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Research article

HOMOCYSTEINE CONCENTRATION IN THE SERUM OF DOGS NATURALLY INFECTED WITH *LEISHMANIA* SPP. – ASSOCIATION WITH THE STAGE OF THE DISEASE, THERAPY, AND CLINICAL PATHOLOGY DATA

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Progressive tissue injury in canine leishmaniosis (CL) is related to the deposition of immune complexes, which induces vasculitis and leads to endothelial dysfunction. Homocysteine (Hcy) increase may worsen endothelial dysfunction, but data concerning its concentration in different CL stages and links to the acute phase response and oxidative stress are missing. We compared Hcy levels between dogs with mild (N=24) and moderate CL without treatment (N=17) and treated with anti-*Leishmania* drugs and vitamin B supplements (N=9). Dogs with moderate CL, regardless of therapy administration, had more distinct clinical signs, lower erythron values, and a higher level of acute-phase proteins (APPs), IgG against *Leishmania* spp., urea and creatinine, than dogs with mild CL. Hcy values did not differ between stages, but treated dogs had the lowest levels of Hcy. An inverse relationship existed between Hcy and the CL stage, therapy, levels of IgG, and clinical pathology data. The only positive relationship existed between Hcy and the erythron state. The disease stage and therapeutic intervention were not related to the oxidative stress level, except in the case of paraoxonase-1/Hcy ratio, indicating favorable conditions for antioxidative defense in treated dogs. In conclusion, changes in Hcy levels indicated its possible involvement with endothelial dysfunction and inverse relationship to tissue injury evaluated by APPs. Finally, Hcy might be an early marker of favorable conditions for endothelium recovery in CL.

Key words: allopurinol, B vitamins, homocysteine, *Leishmania* spp, miltefosin

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INTRODUCTION

Canine leishmaniosis (CL) is a vector-borne parasitic disease that represents one of the most significant zoonoses in the Mediterranean basin and the surrounding geographic locations. CL results from the infection with *Leishmania infantum*, and the female sandflies from the *Phlebotomus* genus act as vectors. Often, the infection can be asymptomatic or with mild signs like minor papular lesions or isolated enlarged lymph nodes. In moderate CL, the signs involve skin lesions, onychogryphosis, multiple lymphadenomegaly, fever, cachexia, ophthalmological disorders and epistaxis. Severe complications like vasculitis, arthritis and glomerulonephritis accompany CL progression towards the severe form. In the most advanced stage of CL, dogs develop the nephrotic syndrome, chronic kidney disease, and an increased risk for pulmonary thromboembolism. In the endemic areas, the prevalence of symptomatic CL ranges between 0.4% and 29% of the total canine population, whereby the number of asymptomatic cases of infection is several times higher. Furthermore, in the last decades, cases of CL spread to the regions previously considered as „CL-free" [1]. Meglumine antimoniate, miltefosine, and allopurinol represent the drugs currently used in CL. According to the current recommendations, pharmacological treatment starts when a dog develops the moderate form of the disease, in which more marked clinical signs occur together with clinical pathological abnormalities like mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, etc. The patients usually receive a combination of allopurinol and meglumine antimoniate or miltefosine, except for the most advanced stage, in which monotherapy with allopurinol remains as the only choice. Nevertheless, the failure to achieve complete clinical remission and the elimination of the parasite, together with the resistance against the abovementioned drugs, remain the actual challenges in the pharmacological treatment of CL [1,2].

Homocysteine (Hcy) is the methionine (Met) metabolite and represents the final product of the set of sequential reactions, which starts with Met conversion to S-adenosyl-Met (AdoMet). In the next step, AdoMet donates the methyl group for methylation of various, whereby itself transforms into S-adenosylhomocysteine (AdoHcy). The pathway finishes with the hydrolysis of AdoHcy and the formation of Hcy. If present in increased concentration, Hcy can impair the endothelial function [3]. The development of vasculitis in CL, occurring consequentially to the endothelial dysfunction caused by immune complexes' deposition [4], brings the rationale to assess Hcy levels in CL. Also, Hcy metabolism requires several B vitamins as the cofactors, and the recent study by de Sousa Gonçalves et al. [5] showed clinical benefits after administration of B vitamins, together with omega-3 polyunsaturated fatty acids, as adjuvants to anti-*Leishmania* drugs.

