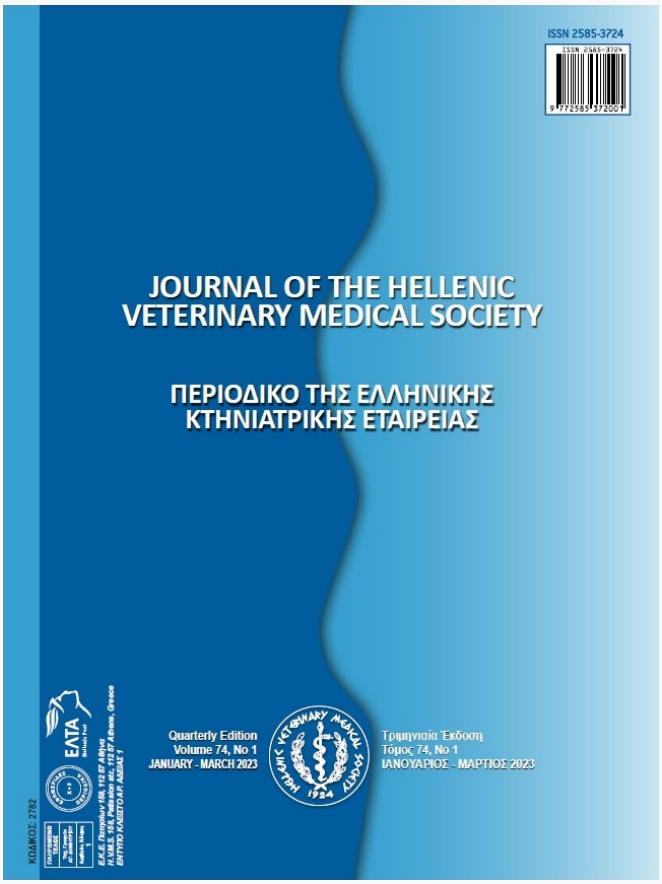
Journal of the Hellenic Veterinary Medical Society

Vol 74, No 1 (2023)



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doi: 10.12681/jhvms.29529

To cite this article:

Ilic Bozovic, A., Đoković, P., Milanović, Z., Janjić, F., Spariosu, K., Radonjić, V., Radaković, M., Magaš, V., Filipović, D., Stanković, S., Kovačević Filipović, M., & Beletić, A. (2023). Could homocysteine represent a negative acute phase reactant in canine infections-a pilot study?. *Journal of the Hellenic Veterinary Medical Society*, *74*(1), 5463–5470. https://doi.org/10.12681/jhvms.29529

J HELLENIC VET MED SOC 2023, 74 (1): 5463-5470
ΠΕΚΕ 2023, 74 (1): 5463-5470

Research article
Ερευνητικό άρθρο

Could homocysteine represent a negative acute phase reactant in canine infections-a pilot study?

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ABSTRACT: Homocysteine (Hcy) was investigated as the biomarker of cardiac, renal, and gastrointestinal disorders in dogs. Data about low Hcy concentrations in the systemic inflammatory response syndrome raised a hypothesis that Hcy in dogs could be a negative acute-phase reactant. This survey compared Hcy concentrations, serum amyloid A (SAA), and the routine laboratory parameters between healthy (HD, N=6) and dogs with inflammation of different extent: mild (dirofilariosis (DIR), N=31), moderate (babesiosis (BAB), N=12), and severe (pyometra (PYO), N=8). The BAB and PYO groups had lower Hcy er than HD. Also, the levels in the PYO group were below those in the DIRO group. SAA had the inverse pattern. Across the groups, Hcy and SAA levels correlated negatively ($\rho = -0.502$, $P<0.001$). Hcy and SAA correlated with the erythrocyte count, hematocrit, hemoglobin and mean cellular hemoglobin concentrations, and neutrophil count, with correlations being positive for Hcy and negative for SAA. Among all dogs, hemoglobin was the only independent predictor of Hcy concentration. Hcy levels in canine infections, decreased as acute-phase reaction (APR) intensified. Also, they were related with the hematology changes accompanying the APR. Further studies will establish the clinical potential of these alterations.

