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30 **Keywords**

31 Urine, microalbuminuria, UAC, electrophoresis, CKD, LUTD

32

Abstract

Objectives: The aims of this study were to validate a semi-automated high resolution electrophoretic (HRE) technique to quantify urinary albumin in healthy and diseased cats and to evaluate its diagnostic performances in cases of proteinuria and renal diseases.

Methods: Urine samples were collected from 88 cats (healthy; chronic kidney disease, CKD; lower urinary tract disease, LUTD; non-urinary tract diseases, OTHER). Urines were routinely analysed and HRE was performed. Within-assay and between-assay variability, linearity, accuracy, recovery and the lowest detectable and quantifiable bands were calculated. Receiver operating curves (ROC) analysis were also performed.

Results: All coefficients of variation were below 10%, percentage recovery was between 97% and 109% with a high linearity (r=0.99). HRE allowed the visualisation of a faint band of albumin and a diffused band between alpha and beta zones in healthy cats, while profiles from diseased cats were variable. Albumin (mg/dl) and urine albumin:creatinine ratio (UAC) were significantly (P <0.05) different between healthy and diseased cats. After ROC analysis, UAC values of 0.035 and 0.074 had a high sensitivity and high specificity, respectively, to classify proteinuria and identify borderline proteinuric cats. Moreover, an UAC of 0.017 had a high sensitivity to distinguish between healthy and diseased cats. However, UAC was not able to distinguish between renal (CKD) and non-renal diseases (LUTD/OTHER), probably

due to the pathophysiology of CKD in cats, which is characterised by low grade proteinuria and less glomerular involvement than in dogs.

Conclusions and relevance. HRE is an accurate and precise method that could be used to measure albuminuria in cats. UAC was useful to correctly classify proteinuria and to discriminate between healthy and disease cats. HRE might also provide additional information on urine proteins with a profile of all proteins (albumin and globulins) to aid clinicians in the diagnosis of diseases characterised by proteinuria.

Introduction

Very low concentrations of proteins are physiologically present in urine of healthy organisms, and all these proteins are collectively termed “proteinuria”. The increase of proteinuria is mainly due to two different mechanisms: loss of selective glomerular filtration and impaired tubular resorption. Traces of albumin are physiologically present in urine of healthy animals. In dogs, albumin concentration greater than 30 mg/dl is defined as overt albuminuria[1,2], while microalbuminuria is defined as a concentration of albumin in urine greater than 1 mg/dl but below the limit of detection of semiquantitative screening tests (30 mg/dl). Albuminuria can also be expressed as urinary albumin:creatinine ratio (UAC) to adjust for differences in urine concentration and volume, as it is used for proteinuria (urine protein:creatinine ratio; UPC). In human medicine UAC values lower than 0.03 are considered normal, microalbuminuria is defined between 0.03 and 0.3, and values greater than 0.3 are considered as overt albuminuria.[3] Microalbuminuria is an important biomarker of glomerular damage and its role in predicting clinical end-point (cardiovascular events, renal events and mortality) and to monitor diabetic nephropathy, cardiovascular disease and hypertension is well documented in humans.[3–6]

In dogs and cats microalbuminuria has been evaluated in correlation with different pathologies and clinical conditions, such as chronic kidney disease (CKD) and renal failure[7,8], leishmaniosis[9], hypertension[10,11], hyperadrenocorticism[12,13], severe

inflammatory response syndrome (SIRS)[14], critically ill dogs in intensive care unit[15,16], diabetes mellitus[17] and a variety of other systemic disorders.[1,18] However, only Syme et al[8] reported the UAC calculated in a population of healthy cats and a reference interval for cats utilising an appropriate number of individuals has not been defined so far. In addition, the methods used to quantify albuminuria present some disadvantages. In particular ELISA[8,19,20] is time consuming and not applicable in routine clinical screening. The immunoturbidimetric assay[21,22] is based on the interaction with an anti-human albumin polyclonal antibody, and the immunoassay Early Renal Damage (ERD)[17,23,24] is semiquantitative.

In this context, there is a need for a fast and reliable method applicable in routine clinical diagnostics. Therefore, the aims of this study were to validate a semiautomatic electrophoretic method on high resolution agarose gel (HRE) to quantify albuminuria in cat urine and test its clinical applications in routine analysis.

Material and methods

Study design

This study was performed using 88 urine samples submitted to the Veterinary Clinical Pathology Service of the Veterinary Teaching Hospital of the University of Bologna for a variety of clinical conditions.