Previous articles reported Hcy levels in dogs with hypothyroidism, systemic inflammatory response syndrome, cardiac, renal, and gastrointestinal disorders [6,7]. Nevertheless, data for Hcy levels in CL are not available. Also, the previous studies pinpointed the utility of laboratory testing for the assessment of the initial effects of

the anti-Leishmania drugs [8]. Therefore, we aimed to assess the relationship between Hcy levels and clinical stage of the infection, short-term administration of anti-*Leishmania* drugs and food supplement containing B vitamins, and clinical pathology data in dogs with CL.

MATERIAL AND METHODS

Animals

Between November and December 2018, the study enrolled 50 dogs (28 males and 22 females) with CL from the private shelter in the Bar area (Montenegro), that is endemic for CL [9]. Also, twenty-two of them were the pure-breed dogs–five Serbian hounds, four Posavac hounds, two britannies, and one dogs of the following breeds: boxer, Serbian tricolor hound, Cane Corso, English setter, Staffordshire bull terrier, dachshund, griffon, shar pei, German shepherd, pinscher, American Staffordshire terrier. The stray dogs represented approximately half of the enrolled dogs, what limited the possibility for a reliable comparison of age in the groups.

The criteria for diagnosis and staging included presence of clinical signs and the level of antibodies against *Leishmania* spp. [4]. Twenty-four dogs had a mild form of CL. In 26 dogs CL progressed towards the moderate stage; nine of them received the treatment once a day, which consisted of peroral administration of 2 mg/kg miltefosine, 10 mg/kg allopurinol [1], and one tablet of the commercial food supplement containing vitamin B complex (Anima Strath, Bio-Strath, Switzerland). The therapy had been administered for three weeks, before the study started. According to the EU Directive 2010/63 and Serbian Law for Animal Welfare, the Ethical Committee at the Faculty of Veterinary medicine, University of Belgrade (Serbia) issued the certificate (01/2021) that confirms the study did not violate animal welfare by using sera surplus, originally collected for diagnostic purposes and the patients' follow-up, for all the analyses.

Laboratory methods

After puncturing *V. cephalica antebrachii*, blood samples had been collected into the EDTA tubes for hematology analyses, and plain tubes for serum separation. Following the centrifugation for 10 minutes at 1500 g, the sera were stored at -20°C until testing.

The chemiluminescent microparticle immunoassay on the ARCHITECT® ci8200 Integrated System (Abbott Diagnostics, Germany) was used to measure Hcy concentration. The level of IgG against *Leishmania* spp. was tested with the canine-specific commercial enzyme-linked immunosorbent assay (ELISA) kit (NovaTec Immundiagnostica GmbH, Germany) according to the manufacturer's instructions.

Complete blood count (CBC) was assessed on BC 2800 hematology analyzer (Mindray Ltd, UK). The routine biochemistry profile included urea, creatinine (CRE), total proteins (TP), albumin (ALB), cholesterol (CHOL), triglycerides (TRIG), alanine

aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT). Their levels were measured using the commercial spectrophotometry kits (BioSystems S.A., Spain) on BA-88A Semi-Auto Chemistry Analyzer (Mindray, China).

Protein fractions were analyzed using agarose gel electrophoresis, following the protocol described by Milanović et al. [10], while the levels of the individual acute phase proteins (APPs) were measured using immuno- and spectrophotometry assays. The commercial ELISA kit (Abcam, UK) was used for the measurement of serum amyloid A (SAA) concentration. The spectrophotometry methods were applied for haptoglobin (HPT) [11], ceruloplasmin (CER) [12], and paraoxonase-1 (PON-1) [13]. The level of thiol groups (THIOLS), measured using the method by Ellman [14], served as an indicator of oxidative damage.

Statistical analyses

Statistical evaluation included the χ^2 test, Kruskal-Wallis test with the post-hoc analysis according to Conover, Spearman's rank correlation, and multiple regression analysis. Test results were significant if the corresponding P-value was less than 0.05. The MedCalc® software v.16.2.1. was used for testing.

RESULTS

The study groups did not differ regarding the sex of the dogs (relative number of males was 10/17, 4/9, and 14/24 respectively in the group with moderate clinical stage no therapy, moderate clinical stage with therapy and mild clinical stage; P=0.742). Similar was present for the number of pure-breed dogs (7/17, 5/9, and 10/24; P=0.742).

