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Utility of urinary markers in the assessment of renal dysfunction in dogs with chronic kidney disease

DAGMARA WINIARCZYK

Department and Clinic of Animal Internal Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland

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Winiarczyk D.

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Summary

Chronic kidney disease is a common and clinically significant disease. This complication leads to a decrease of the glomerular filtration rate and in consequence causes azotaemia and uraemia. The objective of this study was to assess the localization and extent of renal damage in dogs with stage-3 chronic kidney disease using a urinary marker for glomerular dysfunction, proximal tubular dysfunction and distal tubular dysfunction (uIgG, uRBP and uTHP, respectively). The examination was performed in twelve dogs affected in stage-3 chronic kidney disease and ten clinically healthy dogs (female and male of comparable age). The levels of urinary biomarkers were measured by commercially available ELISA-tests. In the infected animals a significant renal excretion of HMW protein uIgG and LMW protein uRBP was observed, indicating a dysfunction of the glomerular and tubular regions of the kidneys. Lower levels of uTHP in dogs with CKD was noticed, which may suggest impaired distal tubular regions of the kidneys.

Keywords: chronic kidney disease, urinary marker, immunoglobulin G, retinol binding protein, uromodulin

Chronic kidney disease (CKD) is an important cause of morbidity and mortality in dogs, and it is often a result of primary glomerular disease (8). The prevalence of CKD increases with age, with 15% of dogs over 10 years old being affected (28). Early diagnosis may allow therapeutic intervention that prevents further damage and progressive decline of renal function. However, only a decrease of > 75% of renal functional mass will be detected by current diagnostic tests such as blood urea nitrogen (BUN) and serum creatinine (sCr) concentrations (10). At present symmetric dimethylarginine (SDMA) is used as a screening tool for early kidney dysfunction and monitoring treatment in cases of chronic kidney disease (CKD). Unfortunately there are no current studies describing the suitability of this test for use with published population-based reference intervals. It is also well know that proteinuria is a marker and mediator of chronic kidney disease and itself can promote further renal damage and CKD progression. Nevertheless, the mechanism by which excess proteins induce renal injury is still not entirely understood (8). In clinical practice, the urinary protein-to-creatinine ratio (UP/C) and microalbuminuria assays are of limited usefulness, because it indicates only the magnitude of proteinuria and not the origin of the loss (glomerular or tubular) (12). Sensitive and specific biomarkers for early prediction and monitoring of CKD in dogs have received increasing attention in recent years (16, 19, 29-35, 38), but they are currently lacking. Urinary proteins of low (LMW) to high molecular weight (HMW) have recently been introduced and have been helpful in assessing the localization, extent and progression of renal injuries (21, 38). Among these biomarkers, HMW proteins such as urinary immunoglobulin G (uIgG) are usually associated with glomerular damage, whereas detection of LMW proteins like retinol binding protein (uRBP) typically reflects proximal tubular damage (1-3, 8, 38). A marker that might be useful in the recognition of distal tubular injury is the Tamm-Horsfal protein (THP), a glycoprotein exclusively synthesized in the cells lining the thick ascending limb and distal convoluted tubules (35).

The objective of this study was to assess the localization and extent of renal damage in dogs with chronic kidney disease using a urinary marker for glomerular (uIgG), proximal tubular dysfunction (uRBP) and distal tubular dysfunction (uTHP).

Material and methods

Animals. The current study was performed at the Faculty of Veterinary Medicine in Lublin. All owners agreed to participate in the study and signed an informed form.

The study involved 22 mixed breeds dogs (12 males and 10 females), weighing 5-8 kg (median 6.2 kg) and aged 2-7 years (median 4.35 years), divided into two groups. Group 1, the study group (n = 12; six males and six females), consisted of dogs with stage –3 chronic kidney disease (according to IRIS classification). Group 2, ten healthy dogs (student-owned and healthy patients referred to the clinic for vaccination purpose). Dogs were judged healthy based on history, physical examination, hematology and biochemical profile, and urinalysis. The diseased group was comprised of dogs with CKD diagnosed on the basis of history, clinical signs, and clinicopathological results. According to the International Renal Interest Society (IRIS) CKD guidelines all dogs in group 1 had clinical finding of CKD in 3 stage, persistent pathological renal proteinuria based on the urine protein to creatinine ration, assessed and confirmed over a 2 month period (UPC > 0.5), and a serum creatinine concentration ≥ 2.1 mg/dl.

All dogs were submitted to a physical examination, arterial blood pressure measurement (by Doppler methods, in accordance with the guidelines American College of Veterinary Internal Medicine), blood and urine sampling.

