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Nephrin and podocin mRNA detection in urine sediment of dogs with chronic kidney disease: preliminary observations

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Abstract

Introduction: Dogs with chronic kidney disease (CKD) may have alterations in the glomerular filtration barrier, including podocyte loss. Detection of podocyte mRNA in urine could be useful for assessing podocyturia in dogs with kidney disease. The objective of this study was to evaluate the presence of nephrin mRNA (*NPHS1*) and podocin mRNA (*NPHS2*) in urine sediments of dogs with naturally occurring CKD and healthy dogs. **Material and Methods:** Twenty-four dogs, 14 with CKD and 10 as healthy controls, underwent clinical evaluation. The dogs with CKD were divided into two groups, according to the International Renal Interest Society criteria: stage 1 or 2 CKD (n = 5) and stage 3 or 4 CKD (n = 9). Urine was collected by catheterisation or free catch and RNA isolation from the urine sediments was optimised using glycogen as a co-precipitant. Detection of *NPHS1* and *NPHS2* in the sediment samples was performed using quantitative real-time PCR. **Results:** Both types of mRNA were detected in samples from all groups, but the percentages of detection were higher in the group of dogs with stage 1 or 2 CKD and lower in the group of dogs with stage 3 or 4 disease. **Conclusion:** Physiological podocyturia was observed in healthy dogs, and the results suggest differential podocyturia in dogs with CKD, according to the stage of the disease, *i.e.* an increase in podocyturia in dogs at stage 1 or 2 and a reduction in podocyturia in dogs at stage 3 or 4.

Keywords: dog, *NPHS1*, *NPHS2*, podocyturia, quantitative real-time PCR.

Introduction

The glomerular filtration barrier (GFB) is formed by glomerular endothelial cells, the glomerular basement membrane (GBM), and podocyte foot processes covering the GBM. Foot processes from neighbouring podocytes interdigitate and are connected by a membrane called the slit diaphragm (SD). The GBM and SD serve as barriers to prevent the filtration of plasma macromolecules such as proteins. Nephrin, encoded by the *NPHS1* gene, is a transmembrane protein in the SD, whereas podocin, encoded by the *NPHS2* gene, interacts with the cytoplasmic tail of nephrin and participates in the connection between the SD and the cytoskeleton of foot processes (20).

Chronic kidney disease (CKD) is characterised by irreversible lesions in the renal parenchyma that compromise the structure and function of nephrons (16). It is commonly reported in dogs aged over 12 years (21); considering that the care of older dogs needs increasingly frequent veterinarian involvement, the number of CKD cases acquires significance in routine clinical care provision.

Dogs suffering from CKD have alterations in the GFB, which include GBM thickening, loss of adhesion between podocytes and the GBM, and podocyte foot process effacement (14). These podocyte changes are frequently accompanied by podocyte detachment, podocyturia and podocytopaenia.

Podocyturia can occur naturally in humans (31) and horses (26); however, an increased extent of podocyturia over what may occur naturally is related to glomerular lesions, with proteinuria as one of the main consequences (29). Several glomerular diseases can

cause damage to podocytes, including degenerative, infectious, and metabolic diseases (12). Recent studies have evaluated podocin detection in urine sediments of dogs to estimate podocyturia in these patients (27, 28). In these studies, dogs with CKD or degenerative mitral valve disease (DMVD) had greater podocyturia than healthy dogs (27, 28). Podocyturia was assessed by urinary podocin concentration using an ELISA test or by podocin detection using liquid chromatography–mass spectrometry, and the CKD group was formed by dogs at different stages of the disease. However, possible differences in podocyturia between CKD stages have not been evaluated.

Nephrin mRNA (*NPHS1*) and podocin mRNA (*NPHS2*) have been used as podocyte markers in urine (9, 33). In experimental models, induced glomerular lesions promoted an increase in urinary *NPHS1* and *NPHS2* excretion, but disease progression was marked only by urine *NPHS2* detection (25). Likewise, in humans with progressive glomerular disease, the amount of urine *NPHS2* was greater than that in healthy individuals (33). However, *NPHS1* and *NPHS2* detection patterns in the urine of dogs with CKD have not yet been described.

