further studies are needed to address this issue.

Approximately, 33% of the DM dogs had overt proteinuria, comparable to or somewhat higher than the affected dogs in previous reports, which can be explained by the fact that some of these initial studies used a cutoff of 1.0 instead of 0.5 of UPC to define proteinuria.[23,24] During follow-up, proteinuria in dogs with DM did not improve significantly. In a previous study of 12 dogs with DM, proteinuria regressed in 7/12, and increased in 4 of these dogs during a 12- to 18-month follow-up.[25] Nevertheless, our results should not be directly compared to the results of this previous study, because it included dogs with concomitant HAC and DM as well as dogs with DM.[25]

Interesting observations in our study were the prevalence of borderline proteinuria in 29% of the healthy elderly control dogs, and the absence of a significant difference for UPC values between dogs with DM and healthy controls at presentation. Therefore, presence of overt proteinuria in dogs with DM should be interpreted with caution. The absence of active sediments or positive urine cultures in the healthy dogs suggests a renal origin of the borderline proteinuria in older animals. The high occurrence of borderline proteinuria in healthy individuals has also been documented in people and cats.[38,39] Age-related proteinuria still needs to be elucidated in veterinary medicine. The relatively high incidence of persistent proteinuria in aging people is caused by underlying diseases such as DM, hypertension, multiple myeloma, amyloidosis, and drug-induced nephropathy.[38] Considering the high occurrence of systemic hypertension (53%) in our healthy control dogs, hypertension might contribute to the development of proteinuria in elderly dogs.

During the 6-month follow-up, both GFR and the urinary renal markers did not change significantly in the DM group. Previous studies in dogs with spontaneous DM have described microalbuminuria in 20–70% of the dogs.[23,24,40] Histopathologic lesions, consistent with human diabetes nephropathy, have been described in dogs with experimentally induced DM.[41] In the current study, no significant differences were observed for the urinary renal markers between the DM group and the healthy controls at initial presentation. In both the groups, there was a wide range in concentrations of all urinary markers indicating large bio-variability between dogs. It is possible that a study including more dogs might reveal significant differences. In our opinion, the observed broad overlap between both the groups limits the clinical relevance of previously observed renal histopathologic alterations. Diabetic nephropathy is a long-term complication of DM in human medicine.[42,43] The majority (72%) of diabetic dogs in our study were symptomatic for less than one month, and the maximum follow-up time was 6 months, which may be too short for the development of relevant renal dysfunction. However, a recent 2-year longitudinal study in DM dogs could not detect a significant effect of time on the prevalence of proteinuria, microalbuminuria, and hypertension either.[40] Therefore, we assume that it is unlikely that serial measurements over a longer time period would detect a renal functional decline and associated changes in the tested markers in DM dogs. Rather, recent biochemical and histopathologic data in cats[44] and biochemical data in dogs[40], like our findings, question the existence of DKD in small animals. The high incidence of systemic hypertension (67%) and persistent overt proteinuria (33%) in DM dogs suggest that they should be monitored. However, in our study, no significant differences were found for SBP and UPC at presentation between dogs with DM and the older healthy control dogs. The simultaneous occurrence of borderline proteinuria and hypertension in the elderly, healthy dogs underlines the need for further research and the development of age-specific reference intervals for biomarkers of renal dysfunction.

The significant decrease of sCysC over time within the DM group is likely not an indication for renal

dysfunction. Rather, we consider this observation to be of questionable clinical relevance because the GFR did not significantly change over time, and the difference between CysC medians in DM and healthy dogs was small, while there was a broad overlap at the different time points. A potential limitation of the current study is that we did not measure GFR using the gold standard of urinary inulin clearance. However, this is a time-consuming, stressful, and potentially harmful procedure, with an increased risk of urinary tract infections. Moreover, Cl_{creat} has been reported to show a good correlation with urinary inulin clearance.[32]

There was no significant difference for sCysC in HAC dogs compared to the controls at initial presentation, which is in contrast to the glucocorticoid-induced increase of sCysC seen in human patients receiving high doses of short-acting steroids.[10] We therefore hypothesize that the prolonged exposure to endogenous increased concentrations of corticosteroids could lead to a suppression of this glucocorticoid-mediated increase in sCysC. Indeed, no effect of long-acting steroids on sCysC has been observed in human medicine, supporting our hypothesis.[12] Moreover, the significant posttreatment decrease in GFR in the HAC group was not associated with an increase of sCysC concentrations. However, renal dysfunction has been described by our group in dogs with HAC.[13–16] Although sCysC has been shown to be more sensitive than sCr to detect GFR changes[9], sCysC did not detect minor but significant changes of GFR in dogs with HAC.

In conclusion, the human PENIA is a valid assay for measuring canine sCysC. The lack of significant changes of sCysC in dogs with DM and HAC in comparison with healthy elderly control dogs suggests that older dogs might need a different reference interval for sCysC due to age-related renal changes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linearity of expected serum cystatin C (sCysC) concentrations (mg/L) vs measured sCysC concentrations (mg/L) after serial dilution of serum from a healthy control dog using the particle-enhanced nephelometric immunoassay (PENIA). Linearity is defined by calculation of the correlation coefficient (r).