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Urinary cystatin C and N-acetyl-beta-D-glucosaminidase (NAG) as early biomarkers for renal disease in dogs with leishmaniosis

Patricia Ruiz [a], Ángela Durán [b], Francisco Javier Duque [a], Mario Alberto González [a], José Ignacio Cristóbal [b], Paloma Nicolás [a], Eva María Pérez-Merino [c], Beatriz Macías-García [a,*,1], Rafael Barrera [a,1]

[a] MINVET Research Group. Departamento de Medicina Animal, Facultad de Veterinaria, Universidad de Extremadura, 10003 Cáceres, Spain
[b] Hospital Clínico Veterinario, Facultad de Veterinaria, Universidad de Extremadura, 10003 Cáceres, Spain
[c] Departamento de Medicina Animal, Facultad de Veterinaria, Universidad de Extremadura, 10003 Cáceres, Spain

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ABSTRACT

Canine leishmaniasis (CanL) is a disease caused by *Leishmania infantum* that can vary from a subclinical infection to a severe disease. Dogs affected with CanL present varying degrees of renal dysfunction. Unfortunately, traditional biomarkers such as urea and creatinine detect renal damage in advanced stages of the disease, so more accurate biomarkers are needed. Hence, we aimed to study how urinary cystatin C (CysC) and N-acetyl-beta-D-glucosaminidase (NAG), behave in dogs with CanL at different stages of the disease. Eighty-six CanL infected dogs were classified according to LeishVet stages: LI (16 dogs), LIIa (12 dogs), LIIb (12 dogs), LIII (16 dogs) and LIV (30 dogs); as a control, 17 healthy dogs were studied. Blood samples were collected for complete haematological and biochemistry analysis including plasma cystatin C. Urine analysis included urine specific gravity (USG), urine protein to creatinine ratio (UPC), CysC and NAG expressed as a ratio with creatinine uCysCc (μg/g) and uNAGc (IU/g). The haematological, biochemical and urinary analysis coincided with the LeishVet guidelines. The statistical study of the uCysCc ratio and the uNAGc, showed significant increase when compared against control starting from group LI ($p < 0.05$). Interestingly, when the cut-off values were calculated using the ROC curve, uCysCc (258.85 μg/g) and uNAGc (2.25 IU/g) 75 % of the dogs included in LI groups surpassed the threshold. Hence our study indicates that uCysCc and uNAGc, could help to detect early renal damage in CanL affected dogs.

1. Introduction

Canine leishmaniasis (CanL) is a vector-borne zoonotic disease, caused by the protozoan parasite *Leishmania infantum*, which is endemic in Spain (Gálvez et al., 2020). In the region of Extremadura, where the present study was carried out, a seroprevalence ranging between 14 % and 34.2 % has been reported (Gálvez et al., 2020). The dogs affected may present different clinical manifestations which can vary from a subclinical infection in which no signs of CanL can be appreciated, to severe disease, with severe risk of death (Solano-Gallego et al., 2011).

Lymphadenopathy and dermal changes are the most common clinical signs observed in CanL (Meléndez-Lazo et al., 2018). However, renal dysfunction is frequently observed and is associated with poor prognosis and increased mortality (da Silva Junior et al., 2014). Renal failure in CanL is associated with the deposition of soluble circulating immune complexes resulting from the exacerbated humoral immune response against the parasite (Dayakar et al., 2019). This deposition results in glomerular disease, which is observed clinically as persistent proteinuria, although it usually progresses to glomerulonephritis and/or interstitial nephritis (Costa et al., 2003).

To detect and assess the progression of chronic kidney disease (CKD) in CanL the Canine Leishmaniosis Working Group (Roura et al., 2021) recommends an array of biomarkers including, creatinine, symmetric dimethylarginine (SDMA), urinary protein to creatinine ratio (UPC) together with a urinary specific gravity (Roura et al., 2021; IRIS staging of CKD (n.d.)). Unfortunately, traditional biomarkers such as urea and creatinine detect renal damage in advanced stages of the disease, when at least 75 % of the nephrons are not functional. As an alternative, SDMA

* Correspondence to: Department of Animal Medicine, Veterinary Faculty, University of Extremadura, Avenida de la Universidad s/n, 10003 Cáceres, Spain.
E-mail address: bemaciasg@unex.es (B. Macías-García).
[1] #These authors contributed equally to the present work and should be regarded as co-last authors.

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has been proposed due to its lower influence by non-renal factors and its ability to detect a decrease in glomerular filtration rate (GFR) earlier (Nabity et al., 2015; McKenna et al., 2020). However, even in the best of scenarios, it is known that at least 40 % of the nephrons are damaged when SDMA values increase (McKenna et al., 2020). Hence, in the daily clinic, more accurate biomarkers are needed for the early detection and location of kidney damage within the nephron.

There are few studies focused on the study of alternative biomarkers to detect early kidney disease in CanL. Until now, several biomarkers of glomerular disease have been investigate including Cystatin C (CysC) in blood, urinary immunoglobulin G (IgG), C-reactive protein and ferritin (García-Martínez et al., 2015; Pardo-Marín et al., 2016). Other biomarkers have been proposed to detect tubular damage including Gamma-Glutamyl Transferase or GGT (Pardo-Marín et al., 2016; Ibba et al., 2016; Paltrinieri et al., 2018), N-acetyl-beta-D-glucosaminidase or NAG (Pardo-Marín et al., 2016), Neutrophil Gelatinase-Associated Lipocalin or NGAL (Peris et al., 2020; Dias et al., 2020), urinary CysC (García-Martínez et al., 2015; Dias et al., 2020) and retinol transporter protein or RBP (Pardo-Marín et al., 2016). Unfortunately, some of these studies aimed to verify their usefulness for the follow up of the treatment of CanL, rather than in the early diagnosis of kidney disease itself (Pardo-Marín et al., 2016).

For the study of these early biomarkers, the LeishVet classification is useful as it allows for the diagnosis of dogs affected with CanL in the absence of azotemia and/or proteinuria (Solano-Gallego et al., 2011). Interestingly, Costa et al. (2003) demonstrated that in CanL positive dogs, glomerular lesions are present in all animals while interstitial nephritis occurs in 78.18 % of the patients, even in the absence of clinical and/or laboratorial abnormalities.