The analysis of urinary excretion of *NPHS1* and *NPHS2* has also been used in human medicine to assess the risk of glomerular injury developing or progressing. A recent study showed that diabetic adults with albuminuria had an increased urinary *NPHS2*:*NPHS1* ratio and urinary *NPHS2*:creatinine ratio compared with non-diabetic subjects. These results are consistent with the increased podocyte loss due to glomerular damage caused by *diabetes mellitus* (10). Similar results were observed in diabetic patients with normoalbuminuria, suggesting that podocyturia increased significantly prior to the onset of albuminuria. Furthermore, in a long-term analysis, an increased urinary *NPHS2*:creatinine ratio was associated with a reduction in the glomerular filtration rate (10). Therefore, urinary podocyte mRNA excretion could be an important parameter in assessing the risk of glomerular disease progression. Podocyte mRNA levels were evaluated in the urinary sediment of obese children, and increased urinary *NPHS2* expression, even in the absence of albuminuria, was considered a possible indicator of early obesity-related glomerulopathy (17).

In veterinary medicine, Szczepankiewicz *et al*. (27, 28) found that dogs with DMVD and symptomatic chronic heart failure showed increased podocyturia compared to control dogs but had serum creatinine, urea and symmetric dimethylarginine values within the reference range, suggesting the identification of early kidney damage secondary to heart disease, which was compatible with cardiorenal syndrome in dogs.

Urine can be obtained in large volumes, and the collection of this type of sample is well tolerated by dogs. In additional recommendation of urine as sample material, it has been increasingly used to search for biomarkers related to different diseases in animals, including CKD (28). Therefore, the aim of this study was to evaluate the detection of *NPHS1* and *NPHS2* in the urine sediment of dogs with CKD by comparing the results of patients with stages 1 or 2 of the disease with those of patients with stages 3 or 4.

Material and Methods

Animals. The study included 24 dogs that were patients at the Veterinary Hospital of the Faculty of Animal Science and Food Engineering of the University of São Paulo, Brazil. Fourteen dogs were diagnosed with CKD after careful evaluation of their clinical history and physical and complementary examinations. The diagnosis was based on the presence of ultrasound findings compatible with CKD, such as increased echogenicity in the cortical and/or medullary regions, loss of corticomedullary differentiation, or irregular renal contour (3, 23). In addition, at least one of the following criteria was considered: serum creatinine concentration ≥1.4 mg/dL, persistent renal proteinuria with urinary protein:creatinine ratio (UPC) > 0.5 or persistent urine specific gravity (USG) < 1.030 (8). Some dogs were diagnosed with CKD even with serum creatinine <1.4 mg/dL, due to their meeting the other criteria mentioned. CKD dogs were divided into two groups, according to the stage of the disease and following the current International Renal Interest Society criteria (15): three dogs and two bitches at CKD stage 1 or 2 (11.2 ± 0.97 years old, 14.5 ± 3.1 kg), the group consisting of an American pit bull terrier, cocker spaniel, Yorkshire terrier, Australian cattle dog, and boxer; and a dog and eight bitches at CKD stage 3 or 4 (11.2 ± 1.24 years old, 19.8 ± 4.3 kg), the group consisting of a Great Dane, beagle, bulldog, Labrador retriever, cocker spaniel, and four mixed-breed dogs. Animals with CKD were required to have been in a stable clinical condition and under veterinary care for at least three months to be included in this study. Five clinically healthy dogs and the same number of healthy bitches (8.9 ± 0.89 years old, 10.2 ± 2.1 kg), this group consisting of one Dachshund and nine mixed-breed dogs, were used as controls. The inclusion criteria for the control group were the absence of any signs of illness during the clinical examination and no changes in the laboratory tests. Dogs with underlying medical conditions (*e.g.* cystitis or neoplasms) or dehydration were excluded from the study.