The control group (H) comprised 22 cats considered healthy on the basis of

history and physical examination and without any other abnormality in serum biochemistry and urinalysis. The diseased cats were assigned to one of three categories of disease based on history, clinical signs, clinicopathological and imaging data as follows: chronic kidney disease (CKD) n=32, lower urinary tract disease (LUTD) n=18, other disease without involvement of the urinary system (OTHER) n=16 (inflammatory/infectious diseases n=6; orthopedic/neurologic diseases n=4; gastrointestinal disease n=3; neoplastic disease n=3). Cats with clinical and cytological evidence of urinary tract infection were included in the LUTD group. CKD cats were diagnosed and staged following IRIS guidelines. [25]

All experimental procedures were approved by the Institutional Scientific Ethical Committee for Animal Testing of the University of Bologna (approval number 8-72-2012; date of approval 01 October 2012) and the owners signed an informed consent form before inclusion of their animal in the study.

Urinalysis

Complete urinalysis, including measurement of the specific gravity by a refractometer and semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) was performed on all samples. After centrifugation at 1,500 x *g* for 10 minutes and microscopic sediment analysis at low (100x) and high (400x) power fields, the supernatants were divided into two aliquots and stored at -80°C for subsequent analysis.

Urine total proteins (UTP) and creatinine were determined using commercial kits (Urinary/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter) on an automated chemistry analyser (AU 400, Olympus/Beckman Coulter) allowing the calculation of the urinary total protein-to-creatinine ratio (UPC).

High Resolution Electrophoresis (HRE)

All urine samples were analysed with high resolution electrophoresis (HRE) on 0.8% agarose gel at pH 8.6 (HydraGel HR 15, Sebia) in combination with the semi-automated system Hydrasys (Sebia), according to the manufacturer instructions. The gels were dried, stained with acid violet solution and band staining density acquired by the Epson Perfection V700 photo scanner/densitometer. The obtained pherograms were analysed by the Phoresis software (version 6.1.2). The relative percentage and absolute concentration of albumin were calculated based on the density determined by the densitometer. As reported by the manufacturer, the limit of detection of this technique for serum albumin is 1.5-2 mg/dl (0.15 to 0.2 micrograms per band), with concentrations linear up to at least 5.8 g/dl and optimal protein concentration of 200 mg/dl. Urine samples with higher protein concentrations were diluted in order to achieve the desired optimal concentration.

HRE validation

144 To validate the HRE method for albumin quantification in cat urine, precision
145 (within and between assay variability), linearity, accuracy and analytical sensitivity
146 were calculated. Within-assay variability was assessed on five cat urine samples with
147 different albumin concentrations ranging from 7.4 to 67.7 mg/dl (7.4; 13.4; 23.5; 38.8;
148 67.7). For within-assay, samples were run six times on the same gel. Between-assay
149 variability was evaluated measuring 4 samples (7.4; 13.4; 23.5; 67.7 mg/dl) in duplicate
150 on five different days.
151 Linearity was tested by serial dilution of one sample (79 mg/dl) until reaching
152 the expected albumin concentration under the limit of detection (LOD) defined by the
153 manufacturer (1.5 mg/dl). All samples were analysed in duplicate. The analytical
154 sensitivity was obtained by the definition of the lowest detectable band (the lowest
155 concentration with weak or barely visible albumin band on the gel) and the lowest
156 quantifiable band (the lowest concentration with a quantifiable peak in the pherogram).
157 To evaluate accuracy, in the absence of a reference method for cat urine, a %
158 recovery study was made. Three solutions were prepared by adding to 100 µl of cat
159 urine, containing 24.5 mg/dl of albumin, 100 µl of saline solution (12.25 mg/dl of
160 expected albumin), (L) and 100 µl of two cat urine samples reaching 32.9 mg/dl (M)
161 and 53.8 mg/dl (H) of expected albumin. Five replicates were made for each solution.
162
163 *Statistical analysis*

Statistical analysis was performed with MedCalc ® 11.3.3.0. Data are reported as median (range). Precision was evaluated by the calculation of the Coefficient of Variation (CV) as follows: CV = (SD/mean)*100. Correlation and regression analysis were performed on expected and observed values for albumin and on UPC and UAC. According to IRIS[25] CKD staging guidelines , cats with UPC >0.4, 0.2-0.4, and <0.2 were classified as proteinuric, borderline proteinuric or non-proteinuric, respectively.

