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Research paper

Evaluation of serum biomarkers and proteinuria for the early detection of renal damage in dogs with heartworm (*Dirofilaria immitis*)

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ABSTRACT

Glomerulonephropathy associated with *Dirofilaria immitis* (heartworm) is relatively frequent in infected dogs. Given the importance and the scarcity of studies focused on its prevalence and diagnosis, the objective was to determine the prevalence of proteinuria and functional indicators of glomerular filtration rate in dogs with heartworm disease and discuss its utility in the detection of renal impairment. Sera and urine from 47 infected dogs were analyzed in a reference laboratory. Urea, creatinine, plasma proteins and serum symmetric dimethylarginine (SDMA) were analyzed in sera, while the UPC ratio was performed in urine. Dogs were further evaluated for the presence/absence of microfilariae, pulmonary and systemic hypertension, and the parasite burden was assessed. The results showed that 19.1 % of dogs showed proteinuria (UPC $>$ 0.5) and 17 % showed borderline proteinuria (UPC 0.2 − 0.5). Creatinine and SDMA were high ($>$ 1.8 mg/dl and $\geq$ 18 µg/dl, respectively) in 4.2 % of dogs. UPC ratio was significantly increased in dogs with high parasite burden and in dogs with microfilariemia ($p < 0.05$). Dogs with pulmonary hypertension showed higher increases in proteinuria as well, which was probably due to the chronicity of the infection. No significant differences were found in serum and urine values regarding systemic blood pressure. Despite the limitations of this study, proteinuria/borderline proteinuria was present in 36.2 % of dogs with heartworm disease, and this may be due to glomerular disease. Therefore, the detection of proteinuria, along with other renal biomarkers in the diagnostic protocols, could help identify kidney alterations or risk of renal damage in heartworm disease.

1. Introduction

Canine heartworm (*Dirofilaria immitis*) is a cardiopulmonary pathology, mainly caused by the presence of the adult parasites in the pulmonary arteries. These trigger the development of proliferative endarteritis, causing vascular damage, pulmonary hypertension and congestive heart failure (McCall et al., 2008; Simón et al., 2012). At cardiopulmonary level the disease is very well studied; however, other organs can be damaged by the presence of *D. immitis* as well, such as the liver (von Lichtenberg et al., 1962; Kramer et al., 2005) and the kidney (Casey and Splitter, 1975; Paes-de-Almeida et al., 2003). The implications of heartworm at this level are less studied and, to some extent, neglected. Although renal damage was believed to be uncommon in infected dogs, studies have demonstrated that the disease may be more frequent than previously recognized (Shirota et al., 1979; Ludders et al., 1988; Paes-de-Almeida et al., 2003).

The pathophysiological findings that can be found in the kidney of dogs parasitized by *D. immitis* are diverse and nonspecific. Normally, functional alterations occur at the level of the glomerular basement membrane, triggering the onset of proteinuria (Buoro and Atwell, 1983). The deposit of immune complexes in the basal membrane due to the parasite infection produces a physical damage to the glomerular endothelium, which leads to the appearance of proliferative glomerulonephritis, histologically characterized by the presence of mesangial, endothelial and inflammatory cells, deposits in the glomerular basement membrane, thickening and vacuolation, and expansion of the mesangial matrix (Casey and Splitter, 1975; Paes-de-Almeida et al., 2003). Chronic interstitial nephritis, with multiple cortical and medullary scarring has been described as well; also, glomerulosclerosis and amyloidosis have been reported (Simpson et al., 1974; Drazner, 1978; Shirota et al., 1979; Grauer et al., 1989; Paes-de-Almeida et al., 2003).

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The presence of microfilaria is an important factor and has been correlated with the loss of proteins in the urine, although azotemia is normally not observed, and other studies suggest that *Wolbachia* may contribute to immune-mediated kidney disease in dogs with heartworm infection (Simpson et al., 1974; Aikawa et al., 1981; Kramer et al., 2005; Morchón et al., 2012; Hormaeche et al., 2014).

There is a lack of studies that determine the prevalence of laboratory abnormalities related to kidney damage, both serological and renal. As has been shown, since it is considered that the presence of renal alterations is greater than estimated, the presence of serological and urinary alterations should also be high. Therefore, from the importance of kidney damage in dogs infected by *D. immitis* and the lack of studies focused on its prevalence and diagnosis, the objective was to determine the prevalence of proteinuria and functional indicators of glomerular filtration rate (GFR) in dogs with heartworm disease and discuss its utility in the detection of renal impairment.