Sample collection, processing, and complementary examinations. Blood was collected from the jugular vein after an 8 to 12 h fasting period into tubes containing K2-EDTA or gel and clot activator for haematological and biochemical examinations, respectively. Blood counts were performed using a BC-2800Vet automatic blood analyser (Mindray Bio-Medical Electronics Co., Shenzhen, China). Serum and urinary biochemical parameters were assessed using specific reagents

(Labtest Diagnostica, Lagoa Santa, MG, Brazil) and a BS-120 biochemical analyser (Mindray Bio-Medical Electronics Co.). Colorimetric methods were used to measure serum albumin (bromocresol green) and creatinine (modified Jaffé method) concentrations, and the serum urea concentration was determined using an ultraviolet enzymatic method. In dogs with CKD, serum inorganic phosphorus concentration was measured using ultraviolet photometry (modified Daly and Ertingshausen method) and serum ionised calcium concentration was measured using a SmartLyte Plus electrolyte analyser (Diamond Diagnostics, Holliston, MA, USA). Morning urine was collected by catheterisation (6 dogs) or free catch (18 dogs), and 10 mL was used in the urinalysis, which comprised a dipstick analysis (Labtest Diagnostica) and sediment examination. Another 10 mL was stored at −80°C for subsequent RNA isolation. Refractometric determination of USG was carried out, and urinary protein and creatinine concentrations were measured in the supernatant using pyrogallol red and modified Jaffé methods, respectively. The UPC ratio was calculated by dividing the urinary protein concentration (mg/dL) by the urinary creatinine concentration (mg/dL). At the time of sample collection, CKD dogs had a stable clinical condition, normal hydration, and were not medicated with vasodilators.

Systolic blood pressure (SBP) was measured in the left ulnar artery using a DV 610V ultrasonic Doppler flow detector (MEDMEGA, Franca, SP, Brazil) after the patient was acclimatised to the clinic, and the average of three measurements was used. A kidney ultrasound scan was performed with a MyLab Class C Vet system (Esaote, Genoa, Italy) using a microconvex 3–9 MHz transducer.

RNA isolation, reverse transcription, and quantitative real-time PCR (qPCR). Urine samples were carefully thawed and centrifuged at 3,000 × *g* for 30 min at 4°C. The supernatant was discarded, and 1 mL of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and 1 µL of glycogen (20 mg/mL) (18) were added to the pellet. The sample was homogenised, transferred to a new 1.5 mL tube, vortexed, and incubated for 5 min at room temperature. Next, 200 µL of chloroform was added, and the solution was vortexed and incubated for 5 min at room temperature. The sample was centrifuged at 10,000 × *g* for 15 min at 4°C. After centrifugation, the aqueous phase was transferred to a new 1.5 mL tube and one volume of isopropanol and 100 µL of sodium acetate (3 M, pH 5.2) were added. The sample was kept on ice for 30 min and then centrifuged at 10,000 × *g* for 20 min at 4°C for RNA precipitation. The supernatant was discarded, and the pellet was resuspended in 1 mL of 70% ethanol. The tube containing the resuspended pellet was vortexed and centrifuged twice at 10,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 35 µL of RNAse-free water and transferred to a new 1.5 mL tube for reverse transcription. Total RNA concentration and RNA purity ratios were estimated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and the presence of 18S and 28S ribosomal bands was analysed by one-dimensional agarose gel electrophoresis. Total RNA was treated with Ambion DNase I (RNase-free; Thermo Fisher Scientific) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). A qPCR was performed using Fast SYBR Green Master Mix and StepOne Real-Time PCR equipment (Applied Biosystems, Foster City, CA, USA). The cycling conditions were as follows: initial denaturation at 95°C for 20 s, 40 cycles of 3 s at 95°C and 30 s at 60°C, followed by a melting curve ranging from 60°C to 95°C (acquiring fluorescence data every 0.3°C). Ribosomal RNA from the 18S gene (18S rRNA) was used as a housekeeping gene, and a fresh, *post-mortem* cortex fragment, sampled from an adult dog's kidney, served as a positive control of the reaction. The primer sequences for *NPHS1* were forward 5'-CCTGTATGATGAAGTGGAAAGG-3' and reverse 5′-AGGGTCCAAGTTTCCTGCTAC-3′ (14). The primers for *NPHS2* and 18S were designed using Primer3Plus software (30), analysed for dimers and hairpins, and verified for multiple alignments with the basic local alignment search tool (BLAST; www.https://blast.ncbi.nlm.nih.gov/Blast.cgi). For *NPHS2* they were forward 5′-GGTTTAG GGGCCTG TGAGTG-3′ and reverse 5′-AGC AGATGTCCCAG TCGAAA-3′ and for 18S forward 5′- CCTGCGGCT TAATTTGACTC-3′ and reverse 5′-CTGTCAATC CTGTCCGTGTC-3′. Detection of *NPHS1* and *NPHS2* was based on the automatic cycle threshold (Step One software, Thermo Fisher Scientific), and 18S was used only as a reference for RNA extraction.