Considering that most dogs with CanL present some degree of impaired renal function, it is of utmost importance to validate adequate laboratory biomarkers that allow for an early diagnosis of CKD, hence contributing to improved clinical management of the disease. The aim of this study was to verify how urinary CysC and NAG and plasma CysC, behave in dogs with CanL at different stages of the disease, and evaluate their potential in the early diagnosis of CKD in the daily clinic.

2. Material and methods

2.1. Animals

This retrospective diagnostic study was performed at the University of Extremadura, Spain. All owners approved and signed an informed consent form. Client-owned dogs of any breed and age with a previous diagnosis of leishmaniasis were included. The dogs included were referred, or presented as primary patients, to the Veterinary Teaching Hospital of the University of Extremadura between the 1st of March of 2019, and 31st of February of 2022.

The approval of the Animal Experimentation Ethics Committee of the University of Extremadura (Spain) was not necessary as the Bioethics Committee considered that no animal experimentation was performed (Ref. 91/2020), according to the RD 53 of 2013.

One hundred and three dogs were studied and divided into two groups: group C (17 healthy control dogs) and group L (86 dogs naturally infected with *L. infantum*).

The control group included clinically healthy dogs presented for elective surgery or annual check-up and were negative to *L. infantum* in laboratorial leishmaniasis tests (described below). Group L is constituted by dogs that met the following inclusion criteria: all presented positive leishmaniasis tests (described below), were above 12 months of age and no concurrent disease was diagnosed. All animals presented correct hydration status, absence of urinary tract infection and had never been administered leishmanicidal and/or leishmaniostatic drugs. All of them underwent physical examination, determination of systolic arterial blood pressure, complete blood count, serum biochemical analysis, urinalysis (including urine culture), faecal examination for parasites and leishmaniasis testing. The animals were classified according to the recommendations of the LeishVet group of experts (Solano-Gallego et al., 2011) following the actualized IRIS guide of 2019 (International Renal Interest Society). The study was not blinded, and investigators and clinicians were aware of the groups the dogs belonged to.

2.2. Blood pressure measurement

Systolic blood pressure (SBP) was measured by a non-invasive method (Veterinary Blood Pressure Monitor Vet20®, SunTech Medical, Morrisville, USA). The patients previously underwent a period of adjustment in a quiet room to avoid, as much as possible, the influence of stress. The size of the cuff was adapted to 40 % of the diameter of the limb, always placed on the same limb and with the animals in the same position. Five measurements were collected, discarding the first and last, and the arithmetic mean of the remaining three was determined. SBP values > 160 mmHg were considered above the reference range as hypertension.

2.3. Clinical pathology testing

Blood samples were collected from the cephalic vein after a 12-hour fasting and placed in tubes containing EDTA for the hematologic determinations and tubes containing heparin for biochemistry analysis. Plasma was prepared by centrifuging blood samples at 540 g for 10 min. The hematologic analyses were performed immediately with an automated analyser (ProCyte DX®; IDEXX Laboratories, USA). The haematological variables determined included red cell count, haemoglobin concentration, haematocrit value, mean corpuscular volume, mean corpuscular haemoglobin concentration, total leukocyte count, differential leukocyte count (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), and platelet count. The biochemical variables determined included urea, creatinine (pCreat), SDMA, CysC, alkaline phosphatase (ALP), total protein, albumin, cholesterol, calcium, alanine aminotransferase (ALT) and phosphorus. Globulin concentration was calculated by subtracting the albumin value from that of total proteins. They were performed using an automated biochemical analyser (Saturno 100 Vet Crony® Instruments, Rome, Italy) not exceeding one hour after the extraction of the sample, with commercial kits (Spinreact® Laboratory Barcelona, Spain), according to the manufacturer's instructions. SDMA was determined using a Catalyst Dx Chemistry Analyzer (IDEXX® Laboratories, Inc, Westbrook, ME). Plasmatic CysC concentration (pCysC) was determined by a latex turbidimetric commercial kit (Cystatin C turbilatex; Spinreact®, Barcelona, Spain) as previously validated by Almy et al. (2002) and verified in our laboratory by Muñoz et al. (2017) for the commercial technique used.

Urine was obtained by ultrasound-guided cystocentesis. 0.5 millilitres of urine were used for urinary culture and three millilitres were used for routine urinalysis (Multistix Reagent Strips®, Bayer Corporation, Madrid, Spain) according to the manufacturer's instructions using an Urispin reader (Spinreact®, Spain). The rest was centrifuged at 130 g for 5 min to perform the urinary sediment, which was evaluated using an optical microscope (40x objective). The sediment was considered inactive if it met the following conditions: < 5 erythrocytes/hpf; < 5 leukocytes/hpf; occasional epithelial cells; absence of bacteria. The urine specific gravity (USG) was determined with a refractometer. In the supernatant, the UPC was determined. The pyrogallol-molybdate red technique was used to determine the concentration of urinary proteins and the Jaffé reaction for creatinine (RAL Diagnostics®, SA, Spain), programmed for an automatic blood chemistry analyser (Saturno 100 VetCrony® Instruments, Rome, Italy). The remaining supernatant was frozen at -80 °C until the determination of the concentration of CysC and NAG, before a month. The samples were subjected to a single thaw cycle.

Urinary CysC also was determined by a latex turbidimetric commercial kit (Cystatin C turbilatex; Spinreact®, Barcelona, Spain). The

commercial technique used was verified. As an indication of assay precision, the intraday coefficient of variation (CV) was calculated from 10 samples assayed on the same day, and the interday CV was calculated from 10 samples assayed on separate days. The accuracy of the assay was investigated by linearity during dilution using the mean of three calibration curves of four standards with known CysC concentrations (Cystatin C Calibrator; Spinreact®, Barcelona, Spain). The critical limit (CL), limit of detection (LOD), and limit of quantification (LOQ) were calculated. The repeatability and reproducibility of the CysC turbidimetric assay had satisfactory variability with a within-day CV = 5.66 % and a between-day CV = 4.06 %, both less than 10 %.