2. Materials and methods

2.1. Study population

For this study, samples from 47 dogs infected by *D. immitis* were used. The dogs lived in a hyperendemic area (Montoya-Alonso et al., 2016), and all were client-owned dogs brought to the Veterinary Medicine Service of the University of Las Palmas de Gran Canaria, Spain. All were dogs presented for routine care and were included in the study based on being positive to *D. immitis* and fulfilling the inclusion criteria, which were: never having received treatment for heartworm disease, no previous history of heartworm infection, no concomitant diseases or receiving medication, and owner consent to participate in the survey.

The heartworm infection was established by the presence of circulating antigens by using a commercial kit (Urano test Dirofilaria®, Urano Vet SL, Barcelona, Spain). Furthermore, presence or absence of microfilariae was determined by using a modified Knott test.

The study was approved by the ethical committee of the Veterinary Medicine Service of the University of Las Palmas de Gran Canaria and was carried out in accordance with the current European legislation on animal protection.

2.2. Tests performed

During the examination of the dogs, an echocardiogram was performed by using an ultrasound machine with spectral and color Doppler and multifrequency probes (5.5 − 10 MHz) (Logic P5, General Electric, New York, USA). All the echocardiographic records, scorings and measurements were carried out by the same researcher. The dogs were placed in right lateral recumbence with the transducer placed in the third intercostal space. Parasite load was determined according to previously established guidelines and scoring the dogs from 1 to 4, from low to high worm burden; specifically, high parasite load (scores 3 or 4) was considered when worm echoes occupied the right pulmonary artery and extended to the main pulmonary artery, or occupied the whole right pulmonary artery and the main pulmonary artery to the level of pulmonary valve, and a classification of low parasite load (scores 1 or 2) was considered when no worms were visualized or only few worm echoes in the distal part of the right pulmonary artery (Venco et al., 2003). The Right Pulmonary Artery Distensibility (RPAD) index was also determined, which is basically calculated as the difference in diameter of the right pulmonary artery in systole and diastole as measured by M-mode, and calculated following the formula: (systolic diameter minus diastolic diameter, divided by systolic diameter) in three different cycles (Venco et al., 2014). This index determines the compliance of the right pulmonary artery and is used as a measure of pulmonary hypertension and endarteritis in dogs with heartworm (Venco et al., 2014; Serrano-Parreño et al., 2017a; 2017b). Moderate to severe pulmonary hypertension was considered with an RPAD index < 29 % (Venco et al., 2014; Visser et al., 2016; Falcón-Cordón et al., 2019).

Systemic blood pressure was also measured using an oscillometric method (Vet HDO Monitor, S + B medVet GmbH, Babenhausen, Germany) with an appropriately sized cuff placed around the front part of the left foreleg, medio-proximal between the elbow and carpal joint, placing the sensor over radial artery, with the dog lying down upright in ventral recumbent position. Three measures were taken every 10 min, excluding from analysis the results if the dogs showed signs of anxiety or excitement. Hypertension was defined as systolic blood pressure > 160 mm Hg, according to the criteria of the American College of Veterinary Internal Medicine (Acierno et al., 2018).

Urine was removed by cystocentesis, placing a needle into the urinary bladder through the abdominal wall, echocardiographically guided and stored at -20 °C. Blood samples were collected from the cephalic vein and serum was stored at -20 °C until analysis. All samples were sent to a reference laboratory (Idexx Laboratories, Barcelona, Spain).

In urine, the presence of proteins was measured by using the Pyrogallol red- Molybdate method, and the protein / creatinine ratio (UPC) was also determined. Proteinuria was defined as a UPC value > 0.5 and borderline proteinuria was defined as a UPC value between 0.2 to 0.5, according to the IRIS guidelines (Anon, 2019). Due to the low number of dogs studied, for the statistical study proteinuric and borderline proteinuric dogs were unified.

In serum, urea, creatinine, total plasma proteins and serum symmetric dimethylarginine (SDMA) were measured. Reference values for urea, creatinine and total plasma proteins were based on the reference range for the machine (21–59 mg/dl, 0.5–1.5 mg/dl and 4.8–7.8 g/dl, respectively). A value of SDMA ≥ 18 µg/dl was established according to a recent research (McKenna et al., 2020).