Keywords: homocysteine; inflammation; dirofilariosis; babesiosis; pyometra

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Date of initial submission: 06-02-2022
Date of acceptance: 09-11-2022

INTRODUCTION

Homocysteine (Hcy) is a nonproteinogenic, sulfur-containing amino acid that participates in the "activated methyl group cycle" and the cysteine biosynthesis from methionine. Folic acid and vitamin B12 are participating in the remethylation of Hcy back to methionine and the transsulfuration pathway, leading to cysteine formation, is dependent on the availability of vitamin B6. In excessive amounts, Hcy forms covalent complexes with proteins which has deleterious structural and functional effects. In man, hyperhomocysteinemia (HHcy) is associated with cardiovascular, gastrointestinal, and neurodegenerative diseases, autoimmune disorders, and inflammatory conditions (Li et al., 2015; Pizzorno, 2014; Schalinske & Smazal, 2012; Wu, 2007).

The results of the previous studies testing Hcy concentration as a marker of specific organ dysfunction or tissue injury in dogs have been contradictory. Some authors reported the presence of HHcy in patients with cardiac disorders (Rossi et al., 2008), but there are also studies in which such findings were absent (Lee et al., 2012). The same ambiguity appeared for renal (Kakimoto et al., 2014; Rossi et al., 2008) and gastrointestinal diseases (Benvenuti et al., 2020). The searching for a common denominator for high and low Hcy levels is challenging due to the variety of investigated conditions and status of the patients. However, dogs with a systemic inflammatory response syndrome had low Hcy concentrations(Patterson et al., 2013). Thus, it seems reasonable to test the hypothesis that Hcy in dogs is a negative acute-phase reactant (APR).

This survey compared Hcy concentrations between healthy and dogs with mild, chronic inflammatory reaction due to Dirofilaria immitis infection, moderate acute inflammation caused by Babesia canis infection, and severe acute inflammation due to pyometra. In parallel, the routine hematology and biochemistry parameters, and serum amyloid A (SAA), as a marker of the inflammation intensity, were compared among the abovementioned groups.

MATERIALS AND METHODS

The study included privately-owned dogs (N=57) admitted between February 2017 and December 2019 at the Small Animal Clinic of the Faculty of Veterinary medicine, University of Belgrade.

Healthy dogs (HD, N=6; 3 males: Mixed breed dogs, German Shorthaired Pointer, Labrador; 3 females: 2 Mixed breed dogs, Wire-haired Pointer) came for annual health check-ups. They had no alterations on physical examination and in the results of hematology, and blood chemistry tests. The median age for this group was 2 (min-max: 1-6 years) years. The group of *D. immitis* positive dogs (DIR, N=31; 17 males: 8 Mixed breed dogs, 2 German Wire-haired Pointers, 2 Golden Retrievers, Labrador Retriever, Epagneul Breton, Staffordshire Terrier, Springer Spanel, Malinoa; 14 females: 5 Mixed breed dogs, 2 Labrador Retrievers, 2 German Shorthaired Pointers, Rottwieler, German Wire-haired Pointer, Epagnuel Breton, Springer Spaniel, German Shepherd) were the dogs that came to health check-ups, and were negative to any other VBD except *D. immitis*, with a median age of 5 (2-11) years. Among them, six dogs showed mild signs of heartworm disease (occasional cough) and others were asymptomatic, what allowed their classification as having the Class 1 of heartworm disease according to the American Heartworm Society classification system (Kosić & Lalošević, 2020). The *B. canis* group (BAB, N=12; 7 males: 3 Mixed breed dogs, Akita Inu, Malinoa, Golden Retriever, Shar Pei; 5 females: 2 Mixed breed dog, Dachshund, Maltese, Golder Retriever) included the dogs admitted with clinical signs (fever, anorexia, hemoglobinuria) and laboratory findings (thrombocytopenia, mild or moderate anemia, large *Babesia* organisms on the blood smear) that corresponded to the acute babesiosis (Spariosu et al., 2021). The median age of the BAB group was 4 (1-15) years. In the pyometra group (PYO, N=8; 3 Mixed breed dogs, Beagle, Golden Retriever, Labrador Retriever, Doberman, German Wirehaired Pointer; median age 4 (2-6) years) there were dogs diagnosed with pyometra based on the clinical signs (polyuria, polydipsia, fever, apathy), results of the laboratory tests, and the ultrasound findings (Hagman, 2018). Exclusion criteria for all groups were the evidence of any other unrelated pathological condition (wounds, neoplasia, endocrinopathies, allergies, etc.).