Minor papular lesions, present in 11 dogs, were the only clinical sign in the group with mild CL. The most common clinical signs among dogs with moderate CL were onychogryphosis and cutaneous ulcerations, whereby almost half of the dogs presented with a combination of two clinical signs (Table 1). Furthermore, the group that received therapy did not differ from the group without the treatment regarding the frequency of individual clinical signs (P=0.261) or the total number of present signs (P=0.404).

The median level of IgG against *Leishmania* spp. in dogs with mild CL was approximately ten times lower than in the group with moderate CL, regardless of whether the dogs received the pharmacological treatment (Figure 1A). The dogs on the therapeutic regimen had a lower concentration of Hcy when compared with the dogs without treatment and dogs with mild CL (Figure 1B).

Table 1. Frequency of clinical signs in dogs with moderate leishmaniosis

	Therapy	
	Yes (N=9)	**No (N=17)**
Individual sign		
Exfoliative dermatitis	3	8
Onychogryphosis	6	14
Ulcerations	5	10
Epistaxis	3	1
Weight loss	4	3
Anorexia	3	7
Total number of signs		
One	1	1
Two	4	8
Three	1	6
Four	3	2

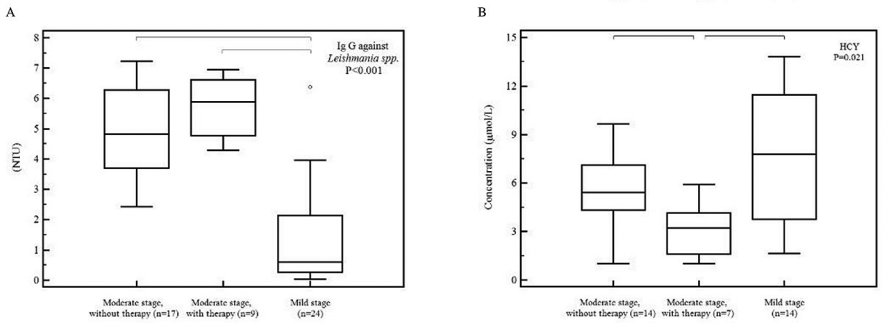

Figure 1. Concentration of **(A)** IgG against *Leishmania* spp. in NTU-units defined by the ELISA kit Manufacturer (NovaTec Immundiagnostica GmbH, Germany), and **(B)** homocysteine (Hcy). Kruskal-Wallis test with the post-hoc analysis according to Conover, $P < 0.05$ is considered significant. Box represents values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles represent outliers. The connectors indicate the significant difference between the groups.

Table 2 presents the values of routine hematology and biochemistry parameters compared between the study groups. In dogs with mild CL the hematocrit and leukocyte count were higher than in both groups with moderate CL. Urea, CRE, TP, and ALT were higher in dogs with moderate CL, regardless of therapy administration, when compared with dogs that had mild CL. An opposite trend occurred for ALB concentration. The group that receiving therapy, showed higher TRIG than the other two groups. Results for AST differed between all three groups, being the lowest in dogs with a mild CL, and highest in the dogs under the pharmacological treatment.

Table 2. Comparison of the hematology and biochemistry results between the dogs with different clinical stages of leishmaniosis. Kruskal-Wallis test with post-hoc analysis according to Conover, $P < 0.05$ is considered significant.