2.3. Statistical analysis

The data were analyzed using the SPSS Base 20.0 software for Windows. A Kolmogoroz-Smirnov test was performed to verify the normal distribution of the data. Continuous variables were expressed as median and range. Qualitative variables were expressed as percentage. The Chi square test or Fisher's exact test was used to assess the association between categorical variables. Due to the low number of dogs studied, it was decided to group proteinuric and borderline proteinuric dogs for the statistical study. When the age variable was evaluated, the dogs were grouped into two groups (1 − 4 years and 5 − 10 years of age). In all cases, a p value < 0.05 was determined as significant.

3. Results

Of the studied dogs, 20 were male and 27 were female, and the age ranged from 1 to 10 years with a mean age of 4.9 years; dogs were further divided into two groups, from 1 to 4 years (group 1, n = 23), and from 5 to 10 years (group 2, n = 24). The results of the laboratory parameters were evaluated, based on the presence / absence of microfilariae, pulmonary hypertension, systemic hypertension, as well as age and parasite load, as can be seen in Table 1.

The results showed that 40.4 % (19/47) of the dogs presented moderate to severe pulmonary hypertension with a RPAD index < 29 %. Systemic hypertension was present in 21.3 % (10/47) of the dogs (Fig. 1).

Based on the modified Knott test, microfilaremia was present in 53.2 % (25/47) of the dogs and, according to the echocardiographic findings, 23.4 % (11/47) showed high parasite load and 76.6 % (36/47) showed low parasite load.

The results of the parameters measured in the laboratory can be seen in Fig. 1. The laboratory analysis showed that 19.1 % (9/47) of the dogs had proteinuria and 17 % (8/47) had borderline proteinuria. Proteinuria or borderline proteinuria was present in 63.6 % (7/11) of

Table 1
Percentages of altered values in each of the parameters evaluated according to the parasite load, as well as the presence / absence of microfilariae, systemic hypertension and pulmonary hypertension. In brackets, the median and range of those parameters that have shown pathological results. Statistically significant differences (*) based on parasite load, microfilaremia, pulmonary tension or age group ($p < 0.05$).

	Parasite burden		Microfilariae		Systemic blood pressure		Pulmonary blood pressure		Age	
Altered parameters	High burden	Low burden	Presence of mf	Absence of mf	Systemic hypertension	Systemic normotension	Pulmonary hypertension	Pulmonary normotension	1 – 4 years	5 – 9 years
UPC ratio	63.6 % (0.61; 0.24 – 9.17)	28.8 %* (0.38; 0.28 – 3.7)	44 % (0.79; 0.27 – 9.17)	27.3 %* (0.43; 0.24 – 5.29)	30 % (0.39; 0.33 – 0.53)	32.4 % (1.38; 0.24 – 9.17)	47.4 % (2.19; 0.33 – 9.17)	28.6 %* (0.28; 0.24 – 5.25)	40 % (0.4; 0.24 – 9.17)	31.8 % (0.69; 0.33 – 3.7)
Urea (mg/dl)	0%	2.8 % (251)	4% (251)	0%	0%	2.7 % (251)	5.3 % (251)	0%	0%	4.5 % (251)
Creatinine (mg/dl)	0%	5.5 % (4.55; 1.8 – 7.3)	4% (7.3)	4.5 % (1.8)	0%	5.4 % (4.55; 1.8 – 7.3)	10.5 % (4.55; 1.8 – 7.3)	0%	4% (1.8)	4.5 % (7.3)
Plasma proteins (g/dl)	18.2 % (9.1; 8.6 – 9.6)	8.3 % (7.9; 7.9 – 8.6)	12 % (8.6; 7.9 – 9.6)	9.1 % (8.2; 7.9 – 8.6)	10 % (7.9)	8.1 % (8.6; 7.9 – 8.6)	15.8 % (8.6; 7.9 – 8.6)	7.1 % (8.7; 7.9 – 9.6)	12 % (8.6; 7.9 – 9.6)	9.1 % (8.2; 7.9 – 8.6)
SDMA (µg/dl)	9.1 % (25)	2.8 % (62)	8% (43.5; 25 – 62)	0%	0%	5.4 % (43.5; 25 – 62)	5.3 % (62)	3.6 % (25)	0%	9.1 % (43.5; 25 – 62)