Receiver operator curves (ROC) were generated to evaluate sensitivity (SE), specificity (SP), positive and negative predictive values (PPV; NPV) and positive and negative likelihood ratios (LR+; LR-) of UAC and dipstick to classify proteinuria, using as a reference cut-off UPC 0.2 (according to IRIS guidelines). ROC curves were also generated to evaluate SE, SP, PPV, NPV, LR+ and LR- of UAC, UPC and dipstick to classify patients as healthy or diseased.

Non parametric statistic (Kruskal-Wallis with post-hoc test for pairwise comparison of groups) were performed to evaluate differences between groups (H, CKD, LUTD and OTHER) for UPC, UAC and age. Values of albumin less than 5 mg/dl (defined as lowest quantifiable band) were set at 2.5 mg/dl and UAC and albumin percentage values were calculated according to this value. Significance was set at P <0.05 unless otherwise specified.

Results

HRE validation and albumin quantification

HRE had within-assay CVs of 2.6% (67.7 mg/dl), 5.8% (38.8 mg/dl), 3.4% (23.5 mg/dl), 7.4% (13.4 mg/dl), and 7.6% (7.4 mg/dl) and between assay CVs of 3.5% (67.7 mg/dl), 1.2% (23.5 mg/dl), 7.4% (13.4 mg/dl) and 9% (7.4 mg/dl). Percentage recovery of 97%, 93% and 109% for high (H), medium (M) and low (L) concentration respectively were calculated and a significant correlation was found between measured and expected albumin concentration ($r = 0.99$; $P < 0.001$). The lowest detectable band of 1.25 mg/dl was set by visual inspection of the gels and the lowest quantifiable band of 5 mg/dl was determined by densitometric analysis.

After the validation, HRE was performed on the 88 urine samples. Nineteen out of the 32 urine samples with UTP <40 mg/dl and 6/56 urine samples with UTP >40 had a barely visible protein band corresponding to an albumin concentration lower than lowest quantifiable band.

Diagnostic performances of HRE and clinical application

Twenty-two cats were included in the study as the healthy group (Table 1; Supplementary Table 1). The median age was 22.5 (6-168) months and median UPC was 0.12 (0.06-0.32). HRE allowed the visualisation of a faint band of albumin and a diffused band between alpha and beta zones (Figure 1a) in most samples. Only in nine samples (41%) albumin concentration was quantifiable, the median concentration was

<5 mg/dl (<5-29.8) and the calculated median UAC was 0.011 (0.004-0.069). No differences were found between males and females.

Sixty-six samples were included in the diseased group (Table 1; Supplementary Table 2) with a median age of 132 (24-268) months and median UPC of 0.51 (0.07-16.15). These samples had variable electrophoretic profiles (Figure 1b). In particular, the albumin band was evident and quantifiable in 54 samples (82%) and the median albumin concentration and UAC were 12 mg/dl (<5-962) and 0.111 (0.009-7.056), respectively, both significantly higher than in healthy cats (P <0.01). Moreover, it was also possible to separate and identify the alpha, beta and gamma globulins zones (Figure 1c).

Albuminuria vs Proteinuria - Using the IRIS guidelines, the 88 samples were classified as non-proteinuric (UPC <0.2; n = 38) borderline proteinuric (UPC 0.2-0.4; n = 11), and proteinuric (UPC >0.4; n = 39). UAC was significantly correlated to UPC (r = 0.967; P <0.0001). ROC curves were generated to evaluate diagnostic performance of UAC and dipstick (considered as positive for trace results) to correctly classify proteinuria, considering as positive samples with UPC >0.2 (borderline proteinuric and proteinuric). Area under curve (AUC) for UAC (0.939) was significantly greater (P <0.01) than dipstick (0.537). Table 2 shows SE, SP, PPV and NPV for the best criteria calculated by the ROC curves for UAC and dipstick. For UAC, two different values demonstrated high sensitivity (0.035) or high specificity (0.074) for overt or borderline

proteinuria, while dipstick showed very low specificity due to the high number of false positive results. Using these two cut-off values, as reported in Table 3, 10 non-proteinuric samples had abnormal UAC values.

Albuminuria vs Diseases – According to serum creatinine concentration and IRIS guidelines, CKD cats were staged as follows: stage I n = 3, stage II n = 8, stage III n = 9 and stage IV n = 12. CKD cats were significantly older with a median age of 163 (24-252) months (P <0.01) and had significantly increased UPC (0.63; 0.10-16.15), albumin concentration (11.4 mg/dl; <5-962) and UAC (0.117; 0.009-7.057) than the H group (P <0.01). LUTD cats were significantly older than H group cats with a median age of 132 (36-268) months (P <0.01) and had significantly increased UPC (0.7; 0.07-4.59). Albumin concentration (20.2 mg/dl; <5-134.2) and UAC (0.184; 0.010-1.487) in LUTD cats were significantly higher than H group (P <0.01). OTHER cats were significantly older than H and younger than CKD group with a median age of 102 (24-192) months (P <0.05) and had UPC (0.21; 0.09-1.52) significantly higher than the H group (P <0.05). Albumin concentration (15.2 mg/dl; 6.6-147.2) and UAC (0.053~~2~~; 0.013-0.465) in this group were higher than in H group (P <0.05). Figure 2 shows the comparison of UAC among H, CKD, LUTD and OTHER groups.