Sample collection. The clinical study involved the collection of blood and urine samples. Each blood sample was collected using a closed vacuum system to a test tube with EDTA and subjected to haematological analysis in an Exigo Vet analyser (Boule Sweden). The serum obtained after centrifuging at 3000 rpm for 15 minutes at a temperature of 4°C was analyzed in an automatic biochemical analyzer (Mindray BS-130). The chemistry panel included: alanine transferase, aspartate aminotransferase, total bilirubin, urea, creatinine, glucose, albumin, and total protein. The urine was collected from the morning midstream in containers with protease inhibitor (20 µl per 5 ml of urine; Protease Inhibitor Cocktail, Roche Diagnostic Corp) and divided into portions, one of which was subjected to a complete routine urinalysis together with sediment examination and quantitative assessment of proteinuria using the Urine Protein to Creatinine Ratio. The UPC was measured twice, in two samples collected at a two-week interval. UPC levels exceeding 0.5 were considered to be proteinuria. The specific gravity was determined on the basis of measurements with a refractometer. The remaining portion of urine was frozen at –80°C for further analysis. The whole procedure of sample preparation was performed within one hour from material collection. Quantification of urinary markers uIgG, uRBP, and uTHP was performed on thawed supernatant.

Blood pressure was measured using the Doppler method. The measurements were made using an Ultrasonic Doppler Flow Detector, Model 811; Parks Medical Electronics, Inc., Aloha, Ore. The blood pressure was measured after the patient was acclimatized in the clinic, as the average of three measurements. Values exceeding 160 mmHg for systolic pressure were considered to be hypertension.

The kidney ultrasound was performed on an Esaote Mylab machine using a microconvex 3-9 MHz transducer.

Urinary markers. All of the urine samples were analyzed using commercially available canine- or human-specific sandwich enzyme-linked immunosorbent assays (ELISA) (Immunology Consultants Laboratory, Newberg, USA, MD Products North America) to determine the concentrations of uIgG, uRBP and uTHP. The absorbance was measured at a wavelength of 450 nm using an ELISA plate reader (SpectraMax M2). A 4-Parameter Logistic Non-Linear Regression Curve-Fitting Model (MasterPlex Software, Hitachi Solutions) was used to generate the standard curve and calculate the concentrations of uIgG, uRBP and uTHP. The results were normalized to urinary creatinine concentrations (uCr) and expressed as ratios in mg/g.

Statistical analysis. The statistical analysis was performed using the Mann-Whitney U test; non-parametric test for independent samples. uIgG/uCr, uTHP/uCr, uRBP/uCr were used as independent variables. Variables were added one by one (forward step) and the model refitted until the p-values were statistically significant ($p < 0.05$). The statistical analyses were performed using Statistica 10.0 software (StatSoft Poland).

Results and discussion

The main pathological finding in hematology analysis in group 1 was anemia (6/12), biochemistry analysis revealed azotemia (the average level of creatinine was 4.3 mg/dl) (Tab. 1). Almost all dogs with CKD had symptoms of hypertension (the average of systolic blood pressure > 160 mmHg). Renal sonographic examinations in group 1 revealed a decrease of parenchymal thickness in both kidney and renal atrophy. The degree of proteinuria in group 1 is presented in Table 2. The macroscopic evaluation of urine in both groups showed yellow sample colors. None of the dogs of the control group had proteinuria and all parameters in hematology, biochemical analysis and ultrasound examination were in the physiological range (Tab. 1). The levels of urinary biomarkers (uIgG/Cr, uTHP/Cr,

Tab. 1. Blood analysis parameters for the healthy and chronic kidney disease dogs (expressed as median and range)

Variable	Group 1 (n = 12)	Group 2 (n = 10)	Reference range
Leukocyte [10^9/L]	12.8 (10.0-19.3)	8.5 (6.3-11.1)	6-17
Limfocyte [10^9/L]	2.8 (1.2-5.1)	2.1 (0.6-3.8)	1.2-5.0
Erytrocyte [10^9/L]	4.8 (3.77-6.18)	7.8 (6.0-9.4)	5.5-8.5
Hematocryt [%]	29.5 (21.1-39.3)	51.1 (40.7-61.3)	37-5
Hemoglobin [g/dL]	11.4 (8.4-14.4)	18.4 (14.2-21.3)	12-18
Plates [10^9/L]	224.1 (25- 342)	93 (164-339)	200-500
ALT [u/l]	111.4 (32-413)	47 (21-101)	3-50
AST [u/l]	52 (33-129)	28 (18-46)	1-37
BIL [T]	7.3 (1.2-18.5)	0.2 (0.1-0.5)	≤ 0.60
UREA [mg/dl]	393.1 (98.2-511.7)	37.7 (27.5-59.7)	20-45
CREA [mg/dl]	4.3 (3.2-5.0)	1.2 (0.6-1.7)	1.00-1.70
GLUC [mg/dl]	103.1 (89-122)	117.2 (66-136)	70-120
ALB [g/dl]	17.1 (3.3-90)	3.7 (3.3-4.5)	3.3-5.6
TP g/dl	6.8 (5.8-7.6)	6.7 (2.2-8.6)	5.5-7.0

Tab. 2. Results of routine urinary parameters for the renal function in dogs with chronic kidney disease (group 1) and healthy dogs (group 2) – expressed as median and range