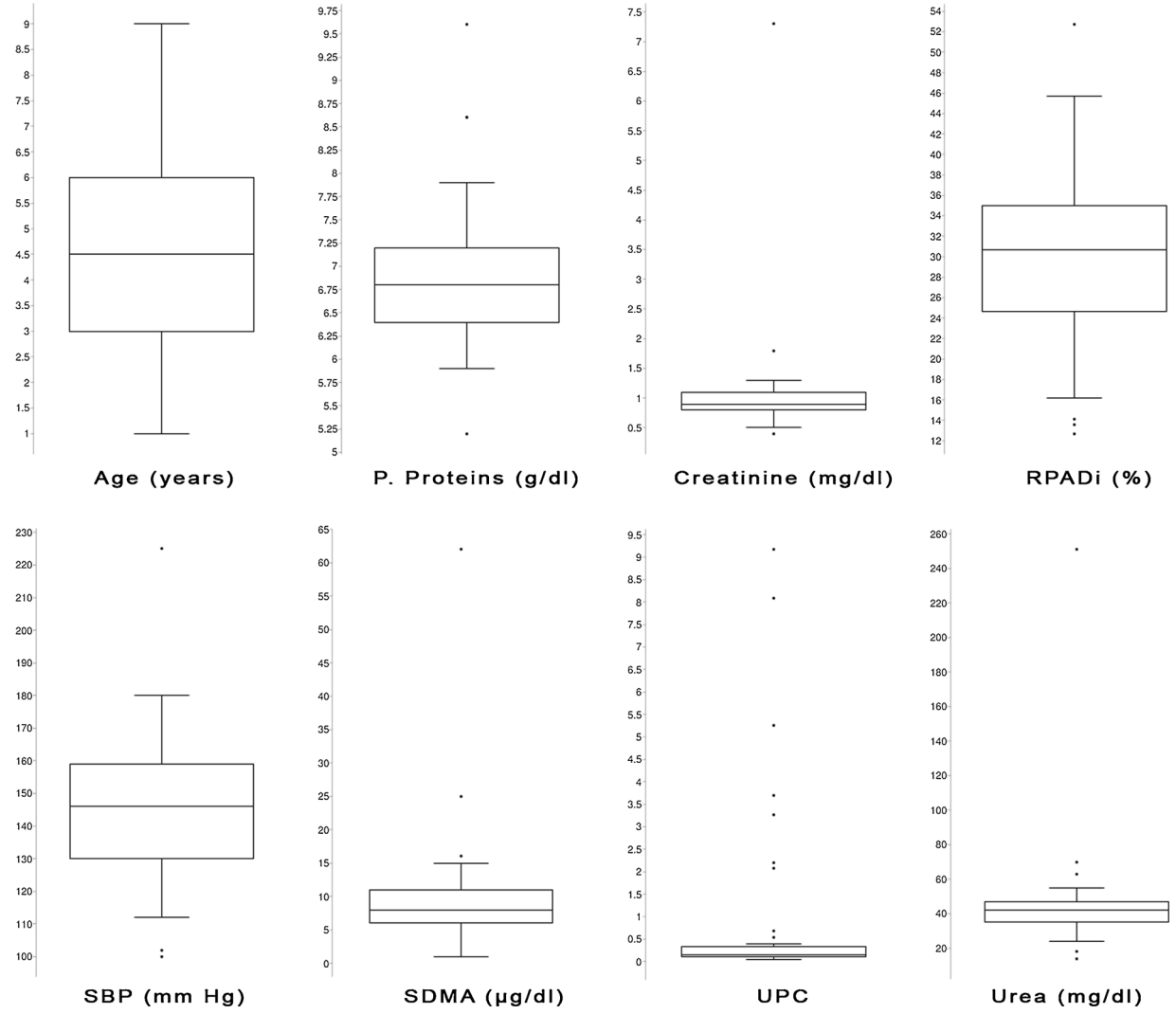

Fig. 1. Results obtained in the laboratory parameters (creatinine, plasma proteins, SDMA, UPC ratio and Urea) as well as results gathered for RPAD index, systolic blood pressure (SBP), and age. The units of each parameter are indicated in brackets. The box plots represent median (horizontal lines within boxes), 25th and 75th percentiles (boxes) and minimum and maximum values (whiskers).