Owners signed informed consent for using the surplus of material and data obtained during diagnostic procedures for scientific purposes. The Ethical Committee at the Faculty of Veterinary Medicine, University of Belgrade, Serbia, approved this research, and based on the Serbian Law of Animal Welfare, permission was acquired from the Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (number 323-07-03455/2015-05/3).

The commercial vacuum tubes (BD Vacutainer with K_2EDTA and BD Vacutainer SST™, BD Franklin Lakes NJ USA) were used to collect blood samples. The complete blood count (CBC) was analyzed on the hematology analyzer (Abacus Junior Vet. Diatron, Austria). All animals were tested with SNAP 4Dx (IDEXX Laboratories Westbrook, ME, USA) test system for *D. immitis*, *Ehrlichia* spp., *Borrelia* spp., and *Anaplasma* spp. Blood smears stained with BioDiff (BioGnost, Croatia) served for the initial detection of the large *Babesia* organisms that were later identified as *B. canis* using the polymerase chain reaction (IDEXX Laboratories, Westbrook, Maine, USA). The concentration of the routine biochemistry parameters (total proteins, albumin, glucose, cholesterol, triglycerides, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyl transferase) was measured with the commercial kits (ELITechGroup Solutions, Puteaux, France) on the automated analyzer (Technicon RA-XT, Bayer, Dublin, Ireland). To measure SAA concentration, we applied the commercial enzyme-linked immunosorbent assay kit (Tridelta Development Ltd. Ireland). Hcy concentration in serum was measured with the commercial chemiluminescent microparticle immunoassay (CMIA) on the ARCHITECT® ci8200 Integrated System (Abbott Diagnostics, Wiesbaden, Germany).

Data were analyzed using MedCalc statistical software (version 16.2.1, Ostend, Belgium). Statistical analysis included the Kruskal-Wallis test with the posthoc analysis according to Conover and Spearman`s rank correlation (Marusteri and Bacarea. 2010). We noticed clots in some samples collected with K_2EDTA, so the obtained results were not suitable for the statistical analyses. Also, in some cases, the volume of serum was not sufficient to complete all assays. The numbers in italic indicate the number of samples included in statistical analyses.

RESULTS

Figure 1A presents the comparison of Hcy levels between the groups. Dogs with babesiosis and pyometra had lower Hcy than the healthy ones. Also, the levels measured in the PYO group were below those measured in the DIRO group. The inverse pattern was present for SAA (Figure 1B). The dogs from the BAB and PYO groups had higher SAA concentrations compared with the HD and DIR groups. Nevertheless, the difference between the HD and DIR groups, as well as between the BAB and PYO groups was absent. Furthermore, there was a strong negative association between the Hcy and SAA across the groups (Spearman's coefficient of rank correlation (95% Confidence Interval) = -0.502 (-0.690 to -0.251), P<0.001).

Table 1 and Table 2 summarizes the additional results. There was no difference in the routine hematology and biochemistry parameters between HD and DIR dogs. The BAB dogs had lower median CBC than the three other groups. Also, the median glycemia value was the highest in the BAB group. All groups enrolling the dogs with infections had lower median albumin level in comparison with the healthy ones. Further, among the dogs with infections, the

