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Characterization of proteinuria in treated and untreated dogs naturally infected with *Leishmania* sp.[1]

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ABSTRACT.- Valle P.G., Veado J.C.C., Ribeiro V.M., Teles P.P.A., Costa Val A.P., Dornelas L.R.S.M., Fonseca L.A. & Paes Leme F.O. 2021. **Characterization of proteinuria in treated and untreated dogs naturally infected with *Leishmania* sp.** *Pesquisa Veterinária Brasileira 41:e06905, 2021.* Departamento de Clínica e Cirurgia Veterinárias, Universidade Federal de Minas Gerais, Av. Presidente Antônio Carlos 6627, Cx. Postal 567, Pampulha, Belo Horizonte, MG 30270-901, Brazil. E-mail: pillarvalle@yahoo.com.br

In the search for an early biomarker of renal injury, this study aimed to determine the urinary protein profile of dogs with leishmaniasis without treatment and treated as determined by Brazilian legislation. The identification of proteinuria, its classification and the circumstances in which it takes place instigated this study. For this, 30 dogs from an outpatient clinic at a Veterinary Hospital in Belo Horizonte were evaluated. All animals underwent clinical and laboratory tests, which included renal biomarkers. The proteins were characterized using the SDS-page electrophoresis technique, and thus, a urinary protein profile was developed comparing patients considered clinically healthy with dogs infected with leishmaniasis that were under treatment and with untreated infected dogs. The results showed that the hematological and biochemical parameters showed similar behavior between the groups of healthy dogs and dogs with leishmaniasis treated, however a very heterogeneous pattern of urinary proteins can be observed and differed between healthy animals and animals with leishmaniasis, as well as between treated and untreated animals. The results suggest that the classification of proteinuria can be a tool that helps in the staging of animals infected with L. infantum and can differentiate them as to the severity of existing kidney injuries.

INDEX TERMS: *Leishmania* sp., dogs, kidney, proteinuria, electrophoresis, leishmaniasis.

RESUMO.- [**Caracterização da proteinúria de cães tratados e não tratados, naturalmente infectados com *Leishmania* sp.**] Na busca por um biomarcador precoce de injúria renal, este trabalho teve como objetivo determinar o perfil proteico urinário de cães infectados com leishmaniose sem tratamento e tratados conforme determina a legislação brasileira. A identificação da proteinúria, sua classificação e as circunstâncias em que ocorrem instigaram este estudo. Para tanto, foram avaliados 30 cães oriundos do atendimento clínico ambulatorial de um Hospital Veterinário em Belo Horizonte. Todos os animais passaram por exame clínico e laboratorial, que incluíram biomarcadores renais. As proteínas foram caracterizadas através da técnica de eletroforese por SDS-PAGE, e assim, foi elaborado um perfil proteico urinário comparando pacientes considerados clinicamente hígidos, com cães infectados por *Leishmania* (*L.*) *infantum* e que estavam sob tratamento e cães infectados não tratados. Os resultados demonstraram que os parâmetros hematológicos e bioquímicos apresentaram comportamento semelhante entre os grupos de cães hígidos e de cães infectados com *L. infantum* tratados, entretanto um padrão muito heterogêneo de proteínas urinárias pode ser observado e diferiu entre animais hígidos e animais com leishmaniose, assim como entre os animais tratados e não tratados. Os resultados sugerem que a classificação da proteinúria pode ser uma ferramenta que auxilia no estadiamento de animais infectados

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por *L. infantum* podendo diferenciá-los quanto à gravidade de lesões renais existentes.

TERMOS DE INDEXAÇÃO: *Leishmania* sp., cães, caninos, rim, proteinúria, eletroforese, leishmaniose.

INTRODUCTION

Advances in understanding the cellular and molecular mechanisms involved in acute kidney injury have clarified previously unknown pathophysiological mechanisms. However, this knowledge has not yet resulted in the application of specific or effective treatments to prevent or eliminate the action of etiologic agents, making it difficult to control the progression of acute kidney injury. This justifies the efforts made in recent decades to recognize and use diagnostic tools and staging, especially in the search for new markers of kidney injury and dysfunction (Magro & Vattimo 2007).

Considering that glomerular changes can be classified as glomerular kidney injury and that the main forms of glomerulonephritis are immune-mediated (Veado et al. 2014), patients with periodontal disease, pyometra, infectious hepatitis, ehrlichiosis, and canine visceral leishmaniasis (CVL) are expected to present glomerular changes. This is due to the production of antibodies, which result in the formation and deposition of glomerular immune complexes, favoring acute renal failure (ARF) and compromising the quality and survival of patients (Grauer 2010, Chew et al. 2011). CVL is considered endemic in the broader geographic area of Belo Horizonte, State of Minas Gerais, Brazil, where this study was conducted. CVL causes a polymorphic clinical picture, in which kidney diseases are the leading causes of death (Zatelli et al. 2003).

In the search for early protein biomarkers of renal impairment, and in the expectation of defining the nephron segment involved, several techniques have been employed, including electrophoresis and proteomics (Thongboonkerd 2004). However, no study was found using these techniques as a discriminatory tool for CVL, making the present study a pioneer in this subject. According to Hokamp et al. (2018), it is possible to classify lesions into glomerular or tubular, and to grade them from the number of protein bands and the molecular weights (MWs) of these proteins. For this purpose, denaturation mediated by sodium dodecyl sulfate and by the negative charge of urinary proteins is used, allowing mass-dependent migration during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). According to Paltrinieri et al. (2016), the results correlate well with the histopathology of kidney biopsies, especially for the differentiation between glomerular damage and severe tubulointerstitial damage.

