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Objectives: Evaluation of urine albumin:creatinine ratio, urine cystatin C:creatinine ratio, urine protein:creatinine ratio and urine specific gravity as screening tests for azotaemic chronic kidney disease in cats.

Methods: Cats (>8 years) were defined as healthy non-azotaemic (n=40) if they had serum creatinine concentration <153 µmol/L and no history of significant disease, or as having azotaemic chronic kidney disease (n=12) if they had serum creatinine concentration >153 µmol/L with urine specific gravity <1.035. Urine albumin:creatinine ratio, urine cystatin C:creatinine ratio, urine protein:creatinine ratio and urine specific gravity were compared between the two groups.

Results: Urine cystatin C:creatinine ratio was significantly lower in cats with azotaemic chronic kidney disease than healthy cats (3.7 [1.4, 4.3]x10^{-6} vs. 13.9 [6.3, 24.7]x10^{-6}; P=0.011). Urine specific gravity was also significantly lower in the azotaemic chronic kidney disease group than in the healthy group (1.022 [1.017, 1.028] vs. 1.043 [1.034, >1.050]; P<0.001). Urine albumin:creatinine ratio and urine protein:creatinine ratio were not significantly different between the groups (P=0.075 and P=0.965 respectively).

Clinical significance: Urine cystatin C:creatinine ratio and urine specific gravity were significantly lower in cats with azotaemic chronic kidney disease than healthy cats, however neither biomarker was an adequate sole screening test for azotaemic chronic kidney disease.

Introduction

Chronic kidney disease (CKD) is a common condition of geriatric cats, which is reported to affect up to 31% of cats aged over 15 years (Lulich et al. 1992). Renal azotaemia is diagnosed by documentation of an elevated serum creatinine concentration in conjunction with evidence of reduced urine concentrating ability (Stockham and Scott 2008). However, routine screening of senior and geriatric cats for azotaemic CKD can be expensive and may be perceived as invasive by owners.

Biomarkers of renal damage and dysfunction might be expected to appear in urine at an early stage of CKD, and so the detection of urinary biomarkers could have the potential to facilitate the diagnosis of CKD in cats. Urine can be collected by non-invasive techniques (Osborne and Stevens 1999), therefore these markers would be an ideal method by which to screen geriatric cats for the presence of CKD.

The detection of small amounts of albumin in urine is abnormal, as albumin is too large to cross the glomerular filtration barrier in large quantities, and any albumin which does cross the glomerular barrier is normally reabsorbed and degraded by tubular epithelial cells (Grauer 2007). CKD in cats is usually associated with tubulointerstitial nephritis and tubular damage, which will impair the tubular reabsorption of albumin (DiBartola et al. 1987, Chakrabarti et al. 2013, McLeland et al. 2015), therefore low level albuminuria might be expected in cats with CKD. Quantitative measurements of albuminuria can be determined by ELISA (Syme et al. 2006, Lyon et al. 2010), however this technique has not been automated to date. An automated method for measuring urinary albumin concentrations has been validated in dogs (Murgier et al. 2009), however its validation has not yet been reported in the cat.

Cystatin C is a low molecular weight protein which is synthesised at a stable rate by most nucleated cells. Cystatin C is freely filtered by the glomeruli, however it is mostly reabsorbed

and catabolised by the proximal tubular cells of the kidney so that only a small amount of cystatin C is excreted in the urine of healthy animals (Uchida and Gotoh 2002). Tubular damage will impair the reabsorption and degradation of cystatin C such that urinary cystatin C excretion would increase, therefore the detection of high concentrations of cystatin C in the urine might be expected to correlate with renal tubular damage (Uchida and Gotoh 2002). An automated particle enhanced turbidimetric assay (PETIA) for the measurement of cystatin C in canine urine has recently been validated, and dogs with evident CKD had markedly greater urinary cystatin C excretion than normal control dogs or dogs with other systemic diseases (Monti et al. 2012). However, the PETIA has not been validated for the measurement of urinary cystatin C in feline urine to date. A human cystatin C particle enhanced nephelometric immunoassay (PENIA) was recently validated for use in cats, and a small pilot study demonstrated that urinary cystatin C:creatinine ratio was higher in cats with CKD than healthy control cats (Ghys et al. 2014). However, the PENIA requires the use of a specialised immunonephelometer, whereas the PETIA can be performed using standard automated analysers that are present in many commercial laboratories.