Parameter (unit)	Reference range	Clinical stage median (min–max)			P-value
		Moderate, no therapy	Moderate, with therapy	Mild	
RBC (10^{12}/L)	5.5–8.5	5.1 (2.3–6.3)[17]	4.1 (1.7–6.5)[9]	5.6 (4.1–6.5)[18]	0.065
HGB (g/L)	120–180	128 (54–219)[17]	118 (40–203)[9]	149 (111–194)[18]	0.082
HCT (%)	37–55	33 (16–43)[17]	26 (12–41)[9]	37 (29–45)[18]	0.042†
MCV (fL)	60–77	73 (66–78)[17]	70 (62–81)[9]	73 (69–77)[18]	0.389
MCHC (g/L)	370–410	393 (314–553)[17]	368 (280–491)[9]	406 (331–456)[18]	0.557
WBC (10^9/L)	6–17	11.0 (3.4–20.3)[17]	10.7 (7.0–25.3) [9]	14.7 (6.9–25.2)[18]	0.021†
NEUT (10^9/L)	3–12	6.4 (1.6–14.1)[17]	7.3 (3.1–18.0)[9]	7.9 (3.5–15.2)[18]	0.085
LYM (10^9/L)	1–4.8	0.5 (0.1–6.1)[17]	3.0 (0.1–7.6)[9]	0.8 (0.3–2.7)[18]	0.634
MID (10^9/L)	0.2–1.5	2.9 (0.1–7.3)[17]	0.3 (0.1–7.3)[9]	5.3 (0.1–10.6)[18]	0.055
PLT (10^9/L)	200–500	391 (248–618)[17]	400 (202–863)[9]	423 (224–868)[18]	0.828
Urea (mmol/L)	2.9–10.0	9.5 (6.9–14.9)[16]	11.9 (8.7–29.6)[9]	4.7 (3.1–9.0)[19]	<0.001†
CRE (μmol/L)	54–150	155 (101–274)[16]	194 (104–754)[9]	86 (60–112)[19]	<0.001†
TP (g/L)	55–75	76 (69–79)[16]	77 (70–80)[9]	68 (62–78)[19]	<0.001†
ALB (g/L)	29–35	23 (19–26)[16]	23 (18–25)[9]	29 (23–33)[19]	<0.001†
CHOL (mmol/L)	3.5–7.5	3.6 (2.7–9.3)[14]	4.5 (3.2–15.8)[8]	3.7 (3.0–6.0)[19]	0.106
TRIG (mmol/L)	0.3–1.5	0.5 (0.3–1.6)[14]	0.7 (0.6–1.3)[8]	0.8 (0.2–0.9)[19]	0.002#
AST (U/L)	13–60	44 (29–62)[16]	56 (44–69)[9]	36 (28–44)[19]	<0.001*
ALT (U/L)	10–109	84 (62–211)[16]	102 (71–375)[9]	46 (29–323)[19]	<0.001†
ALP (U/L)	11–114	121 (14–252)[14]	148 (20–549)[9]	168 (16–560)[19]	0.439
GGT (U/L)	1–12	4 (1–7)[16]	2 (1–6)[9]	4 (1–15)[19]	0.597

Abbreviations: ALB-albumin, ALT–alanine aminotransferase, ALP–alkaline phosphatase, AST–aspartate aminotransferase, CHOL–cholesterol, CRE–creatinine, GGT–gamma-glutamyltransferase, HCT–hematocrit, HGB–hemoglobin, LYM–lymphocytes, MID–medium cells (monocytes and part of eosinophils), MCHC–mean cell hemoglobin concentration, MCV–mean corpuscular volume, NEUT–neutrophils, PLT–platelets, RBC–red blood cells, TP–total proteins, TRIG–triglycerides, WBC–white blood cells, †– $P<0.05$ for mild vs. other two stages, #– $P<0.05$ for moderate with therapy vs. other two stages, *– $P<0.05$ for moderate with therapy vs. other two stages and moderate without therapy vs. mild stage. The number in italics superscript designates the number of analyzed samples.

The only difference in the abundance of the serum proteins' fractions was present for γ-globulins, which had higher levels in both groups of dogs with moderate CL in comparison with the group of dogs with mild CL (Figure 2A). The levels of HPT (Figure 2B) and CER (Figure 2C) showed an analogous pattern, while the difference in SAA level was absent (Figure 2D).