ROC curves were generated to evaluate and compare dipstick, UAC and UPC to correctly classify cats. The performance of UPC, UAC and dipstick is summarised in Table 4. ROC curves comparison for the classification of patients as healthy or diseased

is reported in Figure 3a. AUCs for UPC (0.909), UAC (0.877) and USG (0.865) were not significantly different from each other but significantly different from AUC for dipstick (0.631) (P <0.001). For UPC, the best criterion was set at 0.165, while for UAC, two different values gave high sensitivity (0.017) or high specificity (0.074). Dipstick showed very low specificity. ROC comparison was lastly generated to discriminate cats as affected by CKD or LUTD/OTHER diseases (Figure 3b). AUCs for UPC (0.665) and USG (0.740) were not significantly different from each other, but were both significantly different from AUC for dipstick (0.525) and UAC (0.585) (P <0.05).

Discussion

Several studies suggest that even mild proteinuria/albuminuria can be indicative of the severity of disease and predict poor outcome;[8,19,26,27] in contrast, the difficulty of albumin quantification with dipsticks and the absence of a validated method for cat urine highlight the importance of a sensitive, accurate and precise analytical method for albumin quantification in urine. In this context, our work aimed to validate an electrophoretic method for albumin quantification and to evaluate its clinical application in routine urinalysis.

Validation of HRE

The electrophoretic method validated in the present study is a semiautomatic

technique and the obtained CVs were lower than 10%. As a general rule, CVs for automated assays should be less than 5% and for manual assays less than 10%.[28] Therefore, considering that HRE is a semiautomatic technique, the variability determined in the present study can be considered acceptable. The method was linear in the tested range of concentrations and the results of the recovery test can be considered acceptable for routine diagnostic laboratory use.[28] Regarding the LOD, it was difficult to quantify blank (saline solution, NaCl 0.9%) by densitometry. The scanner reads impurities on the gel generating irregular profiles, not related to the protein content of the samples; the same analytical problem was also reported by other authors.[29] As a consequence, the LOD was set at 1.25 mg/dl of expected albumin, since at this concentration, close to the LOD defined by the manufacturer, a faint band of the protein was optically visible on the gel, though not quantifiable by the software. Furthermore, the software allows the technician/clinician to reduce the interferences and evaluate/validate the correspondence between visual inspection of the gel and the densitometric profile. Therefore, the ease and rapidity of use, the high reproducibility and accuracy and wide range of linearity, combined with the final evaluation of the pherogram by the clinician/technician, allow us to conclude that HRE is a reliable method for quantifying albuminuria in cats.

Diagnostic performance of HRE and clinical application

284 HRE was useful to highlight that urine from healthy cats is characterised by very
285 low albumin concentrations. This result is confirmatory of data reported by Ferlizza et
286 al[30], who pointed out that the most abundant proteins in urine from healthy cats were
287 cauxin (at greater concentration in entire males) and uromodulin, both produced by
288 healthy tubular cells and specifically secreted in urine. Diseased cats presented a greater
289 variability in the electrophoretic profiles with increasing concentration/density of the
290 albumin band and of alpha, beta and gamma zones. The quantification of the globulins
291 was out of the scope of the present paper and they were not correlated to the
292 presence/absence of CKD or other diseases. Nevertheless, Giori et al[29] correlated the
293 reduced percentage of alpha1-globulins to the glomerular origin of proteinuria in dogs.
294 These results, were associated with a clear difference between the pherograms from
295 healthy cats compared with the diseased ones in the present study, and allowed us to
296 hypothesize the potential usefulness of HRE to evaluate albumin, alpha, beta and
297 gamma globulins to correctly classify proteinuria in both dogs and cats.
298 In the present study patients were arbitrarily classified according to IRIS
299 guidelines for proteinuria. Although these guidelines have been created to classify CKD
300 patients, the authors considered the categories proposed as useful to classify proteinuria
301 in cats regardless of the presence of CKD, since cats frequently present low grade
302 proteinuria in the clinical practice.[2,16,17] To evaluate the ability of HRE to correctly
303 classify proteinuria, a comparison was made with dipstick, using an UPC value of 0.2 as