The first aim of this study was to validate two human PETIAs for the measurement of urinary albumin and cystatin C in feline urine. Then using these assays we aimed to investigate various urinary biomarkers (urinary albumin:creatinine ratio [uAlb:Cr], urinary cystatin C:creatinine ratio [uCysC:Cr], UPC and USG) to assess if they could be potentially used as screening tests for the detection of azotaemic CKD in cats. Our hypothesis was that increased urinary excretion of albumin and cystatin C would be superior urinary screening tests for the presence of azotaemic CKD in cats than either UPC or USG.

Materials and methods

The measurements of urinary albumin and cystatin C were obtained using an automated analyser (Olympus AU400, Beckman Coulter). Creatinine concentrations were measured by the Jaffe Kinetic method and urine total protein concentrations were measured by the pyrogallol red method on the same analyser.

Modification of the PETIA for measurement of urinary albumin in feline urine

The measurements of urinary albumin were made using a commercially available human PETIA (Microalbumin Synchron CX Systems, Beckman Coulter Inc.). However, as a previous study, which published the validation of the human PETIA for measurement of urinary albumin in canine urine (Murgier et al. 2009), reported that the monoclonal antibody against human albumin was only partially cross-reactive with canine albumin, initial experiments were performed to establish if calibration with canine albumin was also necessary for use with feline urine samples. Canine specific calibrators were made from a solution of purified canine albumin (MP Biomedicals, Aurora, OH, USA). The purity of the purified canine albumin was assessed by visual assessment of the peak obtained by agarose gel electrophoresis. A five point calibration curve was constructed by diluting the solution of purified canine albumin (7.85 g/L) to concentrations of 392.5, 196.3, 98.3, 49.2, and 12.3 mg/L with a 0.9% NaCl solution. Purified canine albumin was used for these calibrators because purified feline albumin was not commercially available. The calibrators provided with the human PETIA kit were also used to establish a second five point calibration curve. Feline serum samples of known albumin concentration (measured by an automated bromocresol green assay) were diluted to give concentrations within the range of the assay.

Albumin concentrations in these samples were then measured using the assay after calibration with the canine calibrators and human calibrators.

Modification of the PETIA for measurement of urinary cystatin C in feline urine

The measurements of urinary cystatin C were made using a human PETIA method (Gentian, Moss, Norway). The analyser programme was modified by the addition of an additional calibration point (point 0), which was obtained using a 0.9% NaCl solution.

Validation of modified PETIAs for the measurement of urinary albumin and cystatin C in feline urine

Precision of the modified human PETIAs was assessed by evaluating intra- and inter-assay coefficients of variation for urine samples with low, medium and high concentrations of albumin and cystatin C. For intra-assay precision ten replicates of each sample were evaluated within the same run. For assessment of inter-assay variability, pooled feline urine samples were evaluated in triplicate on five consecutive working days. In the absence of purified feline cystatin C or albumin, recovery was evaluated by the addition of increasing amounts of cystatin C calibrator solution (7.5 mg/L) or albumin calibrator (393 mg/L) to a urine sample which contained no detectable cystatin C or albumin. Dilutional linearity was assessed by serial dilution of a urine sample with high cystatin C or albumin with a urine sample containing no detectable cystatin C or low concentrations of albumin, in order to avoid changes to the urine matrix. The limit of blank was determined by measurement of the urinary albumin and cystatin C concentrations in deionised water (diH_2O), which was evaluated in five samples on five consecutive working days. The limit of blank was

calculated as the mean interpolated albumin or cystatin C concentration in diH_2O + 2*standard deviation of urine albumin or cystatin C concentration in diH_2O (Armbruster and Pry 2008). Stability of urinary albumin and cystatin C in four samples following storage at room temperature (approximately 22°C) for 24, 48, 72 and 168 hours, and following storage at -20°C for 28 days was also assessed.