The urinary concentration of NAG was determined using a commercial kit (Diazyme® Laboratories; USA), programmed for an automatic biochemistry analyzer (Saturno 100 VetCrony® Instruments, Rome, Italy). NAG hydrolyses the 2-methoxy-4-(2 întrovinyl)-phenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MNP-GlcNAc) to 2-methoxy-4-(2 întrovinyl)-phenol product. The product formation is confirmed by detection of colour at 505 nm upon addition of an alkaline buffer.

The results of both biomarkers in urine were expressed as a ratio with creatinine: uCysCc (μg/g) and uNAGc (U/g).

2.4. Diagnosis of Leishmania infantum

An ELISA for semi-quantitative detection of specific antibodies against the total soluble antigen of *L. infantum* (obtained from *L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme MON-1) was carried out (Belinchón-Lorenzo et al., 2013). The diagnosis of CanL was achieved based on appropriate clinical signs and the serology titre (at least a three-fold increase above the laboratory's reference cut-off value). This was often complemented with a positive direct microscopical visualisation of *Leishmania* amastigotes in lymph node or bone marrow aspirate, or a positive PCR.

All dogs were tested for the absence of canine heartworm disease, *Anaplasma phagocytophylum*, *Borrelia burgdorferi* and *Ehrlichia canis* antibodies (Canine SNAP 4Dx, IDEXX® Laboratories, USA).

2.5. Statistical analyses

The statistical analyses were performed using SPSS Statistic software, version 27 (IBM Corp., NY, USA). All data were reported as mean ± standard deviation to describe distribution of continuous variables. Parameters were investigated for normality using the Kolmogorov-Smirnov test. Differences with P-value < 0.05 were considered as statistically significant. Non-normal distribution variables were compared with the Mann-Whitney U test followed by a Dunn's post-hoc test. Correlation between investigated markers and conventional parameters were calculated by nonparametric Spearman's rank correlation coefficient. Optimal cut-off points of biomarkers were determined by receiver operating characteristic (ROC) curves by selecting the highest percentage of correct classification. The best sensitivity and specificity were chosen as previously validated (Unal, 2017). Area under the curves (AUC) and 95 % confidence interval (CI) were calculated.

3. Results

The control animals (group C) were from different age (5.26 ± 2.18 years old), sex (10 males and 7 females) and breeds. They were considered healthy based on the medical history, physical examination, blood pressure and results of clinical tests (analytical parameters within the reference range provided).

At the day of enrolment into the study, in order to classify the dogs according to the LeishVet guidelines (Solano-Gallego et al., 2011), dogs underwent physical examination, repeated blood pressure measurements, collection of venous blood and urine and abdominal ultrasound examination. Eighty six dogs met all the necessary requirements and were classified according to the LeishVet guidelines: LI stage (LI) (16 dogs; 6.8 ± 2.3 years old; 7 males and 9 females; different breeds), LII stage (LII), divided into sub stages LIIa (LIIa) (12 dogs; 5.3 ± 2.2 years old; 7 males and 5 females; different breeds) and sub stage LIIb (LIIb) (12 dogs; 7.6 ± 2.4 years old; 8 males and 4 females; different breeds), LIII stage (LIII) (16 dogs; 6.2 ± 2.5 years old; 9 males and 7 females; different breeds) and LIV stage (LIV) (30 dogs; 6.9 ± 3.3 years old; 17 males and 13 females; different breeds).

The measurements corresponding to the SBP were the following: group C: 120.86 ± 9.58 mmHg; LI group: 123.56 ± 20.49 mmHg; group LIIa: 127.82 ± 36.78 mmHg; LIIb group: 130.55 ± 20.01 mmHg; group LIII: 158.70 ± 42.33 mmHg; LIV group: 165.53 ± 59.58 mmHg. A statistically significant increase was observed compared to the group of healthy dogs (group C) in groups LIII and LIV ($p < 0.05$).

The haematological study revealed the presence of normocytic and normochromic anaemia in the LII group which differed statistically from LIIb ($p < 0.05$ becoming more evident as the disease worsened). A statistically significant decrease in the lymphocyte count has also been observed in groups LIIa, LIII and LIV, being statistically significant when compared against control (Supplemental file).

Blood biochemistry showed a statistically significant decrease in plasma albumin concentration when compared against control in all groups. A significant increase in plasma phosphorus concentration in LIV was observed in all animals (100 %) and a statistically significant increase in pCreat and pCysC in groups LIIa, LIIb, LIII, and LIV. A statistically significant increase in the concentration of SDMA was observed in all the dogs affected with leishmaniosis (LI-LIV) compared to healthy dogs ($p < 0.05$; Table 1).

As expected, a progressive increase in the UPC value has been observed in the groups of dogs infected by *Leishmania* spp., with significant differences compared to the control starting from group LI (Table 2; $p < 0.05$). A vivid decrease in the mean values of USG was observed in all CanL affected dogs in study when compared against control (Table 2).

The statistical study of the uCysCc and uNAGc ratio showed significant increase when compared against control starting from group LI (Table 2; $p < 0.05$). Our data demonstrated that pCysC, uCysCc and uNAGc showed a high dispersion of data with respect to the average value as can be observed in the standard deviations provided (Tables 1 and 2). These vivid differences in the standard deviation can be attributed to the varying degrees of glomerular and/or tubular dysfunction that the patients present even when enclosed within the same LeishVet stage. Hence, and to establish if these biomarkers could somehow help to detect early renal damage in the absence of azotemia and proteinuria, the area under the ROC curve (AUC), sensitivity, and specificity of the concentration of the biomarkers studied were determined. To do this, two different approaches were followed. In the first one, all the groups of CanL affected dogs were compared against the control group. In the second one, only dogs included in LeishVet stages LI and LIIa (non-azotemic and non-proteinuric) were studied (Figs. 1A and 1B), and the cut-offs value in the ROC curve analysis to detect early CKD was calculated (Table 3) for pCreat, SDMA, pCysC, UPC, uCysCc and uNAGc.