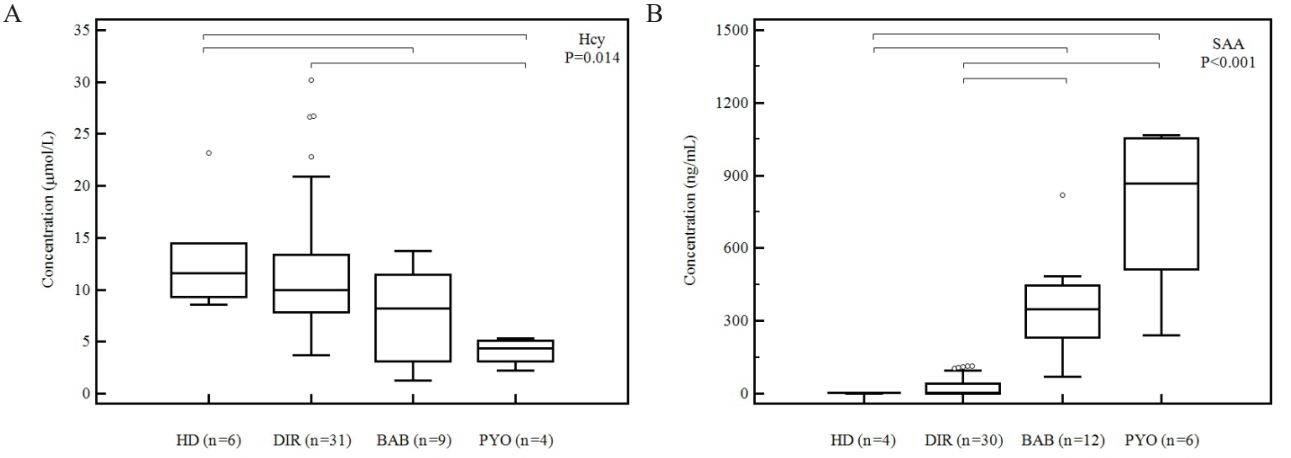

Figure 1. Concentration of (A) homocysteine (Hcy) and (B) serum amyloid A (SAA) in healthy dogs (HD) and dogs with dirofilariosis (DIR), babesiosis (BAB) and pyometra (PYO). "n" indicates the number of tested samples. Kruskal-Wallis test, $P < 0.05$ is considered significant. The connectors extend between the groups with the significant difference, as identified by the *post-hoc* analysis according to Conover. Box represents the 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

Table 1. Hematology parameters (median with minimum and maximum) in healthy dogs (HD) and dogs with dirofilariosis (DIR), babesiosis (BAB) and pyometra (PYO). The number of the analyzed samples is in italics

Parameter	Reference range	HD	DIR	BAB	PYO	P-value
RBC (x10^{12}/L)	5.5-8.5	7.18 (5.66-7.94)^{6}	6.41 (3.44-8.09)^{21}	5.11 (1.64-9.07)^{12}	3.94 (3.51-5.69)^{4}	0.002*
HGB (g/L)	120-180	168 (106-188)^{6}	154 (90-204) ^{21}	115 (51-207)^{12}	98 (70-140)^{4}	0.006*
HCT (%)	37-55	41.1 (27.3-46.4)^{6}	39.1 (23.1-48.6)^{21}	30.7 (10.4-52.2)^{12}	26.3 (19.9- 37.5)^{4}	0.010*
MCV (fL)	60-77	58 (48-59)^{6}	60 (54-71)^{21}	62 (49-68)^{12}	62 (57-74)^{4}	0.178
MCHC (g/L)	310-340	405 (387-408)^{6}	395 (344-426)^{21}	385 (355-495)^{12}	382 (330-442)^{4}	0.545
WBC (x10^{9}/L)	6-17	12.2 (8.0-15.1)^{6}	13.9 (6.8-25.5)^{21}	4.7 (3.3-34.8)^{12}	29.8 (7.0-80.4)^{4}	<0.001$^{\#}$
NEUT (x10^{9}/L)	3-12	8.6 (5.6-12.4)^{6}	10.2 (5.7-19.3)^{18}	2.9 (0.4-27.9)^{12}	21.6 (5.5-72.6)^{4}	<0.001$^{\#}$
LYM (x10^{9}/L)	1-4.8	2.5 (2.0-4.4)^{6}	2.3 (0.9-6.2)^{18}	0.9 (0.1-4.5)^{12}	3.3 (1.4-8.2)^{4}	0.013$^{\#}$
PLT (x10^{9}/L)	200-500	393 (85-523)^{6}	258 (126-437)^{21}	0 (0-35)^{12}	227 (209-433)^{4}	<0.001$^{\#}$

Abbreviations: HCT- hematocrit; HDL-high density lipoproteins; HGB-hemoglobin; LYM-lymphocytes; MCHC-mean cellular hemoglobin concentration; MCV-mean corpuscular volume; NEUT-neutrophils; PLT-platelets; RBC-red blood cells; WBC-white blood cells. Symbols for significant difference: *- BAB vs. HD&DIR and PYO vs. HD&DIR; $^{\#}$- BAB vs. HD&DIR&PYO.