Clinical study

Blood and urine samples were obtained from cats at three UK first opinion practices between 1st March 2013-30th April 2015 as part of a free of charge screening programme. The Ethics and Welfare Committee of our institution approved the diagnostic protocol (CR56). To be included, the cats were ≥8 years old, and had no known significant systemic diseases (e.g. cardiac disease, diabetes mellitus, or hyperthyroidism). Exclusion criteria included the feeding of a low protein low phosphate (renal care) diet, recent or ongoing treatment with corticosteroids, diuretics or angiotensin converting enzyme inhibitors, and recent or concurrent intravenous fluid therapy at the time of sampling. Blood samples (in EDTA and serum tubes) were taken by jugular venepuncture and urine samples were taken by cystocentesis if possible. If cystocentesis was not possible, the owners were asked to obtain a free catch urine sample and submit it for analysis within 3 days of blood sampling. Blood and urine samples were submitted to a commercial laboratory for complete blood count, serum biochemistry including total thyroxine concentration (TT4) and urinalysis including UPC. Urinalysis included evaluation of USG by refractometry, urine dipstick and sediment analysis. Excess urine was stored at -80°C until batch analysis of urine albumin and cystatin C which was performed at approximately 6 monthly intervals.

Samples were excluded from further analysis if; TT4 was >40 nmol/L, there was evidence of bacteriuria, pyuria or gross haematuria, severe systemic illness was apparent on haematology and biochemistry, or if the samples were more than 3 days old at the time of sample analysis. Cats were classified as having azotaemic CKD if they had a serum creatinine concentration >153 μmol/L with concurrent USG <1.035. Cats that were not classified as having azotaemic CKD (non-azotaemic group) were then further sub-classified based on clinical history into either healthy non-azotaemic or non-healthy non-azotaemic groups. Cats included in the healthy non-azotaemic group had no clinical history of disease except for dental disease, arthritis or mild entropion.

Statistical analysis

Statistical analysis was performed using commercially available software (SPSS for Windows 21.0, SPSS Inc, Chicago, Illinois, USA). Correlations between age, serum concentrations of urea, creatinine and TT4, UPC, uAlb:Cr and uCysC:Cr were made by Spearman's correlation. Comparisons between groups (healthy non-azotaemic vs. azotaemic CKD) were made using the Mann Whitney U test. Receiver operator curves were constructed to evaluate the sensitivity and specificity of urinary biomarkers for the detection of azotaemic CKD. Data are presented as median [25^{th}, 75^{th} percentiles] unless otherwise stated and statistical significance was defined as $P \leq 0.05$.

Results

Assay validations

Comparison of the measured albumin concentrations in diluted feline serum samples indicated that the utilisation of the canine calibrators was optimal since the use of canine calibrators resulted in a measured albumin concentration that more closely approximated the expected albumin concentration. When the human calibrators were used, the measured albumin concentration was approximately 50% of the expected albumin concentration (Figure 1). This was consistent with partial cross reactivity between the human monoclonal antibody and feline albumin, which has been described previously in dogs (Murgier et al. 2009).

The PETIA for urine albumin demonstrated excellent precision and reproducibility at all levels tested (Table 1). Mean (± standard deviation) canine albumin recovery was acceptable (92±1%) and the assay was linear in the range 5.6-381.4 mg/L (r^2=0.997). The limit of blank was determined to be <0.1 mg/L and urine albumin was stable for 72 hours at room temperature and following 28 days of storage at -20^{o}C (<10% change in measured urinary albumin concentration).