Interestingly, the cut-off value for pCysC was set at 0.22 mg/l in the total group (sensitivity 79.70 % and specificity 91.00 %; Table 3) and 0.21 mg/l in the non-azotemic non-proteinuric groups (sensitivity 54.50 % and specificity 72.70 %; Table 3). This biomarker was increased in 2 out of 17 dogs in the control group taking 0.22 mg/ml as cut-off value (11.76 %) while 8/16 patients in LI (50 %) and 8/12 in LIIa (66,67 %) had a pCysC value over the threshold.

The cut-off value for uCysCc was calculated to be 258.85 μg/g in the total group (sensitivity 98.40 % and specificity 100 %; Table 3) and 258.85 μg/g in the non-azotemic non-proteinuric groups (sensitivity 95.50 % and specificity 100 %; Table 3). If this value is used as a reference, no dog in the control group presented values above, but 15/16 in LI (93.75 %) and 12/12 in LIIa (100%) exhibited a uCysCc value over the cut-off value.

The cut-off value for uNAGc was calculated to be 2.25 IU/g in the

Table 1
Biochemistry results in healthy dogs (Group C) and dogs with leishmaniasis (Group L) at LeishVet stages included in this study.

	TP (g/dl)	Albumin (g/dl)	Globulins (g/dl)	Cholesterol (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)
Group C	6,56 ± 0,85	3,60 ± 0,28	2,95 ± 0,53	212,29 ± 43.20	0,97 ± 0,08	32.35 ± 7,57
LI	7,36 ± 0,7*[c]	3,21 ± 0,60*[ce]	4.14 ± 0,97*[c]	180,44 ± 43,25*[e]	0,94 ± 0,12[cde]	29,93 ± 12.73[cde]
LIIa	8,00 ± 1,35*	3.17 ± 0,51*	4.83 ± 1,62*	216,33 ± 91,20	1,19 ± 0,21*[de]	53.81 ± 40.41[e]
LIIb	8,12 ± 1,10*[a]	2,87 ± 0,49*[ae]	5,25 ± 1,26*[a]	216,83 ± 146,01	1,20 ± 0,14*[ade]	77,45 ± 39,40*[ae]
LIII	7,33 ± 1.03*	2,89 ± 0,78*	4,45 ± 1,39*	215,94 ± 80,30	1,61 ± 0,28*[abce]	74.25 ± 38.20*[ae]
LIV	7,62 ± 1,52*	2,60 ± 0,66*[ab]	5.05 ± 1,76*	238,67 ± 84,51[a]	5,70 ± 2,46*[abcd]	254,23 ± 137,23*[abcd]
	SDMA (µg/dl)	**Cystatin C (mg/l)**	**Calcium (mg/dl)**	**Phosphorus (mg/dl)**	**ALP (UI/l)**	**ALT (UI/l)**
Group C	6.80 ± 2.11	0,17 ± 0,06	10,25 ± 0.68	4,57 ± 0,72	55.06 ± 15.76	29.88 ± 8.53
LI	10.50 ± 0.56*[de]	0,22 ± 0,06[cde]	10.62 ± 0.78	4,22 ± 0,71[de]	54.06 ± 18.32[cde]	48.47 ± 16.42*
LIIa	10.16 ± 2.09*[cde]	0,27 ± 0,11*[de]	10.63 ± 0.98	4,67 ± 0,75	107.00 ± 69.88	41.25 ± 26.88[e]
LIIb	12.30 ± 2.12*[bce]	0,37 ± 0,22*[ae]	10.04 ± 0.64	4,55 ± 1.66[e]	157.00 ± 79.84*[a]	38.67 ± 10.52*[e]
LIII	19.64 ± 3.58*[abde]	0,38 ± 0,14*[abe]	11.52 ± 2.00	5.20 ± 1.00[ae]	265.44 ± 351.55*[a]	63.81 ± 48.98*
LIV	50.09 ± 13.30*[abcd]	1,07 ± 0,62*[abcd]	11.01 ± 2.74	17,97 ± 6.04*[abd]	457.00 ± 787.13*[a]	62.86 ± 31.40*[bc]

Abbreviations: TP, total proteins. Values are presented as mean ± SD.
*P < 0.05 differ statistically between the Group C and all LeishVet stages.
[a]P < 0.05 differ statistically between LeishVet stage LI and LeishVet stage LIIb.
[b]P < 0.05 differ statistically between LeishVet stage LI and LeishVet stage LIII.
[c]P < 0.05 differ statistically between LeishVet stage LI and LeishVet stage LIV.
[d]P < 0.05 differ statistically between LeishVet stage LIIa and LeishVet stage LIV.
[e]P < 0.05 differ statistically between LeishVet stage LIIb and LeishVet stage LIV.
[f] P < 0.05 differ statistically between LeishVet stage LIII and LeishVet stage LIV.

Table 2
Urinalysis results in healthy dogs (Group C) and dogs with leishmaniasis (Group L) at LeishVet stages included in this study.

	UPC	USG	uCysCc (µg/g)	uNAGc (UI/g)
Group C	0.07 ± 0.03	1044.23 ± 7.30	75.38 ± 50.8	1.64 ± 0.68
LI	0.14 ± 0.05*[bcde]	1031.19 ± 14.98*[e]	882.78 ± 730.57*[cde]	3.94 ± 2.23*[de]
LIIa	0.37 ± 0.15*[acde]	1029.08 ± 8.50*[e]	2921.53 ± 2662.54*[cde]	4.68 ± 2.33*[cde]
LIIb	0.936 ± 0.08*[abde]	1027.58 ± 12.40*[e]	7424.26 ± 7153.91*[abde]	2.84 ± 1.68*[bde]
LIII	2.68 ± 1.89*[abce]	1024.50 ± 10.24*[e]	11,191.90 ± 4659.04*[abce]	23.63 ± 11.39*[abce]
LIV	5.29 ± 3.26*[abcd]	1017.80 ± 6.60*[abcd]	39,982.15 ± 19,028.50*[abcd]	121.90 ± 80.45*[abcd]