Variable	Group 1 (n = 12)	Group 2 (n = 10)
uCrea [mg/dl]	54.9 (26.1-86.2)	135.64 (53.91-212.82)
USG	1.020 (1.020-1.030)	1.030
Protein*	+++ (+-+++)	–
UP/UC [mg/mg]	3.7 (0.65-8.9)	< 0.5

Explanation: *Methods of urine protein measurement: sulphosalicylic acid (SSA); uCrea – creatinine in urine; USG – urine specific gravity; UP/UC – urine protein to urine creatinie ratio

Tab. 3. Concentration of urinary markers in dogs with chronic kidney disease (group 1) and healthy dogs (group 2) – expressed as median and range

Variable	Group 1 (n = 12)	Group 2 (n = 10)
uRBP/uCrea [mg/g]	15.1 (5.13-16.04)*	0.2 (0.09-0.3)
uTHP/uCrea [mg/g]	0.07 (0-0.21)	0.26 (0-0.47)
uIgG/uCrea [mg/g]	305.1 (0-1921.51)*	0

Explanations: uCrea – urinary creatinine; uIgG – urinary immunoglobulin G; uRBP – urinary retinol binding protein; uTHP – urinary Tamm-Horsfal protein; * $p \leq 0.05$

and uRBP/Cr) in dogs of both groups are presented in Table 3.

Immunoglobulins G were undetectable in healthy dogs. The average value of uIgG/uC in the group of dogs with CKD was 305.1. In healthy dogs, the average level of uRBP/uCr was 0.2 mg/g, in the diseased group it was 15.1. The average value of uTHP/uCr in healthy dogs was 0.26 and did not increase significantly, while in the infected animals it dropped to 0.07.

In the present study we investigate protein excretion in the urine of twelve dogs that were suffering from Chronic Kidney Disease (group 1) and ten healthy dogs (group 2). According to the IRIS grading criteria the dogs were classified as CKD grade 3 (moderate renal azotemia, proteinuria, hypertension). Thereby we focused on the identification of urinary markers for glomerular disorders (Immunoglobulin G) and tubular dysfunction (uromodulin, retinol binding protein).

Increased uIgG level is usually an effect of increased glomerular permeability (14). IgG is usually excreted when the selective permeability of the glomerular capillary wall is severely disrupted (8). In dogs with different types of nephropathy, urine IgG level is evaluated to characterize the severity of proteinuria (21, 25, 53). The elevated level of uIgG in dogs with chronic kidney disease used in our study confirms the findings of other researchers and clearly showed damage in the course of glomerular disease.

The Tamm Horsfall protein (uromodulin) is a urinary glycoprotein exclusively synthesized by tubular cells in the distal part of the nephron (13, 23, 25). There are a few small studies in veterinary medicine that have measured the rate of urinary uromodulin excretion in chronic disease states (29, 56). In our study the level of uTHP/Cr in the diseased group was significantly lower compared to the control group. It has been assumed that the decrease in urinary THP expression reflects damage to the thick limb of Henle's loop and distal convoluted tubules, or even the loss of nephrons. Therefore, low urinary expression of THP might act as a marker of progressive renal tubular disease.

However, further investigations in dogs are required to confirm this rationale for measuring urinary uromodulin as a prognostic tool or as a biomarker of kidney impairment.

uRBP was measured as a marker of proximal tubular dysfunction. In most mammals, this LMW protein circulates in the plasma in the form of a complex with another protein, transthyretin. Vitamin A binds this complex and prevents RBP excretion. However, dogs have high concentrations of transthyretin uncomplexed RBP, filtered by the glomeruli. Under physiological conditions, the filtered RBP is almost completely reabsorbed by megalin-mediated endocytosis in the proximal tubular cells, and tubular dysfunction leads to excessive amounts of uRBP (35, 49). In our study uRBP/Cr was significantly higher in dogs with chronic kidney disease compared to the healthy controls. Similar observations concerning dogs with CKD were made by Smets et al. (37), Nabity et al. (26, 27). The presence of RBP in the last fractions of a urine sample may not simply be the result of saturation of the tubular reabsorption mechanisms with MMW/HMW proteins and their competition for receptor-binding sites (49), but could indeed be the result of direct tubular damage induced by other causes associated with CKD. In one study the effect of inflammatory cytokines on the culture efficiency of proximal tubular epithelial cells was investigated. The obtained results indicated that the exposure of tubular epithelial cells to TNFα caused a decrease in megalin expression, the most important receptor for the re-uptake of LMW proteins in the renal tubules (17). Another study (3) investigating the effect of ischemia-reperfusion injury on specific sodium transporters on the apical membrane of the renal tubule showed both their expression and activity to be greatly reduced.

The results of this study suggest that all evaluated markers for glomerular and tubular dysfunction may improve the diagnosis and monitoring of CKD in dogs. However further studies are needed to confirm these result.

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Corresponding author: Dagmara Winiarczyk DVM, PhD, Department and Clinic of Internal Diseases, ul. Głęboka 30, 20-612 Lublin, Poland; e-mail: winiarczykdm@gmail.com