Nucleotide sequencing. The PCR products from *NPHS1* and *NPHS2* were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing of product DNA was performed using 50 ng/µL of PCR products and 100 µM of each primer. The samples were sent for DNA sequencing at the Human Genome and Stem Cell Research Centre within the Biosciences Institute at the University of São Paulo. Nucleobytes 4Peaks v1.8 software (Aalsmeer, the Netherlands) was used to manually check the electropherograms of the forward and reverse sequences. Pairwise alignment and sequence identification were performed using nucleotide BLAST.

Data analysis. Statistical analysis was performed using GraphPad Prism v9.0.0 software (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was used to assess data normality, and means were compared using analysis of variance followed by the Kruskal–Wallis test or Tukey's test. The frequencies of the semi-quantitative parameters were analysed using the GENMOD procedure. Statistical significance was set at $P \leq 0.05$.

Results

Dogs at CKD stage 3 or 4 had anaemia and azotaemia (Tables 1 and 2), hyperphosphataemia (10.0 ± 0.95 mg/dL; reference range: 2.8–6.1 mg/dL), and ionized hypocalcaemia (0.9 ± 0.70 mmol/L; reference range: 1.12–1.40 mmol/L). In addition, SBP was higher in dogs with stage 3 or 4 CKD (138.3 ± 6.17 mmHg) than in dogs with stage 1 or 2 CKD (114.0 ± 8.28 mmHg) and healthy dogs (111.0 ± 5.85 mmHg) ($P < 0.05$). Urinary parameters revealed proteinuria and a lower USG with normal urinary sediment in dogs with CKD (Table 3).

Table 1. Results for blood haematological parameters in healthy dogs and dogs with chronic kidney disease (CKD) at different stages

		CKD dogs	
Parameter (reference range)	Healthy dogs (n = 10)	Stage 1 or 2 (n = 5)	Stage 3 or 4 (n = 9)
RBC (10^6/μL) (5.5–8.5)	6.7 ± 0.35	6.7 ± 0.49	4.7 ± 0.36**
HGB (g/dL) (12–18)	17.3 ± 0.83	16.7 ± 1.17	11.6 ± 0.88**
HCT (%) (37–55)	50.1 ± 2.49	49.7 ± 3.52	34.9 ± 2.63**
MCV (fL) (60–77)	75.2 ± 1.49	75.0 ± 2.11	75.2 ± 1.57
MCH (pg) (21–26)	26.0 ± 0.58	25.2 ± 0.82	25.0 ± 0.61
MCHC (%) (32–36)	34.6 ± 0.44	33.7 ± 0.62	33.2 ± 0.47
PLT (10^3/μL) (200–500)	307 ± 57.60	386 ± 39.7	378 ± 64.0
RBC morphology	normal	normal	normal
WBC (10^3/μL) (6–17)	7.1 ± 0.63	8.8 ± 1.35	7.5 ± 1.02
WBC morphology	normal	normal	normal

Data are mean values ± standard errors of the mean
RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; MCV – mean cell volume; MCH – mean cell haemoglobin; MCHC – mean cell haemoglobin concentration; PLT – platelet count; WBC – white blood cell count; ** $P < 0.01$, compared to healthy dogs and dogs with stage 1 or 2 CKD

Table 2. Results for blood biochemical parameters in healthy dogs and dogs with chronic kidney disease (CKD) at different stages

		CKD dogs	
Parameter (reference range)	Healthy dogs (n = 10)	Stage 1 or 2 (n = 5)	Stage 3 or 4 (n = 9)
Albumin (g/dL) (2.3–3.8)	3.2 ± 0.16	2.8 ± 0.23	2.7 ± 0.20
Urea (mg/dL) (10–60)	39.4 ± 5.36	47.3 ± 13.48	259.4 ± 46.70*
Creatinine (mg/dL) (0.5–1.4)	1.1 ± 0.14	1.0 ± 0.17	7.4 ± 2.34*

Data are mean values ± standard errors of the mean
* $P < 0.05$, compared to healthy dogs and dogs with stage 1 or 2 CKD

Table 3. Results for routine urinary parameters in healthy dogs and dogs with chronic kidney disease (CKD) at different stages