304 reference. Dipstick showed high sensitivity, but very low specificity, due to the high
305 number of false positive results. However, as suggested by Zatelli et al[31] for dogs, the
306 dipsticks should always be interpreted in light of the USG and, in the present study,
307 most of the false positive samples also had high USG values (>1.035). Moreover,
308 healthy entire male cats secrete cauxin into urine and, that has also been demonstrated
309 to be a cause of false positive proteinuria.[32] The lower UAC value, 0.035, was close to
310 the cut-off used for human microalbuminuria (0.03) and using this criterion nine non-
311 proteinuric samples (UPC<0.2) were classified as microalbuminuric. The second one,
312 0.074, is similar to data reported presented by Syme et al[8] who reported an UAC of 0.08
313 as the upper limit of the reference interval in healthy cats, and lower than the value used
314 in human medicine for overt albuminuria (0.3). The population of healthy cats included
315 in the present study was not age-matched with those in the CKD and non-CKD groups
316 differently to what reported by Syme et al.[8] However, the similarity between the data
317 reported by Syme et al[8] and the cut-off reported in the present study, in spite of the
318 different age of the healthy cats (median 12 vs 1.9 years) might be confirmatory of the
319 clinical reliability of the analysis and suggests that UAC is not influenced by age alone.
320 As regards the difference between cats and humans, and also between cats and dogs,
321 could be explained by cats presenting with lower quantities of proteinuria/albuminuria
322 due to a different pathophysiology in cases of renal diseases. These preliminary results,
323 even if obtained on a limited number of cats, suggest that an UAC of 0.035 as a cut-off

of microalbuminuria and an UAC of 0.074 as cut-off for overt albuminuria, if confirmed by future studies with greater numbers of healthy cats, could be considered complementary to UPC to estimate and identify pathologic low grade proteinuria.

As regards the comparison among groups, both UPC and UAC were able to discriminate healthy cats from the diseased ones. Interestingly, though the renal and urinary tract involvement, CKD and LUTD cats did not show different values of proteinuria and albuminuria than the OTHER group. In our study, diseases apparently not involving the kidney or the urinary tract were characterized by albuminuria higher than healthy cats. The pathogenesis of increased urine albumin concentration in these diseases has not been investigated in this study, however different conditions as hypertension, previous treatments or even subclinical renal involvement could be hypothesised. As previously suggested, proteinuria and albuminuria should be classified as renal or pre/post renal and LUTD is considered a cause of post-renal albuminuria in cats.[2,33] To the authors knowledge no data are present in the literature on albuminuria in cats affected by LUTD and these are the first UAC values reported. The best criteria calculated with ROC curve analysis to distinguish between healthy and diseased were for UPC 0.165, close to the well-established value of 0.2, and for UAC 0.017, lower than the previous calculated value of 0.035. However, after the subsequent ROC analysis, UAC was not able to distinguish between CKD and non-CKD diseases. It is well known that cats affected by CKD present with lower values of proteinuria and

albuminuria than dogs[34,35] and that even low UPC and UAC ratios are correlated with poor outcome.[8] Using the calculated cut-offs (UPC 0.165 and UAC 0.017) within the different groups of diseases, the sensitivity to detect low levels of albuminuria/proteinuria is increased suggesting that these values (UPC <0.165; UAC <0.017) could be reliable for early confirmation of disease with possible renal involvement. The low level of albuminuria/proteinuria in cats with CKD seems to be related to chronic tubulo-interstitial nephropathy.[30,36] Nevertheless, 8/32 CKD samples had an UPC>2 considered as indicative of prevalent glomerular diseases that is not the typical but a possible cause of CKD even in cats. Our results therefore suggest that in these patients histopathologic characterisation of CKD should be performed to correlate urinary electrophoresis with histologic findings.

Although the present study validated a fast and reliable technique to quantify albuminuria in cats producing preliminary results of UAC, our study has a few limitations. First, the limited number of healthy cats, since to construct a true reference interval a greater number of samples is needed. Second, HRE was less sensitive to detect low concentrations of albumin (<5mg/dl) than an ELISA (10-200 ng/ml), but fast and easy to use in the routine clinic. Third, UAC was not able to discriminate between CKD and other diseases characterized by proteinuria suggesting its use as an unspecific marker of disease with possible renal involvement rather than a specific marker of CKD as glomerular filtration rate could not be measured and used as the gold standard in this

study. HRE was unable to separate albumin from cauxin, in particular in entire male healthy cats.