There is a consensus that the rate of urine protein excretion is a marker of kidney disease progression. This consensus points out that some urinary proteins play an important role as biomarkers of kidney disease or toxicity (Polzin et al. 2005, Lefebvre 2011). It is known that albuminuria is an important indicator of loss of permeability of glomerular capillaries and, in practice, may indicate early glomerular and tubular damage (Dibartola 2004, Polzin et al. 2005).

However, a diagnosis of kidney injury can only be made by associating the clinical picture with the composition and amount of proteins present in the urine. Thus, the objective of the present study was to trace the urinary protein profile of healthy dogs, of dogs infected with *Leishmania* (*L.*) *infantum* and treated for this infection, and of untreated infected dogs, using the SDS-PAGE technique in association with the renal profile of these patients.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Ethics Committee of the "Universidade Federal de Minas Gerais" (UFMG) under protocol number 88/2018.

Animals studied and site of the study. For this study, 30 dogs were randomly selected among those treated at a veterinary hospital in Belo Horizonte. This hospital is considered a reference in the care of animals with leishmaniasis; it was chosen in part based on the demand for care and availability of data, but also and mainly because it presents a diverse sample with a broad geographic distribution. Samples were collected from dogs of different ages (between 2 and 14 years), breeds (Poodle, Maltese, Pinscher, mixed-breed, German Spitz, Basset Hound, Dachshund, Cocker Spaniel, Bulldog, Golden Retriever, Border Collie, Lhasa Apso, Chihuahua, Fox Paulistinha, Yorkshire, and Schnauzer), and weights (between 2.200 and 31.300kg), neutered and intact, male and female, with tutors living in the Belo Horizonte metropolitan area. The inclusion criteria were animals seen at the study hospital from September 2019 to February 2020 with a positive result for leishmaniasis, whether treated or not; if treated, the protocol established by the "Grupo de Estudo em Leishmaniose Animal" (BRASILEISH - Brazilian Study Group on Animal Leishmaniosis) must have been followed. Animals considered healthy by clinical and laboratorial criteria were also included as a control group. Patients presenting clinical and laboratory changes and testing positive for any blood parasitosis were excluded, as were those with chronic kidney disease (CKD), with changes in ultrasound imaging, or diagnosed with infection. Samples with findings of hemolysis, lipemia, and jaundice were rejected. All patients underwent ultrasound examination.

Experimental groups. Aiming to compare the groups, the 30 dogs included were divided into three study groups with 10 animals each, namely: a control group (CG) composed of CVL-negative dogs; a group of animals infected with *L. infantum* and treated (TLG) according to the current Brazilian legislation, following the BRASILEISH protocol; and a group of animals infected with *L. infantum* that were not treated (NTLG). SNAP® (IDEXX Laboratories, Inc., Westbrook/ME, USA), indirect immunofluorescence reaction, enzyme-linked immunosorbent assay (ELISA), and quantitative polymerase chain reaction (PCR) tests were used to diagnose positivity.

Sample collection. Blood samples were collected after antisepsis and stored in bottles containing ethylenediamine tetra-acetic acid - EDTA (10%) for the blood count, and in bottles with coagulation activator gel to obtain serum immunological and biochemical analyses, according to the procedures adopted in the routine care of the veterinary hospital. Urine samples were aseptically collected by ultrasound-guided cystocentesis and processed within a maximum of 12 hours for the urinalysis, and within 30 minutes or kept under refrigeration for a maximum of 2 hours for the urine culture. After centrifugation (1000 rpm for 10 minutes), the supernatant of the urine samples was stored in microtubes (1-mL aliquots) and stored in an ultra-low temperature freezer at -80°C for the subsequent execution of the SDS-PAGE procedure.

Ultrasound examination. All ultrasound examinations were performed by the same examiner, using a Saevo FT422 device (Alliage S/A Indústrias Médico-Odontológicas, Ribeirão Preto/SP, Brazil). The technique used in the kidney ultrasound evaluation was described by Nyland et al. (2005). Kidney size (normal, increased, decreased), renal contour (regular, irregular), cortical echogenicity

(increased, normal), and corticomedullary definition (normal, decreased, absent) were evaluated.

Hematological assessment. After reaching room temperature, the blood samples with EDTA were homogenized and introduced into a ProcyteDx device (IDEXX Laboratories, Inc.), which through impedance, laser flow cytometry, and optical fluorescence determined the hematocrit, hemoglobin, and the total erythrocyte, leukocyte, and platelet counts. A blood smear was also made from each sample, and after staining (Romanowsky) it was used for blood microscopy and differential leukocyte count.

Serum and urine biochemistry. For serum and urinary biochemical analyses, a Catalyst One biochemical analyzer with a Chem 10 Clip panel (IDEXX Laboratories, Inc.) was used to determine serum glucose, creatinine, blood urea nitrogen (BUN), urea/creatinine blood fraction, total serum proteins, albumin, globulin, and albumin/globulin fraction. In addition to Chem 10 CLIP, a slide was used for serum dimethylarginine.

For urinary biochemistry, the sample was transferred to a graduated conical tube (CRAL Artigos para Laboratório Ltda., Cotia/SP, Brazil). After centrifugation (in a centrifuge provided by Coleman Equipamentos para Laboratório Comércio e Importação Ltda., Santo André/SP, Brazil), the supernatant was analyzed in Catalyst One to determine urinary protein, creatinine, and protein/creatinine ratio.

Urinalysis. Urine samples were analyzed according to the standard protocol, and density was determined by refractometry. Alere® Urofita® urinalysis strips (Abbott Laboratories, Abbott Park/IL, USA) were used for the chemical analysis. Urinary sedimentoscopy and optical microscopy were also performed, and macro elements such as cylinders and micro elements such as cells, bacteria, and crystals were evaluated.