		CKD dogs	
Parameter (reference range)	Healthy dogs (n = 10)	Stage 1 or 2 (n = 5)	Stage 3 or 4 (n = 9)
USG (1.015–1.045)	1.037 ± 0.0040	1.022 ± 0.0074*	1.014 ± 0.0009**
Protein (mg/dL)	29.4 ± 2.44	210.6 ± 11.67***	124.0 ± 35.76*
Creatinine (mg/dL)	206.1 ± 24.00	94.4 ± 21.47**	70.0 ± 21.47**
UPC (< 0.5)	0.15 ± 0.01	2.25 ± 0.68*	1.76 ± 0.50*
Chemical analysis (semiquantitative testing):			
pH (6.0–7.5)	6.5 ± 0.37	6.4 ± 0.52	5.9 ± 0.39
Protein	between 0 and 1+	between 1 and 3+	between 1 and 2+
Glucose	negative	negative	negative
Ketones	negative	negative	negative
Bilirubin	negative	negative	negative
Urobilinogen	normal	normal	normal
Occult blood	negative	1+ in the urinary sediment of 1 dog	1+ in the urinary sediment of 2 dogs
Microscopic sediment examination:			
Epithelial cells/HPF (<3)	1.70 ± 0.26	1.00 ± 0.22	1.44 ± 0.43
Granular cast/HPF (<1)	rare in the urinary sediment of 1 dog	none	rare in the urinary sediment of 1 dog
Triple phosphate crystal	1+ in the urinary sediment of 2 dogs	2+ in the urinary sediment of 1 dog	none
Amorphous phosphate crystal	none	none	2+ in the urinary sediment of 1 dog
RBC/HPF (<5)	0.30 ± 0.13	0.30 ± 0.20	0.56 ± 0.49
WBC/HPF (<5)	0.50 ± 0.18	0.60 ± 0.25	0.83 ± 0.25
Bacteria	none	none	none

Data are mean values ± standard errors of the mean
USG – urine specific gravity; UPC – urine protein:creatinine ratio; + – subjective scale ranging from 1+ to 4+; HPF – high-power field (40 × objective); RBC – red blood cell count; WBC – white blood cell count; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to healthy dogs

All dogs with CKD showed renal ultrasonographic abnormalities (Table 4). Loss of corticomedullary differentiation and increased cortical echogenicity were equally frequent in patients suffering stage 1 or 2 CKD and patients diagnosed with stages 3 or 4. Irregular contours of the kidneys were more frequently observed in stage 1 or 2 dogs, whereas increased medullary echogenicity was more frequent in stage 3 or 4 subjects.

The mean total RNA concentration and 260/280 purity ratio of RNA extracted from the urine sediments did not differ between the groups of healthy dogs (33.4 ± 8.9 ng/µL and 1.41 ± 0.045) and those with CKD (31.0 ± 14.2 ng/µL and 1.46 ± 0.047). Electrophoretic analysis clearly showed 28S and 18S ribosomal RNA bands in samples from urine sediments (Fig. 1).

The housekeeping gene (18S rRNA) was detected in all urine sediment samples. Nephrin mRNA was more frequently detected in urine sediments from dogs with stage 1 or 2 CKD and less frequently in urine sediments from dogs with stage 3 or 4 CKD compared to healthy dogs. Podocin mRNA was also more often observed in urine sediment samples from dogs with stage 1 or 2 CKD than in healthy dogs and dogs with stage 3 or 4 CKD (Table 5).

A representative image of the qPCR-amplified cDNA products in the present study (18S, *NPHS1*, and *NPHS2*) is shown in Fig. 2.

Nucleotide sequencing of representative PCR products confirmed the identity of the *Canis lupus familiaris NPHS1* and *NPHS2* mRNA sequences. The GenBank accession numbers are XM_541685.7 and XM_038565127.1, respectively.