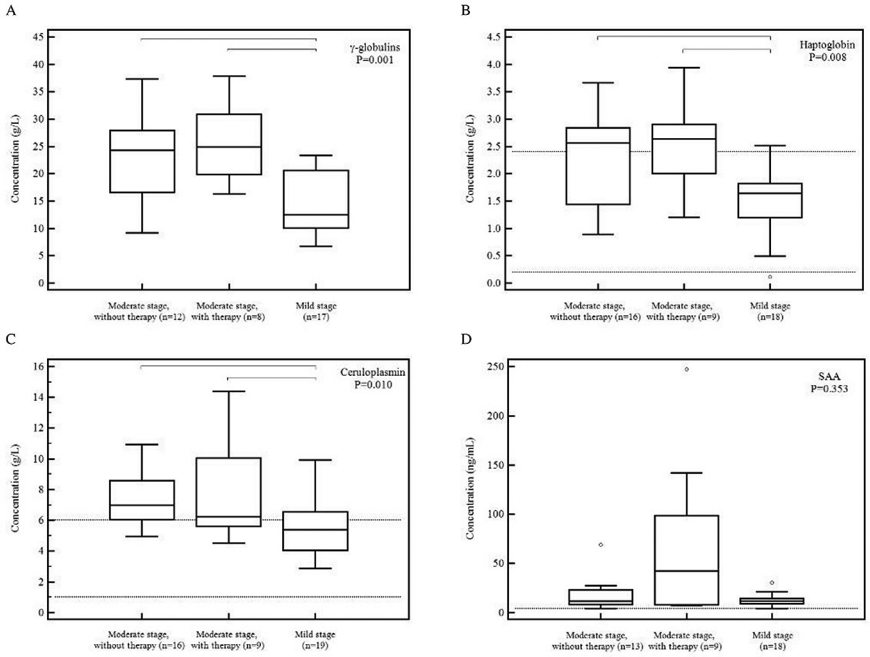

Figure 2. Concentration of **(A)** γ-globulins, **(B)** haptoglobin, **(C)** ceruloplasmin, and **(D)** serum amyloid A (SAA). Kruskal-Wallis test with the post-hoc analysis according to Conover, $P < 0.05$ is considered significant. Box represents values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles represent outliers. The connectors indicate the significant difference between the groups. The dotted line gives the reference values from the clinical laboratory at the Faculty of Veterinary Medicine, University of Belgrade, Serbia.

The dogs with the mild stage of CL had higher PON-1 activity only when compared with the untreated dogs with the moderate stage of the disease (Figure 3A). Nevertheless, the ratio between PON-1 and Hcy in the dogs under the treatment was higher than in the other two groups (Figure 3B). On the contrary, similar differences did not occur for THIOLS (Figure 3C) and THIOLS/Hcy ratio (Figure 3D).

Hcy levels were in correlation with numerous clinical and laboratory parameters (Table 3). However, only five of them, one clinical and four laboratory parameters, independently predicted Hcy values and could explain approximately 2/3 of the total variation in Hcy concentration among all dogs enrolled in the study (Table 4).

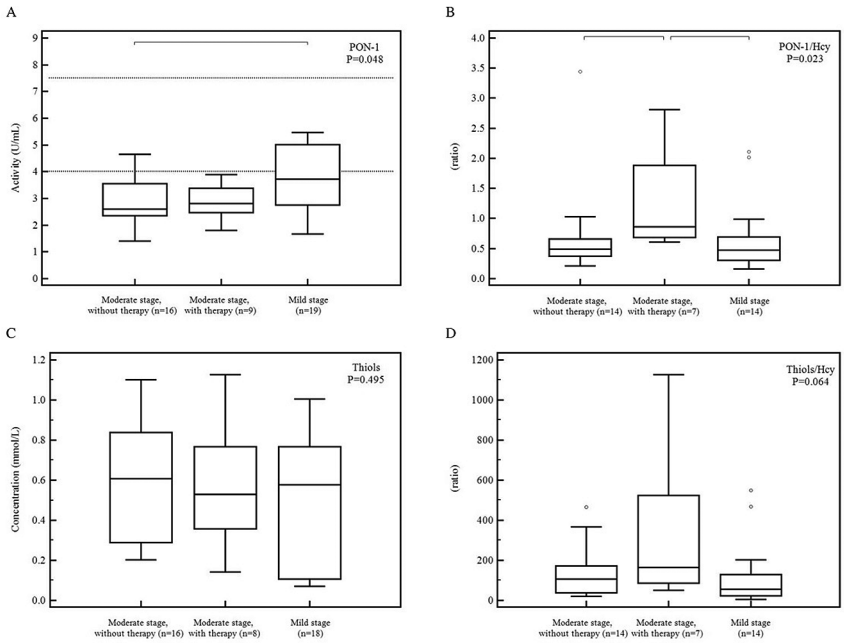

Figure 3. Activity of **(A)** paraoxonase-1 (PON-1), ratio of **(B)** paraoxonase-1/homocysteine (PON-1/Hcy), concentration of **(C)** free thiol groups (Thiols), and ratio **(D)** free thiol groups/homocysteine (Thiols/Hcy). Kruskal-Wallis test with the post-hoc analysis according to Conover, $P < 0.05$ is considered significant. Box represents values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles represent outliers. The connectors indicate the significant difference between the groups. The dotted line gives the reference values from the clinical laboratory at the Faculty of Veterinary Medicine, University of Belgrade, Serbia.