Serology for leishmaniasis. Rapid tests (SNAP® *Leishmania*, IDEXX Laboratories, Inc.) were used to determine serum reactivity, following the manufacturer's instructions. Tests for dirofilariasis, anaplasmosis, ehrlichiosis, and Lyme disease were also performed using SNAP® 4Dx Plus® (IDEXX Laboratories, Inc.).

SDS-PAGE of urinary proteins. Urine samples were analyzed to determine the protein profile at the "Laboratório de Pesquisa em Patologia Clínica Veterinária" (LPPCV - Veterinary Clinical Pathology Research Laboratory) of the "Universidade Federal de Viçosa" (UFV), Brazil. The SDS-PAGE method was used for the qualitative evaluation of urinary proteins based on molecular weight, using a modification of the technique described by Laemmli (1970).

The molecular weights and concentrations of protein fractions were determined by computerized densitometric analysis, processing scans of the samples with LabImage 1D software (Loccus do Brasil Ltda., Cotia/SP, Brazil). Molecular weight markers of 200, 116, 97, 66, 45, 31, 21, 14, and 6kDa, in addition to purified proteins, were used to calculate molecular weights. Reference curves were created for the densitometric evaluation of the protein bands by reading a reference marker.

Once the reading area was defined based on the graph (the peak determines the true band, that is, the area closest to the molecular weight markers; the absence of a peak determines a shadow), the profile windows corresponding to the molecular weights that defined the measurement scores were separated, as shown in Figure 1.

Individual regions of the gel presented "tracks," and each track represented one animal, which presented different bands of proteins with different molecular weights, as shown in Figure 2. The areas under the curve for each band were selected, represented by the total optical density, allowing the relative percentage of the density of each protein band to be determined in relation to the total optical density.

When molecular weight proteins below 45kDa were present, scores of tubular proteins were considered, and when above 45kDa, scores of proteins indicating glomerular lesion were considered, according to Hokamp et al. (2018). Glomerular severity scores ranged from 0 to 3, where 0 represented normal or absent glomerular proteinuria; 1 = mild glomerular proteinuria; 2 = moderate glomerular proteinuria; and 3 = severe glomerular proteinuria. Tubular severity scores ranged from 0 to 4, where 0 represented normal or absent tubular proteinuria; 1 = minimal tubular proteinuria; 2 = mild tubular proteinuria; 3 = moderate tubular proteinuria; and 4 = severe tubular proteinuria.

For comparison between groups, the diversity and number of bands were also evaluated, as well as the molecular weight range in which the highest protein concentration (in mg/dL) was found. The selected intervals were 45-55kDa, 56-90kDa, 91-150kDa, 151-200kDa, and above 200kDa. The objective of these comparisons was to establish which interval had the best discriminatory pattern between groups.

Statistical analysis. A descriptive analysis of the patients' profile was performed based on the data table. This descriptive analysis

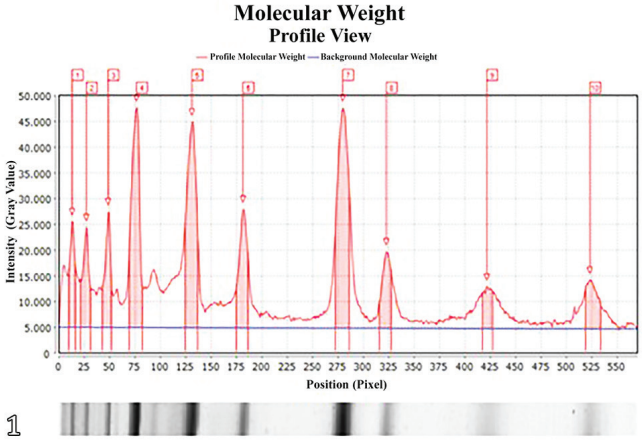


Fig.1. Molecular weight profile window in a reading obtained from dog urine samples in SDS-PAGE gel.

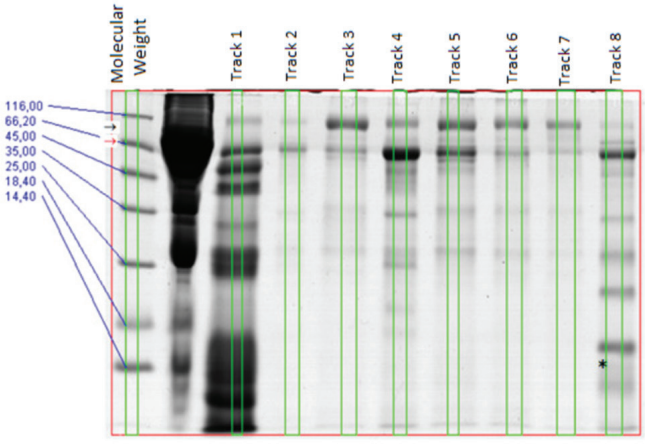


Fig.2. Representative urine samples from dogs with a broad spectrum of proteinuria, presented using SDS-PAGE to demonstrate the application of the gel scoring system. Tracks 1 to 8 represent urine samples from dogs with proteinuria. Track 1 (NTLG): glomerular score = 1, tubular score = 4. Tracks 2, 3, 5, and 6 (CG): glomerular score = 1, tubular score = 1. Tracks 4, 7, and 8 (TLG): glomerular score = 1, tubular score = 2. Prostatic fluid proteins observed in intact male dogs (asterisk). Albumin band (red arrow), Tamm-Horsfall protein band (black arrow).

aimed to evaluate all numerical and categorical variables. The normality of each variable was tested in its original unit and, when possible, a logarithmic transformation was applied. Relative and absolute frequencies for each class were calculated for categorical variables. Then, difference tests were applied individually for each numerical variable, seeking to identify which of these variables differed between the evaluated groups.