Table 4. Semi-quantitative analysis of renal ultrasonographic abnormalities in dogs with chronic kidney disease (CKD) at different stages

	CKD dogs		
Parameter	Stage 1 or 2	Stage 3 or 4	P value
Irregular contour	5/5 (100%)	6/9 (67%)	<0.0001
Loss of corticomedullary differentiation	4/5 (80%)	8/9 (89%)	0.1170
Increased cortical echogenicity	4/5 (80%)	8/9 (89%)	0.1170
Increased medullary echogenicity	0/5	5/9 (56%)	0.0197

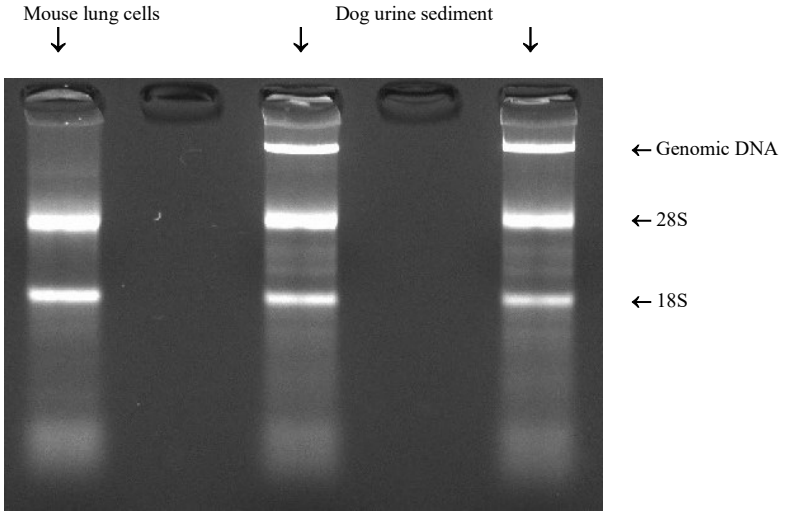

Fig. 1. Electrophoresis of total RNA in agarose gel (2%). Column 1 – total RNA isolated from mouse lung cells; Columns 2 and 3 – total RNA isolated from urine sediment of subject dogs. Ribosomal RNA bands (28S and 18S) were observed in all samples, indicating adequate RNA isolation. The genomic DNA band appeared in samples 1 and 2 because DNAse had not yet been used in the reaction

Table 5. Semi-quantitative analysis of detection of *NPHS1* (nephrin mRNA) and *NPHS2* (podocin mRNA) in urine sediment samples from healthy dogs and dogs with chronic kidney disease (CKD) at different stages

		CKD dogs		
Parameter	Healthy dogs	Stage 1 or 2	Stage 3 or 4	P value
NPHS1 detection	4/10 (40%) [a]	3/5 (60%) [b]	1/9 (11%) [c]	0.0009
NPHS2 detection	2/10 (20%) [a]	4/5 (80%) [b]	1/9 (11%) [a]	0.0188

Different superscript letters on the same line indicate difference between groups