Conclusions

The electrophoretic method validated in this work is precise and accurate for measuring albuminuria in cats. UAC was useful to correctly classify proteinuria and to discriminate among healthy and diseased cats. Values for UAC of 0.035 and 0.074 are suggested as the thresholds of microalbuminuria and overt albuminuria respectively in cats, while a value of >0.017 could be indicative of disease with possible renal involvement. Moreover, we reported the first data on albuminuria in cats affected by LUTD. In addition, HRE can provide additional information on urine proteins and the profile (including albumin and globulins) should be further analysed to aid clinicians in the diagnosis of kidney diseases.

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Conflict of interest

384 The authors declared no potential conflicts of interest with respect to the research,
385 authorship, and/or publication of this article.

References

1. Whittemore JC, Gill VL, Jensen WA, et al. **Evaluation of the association between microalbuminuria and the urine albumin-creatinine ratio and systemic disease in dogs**. *J Am Vet Med Assoc* 2006; 229: 958–963.
2. Grauer GF. **Proteinuria: measurement and interpretation**. *Top Companion Anim Med* 2011; 26: 121–127.
3. Guh JY. **Proteinuria versus albuminuria in chronic kidney disease**. *Nephrology* 2010; 15: 53–56.
4. de Jong PE, Gansevoort RT. **Focus on microalbuminuria to improve cardiac and renal protection**. *Nephron Clin Pract* 2009; 111: 204–211.
5. Erdmann E. **Microalbuminuria as a marker of cardiovascular risk in patients with type 2 diabetes**. *Int J Cardiol* 2006; 107: 147–153.
6. Dobre D, Nimade S, de Zeeuw D. **Albuminuria in heart failure: what do we really know?** *Curr Opin Cardiol* 2009; 24: 148–154.
7. Smets PMY, Meyer E, Maddens BEJ, et al. **Urinary markers in healthy young and aged dogs and dogs with chronic kidney disease**. *J Vet Intern Med* 2010; 24: 65–72.
8. Syme HM, Markwell PJ, Pfeiffer D, et al. **Survival of cats with naturally occurring chronic renal failure is related to severity of proteinuria**. *J Vet Intern Med* 2006; 20: 528–535.
9. Cortadellas O, Fernández del Palacio MJ, Talavera J, et al. **Glomerular filtration rate in dogs with leishmaniasis and chronic kidney disease**. *J Vet Intern Med* 2008; 22: 293–300.
10. Bacic A, Kogika MM, Barbaro KC, et al. **Evaluation of albuminuria and its relationship with blood pressure in dogs with chronic kidney disease**. *Vet Clin Pathol* 2010; 39: 203–209.
11. Jepson RE, Elliott J, Brodbelt D, et al. **Effect of control of systolic blood pressure on survival in cats with systemic hypertension**. *J Vet Intern Med* 2007; 21: 402–409.
12. Lien YH, Hsiang TY, Huang HP. **Associations among systemic blood pressure, microalbuminuria and albuminuria in dogs affected with pituitary- and adrenal-dependent hyperadrenocorticism**. *Acta Vet Scand* 2010; 52: 61. DOI: http://www.actavetscand.com/content/52/1/61
13. Smets PMY, Lefebvre HP, Meij BP, et al. **Long-term follow-up of renal**

function in dogs after treatment for ACTH-dependent hyperadrenocorticism. *J Vet Intern Med* 2012; 26: 565–574.

14. Schaefer H, Kohn B, Schweigert FJ, et al. **Quantitative and qualitative urine protein excretion in dogs with severe inflammatory response syndrome**. *J Vet Intern Med* 2011; 25: 1292–1297.

15. Whittemore J, Marcum B, Mawby D, et al. **Associations among albuminuria, C-reactive protein concentrations, survival predictor index scores, and survival in 78 critically ill dogs**. *J Vet Intern Med* 2011; 25: 818–824.

16. Vaden SL, Turman CA, Harris TL, et al. **The prevalence of albuminuria in dogs and cats in an ICU or recovering from anesthesia**. *J Vet Emerg Crit Care* 2010; 20: 479–487.

17. Al-Ghazlat SA, Langston CE, Greco DS, et al. **The prevalence of microalbuminuria and proteinuria in cats with diabetes mellitus**. *Top Companion Anim Med* 2011; 26: 154–157.

18. Whittemore J, Miyoshi Z, Jensen WA, et al. **Association of microalbuminuria and the urine albumin-to-creatinine ratio with systemic disease in cats**. *J Am Vet Med Assoc* 2007; 230: 1165–1169.