Abbreviations: UPC, urinary ratio proteins/creatinine; USG, urine specific gravity; uCysCc, urinary ratio cystatin C/creatinine; uNAGc, urinary ratio NAG/creatinine. Values are presented as mean ± SD.
*P < 0.05 differ statistically between the Group C and all LeishVet stages.
aP < 0.05 differ statistically between LeishVet stage LI and all LeishVet stages.
bP < 0.05 differ statistically between LeishVet stage LIIa and all LeishVet stages.
cP < 0.05 differ statistically between LeishVet stage LIIb and all LeishVet stages.
dP < 0.05 differ statistically between LeishVet stage LIII and all LeishVet stages.
eP < 0.05 differ statistically between LeishVet stage LIV and all LeishVet stages.

total and non-azotemic non-proteinuric groups (sensitivity over 82.80 % and specificity over 72.70 % in total groups and sensitivity and specificity over 72,70 % in non-azotemic non-proteinuric group; Table 3). When this threshold is used, 3/17 of the dogs (17,65 %) surpassed the limit in the control group, 12/16 in LI (75 %) and 9/12 in LIIa (75 %).

Hence, uCysCc, uNAGc and to a lesser extent pCysC could be valuable diagnostic biomarkers of kidney disease when combined with positive CanL diagnosis compared to pCreat which remains below 1.4 in all the dogs included in control, LI and LIIa groups. To carry out the correlation study between the biomarkers, the dogs were divided into two groups: non-proteinuric dogs (UPC ≤ 0.5; LI and LIIa; Table 4), and proteinuric dogs (UPC > 0.5; LIIb, LIII and LIV; Table 5). In the group of non-proteinuric dogs, a positive correlation (r = 0.555) was found between UPC and uCysCc (p < 0.01), while no correlation was observed among UPC and uNAGc (r = 0.346; p > 0.05). In proteinuric dogs, a correlation (Table 5) was observed between UPC and uCysCc (r = 0.703; p < 0.001) and uNAGc (r = 0.708; p < 0.001). These dogs also exhibit a strong correlation between uCysCc with pCysC, (r = 0643; p < 0.001; Table 5) and with SDMA (r = 0.743; p < 0.001; Table 5). SBP was not significantly correlated with any of the biomarkers studied.

4. Discussion

The incidence of CKD in dogs with leishmaniasis varies according to the bibliography and depends on the criteria used. An elevated pCreat is often used to determine and monitor the degree of azotemia.

Increased pCreat and urea are indicative of advanced kidney damage, a frequent complication in dogs with CanL (Paltrinieri et al., 2016). The increase in pCreat has been observed in dogs included in stages LIIa, LIIb, LIII and LIV compared to the control group (Table 1). Kidney disease secondary to glomerulonephritis related to the deposition of immune complexes is known to be the main cause of mortality in dogs (Baneth et al., 2008). Besides, disorders in the plasma phosphorus concentration are often seen in CKD and have been associated with increased morbidity and mortality (Lucero et al., 2019). Coinciding with the previously mentioned literature, in our study plasma phosphorus values increased as the disease progressed, with the highest mean concentration observed in the LIV group (Table 1).

The results obtained in both hematology and blood biochemistry in our cohort of dogs are those expected in CanL. Anemia is one of the most common laboratorial findings. It has been described that approximately 60 % of infected dogs present this alteration, being generally a normocytic and normochromic anemia (Solano-Gallego et al., 2009). Regarding leukocyte alterations, they vary with the immune status of the animal, the severity of the clinical signs, as well as the presence of parasitosis or associated infections, and can consist in leukocytosis or leukopenia (Lacerda et al., 2017). Lymphopenia has been described as a response to the action of the parasite and is associated to its migration to target organs (Torrecilha et al., 2016). The data obtained in this study coincide with those found in the previously mentioned bibliography, since a decrease in the mean values of these cells has been observed from LIIa (Supplemental file 1).

The most common protein-related disorders are in CanL affected dogs is hypoalbuminemia and hyperglobulinemia. The hypoalbuminemia is primarily attributable to the fact that albumin behaves as negative acute phase proteins and, conversely, globulins as positive acute phase proteins. Secondarily, the hypoalbuminemia can be attributable to renal loss in cases with glomerulopathy and severe proteinuria. This latter, contributes to a more unfavorable evolution of the disease