the dogs with high parasite burden, and 27.8 % (10/36) of dogs with low parasite burden (p = 0.03). According to microfilaremia, proteinuria or borderline proteinuria was more frequent in microfilaremic dogs (44 %, 11/25) versus amicrofilaremic (27.3 %, 6/22) (p = 0.048). When pulmonary pressure was assessed, dogs with pulmonary hypertension presented higher presence of proteinuria or borderline proteinuria (47.4 %, 9/19) than normotensive dogs (28.6 %, 8/28) (p = 0.036). Regarding systemic blood pressure, no significant differences were found in proteinuria/borderline proteinuria between hypertensive (30 %, 3/10) or normotensive dogs (37.8 %, 14/37).

When age was considered, no significant differences were found in proteinuria between both groups (40 % and 31.8 %, respectively).

SDMA was high in 4.3 % (2/47) of dogs. Both showed proteinuria and were microfilaremic, 50 % presented high parasite burden, 50 % showed moderate-severe pulmonary hypertension and both dogs showed normal systemic pressure; the ages were 7 and 9 years old.

Serum creatinine was increased in 4.3 % (2/47) dogs; both dogs showed proteinuria, and one of them showed increases of urea (251 mg/dl) and SDMA (62 μg/dl), as well as presence of microfilariae. Both dogs presented moderate-severe pulmonary hypertension, low parasite burden and absence of systemic hypertension.

Plasma proteins were increased in 10.6 % (5/47) of the dogs; all of them were proteinuric or borderline proteinuric. Pulmonary hypertension was present in 60 % (3/5) of these dogs and systemic hypertension in 20 % (1/5). Microfilariemia was present in 60 % (3/5) of the dogs and high parasite burden in 40 % (2/5). The age of these dogs ranged from 2 to 6 years.

4. Discussion

In heartworm, functional alterations occur in the basement membrane of the glomerulus, which causes the presence of proteinuria. Furthermore, persistent proteinuria alone is believed to cause glomerular and tubulo-interstitial damage or impairment, so its presence in heartworm-infected dogs is a risk (Burton and Harris, 1996; Harley and Langston, 2012). In this study, proteinuria was present in 19.1 % of the dogs and borderline proteinuria was present in 17 % of them. As this was a single UPC measurement, no serial measurements were performed (Nabity et al., 2007) and no urinalysis was performed, the presence of post-renal proteinuria and other undiagnosed causes of renal proteinuria cannot be ruled out. However, due to the high prevalence of proteinuria found, the evaluation of urine proteins could be very useful for the early detection of renal alterations. Therefore, the use of this method should be considered among those of first choice to evaluate the renal status in dogs infected by *D. immitis*. Proteinuria can be patent before there is a significant alteration of other markers of renal function, so it can have great value as an indicator of kidney damage (Jacob et al., 2005; Lees et al., 2005; Harley and Langston, 2012). In addition, renal loss of proteins may be a cause of antithrombin III loss, a coagulation inhibitor. This situation could worsen the clinical presentation in dogs with heartworm, which are at high risk of developing pulmonary thromboembolism due to the death of worms (Carretón et al., 2011, 2013).

SDMA correlates well with GFR; some studies concluded that it is more sensitive than creatinine, increases in earlier stages of decrease in GFR, and is useful for the early detection of renal dysfunction (Hall et al., 2014, 2016; Nabity et al., 2015). However, other studies concluded that there are no significant differences between SDMA and creatinine in the early detection of decreased GFR (Pelander et al., 2019). In this study, similar increases were found in creatinine and SDMA; this contradicts the results obtained by other authors in heartworm disease, although different reference values for SDMA were used (Choi et al., 2017). Due to the low number of dogs studied and the lack of other diagnostic studies (i.e. to study the effect of hydration on both markers), further studies are needed to verify the utility of SDMA and creatinine in the early detection of renal dysfunction in dogs infected by *D. immitis*.

Plasma proteins were increased in 10.6 % of the dogs studied. This increase could be due to an increase in globulins, caused by the presence of the parasite (Sharma and Pachauri, 1982). It was not possible to perform a proteinogram or the breakdown of albumin and globulin; therefore, loss of albumin through the glomerulus or antigenic stimulation of globulins could not be assessed. Therefore, no further conclusions can be drawn, but they do invite deeper examination of plasma proteins in heartworm in future studies, especially considering that all dogs with increases in plasma proteins had proteinuria in this study.