The PETIA for urine cystatin C demonstrated good precision at all levels tested and good reproducibility at medium and high concentrations, however the inter-assay variability of samples with low concentrations of cystatin C was high (37%, Table 2). Mean (± standard deviation) cystatin C calibrator recovery was good (96±8%) and the assay was linear in the range 0.044-3.846 mg/L (r^2=0.996). The limit of blank was determined to be <0.01 mg/L and urine cystatin C was stable for 72 hours at room temperature and following 28 days of storage at -20^{o}C (<15% change in measured urinary albumin concentration).

197 ***Clinical study***

198

199 Samples from 139 cats were submitted for geriatric screening during the study period. Eight
200 cats were excluded because they had a TT4 >40 nmol/L, eight cats were excluded because of
201 other significant systemic diseases, 28 cats were excluded because they had pyuria and/or
202 bacteriuria, and 21 cases were excluded because the samples were >3 days old at the time of
203 analysis.

204 Sixty two cases were non-azotaemic, 22 of which had a clinical history of disease and were
205 excluded. Forty cats were thus included in the healthy non-azotaemic group. Twelve cats
206 were diagnosed with azotaemic CKD. The healthy non-azotaemic group comprised 23 female
207 neutered and 17 male neutered cats. Breeds represented in the healthy non-azotaemic group
208 included thirty-four domestic short or long haired cats, two Siamese cats and one Bengal,
209 Devon Rex, Persian and Russian Blue cat. The azotaemic CKD group consisted of four
210 female neutered and eight male neutered cats. Breeds represented in the azotaemic CKD
211 group included nine domestic short or long haired cats plus one British Short Hair, Burmese
212 and Tonkinese cat. Samples were obtained by cystocentesis in 31/40 healthy non-azotaemic
213 cats and 11/12 cats with azotaemic CKD.

214 There were no significant differences in age, packed cell volume (PCV), and serum TT4
215 concentrations between the healthy non-azotaemic and azotaemic CKD groups (Table 3).

216 Urine albumin:creatinine ratio was weakly positively correlated with age (r_s=0.308, n=74;
217 P<0.001), and serum urea concentration (r_s=0.288, n=74; P=0.013). Urine albumin:creatinine
218 ratio was also strongly positively correlated with UPC (r_s=0.756, n=74; P<0.001), but was not
219 significantly correlated with serum creatinine concentration. Urine cystatin C:creatinine ratio
220 (uCysC:Cr) was not significantly correlated with any parameter including serum urea and
221 creatinine concentrations.

Urine cystatin C:creatinine ratio (uCysC:Cr) was significantly lower in cats with azotaemic CKD than healthy non-azotaemic cats (3.7 [1.4, 4.3]x10^{-6} vs. 13.9 [6.3, 24.7]x10^{-6}; P=0.011, Figure 2). USG was also significantly lower in the azotaemic CKD group than in the healthy non-azotaemic group (1.022 [1.017, 1.028] vs. 1.043 [1.034, >1.050]; P<0.001, Figure 3). In addition, urine albumin:creatinine ratio (uAlb:Cr) was numerically higher in the azotaemic group than the healthy non-azotaemic group (21.7 [8.3, 87.9]x10^{-3} vs. 11.7 [5.7, 19.0]; P=0.075, Figure 4), although this did not reach statistical significance. However, no significant difference in UPC was present between the two groups (azotaemic CKD group 0.18 [0.12, 0.36], healthy non-azotaemic group 0.21 [0.15, 0.24]; P=0.965, Figure 5). Receiver operator curve analysis indicated that both USG (area under curve = 0.905, 95% CI 0.836-0.974) and uCysC:Cr (area under curve = 0.728, 95% CI 0.558-0.899) had an area under the curve which was significantly different from 0.5 (P<0.001 and P=0.013 respectively, Figure 6). In the entire group of cats (healthy non-azotaemic and azotaemic CKD group combined) and the group of healthy non-azotaemic cats only, there was no significant difference in USG, UPC, uAlb:Cr or uCysC:Cr between all cats which had urine samples taken by cystocentesis or by free catch (data not shown). This could not be assessed in the group of cats with azotaemic CKD because only one cat had a sample collected by free catch. If cats which had urine samples taken by free catch were excluded from the analyses, then USG and uCysC:Cr remained significantly lower in the azotaemic CKD group than the healthy non-azotaemic group (data not shown).