Table 3. Spearman's correlation analysis between Hcy and other investigated variables.

Variable	ϱ_s (95% confidence interval)	P-value
Stage (mild vs. moderate)#	-0.347 (-0.609 to -0.015)	0.041
IgG against *Leishmania* spp.#	-0.448 (-0.680 to -0.135)	0.007
Therapy (no vs. yes)#	-0.442 (-0.676 to -0.128)	0.008
RBC#	0.384 (0.058 to 0.636)	0.023
HGB#	0.339 (0.007 to 0.604)	0.046
LYM#	-0.451 (-0.682 to -0.139)	0.006
MID#	0.519 (0.225 to 0.727)	0.001
Urea#	-0.389 (-0.639 to -0.064)	0.021
CRE#	-0.369 (-0.625 to -0.040)	0.029
TP#	-0.338 (-0.603 to -0.005)	0.044
CHOL#	-0.415 (-0.667 to -0.078)	0.018
TRIG#	-0.426 (-0.675 to -0.091)	0.016
AST#	-0.456 (-0.685 to -0.145)	0.006
α2-globulins†	-0.407 (-0.669 to -0.055)	0.026
γ-globulins†	-0.558 (-0.764 to -0.247)	0.001
SAA*	-0.620 (-0.797 to -0.347)	<0.001
CER#	-0.392 (-0.641 to -0.067)	0.020
THIOLS#	-0.517 (-0.725 to -0.222)	<0.001

Abbreviations: AST – aspartate aminotransferase, CER – ceruloplasmin, CHOL – cholesterol, CRE – creatinine, HCT – hematocrit, Hcy – homocysteine, HGB – hemoglobin, LYM – lymphocytes, MID – medium cells (monocytes and part of eosinophils), PLT – platelets, RBC – red blood cells, SAA – serum amyloid A, TP – total proteins, TRIG – triglycerides, ϱ_s – Spearman's rho rank correlation coefficient. The symbol in italics superscript respectively designates the number of analyzed samples, in the groups with moderate clinical stage no therapy, moderate clinical stage with therapy, and mild clinical stage: # – 14/7/14; † – 12/7/14; * – 13/7/14.

Table 4. Predictors of Hcy concentration identified with the multiple regression analysis.

Independent variable	Coefficient	P-value
Therapy (no vs. yes)	-2.6702	0.026
RBC (10^{12}/L)	3.190	0.022
HGB (g/L)	-0.092	0.019
LYM (10^9/L)	-1.116	0.004
Thiols (mmol/L)	-4.703	0.005
Coefficient of determination R^2		0.6346

Abbreviations: Hcy–homocysteine, HGB–hemoglobin, LYM–lymphocytes, RBC–red blood cells.

DISCUSSION

Our study brought the pioneer data about the relationship between Hcy level and stage of CL, clinical pathology features, and administration of anti-*Leishmania* drugs together with vitamin B supplementation. Dogs with a mild form of CL and those with moderate CL without pharmacological treatment had similar Hcy concentrations. On the contrary, the dogs on three-week therapy (miltefosine, allopurinol, and food supplement containing B vitamins) had lower Hcy levels than the former two groups. However, we did not observe the therapy-related differences among the dogs with moderate CL when we statistically analyzed the data about the presence of clinical signs, hematology parameters, APPs, and laboratory tests for renal and liver function. When analyzing all three groups together, Hcy levels had four negative predictors: therapy, lymphocyte count, hemoglobin concentration, and THIOLS level. In addition, erythrocyte count appeared as a positive predictor of Hcy.

Literature data about Hcy levels in healthy dogs are not uniform and may depend on the criteria for the enrollment of dogs, breed, the method for measuring Hcy, etc. According to the results by Rossi et al. [15], Lee et al. [16], and Benvenuti et al. [7], the concentration in healthy dogs could be extrapolated to the range 1.7-10.5 μmol/L. If we rely on this range when interpreting our results, the dogs from both groups with the moderate CL had Hcy concentration in the reference range. The same was true for the majority of dogs with the mild form of the disease, and only few dogs from this group showed slightly increased Hcy. Findings of Grützner et al. [17] for healthy dogs advocate the Hcy level between 5.2 and 25.9 μmol/L, and the data from two more studies [6,18] fit within this range. Using this range would classify the majority of the

dogs receiving therapy as having hypoHcy, while almost all dogs from the other two groups had Hcy levels similar to healthy dogs. All of these considered points indicate that increased Hcy levels did not appear in the majority of the studied dogs.