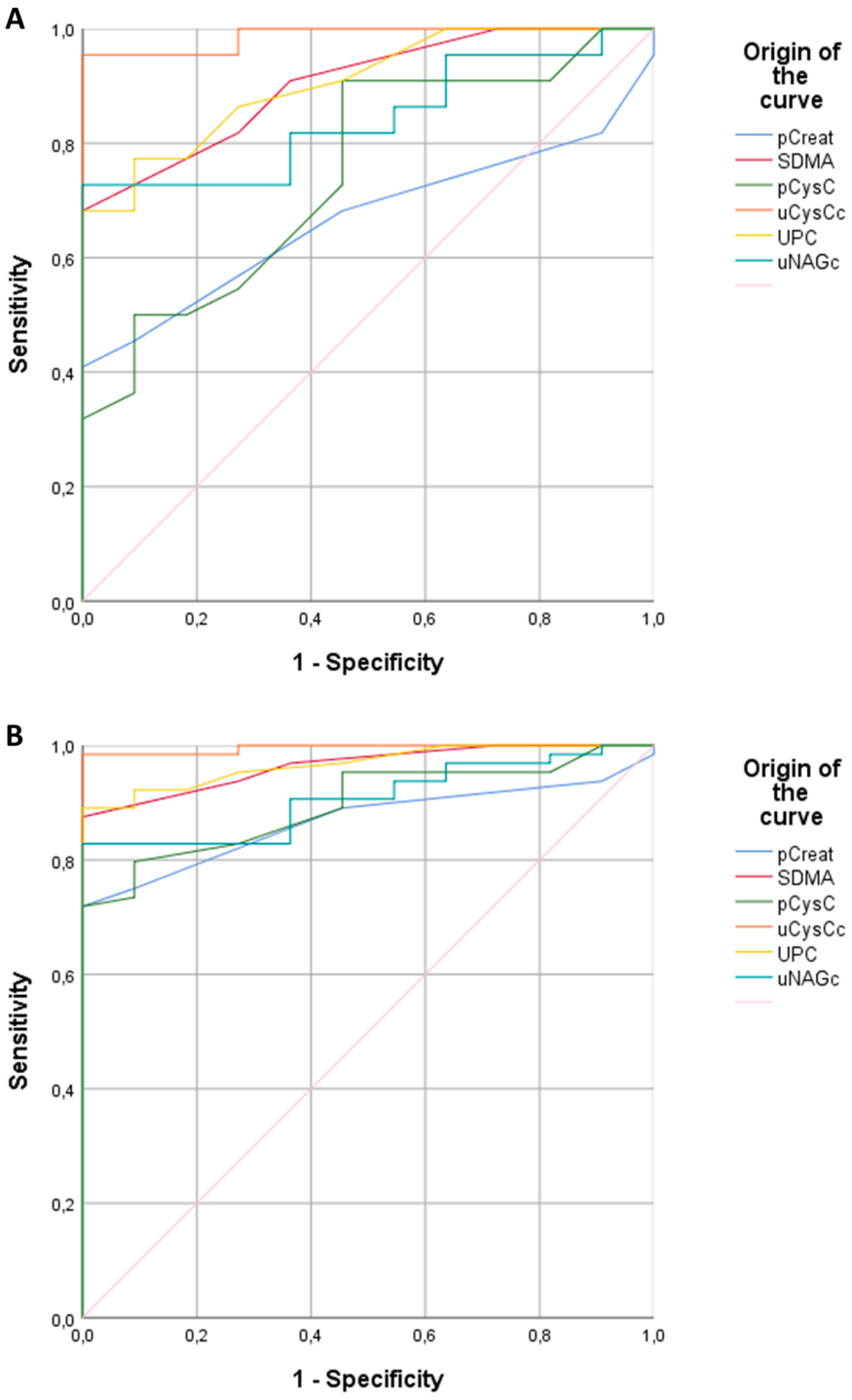

Fig. 1. Nonparametric receiver operating characteristics (ROC) plots of sensitivities and specificities of pCrea, SDMA, pCysC, uCysCc, UPC, and uNAGc levels for detecting kidney damage in the group of dogs with non-proteinuric and non-azotemic leishmaniasis (LeishVet stages LI and LIIa) (A) and in the group of dogs with proteinuric non-azotemic or azotemic leishmaniasis (LeishVet stage LIIb, LIII and LIV) (B).

(Meléndez-Lazo et al., 2018) and in our study both alterations were observed in all groups (Table 1).

Increasing interest in veterinary medicine on the usefulness of novel biomarkers of early kidney disease is being developed. Early diagnosis is crucial to set up a treatment as soon as possible when the chances of success are greatest. This goal is even more important in CanL, since it has been shown that the majority of infected dogs present kidney damage that cannot be detected using conventional analysis (Costa et al., 2003). One of the biomarkers included in our study was pCysC, as it has been demonstrated that the instauration of an early treatment in asymptomatic dogs using angiotensin-converting enzyme (ACE) inhibitors and low protein diets following the IRIS guidelines when a pCysC increase was observed in the absence of azotemia, enhanced the patient́s survival (Iwasa et al., 2019).

However, conflicting results regarding the ability of blood CysC to detect early impaired kidney function compared to creatinine have been reported. De Oliveira et al., in 2018, in a study carried out in 59 untreated dogs naturally infected with *Leishmania infantum*, did not find CysC useful in the diagnosis of CKD, since all the animals that presented increased CysC also presented a creatinine concentration > 1.4 mg/dl

Table 3
The cut-off values, sensitivity, and specificity of each parameter for the prediction of renal disease in dogs with leishmaniasis divided based on the presence or absence of proteinuria and azotemia.

	All dogs in study (Groups LI to LIV)					
	pCreat	SDMA	pCysC	UPC	uCysCc	uNAGc
AUC	0.868	0.964	0.896	0.967	0.996	0.908
95% IC	0.788–0.948***	0.927–1000***	0.822–0.970***	0.931–1000***	0.986–1000***	0.840–0.975***
Cut-offs	>1.37 mg/dL	>13 μg/dl	>0.22 mg/l	>0.20	> 258.85 μg/g	>2.25 IU/g
Sensitivity (%)	50.00	67,80	79.70	82.80	98.40	82.80
Specificity (%)	100	100	91.00	100	100	72.70
	Non-proteinuric[1] and non-azotemic[2] dogs (Groups LI and LIIa)					
AUC	0.667	0.903	0.748	0.905	0.988	0.843
95% IC	0.487–0.848	0.803–1000***	0.575–0.921*	0.806–1000***	0.959–1000***	0.710–0.976**
Cut-offs	>1.05 mg/dL	>10.50 μg/dl	>0.21 mg/l	>0.20	>258.85 μg/g	>2.25 UI/g
Sensitivity (%)	45.50	58.50	54.50	50.00	95.50	72.70
Specificity (%)	90.90	100	72.70	100	100	72.70

Abbreviations: pCreat, plasmatic creatinine; pCysC, plasmatic cystatin C; UPC, urinary ratio proteins/creatinine; uCysCc, urinary ratio cystatin C/creatinine; uNAGc, urinary ratio NAG/creatinine; AUC = area under the ROC curve. IC = confidence interval.
[1]UPC < 0.5.
[2]pCreat < 1.4 mg/dl.
* P < 0.05.
* * P < 0.01.
* ** P < 0.001.

Table 4
Correlation between the biomarkers studied in the group of dogs with non-proteinuric[1] and non-azotemic[2] leishmaniasis (LeishVet stages LI and LIIa).