To test the differences between groups, regression models for categorical predictive variables were adjusted. For well-adjusted variables with normal distribution, a classic linear regression model (ANOVA) was applied. For variables with positive values and asymmetric distributions, but discrepant values, adjustments were made using generalized linear models with gamma distribution. To verify the significance of the difference between pairs, it was necessary to apply multiple (pairwise) comparison tests. For these tests, Tukey's correction was applied.

From the SDS-PAGE data, the molecular weight and concentration values for each animal were extracted from each of the bands. These values were then evaluated through a nonlinear regression method (local polynomial regression adjustment or local polynomial regression fitting), which smoothened the lines, allowing the visualization of the protein weights that presented the highest concentrations for each group.

The graphs show the means and their respective 95% confidence intervals for each group. The letters above each average represent multiple (pairwise) comparisons. Points that share at least one equal letter do not have significant differences between each other at 5% significance. All statistical analyses were performed using R software version 3.6.1 (R Core Team 2019).

RESULTS

The values of erythrocyte, hematocrit, and hemoglobin of NTLG dogs differed statistically from those of CG and TLG dogs, demonstrating the higher incidence of anemia in NTLG dogs. Although the mean number of dogs in the TLG was lower than that of healthy dogs, no statistical difference was observed.

The total and differential leukocyte counts did not follow any pattern in dogs with CVL. One animal presented leukocytosis with a left shift of the regenerative type; two dogs presented leukopenia from neutropenia, and 17 had a normal leukocyte profile. Neither the total leukocyte counts, nor the platelet counts differed statistically between the groups.

In the plasma protein profile of dogs with CVL, changes were observed mainly regarding the serum albumin/globulin ratio. In the NTLG, 30% of the animals had hypoalbuminemia, and 20% had hyperalbuminemia. However, three animals in this group showed an inversion of the A/G values, with ratios below 0.6. Differences were observed in the serum albumin concentration of NTLG dogs in relation to CG and TLG dogs, but the concentration was similar between CG and TLG dogs. Differences in the serum globulin concentration could only be observed between TLG and NTLG dogs; the globulin values of both groups of animals with CVL did not differ from the CG.

Only one TLG animal showed a very slight increase in the creatinine value (1.5mg/dL), but with a normal urea value. However, three NTLG animals had very high BUN values (171, 160, and 78mg/dL), and two of them also presented creatinine values (1.8 and 3.9mg/dL) above the reference range for canines (<1.4mg/dL). Urea values differed statistically between the dogs of all three groups, but creatinine values showed no difference. All animals in the CG were within the normal range for these variables.

A statistical difference was found between NTLG dogs and those of the CG and TLG in the analysis of the symmetrical dimethylated arginine (SDMA) variable. Three NTLG animals had elevated SDMA values (19, 22, and 29µg/dL); two of these animals also had increased creatinine levels (1.8 and 3.9mg/dL). Only one dog in the TLG presented slightly increased SDMA (17µg/dL), but without elevated creatinine.

The animals in this study retained the capacity to conserve urine concentration in all groups, and neither the pH nor the urinary density showed any statistical difference. Glycosuria and hemoglobinuria were observed in 10% of TLG animals, and hemoglobinuria in 10% of the CG. Therefore, these variables could not be statistically compared. Bilirubinuria at one cross (+) was observed in 90% of the CG animals, while 40% of the NTLG dogs had three crosses (+++). In the microscopic analysis, active sediment was observed in some CG animals.

Only one animal in the TLG and another one in the NTLG showed cylindruria. Transition cells were observed in urine samples from 60% of healthy dogs (CG), 30% of TLG dogs, and 17% of NTLG dogs. Kidney cells at one cross (+) could be observed in 10% of the urine samples analyzed in the CG and TLG, and in 50% of the NTLG samples.

One animal in the CG had a protein/creatinine ratio of 2.11; however, this animal presented active sediment in the urine and was therefore removed from the statistical analysis. Three NTLG animals had much higher protein/creatinine ratios (3.02, 16.88, and 15) than the range considered normal for dogs. A statistical difference was found in this ratio between NTLG and CG dogs, demonstrating that NTLG animals presented significant proteinuria.

CG animals present a smaller and less heterogeneous amount of urinary proteins, similar to TLG animals, but clearly differing from the urinary protein loss of NTLG dogs, as observed in Figure 3. A separation of molecular weight scores was performed to differentiate the origin of the proteinuria, whether glomerular