The dogs with mild CL had only minor clinical signs and alterations in laboratory tests, so the absence of a significant increase in Hcy levels may not be surprising. On the contrary, it seemed rational to expect somewhat higher Hcy in dogs with the moderate CL, which had numerous indicators of disease progression: more protruding clinical signs, higher level of IgG against *Leishmania* spp., mild non-regenerative anemia, increased APPs, urea and CRE [1,19,20]. On the contrary, an inverse relationship existed between the Hcy and the majority of laboratory parameters. At least two features appearing in systemic inflammation might explain the absence of Hcy increase. First, the decreased appetite and the consequential lower dietary intake of Met [6] could backlash its conversion into Hcy. Another explanation might be dysproteinemia, which is primarily evidenced as protruding hypoalbuminemia. Namely, ALB binds approximately 90% of Hcy in plasma [21], so the depleted ALB synthesis in synergism with the nephropathy, suspected on the basis on the increased urea and CRE, could diminish the amount of Hcy available for measurement. Nevertheless, this feature might raise an additional question regarding the (patho)physiological route(s) for the elimination of the unbound Hcy. Also, our results showing the negative correlation between Hcy and several APPs, could be evidence in favor of considering Hcy as a negative acute-phase reactant in dogs, as indicated by Patterson et al. [6]. Finally, among numerous negative correlations shown for Hcy in this study, the one with the IgG against Leishmania spp. might be challenging regarding the eventual Hcy-mediated endothelial injury. Although it presumably indicated the lowering blood Hcy level concomitant with the strengthening of the inefficient and deleterious compensatory humoral response against Leishmania spp., the involvement of Hcy in the endothelial injury might not be straightforward. Plausibly, the Hcy-associated endothelial damaging mechanisms might be more potential in the mild form, thus causing the further progression to the moderate stage. Nevertheless, the opposite scenario might be possible-the Hcy from the blood might be consumed during the endothelial damage *in situ*.

In this study, lower Hcy was the only indicator of the beneficial effects after three weeks of combined therapy with miltefosine, allopurinol, and B vitamins supplement. For the therapy-associated differences in other parameters, like APPs, the three-week period might be too short for occurrence [22]. Lower Hcy levels presumably resulted from the dietary supplementation with vitamin B_{12} [23]. The ratio between the median Hcy values in the moderate CL groups (with and without therapy) in this study was similar to the proportion between Hcy previously measured in dogs with hypocobalaminemia (8.70 μmol/L) and those without (4.25 μmol/L) [7]. Considering the absence of difference in Hcy between the untreated dogs with moderate CL and mild CL, it might even provide a rationale for starting with the vitamin B supplementation in the early stage of CL. Also, miltefosine pharmacology actions might be a possible

cause of lower Hcy. Miltefosine inhibits the phosphatidylcholine synthesis [24], in turn, the transfer of methyl groups from S-adenosylmethionine may impede and reduce the yield of adenosylhomocysteine, the direct metabolic precursor of Hcy [25]. Hcy level was in positive correlation with erythrocyte count and hemoglobin, which brings the possibility that eventual iatrogenic erythrocyte destruction, due to the miltefosine hemolytic properties [26], might be partly responsible for the depletion of the Hcy depot in erythrocytes [27]. Nevertheless, it is very challenging to differentiate this „scenario" from Hcy erythrocyte pool reduction due to the anemia of chronic inflammation, which is a usual finding in the moderate CL [1].

Oxidative stress represents one of the CL hallmarks [5]. Nonetheless, THIOL levels and THIOL/Hcy in all three study groups assured that the serum antioxidant properties do not decrease if a moderate form of the disease occurs, nor do the antioxidant capacities improve with therapy administration. However, the difference in PON-1 activity and PON-1/Hcy ratio reflects an additional, potentially protective mechanism of vitamin B supplementation. Beside acting as a part of a general antioxidative system, PON-1 also hydrolyzes Hcy thiolactone, Hcy derivative that is responsible for deleterious effects of hyperhomocysteinemia [28]. Increased consumption of PON-1 in the untreated dogs with moderate CL resulted in lower PON-1 activity after the comparison with those having mild CL. The supplementation with B vitamins and consequential lowering of Hcy yielded a higher PON-1/Hcy ratio, possibly indicating less expenditure of PON-1 on Hcy thiolactone hydrolysis and leaving more PON-1 available for neutralizing reactive oxygen species.