	Albumin	pCreat	SDMA	pCysC	USG	UPC	uCysCc	uNAGc
Albumin		-0.027	-0.121	-0.331	0.302	-0.171	-0.478*	-0.013
pCreat	-0.027		-0.027	0.464**	-0.018	0.616***	0.204	0.160
SDMA	-0.121	0.027		0.192	-0.127	0.112	0.164	0.148
pCysC	-0.331	0.464**	0.192		-0.130	0.269	0.317	0.129
USG	0.302	-0.018	-0.127	-0.130		-0.142	-0.546**	-0.230
UPC	-0.171	0.616***	0.112	0.269	-0.142		0.555**	0.346
uCysCc	-0.478*	0.204	0.164	0.317	-0.546**	0.555**		0.196
uNAGc	-0.013	0.160	0.148	0.129	-0.230	0.346	0.196	

Abbreviations: pCreat, plasmatic creatinine; pCysC, plasmatic cystatin C; UPC, urinary ratio proteins/creatinine; uCysCc, urinary ratio cystatin C/creatinine; uNAGc, urinary ratio NAG/creatinine.
[1]UPC < 0.5.
[2]pCreat < 1.4 mg/dl.
* P < 0.05.
* * P < 0.01.
* ** P < 0.001.

Table 5
Correlation between the biomarkers studied in the group of dogs with proteinuric[1] non-azotemic or azotemic leishmaniasis (LeishVet stage LIIb, LIII and LIV).

	Albumin	pCreat	SDMA	pCysC	USG	UPC	uCysCc	uNAGc
Albumin		-0.171	-0.279	-0.313*	0.101	-0.273*	-0.160	-0.098
pCreat	-0.171		0.981***	0.776***	-0.454***	0.623***	0.756***	0.777***
SDMA	-0.279	0.981***		0.773***	-0.490***	0.617***	0.743***	0.791***
pCysC	-0.313*	0.776***	0.773***		-0.529***	-0.474***	0.643***	0.617***
USG	0.101	-0.454***	-0.490***	-0.529***		-0.439***	-0.398**	-0.399**
UPC	-0.273*	0.623***	0.617***	0.474***	-0.439***		0.703***	0.708***
uCysCc	-0.160	0.756***	0.743***	0.643***	-0.398**	0.703***		0.741***
uNAGc	-0.098	0.777***	0.791***	0.617***	-0.399**	0.708***	0.741***	

Abbreviations: pCreat, plasmatic creatinine; pCysC, plasmatic cystatin C; UPC, urinary ratio proteins/creatinine; uCysCc, urinary ratio cystatin C/creatinine; uNAGc, urinary ratio NAG/creatinine.
[1]UPC > 0.5.
* P < 0.05.
* * P < 0.01.
* ** P < 0.001.

(de Oliveira Frazilio et al., 2018). However, Ko et al. (2021), recommend its use for early detection of CKD and Kim et al. (2020), demonstrated a statistically significant increase in IRIS 1 in dogs, before creatinine concentration was elevated. Miyagawa et al. (2020), demonstrated the usefulness of this biomarker to detect decreases in GFR in dogs but mostly when the dog's weight was below 20 Kg. In our setting, a progressive increase in the concentration of pCysC as CanL progressed was observed starting from the LIIa group in which non-azotemic and non-proteinuric dogs were included (pCreat < 1.4 mg/dl and UPC < 0.5). Liu et al. (2018), demonstrated that serum CysC shows high

individual variability coinciding with our findings. Consequently, Liu et al. (2018) performed their study in healthy dogs and recommend caution when interpretating CysC at limit values, as the altered results overlapped those of CKD affected animals.

It is worth mentioning that in the present report, pCysC concentrations were determined using a latex turbidimetric commercial kit that yielded a mean value of 0.17 ± 0.06 mg/l in healthy dogs. Our results were similar to those measured with a particle-enhanced nephelometric immunoassay (Jonkisz et al., 2010) and with similar methods (Muñoz et al., 2017; Iwasa et al., 2019; Miyagawa et al., 2020). This value, as previously observed Miyagawa et al. (2020), is lower that than those determined by ELISA or by polysterene particle-enhanced turbidometric assay (Wehner et al., 2008; Miyagawa et al., 2009) and hence, when evaluating pCysC, the technique used needs to be taken into account.

It has been previously described that serum CysC sensitivity is higher than that of creatinine, while the latter presents greater specificity (Wehner et al., 2008; Ko et al., 2021), so it is recommended to interpret both parameters together. Interestingly, this observation is overall true in non-proteinuric dogs (pCreat: sensitivity = 45.50 % and specificity = 90.90 %; pCysC: sensitivity = 54.50 % and specificity = 72.70 %; Table 3). This becomes more evident when studying the entire population of dogs with leishmaniasis (pCreat: sensitivity = 50.00 % and specificity = 100 %; pCysC: sensitivity = 79.70 % and specificity = 91.00 %; Table 3). pCysC demonstrates a moderate correlation with pCreat in non-proteinuric dogs (0.464; $p < 0.01$; Table 4), and a strong correlation with pCreat (0776; $p < 0.001$; Table 5) and SDMA (0773; $p < 0.001$; Table 5) in proteinuric dogs, biomarkers that are related to GFR.

The existing data in canine medicine on the concentration of CysC in urine are scarce. The kidney disease generally observed in CanL is mainly related to impaired glomerular function but altered tubular function has also been demonstrated (Zatelli et al., 2003; Solano-Gallego et al., 2007). Due to its small size, CysC is freely filtered through the glomerulus and is reabsorbed in the proximal tubules by megalin-mediated endocytosis where it is completely catabolized (Kaseda et al., 2007). The concentration of CysC increases in the urine when the tubular cells are not able to metabolize or absorb the protein, mainly the ones of the proximal convoluted tubule (Tenstad et al., 1996). Monti et al. (2012), stated that urinary CysC in dogs should be considered as a complementary assay to estimate tubular function instead of altered glomerular function.

In our work, an increase in uCysCc has been observed already in LI and LIIa groups (93,75 % and 100 % of the dogs respectively presented a higher value than those obtained in the dogs in the control group). This fact is interesting considering the high individual variability observed for uCysC and uNAG in LI and LIIa stages obtained in the present study reflecting that varying degrees of renal damage which develop among patients even in the initial stages of CanL. Hence, its sensitivity to detect impaired renal function in dogs with CanL is high and significantly better than the rest of the biomarkers studied in the group of non-proteinuric dogs (pCreat, SDMA, pCysC, UPC and uNAGc), even increasing its usefulness in dogs with proteinuria. These results coincide with those described by Dias et al. (2020) and disagree with those provided by García-Martínez et al. (2015).