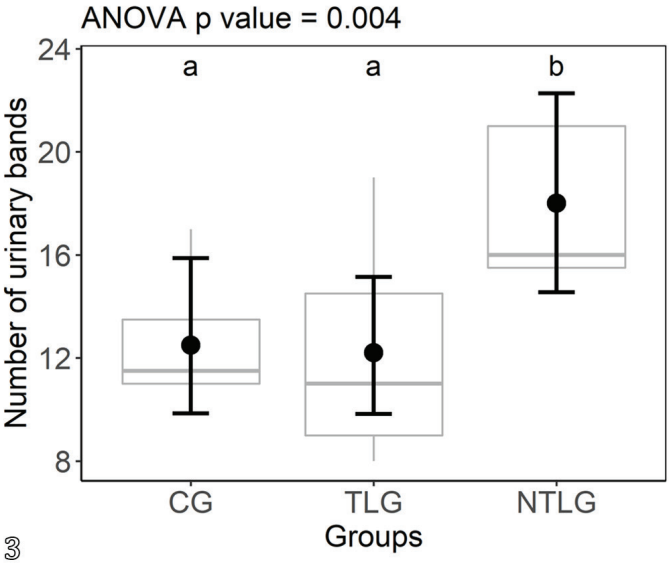


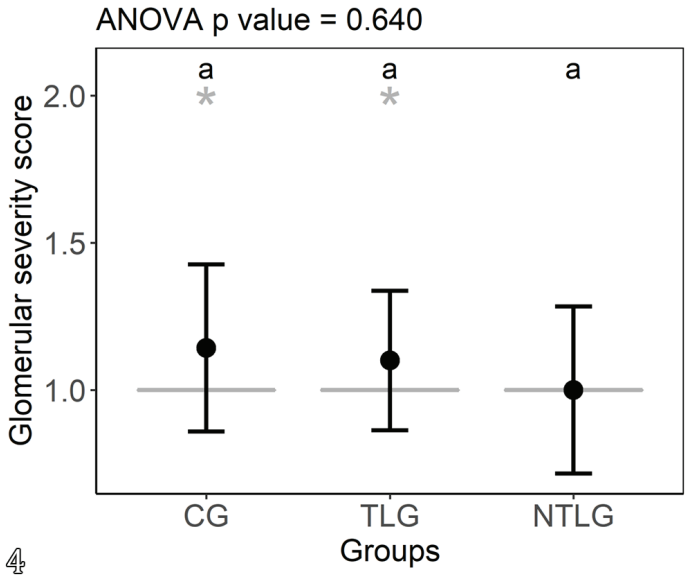
Fig.3. Graph demonstrating the means and their respective 95% confidence intervals for the number of urinary electrophoresis bands of healthy dogs (CG), treated *Leishmania infantum*-infected dogs (TLG), and untreated infected dogs (NTLG). Points that share at least one equal letter do not have significant differences between each other at 5% significance. Outlier individuals (asterisk).

(≥45kDa) or tubular (<45kDa). When analyzing the glomerular injury severity score in Figure 4-5, it can be observed that there was no statistical difference between the groups. Although the TLG score did not differ statistically from the NTLG score, the mean tubular severity score of dogs in the TG is very close to the CG score, as can also be observed in Figure 4-5.

Figure 6 shows the graph with the adjusted curves for each group, in which it can be observed that all three groups had a peak between 60 and 70kDa due to the presence of albumin, and the NTLG had the highest values. The CG also peaked at 40kDa, while the NTLG peaked at 50kDa. Other peaks of lower molecular weights could be verified in NTLG. For this reason, an analysis was performed refining the bands to better visualize the difference between the groups. This statistical analysis showed a significant difference for the presence of proteins with molecular weights in the 9-18kDa and 24-30kDa ranges (Fig.7-8), suggesting the presence of proteins such as cystatin C, alpha-1-microglobulin (α1M), retinol-binding protein (RBP), and interleukin-18 (IL-18) in this group of animals.

DISCUSSION

Nonspecific hematological and clinical changes are commonly observed in dogs with various diseases. For this reason, the diagnosis of leishmaniasis in dogs should be confirmed through PCR-based tools or through the identification and subsequent characterization of the parasite by reference isoenzymic methods. This is important to avoid incorrect diagnoses of other diseases with overlapping clinical and laboratory findings (Santos et al. 2007). However, changes in blood tests are important to follow the progression of



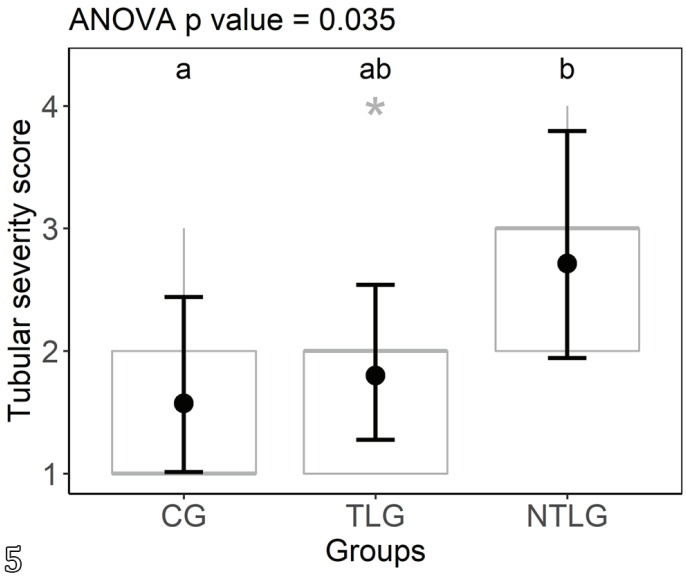


Fig.4-5. Graph demonstrating the means and their respective 95% confidence intervals for the glomerular and tubular severity scores of urinary electrophoresis bands of healthy dogs (CG), treated *Leishmania infantum*-infected dogs (TLG), and untreated infected dogs (NTLG). Points that share at least one equal letter do not have significant differences between each other at 5% significance. Outlier individuals (asterisk).