Since azotaemic CKD can usually be excluded in cats with USG ≥1.035, the utility of USG and uCysC:Cr as predictors of the presence of azotaemic CKD in cats with USG <1.035 (n=24) was also evaluated. When only samples with USG <1.035 were included, uCysC:Cr tended to be lower in cats with azotaemic CKD than healthy non-azotaemic cats (azotaemic CKD group 3.7 [1.7, 4.3] x10^{-6}, n=12 vs. healthy non-azotaemic group 20.1 [6.3, 45.9] x10^{-6},

n=12; P=0.052). There was no significant difference in USG (P=0.114), uAlb:Cr (P=0.291) and UPC (P=0.977) between the two groups. Receiver operator curve analysis indicated that uCysC:Cr had an area under the curve (0.736, 95% CI 0.503-0.969) which was significantly different from 0.5 (P=0.05, Figure 7).

Discussion

The human PETIA for urine albumin demonstrated excellent precision and reproducibility and appeared linear with adequate recovery of albumin and cystatin C when samples containing no detectable albumin or cystatin C were spiked. The human PETIA for urine cystatin C also demonstrated good precision and reproducibility at medium and high concentrations of cystatin C, however the inter-assay variability was high (37%) at low concentrations of cystatin C. The low concentrations of cystatin C (0.05-0.09 mg/L) also represents the working range of the assay for most cats (see Table 3) which may limit the utility of this test for individual cats. Both urine albumin and urine cystatin C appeared stable at room temperature for up to 72 hours, which means that these tests could be performed in samples shipped to a commercial laboratory for analysis. Binding of the monoclonal anti-cystatin C antibody to feline cystatin C was not definitively demonstrated by western blotting in the present study, however biological validity of the assay has been confirmed in serum (data not shown), which suggests that the avian antihuman cystatin C antibody does cross react with feline cystatin C.

Contrary to our hypothesis, and the findings in previous studies of cats and dogs with CKD (Ghys et al. 2014, Monti et al. 2012), urinary excretion of cystatin C (uCysC:Cr) appeared to be lower in cats with azotaemic CKD, than in healthy non-azotaemic cats. This was an unexpected finding, given that the predominant pathology in cats with CKD is

tubulointerstitial nephritis (DiBartola et al. 1987, McLeland et al. 2015, Chakrabarti et al. 2013) which would in turn be expected to cause tubular dysfunction, reduced cystatin C reabsorption in the proximal tubule and increased urinary excretion of cystatin C (Uchida and Gotoh 2002). The reason for the discordant results between the present study and the previous study in cats (Ghys et al. 2014) could be that the cats in the aforementioned study had more advanced CKD and were more proteinuric than the cats included in this study. Cystatin C is reabsorbed in the proximal tubules by megalin-mediated endocytosis, which is also the pathway for albumin reabsorption in the proximal tubule (Christensen et al. 2012). Albuminuria has also been demonstrated to reduce tubular reabsorption of cystatin C (Thielemans et al. 1994), therefore increased proteinuria might cause increased urinary cystatin C excretion. In the present study, cats with azotaemic CKD were predominantly non-proteinuric or borderline proteinuric, whereas in the study by Ghys and others (2014), the median UPC of the cats with CKD was 0.63 (Ghys et al. 2014). In addition, the majority of cats in the previous study by Ghys (Ghys et al. 2014) had a serum creatinine concentration consistent with International Renal Interest Society (IRIS) stage 3 or 4 (Elliott and Grauer 2007), whereas in the present study, the majority of cats had a serum creatinine concentration consistent with IRIS Stage 2. It is possible that increased urinary cystatin C excretion does not occur until the later stages of feline CKD. In the present study, most cats diagnosed with azotaemic CKD did not have isosthenuric urine, which implies that some tubular function was still present. It is possible that the remaining tubular function present in these cats was adequate to allow tubular reabsorption of cystatin C, which might explain the lack of increased urinary cystatin C excretion in cats with azotaemic CKD in this study.