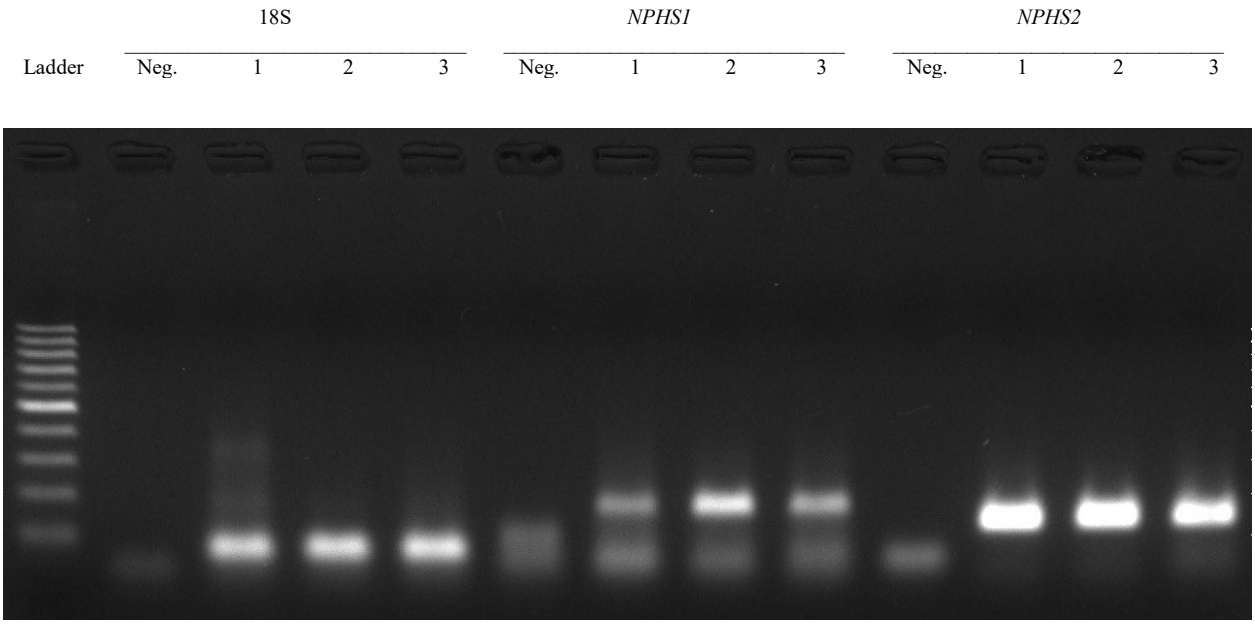

Fig. 2. cDNA product electrophoresis in 1.5% agarose gel. Neg. – negative control; Column 1 – sample from a dog with stage 3 chronic kidney disease; Column 2 – sample from a dog of the control group; Column 3 – sample from a dog with stage 1 chronic kidney disease. The length of 18S was 80 bp, that of *NPHS1* was 156 bp, that of *NPHS2* was 140 bp, and the DNA ladder was 100 bp long

Discussion

The presumptive diagnosis of stage 1 or 2 CKD was based on the normal or slightly elevated serum creatinine value, the presence of abnormalities in renal ultrasound, and the detection of renal proteinuria whether associated or not with lower USG. Dogs with stage 3 or 4 CKD showed a significant decline in renal function, with azotaemia, hyperphosphataemia, ionised hypocalcaemia, anaemia, proteinuria, and an inability to concentrate urine. Anaemia is a common disorder in dogs with stage 3 and 4 CKD and can be a consequence of hyporexia or anorexia, intestinal blood loss, or deficient erythropoietin production by the kidneys (1, 6). The study dogs with CKD had advanced age, and clinical changes persisted throughout the execution of this research.

In dogs with CKD, a low glomerular filtration rate reduces urinary phosphorus excretion, generating hyperphosphataemia, which in turn contributes to the development of renal secondary hyperparathyroidism, which has been associated with an increased risk of death in dogs with azotaemic CKD (24). Both azotaemia and hyperphosphataemia are related to the severity of kidney disease; therefore, they are more commonly observed in more advanced CKD stages (8). Moderate hypocalcaemia (ionised calcium <1 mmol/L) (5) also corresponds to a later event in CKD, possibly caused by reduced calcitriol production in the kidneys (22). This disorder was noted in study dogs with stage 3 or 4 CKD.

The decline in renal function can lead to an increase in blood pressure; therefore, in dogs with stage 3 or 4 CKD, the mean SBP value was higher. Blood pressure increases because of sodium and water retention related to a low glomerular filtration rate, but also because of vasodilator (*e.g.* nitric oxide) level reduction, renin-angiotensin-aldosterone system activation, and sympathetic activity intensification (1, 19).

Renal ultrasonographic abnormalities observed in the study dogs with CKD such as increased cortical and medullary echogenicity, loss of corticomedullary differentiation, and irregular contours are indicative of the chronicity of the disease (3, 23), and most dogs with stage 3 and 4 CKD showed three or more abnormalities, which are compatible with the progression of the disease and the decline of renal function in these animals (23).

Isolation of RNA from urinary sediments of dogs is hampered by low urinary pH, which could facilitate RNA degradation (18), and the lowest USG value in dogs with CKD hinders precipitation and RNA isolation. It is possible that urine characteristics contributed to the 260/280 RNA purity ratio being lower than the accepted value for other biological samples (approximately 1.8). Corroborating this information, Bradley *et al*. (2) isolated RNA from human urinary sediments using TRizol reagent, and the 260/280 RNA purity ratio values were similar to our results. Even with the values obtained for the 260/280 RNA purity ratio, the 28S and 18S ribosomal RNA bands were evidenced by electrophoresis, and the qPCR amplification of the housekeeping gene was effective, demonstrating that the isolated RNA had the necessary quality for gene expression experiments.