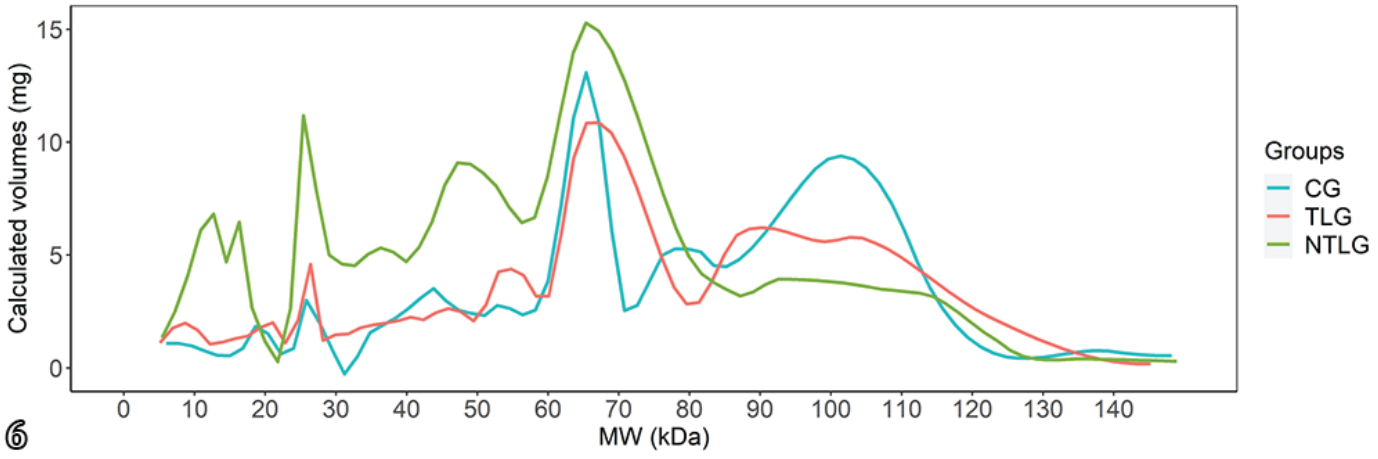


Fig.6. Average volumes calculated for each molecular weight of urinary proteins of healthy dogs (CG), treated *Leishmania infantum*-infected dogs (TLG), and untreated infected dogs (NTLG).

numerous diseases, functioning as a biomarker of clinical, laboratory, and post-therapeutic prognosis, as described by Cardoso (2018) and Braz et al. (2018).

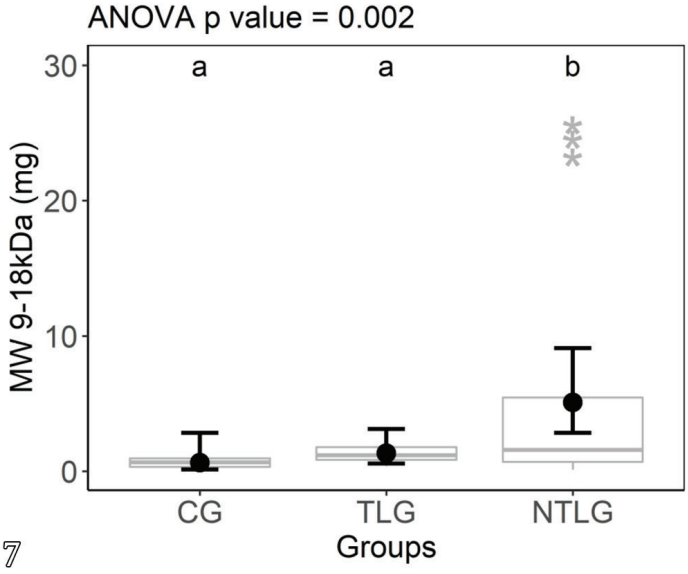
Although the mean erythrogram values of TLG dogs were lower than in CG dogs, no statistical difference was observed between these two groups. On the other hand, NTLG dogs showed statistically lower erythrogram results than those of the other two groups, which leads to the consideration that treatment is important in both the prevention and the progression of systemic diseases that are known to cause anemia and may be caused by this trypanosomatid. Other authors have also reported decreased erythrocyte counts, hematocrit, and hemoglobin in dogs with symptomatic leishmaniasis when compared to healthy animals (Coura-Vital et al. 2011, Freitas et al. 2012, Braz et al. 2015).

In the present study, the mean values for total and differential leukocyte counts and for platelets were generally not significantly influenced by the treatment or the evolution of the disease. Inflammatory infiltrates and spinal-cord parasitism do not seem to negatively influence leukocyte and platelet precursor cells, as is supposed to occur with red blood cells. Similar findings and discussions were conducted by Costa Val (2004).

Some authors have mentioned that proteinuria in dogs with CVL can be so severe that it alters proteinemia (Amusategui et al. 2003, Bonfanti & Zatelli 2004, Costa Val 2004), but such significant proteinuria would only occur in patients at more advanced stages of CKD (Less et al. 2005, Grauer 2010). Therefore, due to the selection criteria of the animals in this study, no significant changes were observed in the animals' serum proteins.

Of the NTLG animals, 20% presented azotemia and 30% uremia, with very high BUN results (171, 160, and 78mg/dL). No azotemia or uremia was observed in any TLG or CG animals. These findings corroborate the assertion that changes in kidney function, represented by increased serum concentrations of BUN and creatinine, are a relatively common finding in dogs with CVL (Paltrinieri et al. 2016). It is well documented in the literature consulted that BUN has some limitations in the assessment of renal function, since it is not steadily produced, and its reabsorption in the renal collecting ducts can be variable. Extrarenal factors can raise BUN levels, such as a high-protein diet and elevated tissue or protein catabolism. Conversely, a low-protein diet, liver disease, and malnutrition can lead to decreased BUN levels (Lefebvre 2011). Creatinine, still considered the best marker of glomerular filtration rate (GFR), has little variation because it is produced at a constant rate, depends exclusively on muscle mass and integrity, is freely filtered in the glomeruli, and is not reabsorbed in the renal tubules (Grauer 2010, Squires 2014). For all their advantages and limitations, BUN and creatinine have been considered late biomarkers. They generally change their serum concentration only when there is a loss of more than 60% of glomerular filtration capacity (Grauer 2010, Lefebvre 2011). Thus, it can be considered that NTLG animals had ARF in addition to kidney injury. According to some studies, approximately 50% of animals with CVL have azotemia (Amusategui et al. 2003). This value is slightly lower than what was observed in the present study. This finding can be justified by the difference in the staging of leishmaniasis. The present study included stage II and III dogs according to BRASILEISH.