The reason why cats with azotaemic CKD in the present study had lower urinary cystatin C excretion is unclear. This could either reflect a rare statistical type I error, or could reflect decreased filtration of cystatin C at the glomerulus, or increased cystatin C reabsorption or

297 metabolism in the kidney. Serum cystatin C concentrations in cats with CKD are increased
298 compared to healthy cats (Ghys et al. 2014), therefore, the decreased urinary cystatin C
299 excretion in cats with azotaemic CKD in the present study may reflect increased renal
300 reabsorption or metabolism of cystatin C in cats with early CKD. The reason why this would
301 occur in the cat is unknown, however if increased renal reabsorption of cystatin C did occur
302 in the cat, this may explain why serum cystatin C concentrations do not appear to be a robust
303 marker of CKD in cats (Ghys et al. 2014). Further studies to directly compare urinary cystatin
304 C measured by PETIA and PENIA would also be warranted since the differences observed in
305 urinary cystatin C excretion of cats with azotaemic CKD between this study and the previous
306 study in cats (Ghys et al. 2014) might reflect differences in analytical methodology.
307 Azotaemic CKD can usually be excluded in a cat with USG ≥1.035 since azotaemia in
308 these cases would usually be categorised as being consistent with pre-renal azotaemia,
309 although a small minority of cats with CKD may be azotaemic whilst maintaining urine
310 concentrating ability. However, the sensitivity and specificity of USG <1.035 for the
311 detection of azotaemic CKD has not been previously established. In the present study, there
312 was no difference in USG between azotaemic and healthy non-azotaemic cats when the USG
313 was <1.035, suggesting that assessment of USG <1.035 alone in a 'spot' urine sample is not
314 helpful for the diagnosis of azotaemic CKD. Documentation of a USG <1.035 could indicate
315 the presence of early non-azotaemic CKD, and it is possible that serial monitoring of USG,
316 and documentation of a persistent USG <1.035 is a more sensitive and specific marker of
317 CKD, however this could not be tested in the present study.
318 In contrast, lower uCysC:Cr tended towards a significant association with the presence of
319 azotaemic CKD, however this did not reach statistical significance. On ROC analysis, the
320 AUC for uCysC:Cr was significantly different from 0.5, and the AUC value was consistent
321 with a fair degree of accuracy. It is likely that the sensitivity and specificity of uCysC:Cr for

322 the detection of cats with early azotaemic CKD would be even lower in a population of cats
323 which included non-healthy cats. Furthermore, the poor repeatability of urine cystatin C
324 measurements at low concentrations that are typically found in cats (with and without CKD)
325 is likely to further limit the utility of uCysC:Cr as a screening test for CKD in individual cats.
326 Urinary excretion of albumin (uAlb:Cr) tended to be higher in cats with azotaemic CKD
327 than healthy non-azotaemic cats, although this did not reach statistical significance.
328 Nevertheless, there was significant overlap in the uAlb:Cr between the healthy non-azotaemic
329 and the azotaemic CKD groups, which indicates that uAlb:Cr would not be a sensitive or
330 specific marker of the presence of azotaemic CKD. UPC was also not significantly different
331 between the cats with azotaemic CKD and healthy non-azotaemic cats. Both of these findings
332 indicate that urinary excretion of albumin and total protein is not increased in cats with early
333 azotaemic CKD compared with healthy non-azotaemic cats.
334 In the present study, more than 50% of cats in the healthy non-azotaemic group had a UPC
335 >0.2, which the IRIS have defined as borderline proteinuria (www.iris-kidney.com). This is
336 consistent with the findings of a previous study of apparently healthy cats (Paepe et al. 2013).
337 It is possible that some cats with subclinical non-azotaemic CKD were included in the
338 healthy non-azotaemic group, which could have confounded the results of this study.
339 However, since the aim of this study was to identify urinary biomarkers of *azotaemic* CKD,
340 the inclusion of cats with early *non-azotaemic* CKD in the healthy non-azotaemic group
341 should not have been a confounding factor. Measurement of glomerular filtration rate (GFR)
342 would be useful to exclude early CKD in healthy non-azotaemic cats, however this was not
343 possible in the first opinion practices which submitted the samples to us.
344 This study was limited by the relatively low number of cats in the azotaemic CKD group
345 and the high number of samples which demonstrated evidence of bacteriuria and pyuria.
346 Urine samples were sometimes not taken contemporaneously with blood samples, however