The study limitations include a rather small number of the dogs, the observational design, the lack of a control group, consisting of clinically healthy dogs with negative tests for IgG against *Leishmania* spp., and the uncomplete information about the age of dogs. Regardless, the obtained results assured the reliable basis for the further investigation of molecular and clinical aspects of this zoonosis.

CONCLUSION

Hcy concentration did not differ between mild and the untreated moderate form of CL, but lower Hcy values were present in the group of dogs with the moderate CL treated with miltefosine, allopurinol, and vitamin B complex during three weeks. However, this treatment neither affected the synthesis of IgG specific for *Leishmania* spp. and total γ-globulins, nor the concentration of major (SAA) and moderate (HPT and CER) positive APPs. This finding indicated the existence of the tissue injury both in non-treated and in treated dogs, and the beneficial effect of therapy and supplementation with vitamins possibly created favorable conditions for the recovery of the endothelium. The therapy administration, erythron state, lymphocyte count, and the serum antioxidant capacity, identified as the independent Hcy predictors, implicate that comprehensive interaction „controls" Hcy levels in dogs with CL. Whether that

interaction has a potential for CL management or presents just an epiphenomenon of the CL natural course, remains a task for future studies.

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Authors' contributions

MKF, DT and AB designed the study; MA collected the samples; MA, AB, MR, KS and SS performed the laboratory analyses; AB performed and interpreted the statistical analyses; MA, AB, MKF and DT interpreted the data and equally contributed in the writing of the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The authors do not have any financial or personal conflicts of interest that could bias the study.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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KONCENTRACIJA HOMOCISTEINA U SERUMU PASA PRIRODNO INFICIRANIH *LEISHMANIA* SPP. – VEZA SA STADIJUMOM BOLESTI, TERAPIJOM I KLINIČKO-PATOLOŠKIM PODACIMA

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Progresivno oštećenje tkiva kod lajšmanioze pasa nastaje zbog taloženja imunskih kompleksa, koji indukuju vaskulitis i dovode do disfunkcije endotela. Iako je poznato da porast homocisteina (Hcy) može negativno uticati na funkciju endotela, još uvek nije istraženo da li je koncentracija Hcy u različitim stadijumima lajšmanioze povezana sa intenzitetom odgovora akutne faze i oksidativnim stresom. U ovom radu, upoređen je nivo Hcy kod pasa sa blagom (N=24) i umerenom formom bolesti u uslovima bez terapije (N=17) i sa terapijom uz dodatak kompleksa B vitamina (N=9). Psi sa umerenom formom bolesti, bez obzira na uslove terapije, imali su izraženiju kliničku sliku, niže vrednosti parametara crvene krvne loze i povišene nivoe proteina akutne faze, IgG protiv *Leishmania* spp, uree i kreatinina, u odnosu na grupu sa blagom formom lajšmanioze. Nivo Hcy se nije razlikovao među grupama sa umerenom formom bolesti, dok su tretirani psi imali značajno niže koncentracije Hcy u odnosu na druge dve grupe. Recipročan odnos je postojao između nivoa Hcy i stadijuma bolesti, terapije, nivoa IgG i kliničko-patoloških podataka. Jedina pozitivna korelacija je postojala između Hcy i parametara crvene krvne loze. Forma bolesti i primena terapije nisu bili u vezi sa nivoom oksidativnog stresa, osim u slučaju odnosa paraoksonaze 1 i Hcy, ukazujući na delimično poboljšanje antioksidativne zaštite kod tretiranih pasa. Promene u koncentraciji Hcy pokazale su da ovaj molekul može imati ulogu u disfunkciji

endotela kod lajšmanioze pasa, kao i da je njegova koncentracija obrnuto srazmerna oštećenju tkiva, posmatrano kroz nivo proteina akutne faze. Konačno, Hcy može biti rani marker oporavka endotela kod lajšmanioze pasa.