Table 2. Biochemistry parameters (median with minimum and maximum) in healthy dogs (HD) and dogs with dirofilariosis (DIR), babesiosis (BAB) and pyometra (PYO). The number of the analyzed samples is in italics

Parameter	Reference range	HD	DIR	BAB	PYO	P-value
GLU (mmol/L)	3-6.7	3.1 (2.3-3.8)^{6}	4.0 (2.2- 6.7)^{31}	5.3 (4.5-7.6)^{11}	4.2 (2.3-5.8)^{8}	0.001$^{\#}$
Urea (mmol/L)	3.3-9.2	5.9 (2.3-12.4)^{6}	4.7 (1.3-32.4)^{31}	4.9 (3.1-18.4)^{11}	2.8 (2.0-6.1)^{7}	0.149
CRE (µmol/L)	50-169	58 (47-150)^{6}	66 (27-209)^{30}	84 (53-240)^{11}	56 (28-152)^{7}	0.442
TP (g/L)	55-80	60 (58-75)^{6}	63 (44-74)^{31}	54 (39-65)^{11}	73 (46-76)^{8}	0.009†
ALB (g/L)	25-40	31 (27-39)^{6}	27 (18-38)^{31}	26 (17-32)^{11}	26 (18-29)^{8}	0.032‡
CHO (mmol/L)	2.5-6.5	4.3 (3.7-7.3)^{6}	5.4 (2.8-8.3)^{26}	4.4 (2.5-5.9)^{11}	6.3 (3.8-10.6)^{8}	0.031†
TRG (mmol/L)	0.6-1.3	0.80 (0.49-1.94)^{6}	0.80 (0.33-1.33) ^{26}	0.88 (0.04-1.79)^{11}	0.68 (0.44-1.30)^{8}	0.545
HDL (%)		71.3 (38.4-79.4)^{5}	59.5 (16.4-87.4)^{15}	50.4 (42.1-57.8)^{10}	71.9 (57.1-79.6)^{8}	0.017$^{\square}$
TRL (%)		28.7 (20.6-61.6)^{5}	40.5 (12.-83.6)^{15}	49.6 (42.2-57.9)^{10}	28.1 (20.4-42.8)^{8}	0.018$^{\square}$
ALT (U/L)	10-50	36 (16-166)^{6}	46 (7-527)^{27}	27 (11-101)^{11}	13 (7-84)^{8}	0.276
AST (U/L)	10-58	27 (18-104)^{6}	28 (17-107)^{31}	34 (23-90)^{11}	18 (7-27)^{8}	0.010‡
ALP (U/L)	0-190	87 (31-178)^{6}	98 (27-701)^{28}	282 (138- 411)^{11}	203 (110-723)^{8}	<0.001*
GGT (U/L)	0-7	6 (3-12)^{6}	7 (2-21)^{24}	2 (2-8)^{11}	3 (2-7)^{4}	0.006$^{\Delta}$

Abbreviations: ALB-albumin; ALP-alkaline phosphatase; ALT-alanine aminotransferase; AST-aspartate aminotransferase; CHO-cholesterol; CRE-creatinine; GGT-gamma-glutamyltransferase; GLU-glucose; HDL - high density lipoproteins; TP-total proteins; TRG-triglycerides; TRL - triglyceride rich fraction. Symbols for significant difference: *- BAB vs. HD&DIR and PYO vs. HD&DIR; $^{\#}$- BAB vs. HD&DIR&PYO; †- BAB vs. DIR&PYO; ‡-HD vs. DIR&BAB&PYO; $^{\square}$- BAB vs. HD&PYO and PYO vs. DIRO&BAB; ‡-PYO vs. DIR&BAB; $^{\Delta}$- BAB vs. HD&DIR.