19. Jepson R, Brodbelt D. **Evaluation of predictors of the development of azotemia in cats**. *J Vet Intern Med* 2009; 23: 806–813.

20. Lyon S, Sanderson M, Vaden S, et al. **Comparison of urine dipstick, sulfosalicylic acid, urine protein-to-creatinine ratio, and species-specific ELISA methods for detection of albumin in urine samples of cats**. *J Am Vet Med Assoc* 2010; 236: 874–879.

21. Kuwahara Y, Nishii N, Takasu M, et al. **Use of urine albumin/creatinine ratio for estimation of proteinuria in cats and dogs**. *J Vet Med Sci* 2008; 70: 865–867.

22. Williams TL, Archer J. **Evaluation of urinary biomarkers for azotemic chronic kidney disease in cats**. *J Small Anim Pract* 2016; 57: 122–129.

23. Mardell EJ, Sparkes AH. **Evaluation of a commercial in-house test kit for the semi-quantitative assessment of microalbuminuria in cats**. *J Feline Med Surg* 2006; 8: 269–278.

24. Hanzlicek AS, Roof CJ, Sanderson MW, et al. **Comparison of urine dipstick, sulfosalicylic acid, urine protein-to-creatinine ratio and a feline-specific immunoassay for detection of albuminuria in cats with chronic kidney disease**. *J Feline Med Surg* 2012; 14: 882–888.

25. International renal interest society. **IRIS Staging of CKD (modified 2015)**.

http://iris-kidney.com/guidelines/staging.html (2015, accessed January 15, 2016)

26. King JN, Tasker S, Gunn-Moore D a, et al. **Prognostic factors in cats with chronic kidney disease**. *J Vet Intern Med* 2007; 21: 906–916.

27. Walker D, Syme H, Markwell P, et al. **Predictors of survival in healthy, non-azotaemic cats**. *J Vet Intern Med* 2004; 18: 417.

28. Jensen AL, Kjelgaard-Hansen M. **Diagnostic test validation**. In: Weiss DJ and Wardrop JK (eds) Schalm's veterinary hematolgy. 6th ed. Ames, USA: Wiley-Blackwell, 2010, pp 1027–1033.

29. Giori L, Tricomi FM, Zatelli A, et al. **High-resolution gel electrophoresis and sodium dodecyl sulphate-agarose gel electrophoresis on urine samples for qualitative analysis of proteinuria in dogs**. *J Vet Diagn Invest* 2011; 23: 682–690.

30. Ferlizza E, Campos A, Neagu A, et al. **The effect of chronic kidney disease on the urine proteome in the domestic cat (*Felis catus*)**. *Vet J* 2015; 204: 73–81.

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

32. Miyazaki M, Fujiwara K, Suzuta Y, et al. **Screening for proteinuria in cats using a conventional dipstick test after removal of cauxin from urine with a Lens culinaris agglutinin lectin tip**. *Vet J* 2011; 189: 312–317.

33. Langston C. **Microalbuminuria in cats**. *J Am Anim Hosp Assoc* 2004; 40: 251–254.

34. Grauer GF. **Measurement, interpretation, and implications of proteinuria and albuminuria**. *Vet Clin North Am Small Anim Pract* 2007; 37: 283–295.

35. Harley L, Langston C. **Proteinuria in dogs and cats**. *Can Vet J* 2012; 53: 631–638.

36. Syme HM. **Proteinuria in cats. Prognostic marker or mediator?** *J Feline Med Surg* 2009; 11: 211–218.

485 **Table 1** Age and results of urine specific gravity (USG), proteinuria and albuminuria for

486 the samples from healthy and diseased cats. Data are reported as median (min-max)

	AGE	sCREA	sUREA	USG	UTP	uCREA	UPC	ALB	ALB	UAC
	Months	mg/dl	mg/dl		mg/dl	mg/dl		%	mg/dl	
RI		**0.8-1.8**	**15-60**	**>1.035**			**<0.2**			
HEALTHY n = 22	22.5 (6-168)	1.5 (1.03-1.8)	48.4 (32-86)	1.071 (1.036-1.090)	35.0 (16-159.6)	344 (162-766)	0.12 (0.06-0.32)	9.8 (5.5-42)	2.5 (2.5-29.8)	0.011 (0.004-0.069)
CKD n = 32	163 (24-252)	3.55 (1.33-12.7)	169 (57.8-501.7)	1.017 (1.006-1.068)	75.4 (12.8-1956.3)	92 (23-583)	0.63 (0.10-16.15)	22.3 (3.9-55.3)	11.4 (2.5-962.0)	0.117 (0.009-7.057)
LUTD n = 18	132 (36-268)	1.75 (1.37-10.78)	108.9 (41.1-479.5)	1.029 (1.008-1.072)	68.3 (30.6-280.2)	162 (91-672)	0.70 (0.07-4.59)	20.7 (5.3-59.1)	20.2 (2.5-134.2)	0.184 (0.010-1.487)
OTHER n = 16	102 (24-192)	1.59 (0.96-3.07)	53.2 (32.3-199.2)	1.058 (1.017-1.080)	74.9 (27.1-881.8)	338 (94-813)	0.21 (0.09-1.52)	20.7 (11.3-44.9)	15.2 (6.6-147.2)	0.052 (0.013 -0.465)