The dosage of SDMA has already been used in the routine care of dogs and cats as an early biomarker of renal dysfunction and in the monitoring of nephropathic patients, especially in those whose serum creatinine and urea values are still within the reference range (CKD stage 1) (Nascimento et al. 2017). As with urea and creatinine, SDMA should always be interpreted in the light of clinical examination findings to rule out causes responsive to azotemia. In cases of subtle increases in serum SDMA, IRIS (2019) recommends studying other factors to corroborate or refute the diagnosis of CKD. Two NTLG animals with elevations of both SDMA and creatinine



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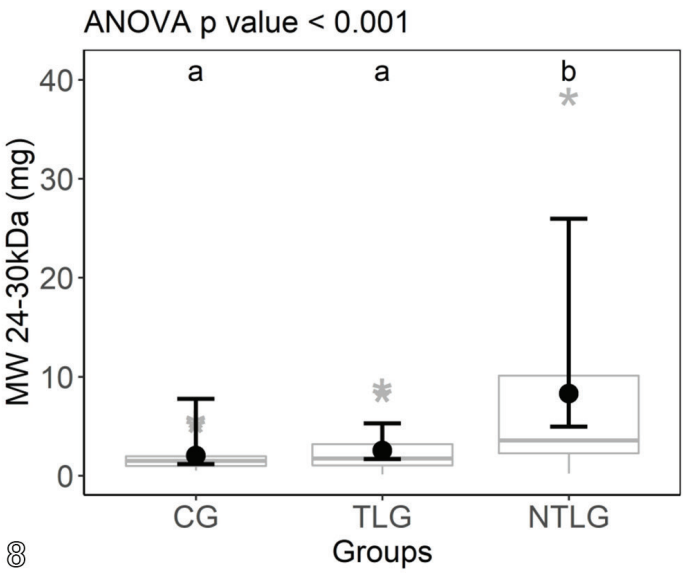

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Fig.7-8. Graph demonstrating the means and their respective 95% confidence intervals for the presence of proteins with molecular weight between 9 and 18kDa and between 24 and 30kDa in healthy dogs (CG), treated *Leishmania infantum*-infected dogs (TLG), and untreated infected dogs (NTLG). Points that share at least one equal letter do not have significant differences between each other at 5% significance. Outlier individuals (asterisk).

values also presented the highest protein/creatinine ratios, showing that even though these animals did not have changes in renal ultrasound, they already had grade II and III ARF, respectively, in addition to initial-stage CKD. One dog in the TLG group showed a slightly increased value of SDMA, but without an increase in creatinine. According to the IRIS recommendations (IRIS 2019), this dog should be monitored, and the results compared with those of the urinalysis, but the findings suggest that this dog suffers from kidney disease that has not yet led to an increase in creatinine, but may have caused a reduction in the GFR.

Among the urinalysis variables, it is essential to check the density, which is commonly normal or increased in ARF due to both pre- and postrenal causes, unlike what is observed in dogs with CKD, which are generally isosthenuric as a result of loss of functional reserve, leading to a reduced ability to concentrate urine that characterizes mild kidney failure if azotemia is not yet present (Squires 2014, Veado et al. 2014). Changes such as proteinuria, cylindruria, hematuria, and altered pH can be observed in both ARF and CKD (Veado et al. 2014). The animals in this study preserved their ability to concentrate urine in all groups. However, five animals (17%) showed an increase in urinary density (>1.045), but this increase should be evaluated in conjunction with the other parameters in the urinalysis. It is important to remember that variations in density and pH may also occur due to the patient's hydration status and high dietary protein consumption, respectively (Nakamae et al. 1980).

Bilirubinuria occurs whenever blood bilirubin is increased to levels higher than the renal threshold (Nakamae et al. 1980), which suggests liver function disorders or excessive hemolysis. However, unlike in other species, in dogs the kidneys play an active role in bilirubin metabolism. Renal tubular cells have all the necessary enzymes to produce bilirubin from the heme group and conjugate it, enabling its excretion. Consequently, the urine of a normal dog may contain minimal but detectable amounts of bilirubin (Grauer 2010, Squires 2014). This explains why 90% of the CG animals had one cross (+) of bilirubinuria, while 40% of NTLG dogs had three crosses (+++), signaling a disorder of the excretory liver function. Godoy et al. (2017) suggest that CVL should be included in the differential diagnosis of liver diseases, especially in areas where this disease is endemic.

Hyperphosphatemia occurs regularly in dogs with decreased GFR. Serum urea concentrations generally follow those of phosphorus. Oliveira et al. (2020) stressed the importance of quantifying phosphorus in these patients, even without azotemia, to direct an early therapeutic intervention when necessary. Regarding the serum phosphorus dosage, the TLG differed statistically from the NTLG, although all groups presented serum phosphorus levels within the reference values for the species (Kirsztajn 2007).