this would only have influenced the categorisation in azotaemic cats which had pre-renal azotaemia at the time of blood sampling that subsequently resolved prior to the time of urine sample collection by the owner. Furthermore, if only cases with contemporaneous urine samples were included in the study, this would bias the study towards the inclusion of cats with palpable bladders at the time of blood sampling, which are in turn more likely to be those that are polyuric. In addition, evaluation of the utility of markers for the detection of azotaemic CKD in cats with USG <1.035 was limited by the relatively low number of healthy cats with USG <1.035 in this study.

Both cystocentesis and free catch urine samples were included in the present study, however it is currently unknown whether the sample collection method will affect the measured urinary albumin and cystatin C concentrations, and further studies are needed to investigate this. The present study was not capable of answering this question since this would require samples to be taken from the same cats by both free catch and then cystocentesis. This was not practical for the submitting veterinarians and would require a separate specific study. It is, however known that there is no influence of sample collection method on the UPC in dogs and cats (Vilhena et al. 2015, Beatrice et al. 2010). Exclusion of cats in which free catch urine samples were obtained did not result in a change in the conclusions of the study, therefore it appears unlikely that differences in the method of sample collection would account for the significant differences observed between the healthy non-azotaemic group and cats with azotaemic CKD.

Assessment of systolic blood pressure was not performed in the present study, because this was not possible within the routine appointments at our collaborator practices. This could have resulted in some hypertensive cats being included in the healthy non-azotaemic and azotaemic CKD groups which might have confounded the analysis of urinary albumin and total protein excretion, since systolic hypertension can contribute to proteinuria (Jepson et al.

2007). However, no effect of hypertension on urinary cystatin C excretion has been documented to date.

The assessment of these markers as a screening test for the presence of azotaemic CKD in a population of non-healthy non-azotaemic cats was also not performed. In practice, it is more likely that screening tests for CKD would be used in non-healthy cats rather than in healthy cats, however since the tested biomarkers appeared inadequate at distinguishing healthy non-azotaemic cats from cats with azotaemic CKD, further assessment of the diagnostic performance of these biomarkers in a population of non-healthy cats was not necessary, as it is likely to be poor.

In conclusion, the human PETIAs for albumin and cystatin C were successfully validated for use in feline urine, however the findings of this study indicate that assessment of uAlb:Cr, uCysC:Cr, USG and UPC alone are not useful screening tests for the presence of azotaemic CKD in older cats. Routine screening of senior and geriatric cats by evaluation of both blood and urine samples is necessary in order to definitively diagnose azotaemic CKD. A USG ≥1.035 can probably be used to exclude a diagnosis of azotaemic CKD in most cases, however documentation of a USG < 1.035 is not a sensitive or specific test for the presence of azotaemic CKD. Decreased urinary excretion of cystatin C appeared to be associated with the presence of azotaemic CKD, perhaps suggesting increased renal tubular metabolism or reabsorption of cystatin C in early feline CKD.

Table 1. Intra- and inter-assay coefficients of variation (CV) at low, medium and high concentrations of urine albumin calculated using a human particle enhanced turbidimetric immunoassay.

Albumin concentration (mg/L)	Intra-assay CV (%)	Inter-assay CV (%)
Low (11.5-12.2)	1.7	6.2
Medium (65.2-71.6)	1.4	3.8
High (164.0-164.2)	1.3	0.6

Table 2. Intra- and inter-assay coefficients of variation (CV) at low, medium and high concentrations of urine cystatin C calculated using a human particle enhanced turbidimetric immunoassay.