Moderately concentrated urine (USG 1.013 to 1.029) concomitant with azotaemia is an indicator of

impaired renal function due to the loss of functional nephrons and the inability of the remaining tubular cells to properly conserve water (32), and a critical achievement in experiments involving gene expression is obtaining sufficient amounts of total RNA from diluted urine samples. Therefore, we opted for the use of glycogen, an inert co-precipitant that maximises RNA recovery (11, 18); thus, the total RNA concentration in urine sediments of dogs was satisfactory.

From the standardisation of the RNA isolation protocol, we proceeded to analyse *NPHS1* and *NPHS2* detection in urine sediment samples. In most positive samples, both *NPHS1* and *NPHS2* were detected, while *NPHS1* alone was detected in two samples of healthy dogs, and *NPHS2* alone was detected in one sample from a dog at CKD stage 1. The presence of *NPHS1* and/or *NPHS2* is indicative of podocyturia in dogs under any of these conditions. Both *NPHS1* and *NPHS2* were detected in urine sediments of clinically healthy dogs. Recent studies have shown that podocin was detected in the urine sediment of healthy dogs by ELISA (28), but not by liquid chromatography–mass spectrometry in multiple reaction monitoring mode (27). Certainly, detection methods vary in terms of sensitivity, but the presence of physiological podocyturia in dogs, as in horses (26) and humans (31), remains under discussion.

More frequent detection of *NPHS1* and *NPHS2* in urine sediment was noted from dogs with stage 1 or 2 CKD, and this result combined with high proteinuria suggests active kidney injury, with particular impairment of the GFB structure and function but with minimal changes in the glomerular filtration rate. In contrast, the frequency of *NPHS1* and *NPHS2* detection in urinary sediments of dogs at CKD stage 3 or 4 was low, which could be a consequence of the reduction in the number of nephrons and podocytes adhering to the GBM. Therefore, both clinical changes and low *NPHS1* and *NPHS2* detection in the urinary sediment were consistent with advanced CKD stages in these dogs.

Ichii *et al*. (14) identified low *NPHS1* expression in *post-mortem* kidney samples of dogs with CKD at different stages. However, they were unable to identify *NPHS1* in urine sediments and *NPHS2* was identified in only one sample (1/10). It is possible that differences in the experimental protocol could explain the discrepancy between the results obtained by Ichii *et al*. (14) and those of the present study. Conversely, similarly to this research, *NPHS1* relative quantitation was lower in more advanced stages of renal disease in dogs suffering from CKD associated with visceral leishmaniosis (7).

The association between proteinuria and urinary *NPHS1* and *NPHS2* levels has been evaluated in animal models and humans. Proteinuria and increased urinary *NPHS1* and *NPHS2* excretion were observed in rats with toxin-induced glomerular damage; however, glomerulopathy progression in these animals was accompanied by persistent proteinuria, and only *NPHS2* was detected in urine (25). In humans, UPC is highly correlated with urine *NPHS2* in nephrotic syndrome and weakly correlated with urine *NPHS2* in membranous glomerulonephritis; therefore, the relationship between proteinuria and podocyturia varies according to the type of glomerular disease (33).

In early CKD, despite the reduction in the number of nephrons, the total glomerular filtration rate remains close to normal owing to the haemodynamic changes that occur in the glomeruli of the remaining nephrons. These haemodynamic changes include a decrease in the resistance of the afferent arteriole and/or an increase in the resistance of the efferent arteriole, which results in increased pressure in the glomerular capillaries and, consequently, in the filtration rate (13). Glomerular hyperfiltration creates mechanical stress on podocytes, leading to podocyte injury and detachment, which contributes to podocyturia and proteinuria (4). These mechanisms could explain the presence of high proteinuria and higher *NPHS1* and *NPHS2* detection in the urine sediment of dogs with stage 1 or 2 CKD. In contrast, dogs with stage 3 or 4 CKD presented high proteinuria and lower *NPHS1* and *NPHS2* detection in urine sediment, and this result is consistent with the reduction seen in CKD dogs in the number of podocytes (14), and the contribution of podocytopaenia to proteinuria.

Considering that podocyte loss can be monitored by detecting *NPHS1* and *NPHS2* in urine (9, 25), our results suggest differential podocyturia in dogs with CKD according to the stage of the disease, *i.e.* an increase in podocyturia in dogs at CKD stage 1 or 2 and a reduction in podocyturia in dogs at CKD stage 3 or 4.

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