The evaluation of proteinuria is mandatory because it is a risk factor for the progression of kidney disease. The American College for Veterinary Internal Medicine (ACVIM) guidelines state that proteinuria should be evaluated in any dog suffering from a predisposing disease such as CVL (Paltrinieri et al. 2016). According to the literature, proteinuria is the most frequently described change in dogs with CVL, ranging from 70% to 100% of cases (Less et al. 2005, Harley & Langston 2012). The IRIS classification (2019) defines non-proteinuric dogs as having a protein/creatinine ratio <0.2; borderline proteinuria ranges from 0.2 to 0.5, and proteinuric dogs have a protein/creatinine ratio >0.5. However, it is essential to confirm the persistence of the increased protein/creatinine ratio on two or three different occasions, with a minimum 15-day interval, for proteinuria to be confirmed (Polzin et al. 2005). When evaluating the protein/creatinine ratio, although the groups with leishmaniasis did not show statistical difference between each other due to the heterogeneity of the NTLG results, 30% of the dogs in this group showed significant proteinuria, pointing to significant glomerular damage.

The analysis of urinary proteins by electrophoresis can guide clinicians in identifying the compromised renal compartment and assessing the severity of kidney damage by analyzing the characteristics and patterns of the bands that appear in the electrophoresis gel (Hokamp et al. 2018). Few studies with urinary electrophoresis are found in the literature for dogs, and none including dogs treated for CVL have been found in recent searches of available databases such as PubMed. The present study is, therefore, the first to brings results like these, in the expectation of characterizing proteinuria both quantitatively and qualitatively, predicting the location and severity of kidney injury in dogs infected with *Leishmania infantum*, both treated and untreated according to the BRASILEISH recommendations. The total number of bands differed among the groups, and this is by itself a significant finding, since animals in the control group had fewer bands and a less heterogeneous urinary protein profile.

Some studies have reported that up to 100% of dogs with CVL have proliferative glomerulonephritis, and almost 80% have interstitial nephritis (Albuquerque et al. 2008, Gomes et al. 2008). The mean molecular weight of the proteins behaved similarly, suggesting a tendency of NTLG dogs to have a higher mean loss of tubular proteins than dogs from the CG and TLG. Although the statistics showed no difference among the groups regarding the loss of proteins with a molecular weight greater than 151kDa, the highest number of animals with proteins with this molecular was also found in the NTLG, characterizing the presence of more intense glomerular lesions in this group's dogs.

Zatelli et al. (2003) state that the glomerular lesions that characterize kidney involvement in dogs with CVL determine the appearance of proteinuria, ranging from normal to pathological. The qualitative evaluation of these proteins plays a central role in the early diagnosis of glomerular conditions, which, according to those authors, are typically primary lesions detected during the course of immune-mediated pathological conditions. When analyzing the glomerular injury severity score in Figure 6, it can be observed that there was no statistical difference among the groups. The presence of significant values for this score in CG animals may indicate insidious, subclinical, or underdiagnosed processes. However, it is important to remember that these data should be analyzed in conjunction with other markers of kidney injury, such as the protein/creatinine ratio, which was within physiological parameters in the CG.

In the present study, NTLG animals showed a higher tubular severity score when compared to the other groups, corroborating literature reports that demonstrated that CVL causes disease-specific interstitial and tubular renal lesions regardless of glomerular alterations, similarly to what was

observed by Albuquerque et al. (2008) and Gomes et al. (2008). Zatelli et al. (2003) observed non-selective glomerular proteinuria (evidenced by the detection of albumin, transferrin, and IgG), and only 5% mixed proteinuria (both tubular and glomerular) in 95% of the dogs infected with CVL evaluated in that study. Paltrinieri et al. (2016) stated that low-molecular-weight proteinuria without signs of glomerular disease can be observed in CVL, possibly due to free light-chain proteins - that is, prerenal proteinuria associated with the production of highly activated antibodies, as opposed to tubular damage.

The values of the tubular score in the NTLG show the importance of measuring urinary gamma glutamyl transferase (GGT) in dogs with CVL, since its presence in the urine reflects losses from the microvilli of the proximal tubule cells, and is therefore an important marker of kidney injury that can demonstrate injury at an early stage. This injury may even precede glomerular damage, since kidney disease is always progressive and can become irreversible (Grauer 2010, Cowgill et al. 2016). Thus, urinary GGT can help to differentiate between tubular damage and prerenal proteinuria (that is, associated with antibody production, as suggested by Paltrinieri et al. [2016]). Frazilio et al. (2018) also stressed the importance of assessing the protein/creatinine ratio and enzymuria in dogs with CVL, even with normal renal excretion. Although the TLG did not differ statistically from the NTLG, the mean TLG score was very close to the mean CG score, indicating that treatment tends to reduce the degree of severity of the tubular injury caused by CVL.

As observed, the group of untreated animals showed statistical differences for the presence of urinary protein with molecular weights in the 9-18kDa and 24-30kDa ranges. Discriminating the proteins in these bands through proteomics may favor diagnosis and prognosis in these patients.

In humans, final urine is composed of approximately 40% albumin, 40% Tamm-Horsfall protein, and the remaining 20% consist of IgA, IgG, and κ and λ light chains (Morales 2002). No study defining the protein composition of the urine of healthy dogs was found, and it is fundamental to understand the protein dynamics in diseases. Thus, the starting point obtained through samples of dogs considered healthy in the present study is considered promising.

CONCLUSIONS

Under the conditions in which this experiment was conducted, and in the sample selected, it can be concluded that the renal injury caused by leishmaniasis was predominantly of glomerular grade 1 and tubular grade 3 origin, although treatment following the BRASILEISH guidelines keeps proteinuria close to the values of animals negative for leishmaniasis.

It is essential to include enzymuria and proteinuria assessment tests in the protocol for diagnosis, staging, and follow-up of leishmaniasis, even in patients with normal excretory function.

Conflict of interest statement.- The authors declare that there is no conflict of interest related to this research.

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