Thielemans et al. (1994), demonstrated in a rat model that albuminuria can lead to dysfunction in the reabsorption of low molecular weight proteins, including CysC through a competitive mechanism at the tubular level. Tkaczyk et al. (2004), described the association between proteinuria and urinary CysC excretion in the idiopathic nephrotic syndrome in children. In addition to the mechanisms described, the “solvent drag phenomenon” has also been suggested, in which CysC in the ultrafiltrate is transported back from the renal tubule by the flow of water (Kim et al., 2020).

Although in the present study a statistically significant correlation has been observed between the UPC and the uCysCc ratio, it is moderate in non-proteinuric dogs ($r = 0.555$; $p < 0.01$, Table 4) compared to that in dogs with proteinuria ($r = 0.703$; $p < 0.001$, Table 5). Therefore, the increase in the concentration of uCysCc can be considered an indicator of tubular alteration at least in non-proteinuric non-azotemic dogs diagnosed with CanL. Besides, this biomarker has also demonstrated high sensitivity and specificity in dogs with CanL, both in non-azotemic animals with UPC < 0.5 (sensitivity = 95.50 % and specificity = 100 %; Table 3), as well as in azotemic and proteinuric dogs (sensitivity = 98.40 % and specificity = 100 %; Table 3), demonstrating its usefulness.

The uNAGc values obtained in the present study in healthy dogs varied from 0 to 2.60 IU/g, coinciding with those provided by Brunker et al. (2009), that ranged between 0.02 and 3.63 IU/g.

NAG is a tubular lysosomal enzyme which is a well-known biomarker of tubular injury. When tubular damage occurs, NAG is released resulting in subsequent increases in the enzyme concentration in urine (Sato et al., 2002). NAG has a high molecular weight (150 kDa) and, therefore, in physiological conditions, it does not cross the glomerular barrier. This biomarker has been evaluated in dogs with CKD, demonstrating a urinary increase compared to healthy dogs (Sato et al., 2002; Smets et al., 2010; Hokamp and Nabity, 2016). Interestingly, several reports establish a better correlation between the uNAGc ratio and glomerular injury compared to interstitial damage in proteinuric animals, demonstrating the existence of a correlation between the UPC and the uNAGc ratio by means of a not well understood mechanism (Nabity et al., 2012). This phenomenon, has been suggested to be related with an increased lysosomal turnover secondary to proteinuria (Sato et al., 2002; Smets et al., 2010; Nabity et al., 2012). However, Hokamp et al. (2016), demonstrated an increase in uNAGc in dogs in the absence of histological evidence of tubulointerstitial damage, and proposed that the increase observed could be related to abnormal pass of NAG by the injured glomerular filtration barrier (Hokamp and Nabity, 2016). This theory could in part explain our results, as in dogs with proteinuria (UPC>0.5), a statistically significant correlation ($r = 0.708$; $p < 0.001$) has been demonstrated between UPC and the uNAGc ratio (Table 5). Furthermore, these authors also described that while uNAGc has been used to detect tubular damage in cases of acute kidney injury, it might also be useful to detect glomerular damage in proteinuric patients affected with chronic nephropathies (Hokamp and Nabity, 2016).

Interestingly, in our study the aforementioned correlation was not observed in non-proteinuric dogs (UPC <0.5) with a correlation among uNAGc and UPC of $r = 0.346$ ($p > 0.05$; Table 4). Besides, an increase in uNAGc was observed in 75 % of the dogs in LI and in 83,34 % of the dogs in LIIa. The cuttoff value of NAG in the group of dogs with leishmaniasis studied was 2.25 UI/g, presenting a sensitivity of 82.80 % and specificity of 72.70 % (Table 3). Hence, as the majority of dogs infected with leishmaniosis develop renal damage, these patients should be subjected to periodic revisons. In these exams, aside from the classical biochemical parameters (including urea, creatinine and SDMA), we strongly recommend to complete the analysis with the use of new renal biomarkers of renal disease such as uCysCc and uNAGc. The use of these novel biomarkers may help to better assess the varying degrees of glomerular and/or tubular dysfunction that the patients present even when enclosed within the same LeishVet stage.

5. Conclusions

Cystatin C in plasma, uCysCc and uNAGc have been demonstrated to be reliable biomarkers in advanced CKD in CanL affected dogs. uCysCc and uNAGc have proved their usefulness to detect early renal damage in dogs in which a positive CanL diagnosis has been provided (Stages LI and LIIa of LeishVet classification). pCysC can detect alterations in the GFR, while uCysCc and uNAGc are related to interstitial, glomerular and tubular injury. uCysCc presents better sensitivity to detect impaired renal function in non-proteinuric dogs diagnosed with CanL compared to the rest of the biomarkers included in the present work. Hence, we recommend to complete the routine analysis of dogs diagnosed with leishmaniosis using new renal biomarkers of renal disease such as uCysCc and uNAGc.

Limitations

Direct measurement of GFR has not been performed. Instead, it was estimated by using indirect GFR markers. The weight of the dogs studied was also not considered for the interpretation of the concentration of plasmatic CysC and renal biopsies were not available.

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CRediT authorship contribution statement

Rafael Barrera, Beatriz Macías-García and **Patricia Ruiz:** Conceptualization. **Rafael Barrera** and **Patricia Ruiz:** Formal analysis. **Beatriz Macías-García** and **Rafael Barrera:** Funding acquisition. **Patricia Ruiz, Ángela Durán, Francisco Javier Duque, Mario Alberto González, José Ignacio Cristóbal, Paloma Nicolás, Eva María Pérez-Merino, Beatriz Macías-García, Rafael Barrera:** Investigation. **Rafael Barrera** and **Patricia Ruiz:** Writing – original draft. **Patricia Ruiz, Ángela Durán, Francisco Javier Duque, Mario Alberto González, José Ignacio Cristóbal, Paloma Nicolás, Eva María Pérez-Merino, Beatriz Macías-García, Rafael Barrera:** Validation. All the authors reviewed and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Beatriz Macias-Garcia reports financial support was provided by Spain Ministry of Science and Innovation.

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Appendix A. Supporting information

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