Table 3. Correlation between hematology and biochemistry parameters (Spearman's rank correlation coefficient (ρ_S) with 95% confidence interval (CI)) with Hcy and SAA in the investigated dogs. The number of the analyzed samples is in italics

Parameter	Hcy (µmol/L)		SAA (ng/mL)	
	ρ_S (95% CI)	P-value	ρ_S (95% CI)	P-value
RBC (x10^{12}/L)	0.542 (0.265 to 0.737)^{37}	<0.001	-0.428 (-0.655 to -0.130)^{39}	0.007
HGB (g/L)	0.494 (0.202 to 0.705)^{37}	0.002	-0.382 (-0.622 to -0.075)^{39}	0.016
HCT (%)	0.432 (0.125 to 0.663)^{37}	0.008	-0.340 (-0.592 to -0.028)^{39}	0.033
MCHC (g/L)	0.470 (0.172 to 0.689)^{37}	0.003	-0.332 (-0.586 to -0.018)^{39}	0.039
WBC (x10^{9}/L)	0.395 (0.081 to 0.637)^{37}	0.015	0.240 (-0.035 to 0.482)^{52}	0.086
NEUT (x10^{9}/L)	0.392 (0.068 to 0.642)^{35}	0.020	-0.374 (-0.623 to -0.057)^{37}	0.022
GLU (mmol/L)	-0.442 (-0.643 to -0.183)^{49}	0.001	0.426 (0.171 to 0.628)^{51}	0.002

Abbreviations: GLU-glucose; HCT- hematocrit; HGB-hemoglobin; MCHC-mean cellular hemoglobin concentration; NEUT-neutrophils; RBC-red blood cells; WBC-white blood cells.

BAB group had the lowest median concentrations of total proteins and cholesterol. The ALP activity was higher in the BAB and PYO groups than in HD and DIR.

As presented in Table 3, almost every parameter that was in positive or negative correlation with homocysteine was in the reversed correlation with SAA. The only exception was WBC count, which was in positive correlation with both Hcy and SAA. Finally, the multiple regression analysis marked the hemoglobin level as the only independent predictor of the Hcy concentration among the investigated dogs (regression coefficient=0.094, P=0.012).

DISCUSSION

Our results showed that Hcy concentrations were lower in dogs with infections that involved moderate or severe APR (acute babesiosis and pyometra) in comparison with the healthy dogs and dogs with infections characterized with mild, chronic APR (dirofilariasis). Moreover, concentrations of Hcy and SAA were in a negative correlation. As SAA represents a major positive acute-phase protein in dogs, the increase in concentration could discriminate different degrees of inflammation (Christensen et al., 2014). That was the case in our study-the dogs with acute inflammation (BAB and PYO groups) had higher SAA levels than dogs with chronic inflammation due to the infection with D. immitis. The Hcy level was less discriminatory as the difference was evident only between the dirofilariasis and pyometra cases. Our results are concordant with the findings of Patterson et al. (2013.) that Hcy was lower in the dogs with SIRS and sepsis when compared to the healthy dogs. Therefore, the results of this study allow considering that Hcy, in dogs, could be a negative acute phase reactant, when infective agents are "driving" the inflammation.

In their study, Patterson et al. (2013) marked poor food intake as the possible cause of low Hcy level in dogs with the complicated form of severe APR. Banton et al. (2021) showed the direct relationship between six hours postprandial Hcy level in blood and the methionine content in the pet food. Inapatence lasting several days is a common sign in babesiosis and pyometra, while it does not occur often in asymptomatic dirofilariasis (Leschnik, 2020). Therefore we could hypothesize that decrease in the Hcy level in blood resulted from the reduction in the dietary availability of methionine that occurred as the APR intensified, thus dogs from healty group and dirofilaria group were fasted for 12 hours at least, before sampling. Also, low albumin concentration in blood might represent another reason for the decreased Hcy level because almost all Hcy in the circulation is in complex with albumin (Hortin et al., 2006). Our results show both lower Hcy and albuminemia levels in the BAB group in comparison with the HD. Nevertheless, the dogs in the PYO group had lower Hcy concentrations than dogs in the DIRO group despite the absence of difference in albuminemia between them. The observed heterogeneity implies the necessity for further studies to decipher the multifactorial nature of the alterations in the Hcy metabolism during canine infections.