Dogs with peripheral blood microfilaria were found to have more frequent abnormalities during the urine and serum analysis. Previous studies have suggested that kidney damage could occur as a result of the deposit of microfilariae in the renal glomeruli, but also due to the release of *Wolbachia* and the immune-mediated glomerulopathy produced by its presence (Aikawa et al., 1981; Grauer et al., 1988; Morchón et al., 2012). Furthermore, dogs with high parasitic burdens presented greater alterations of SDMA, proteins, and presence of proteinuria. A greater presence of worms produces greater antigenemia in the dog and, therefore, may cause a greater deposit of antigens in the organism; hence, increased glomerular formation and deposition of antigen-antibody complexes in the kidneys (Nakagaki et al., 1990, 1993). Experimental studies on immune-complex glomerulonephritis have shown that the formation of large immune complexes depends on a delicate balance of the antigen-antibody concentration, and that the complexes are formed most frequently when there is an excess of circulating antigens (Dixon et al., 1961).

Dogs with pulmonary hypertension showed greater alterations in serological and proteinuria values. Although a relationship between the presence of pulmonary hypertension and renal damage has been described (Husain-Syed et al., 2015), the results are more likely to refer to the chronicity of heartworm infection. Pulmonary hypertension in dogs with *D. immitis* develops chronically (McCall et al., 2008; Simón et al., 2012), so that although the time of infection in the studied dogs is unknown, chronic infections are more likely to correspond to dogs that have developed pulmonary hypertension.

No relationship was observed between the presence of systemic blood pressure and the increase in urine and serum parameters studied. Hypertension has been associated with proteinuria and histological renal injury in dogs (Cortadellas et al., 2006; Bacic et al., 2010); however, the results from this study do not match these statements. A possible justification could be that the hypertension of these dogs was related to White-Coat Hypertension, due the effects of excitement or anxiety in the veterinary clinic (Acierno et al., 2018). Although the study only considered the results of systemic tension in dogs when they showed no signs of anxiety or stress, the effects of anxiety on blood pressure are not predictable, as some dogs may exhibit a dramatic increase in blood pressure while others may not, even some dogs may exhibit a decrease in blood pressure as a result of the measurement process due to parasympathetic nervous system overactivity (Brown et al., 2007). Furthermore, depending on the size of the dog, the distance between the cuff and level of the right atrium will vary, which can affect blood pressure measurements (Rondeau et al., 2013).

When the age of the dogs was evaluated, no significant differences were observed between groups in any of the analytes studied.

5. Conclusions

Although more studies are needed, detecting proteinuria in dogs with heartworm could help us identify the risk or presence of renal damage, while SDMA and creatinine help diagnose ongoing damage from loss of GFR in dogs, especially when tubulointerstitial nephritis is present, which also occurs in heartworm and can manifest with mild proteinuria or without proteinuria (Ludders et al., 1988; Bartges, 2012). Renal damage in dogs with heartworm is little studied, both scientifically and clinically, and there is not a proper protocol for its diagnosis,

staging, management and treatment. Proteinuria can be of post-renal origin, or the causes of renal proteinuria can be others than heartworm; in addition, other factors can cause the presence of transient proteinuria, such as fever, heat, strenuous exercise, or stress (Acierno et al., 2018). Also, low increases of the evaluated parameters should be monitored over time. However, the results suggest the need for further studies to establish the prevalence of kidney damage in dogs with heartworm and determine the usefulness of the markers evaluated in this study, especially proteinuria in the detection of renal disorders in these dogs. Assessing the presence of early kidney damage, or the risk of damage, may allow the implementation of measures to try to avoid further damage (i. e. specific renal treatment). Moreover, according to the results, special attention should be paid to renal function in dogs with presence of microfilariae, high parasite burden, or clinical signs that suggest chronicity, such as the presence of pulmonary hypertension.

CRediT authorship contribution statement

E. Carretón: Conceptualization, Methodology, Supervision, Formal analysis, Writing - original draft, Writing - review & editing. **Y. Falcón-Cordón:** Investigation, Writing - review & editing. **J. Rodon:** Investigation, Writing - review & editing. **J.I. Matos:** Investigation, Writing - review & editing. **R. Morchón:** Formal analysis, Writing - original draft, Writing - review & editing. **J.A. Montoya-Alonso:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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