Cystatin C concentration (mg/L)	Intra-assay CV (%)	Inter-assay CV (%)
Low (0.05-0.09)	11.1	37.0
Medium (0.84-0.94)	4.9	7.7
High (2.59-4.21)	1.5	6.5

405 **Table 3. Comparison of selected clinicopathological variables between healthy non-**
406 **azotaemic cats (n=40) and cats diagnosed with azotaemic CKD (n=12). Data are**
407 **presented as median [25th, 75th percentile].**
408

Parameter	Healthy non-azotaemic group	Azotaemic CKD group	Sig.
Age (years)	12.0 [11.0, 13.4]	12.5 [11.3, 16.1]	0.283
PCV (%)	39 [32, 44]	37 [32, 43]	0.828
Serum urea concentration (mmol/L)	10.4 [8.8, 12.3]	15.1 [10.6, 20.9]	**0.003**
Serum creatinine concentration (µmol/L)	125.0 [110.8, 141.0]	190.0 [169.0, 245.3]	**<0.001**
Serum total thyroxine concentration (nmol/L)	21.7 [16.3, 27.7]	23.0 [18.6, 27.5]	0.798
Urine cystatin C concentration (mg/L)	0.032 [0.013, 0.061]	0.039 [0.013, 0.064]	0.609
Urine albumin concentration (mg/L)	24.8 [10.5, 50.2]	29.0 [12.2, 67.5]	0.595
Urine creatinine concentration (µmol/L)	20069 [13873, 24925]	8946 [6825, 13652]	**<0.001**

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Figure 1. Graph illustrating the observed and expected albumin concentrations of samples measured using a human particle enhanced turbidimetric immunoassay following calibration with human calibrators (provided with the assay kit) and canine calibrators (made from a solution of purified canine albumin). The black line represents the line of equality.

Figure 2. Box and whisker plots showing urine cystatin C: creatinine ratio in a group of healthy non-azotaemic cats (n=40) and cats with azotaemic CKD (n=12). Whiskers represent the 5^{th} and 95^{th} percentiles and circles represent outliers. Urine cystatin C:creatinine ratio was significantly lower in cats with azotaemic CKD than healthy non-azotaemic cats (P=0.011).

Figure 3. Box and whisker plots showing urine specific gravity in a group of healthy non-azotaemic cats (n=40) and cats with azotaemic CKD (n=12). Whiskers represent the 5^{th} and 95^{th} percentiles and circles represent outliers. Urine specific gravity was significantly lower in cats with azotaemic CKD than healthy non-azotaemic cats (P<0.001).

Figure 4. Box and whisker plots showing urine albumin: creatinine ratio in a group of healthy non-azotaemic cats (n=40) and cats with azotaemic CKD (n=12). Whiskers represent the 5^{th} and 95^{th} percentiles and circles represent outliers. Urine albumin :creatinine ratio tended to be significantly higher in cats with azotaemic CKD than healthy non-azotaemic cats (P=0.075).

Figure 5. Box and whisker plots showing urine protein:creatinine ratio in a group of healthy non-azotaemic cats (n=40) and cats with azotaemic CKD (n=12). Whiskers represent the 5th and 95th percentiles and circles represent outliers. Urine protein:creatinine ratio was not significantly different between cats with azotaemic CKD and healthy non-azotaemic cats (P=0.965).

Figure 6. Receiver operator curve demonstrating the sensitivity and specificity of urine cystatin C: creatinine ratio and urine specific gravity as a test for the detection of azotaemic chronic kidney disease in a group of healthy non-azotaemic cats (n=40) and cats with azotaemic CKD (n=12).

Figure 7. Receiver operator curve demonstrating the sensitivity and specificity of urine cystatin C: creatinine ratio and urine specific gravity as a test for the detection of azotaemic chronic kidney disease in a group of healthy non-azotaemic cats (n=12) and cats with azotaemic CKD (n=12) all with a urine specific gravity < 1.035.

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