Further, we demonstrated that both Hcy and SAA correlated with the same parameters (erythrocyte count, hemoglobin, hematocrit, mean cellular hemoglobin concentration, neutrophil count, and glycemia) with correlations being of opposite direction. Anemia, when present in dogs with acute or chronic inflammatory conditions, resulted from a combination of functional iron deficiency, the effect of proinflammatory cytokines, and autoantibodies (Nairz et al., 2016). In pyometra, the high SAA levels accompany the marked leucocytosis (Jitpean et al., 2014), while the concomitant occurrence of the increased SAA concentration and leukopenia is common in acute babesiosis (Milanović et al., 2017). Also, the dogs from the BAB group in our study had the Hcy levels higher than those measured in the PYO group, but lower than those in the DIRO and HD groups. These comparisons might explain the overall positive correlation of WBC with both Hcy and SAA. The negative correlation between the Hcy levels and glycemia, together with the positive relationship among the concentrations of glucose and SAA, could mirror the stress response that developed in parallel with the APR (Yuki et al., 2019). Also, the finding that healthy greyhounds had higher Hcy and hematocrit when compared with the other dog breeds (Heilmann et al., 2017) corroborated our results that marked hemoglobin level was the only independent Hcy predictor among the investigated parameters, altough we had not have any greyhound, in our research.

The range of the Hcy concentrations in the HD group was similar to those that the other authors measured in the healthy dogs using high-performance liquid chromatography (Kakimoto et al., 2014) or gas chromatography with mass spectrometry detection (Grützner et al., 2013; Patterson et al., 2013). Howev-

er, when authors decided to use enzyme-linked immunosorbent (Çayir & Kozat, 2016), immunochemiluminiscent (Gołyński et al., 2017; Lee & Hyun, 2012), or enzymatic methods (Rossi et al., 2008), the Hcy concentrations in healthy dogs were lower than in our survey. Apart from different analytic performances, the bias can also result from differences in breed, gender, dietary availability of vitamin B_{12} and folate, etc.

The relatively small total number of dogs and their unequal distributions among the groups represented the limitations of this survey. Also, the availability of vitamin B_{12} and folate levels in the blood would enhance the interpretation of the results. Regardless, the results provide a reliable rationale for further studies on altered Hcy homeostasis in dogs under inflammatory conditions. They raise a hypothesis that, besides the host factors, the parasites *per se* might interfere with the Hcy homeostasis. *Plasmodium falciparum*, another apicomplexan parasite like *B.canis*, utilizes the methionine metabolites to synthesize polyamines (Chillemi et al., 2004). Also, the presence of the non-mammalian form of cystatione-β-synthase in the filarial worms (Gupta & Srivastava, 2005) implicates that *D. immitis* could metabolize Hcy.

CONCLUSION

In the canine infections, the Hcy level lowered as the APR intensified. Also, a relationship existed between the hematology and biochemistry changes that accompanied the APR and the Hcy concentration in blood. Further studies will establish whether the alterations in the Hcy metabolism have clinical potential or might represent just an additional epiphenomenon in the omni-complex inflammation “puzzle”.

FUNDING SOURCES

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (contract number: 451-03-9/2021-14/200143).

AUTHORS’ CONTRIBUTION

AB and MKF designed the study; ABI, FJ, KS collected the samples and anamnestic data; PĐ, VR, VM, and DF referred the animals, with the anamnestic data, to the Small Animal Clinic of the Faculty of Veterinary medicine, University of Belgrade. ABI, ZM, MR, SS, and AB performed the laboratory analyses; AB and ABI performed and interpreted the statistical analyses; ABI, AB, MKF, and SS interpreted the data; ABI, MKF, and AB equally contributed in the writing of the manuscript. All authors have read and approved the manuscript.

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