487

488 RI = laboratory reference intervals; sCREA = serum creatinine; sUREA = serum urea;

489 UTP = urine total protein; uCREA = urine creatinine; UPC = urine protein:creatinine

490 ratio; ALB = albumin; UAC = urine albumin:creatinine ratio; CKD = chronic kidney

491 disease; LUTD = lower urinary tract disease; OTHER = other diseases not involving the

492 urinary system

493

Table 2 Sensitivity, specificity, positive and negative predictive values (PPV; NPV) and positive and negative likelihood ratios (LR+; LR-) for the best criteria calculated for UAC (0.035 and 0.07) and dipstick (30 mg/dl, lowest level of positivity) using UPC 0.2 as reference cut-off

	UAC 0.035	UAC 0.074	Dipstick 30
Sensitivity (%)	94.00	76	82.00
Specificity (%)	76.30	100	23.70
PPV (%)	82.50	97.4	58.60
NPV (%)	90.30	74.0	50.00
LR+	3.97	--	1.07
LR-	0.079	0.26	0.76

UPC = urine protein:creatinine ratio; UAC = urine albumin:creatinine ratio;

Table 3 Sample distribution in the different classes of proteinuria, according to IRIS, and albuminuria, according to the values determined in the present study

		NA UAC<0.035	MA 0.035<UAC<0.074	A UAC>0.074	TOT
NP	UPC<0.2	28	9	1	38
BP	0.2<UPC<0.4	2	4	5	11
P	UPC>0.4	1	6	32	39
	TOT	31	19	38	88

NA = non-albuminuric; MA = microalbuminuric; A = Overt albuminuric; NP = non-proteinuric; BP = borderline proteinuric; P = proteinuric; UPC = urine protein:creatinine ratio; UAC = urine albumin:creatinine ratio.

510 **Table 4** Sensitivity, specificity, positive and negative predictive values (PPV; NPV) and
511 positive and negative likelihood ratios (LR+; LR-) for the best criteria calculated for
512 UAC (0.027 and 0.07), UPC (0.165 and 0.2) and dipstick (30 mg/dl, lowest level of
513 positivity) to distinguish between healthy and diseased cats
514

	UPC>0.165	UPC>0.2	UAC>0.017	UAC>0.074	Dipstick>30
Sensitivity (%)	84.8	72.7	87.9	59.1	87.9
Specificity (%)	86.4	90.9	63.6	100.0	22.7
PPV (%)	96.6	96.0	87.9	100.0	70.0
NPV (%)	66.7	52.6	63.6	44	5.6
LR+	6.2	16.0	2.42	-	1.14
LR-	0.18	0.29	0.19	0.041	0.53

515

516 UPC = urine protein:creatinine ratio; UAC = urine albumin:creatinine ratio;

517

Figure captions

Figure 1 Representative HRE gels (a, b) and pherogram (c) of urine samples from healthy (a) and diseased (b, c) cats. a) HRE of healthy samples; b) HRE of diseased samples (CKD lanes 3,7; LUTD lanes 5, 9-14; OTHER 1, 2, 4, 6; H lane 8); c) as an example, pherogram of lane 3 (CKD)

Figure 2 UAC values for healthy and diseased groups. Different lower cases indicate significant difference (P <0.05). UAC values greater than 2 were excluded from graphic visualisation. UAC = urine albumin:creatinine ratio; CKD = chronic kidney disease; H = healthy; LUTD = lower urinary tract disease; OTHER = other diseases not involving the urinary system

Figure 3 ROC curve comparison for UPC, UAC, dipstick and USG to classify cat (a) as healthy or diseased and (b) as renal (CKD) or non-renal (LUTD/OTHER). UPC = urine protein:creatinine ratio; UAC = urine albumin:creatinine ratio; STICK = dipstick; USG = urine specific gravity