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RESEARCH ARTICLE

Association of Increased Osmotic Fragility of Red Blood Cells with Common Systemic Inflammatory Diseases in Dogs

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A B S T R A C T

Red blood cell osmotic fragility (RBCOF) refers to susceptibility to hemolysis *in vivo* and *in vitro*. An increase in mean cellular hemoglobin concentration (MCHC) also reflects *in vivo* and *in vitro* hemolysis. Common inflammatory infectious diseases in dogs are often characterized by an increase in MCHC; however, it is not known if there is a concomitant increase in RBCOF. The objective of this study was to determine RBCOF in dogs with babesiosis, dirofilariosis, parvovirosis, pyometra, and canine distemper and to investigate whether RBCOF correlates with routine hematologic and biochemical parameters and lipoprotein fractions. The diagnosis of canine infectious disease was made and complete blood count, serum biochemical profiles, and lipoprotein electrophoresis were determined according to current institutional protocols. RBCOF of healthy control (n=8) and infected dogs (n=73) was measured with a phosphate-buffered saline dilution test, and mean corpuscular fragility (MCF) was calculated. MCF was increased in dirofilariosis, pyometra, and distemper; MCHC was increased in all infected groups; HDL was decreased in babesiosis, pyometra, and distemper compared with healthy controls. However, no correlation was found between these parameters. On the other hand, hematological and biochemical parameters, that are highly predictive of specific infections, correlated with MCF: ALP for babesiosis (r=0.415, *P*=0.035), urea (r=-0.488, *P*=0.034) for dirofilariosis, and leukocyte count (r=-0.820, *P*=0.046) for pyometra. Although elevated MCHC levels in all samples from infected dogs indicate hemolysis, RBCOF was elevated only in dirofilariosis, pyometra, and canine distemper, leading to the conclusion that these apparently related phenomena are controlled by different mechanisms.

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INTRODUCTION

Red blood cell osmotic fragility (RBCOF) depends roughly on its shape and hydration and reflects, within certain limits, its susceptibility to destruction *in vivo* and/or *in vitro*. In addition to various inherited disorders, a spectrum of acquired disorders increases RBCOF, leading to premature hemolysis (Igbokwe, 2018). Acquired disorders such as secondary immune-mediated hemolytic anemia, structural alterations of the erythrocyte membrane and/or cytoplasm, hypotonic dehydration or hyperhydration, and systemic metabolic alterations leading to a decrease in ATP production, have been described (Igbokwe, 2018). The effects of an increase in triglyceride-rich β-lipoproteins on RBCOF in dogs have been documented (Buranakarl *et al.,* 2009; Behling-Kelly and Collins-Cronkright, 2014). In addition, infectious diseases of dogs, such as babesiosis and ehrlichiosis, have been shown to increase RBCOF (Makinde and Bobade, 1994). However, it is not known whether common inflammatory diseases typical of the broad canine population are also characterized by dyslipidemia and changes in RBCOF.

The increase in mean cellular hemoglobin concentration (MCHC) reflects *in vivo* hemolysis and/or *in vitro* hemolysis due to incorrect blood sampling (Gilor and Gilor, 2011). Hemolytic anemia due to immune-mediated mechanisms or the formation of Heinz bodies are examples of high MCHC values due to an increase in RBCOF (O'Rourke, 2022). Although high MCHC levels are not uncommon in clinical situations, elevated RBCOF remains undetected in many pathologic conditions. However, in some cases, *in vitro* hemolysis occurs more frequently as an epiphenomenon. For example, coinfection with *Babesia canis* and *Dirofilaria immitis* in dogs often results with a higher MCHC than mono-infection with *B. canis* (Milanović *et al.*, 2017). Other common diseases in the canine population that show an increase in MCHC are parvovirosis and canine distemper, which are caused by parvoviruses and morbilliviruses, respectively. Pyometra is an acute or chronic purulent bacterial infection of the uterus that is associated with hormonal disruption and often results in elevated MCHC with *E. coli* as the most common pathogen (Hagman, 2022). All of the above diseases are characterized by acute, subacute, or chronic systemic inflammation, but etiologic agents range from intraerythrocytic protozoa in the case of *B. canis*, intravascular nematodes in the case of *D. immitis*, two viruses, and bacterial infections in pyometra. Although dyslipidemia has been described in babesiosis and dirofilariosis in dogs (Milanović *et al.*, 2019), it has not been investigated in other diseases of interest in this study. The aim of this study was to evaluate RBCOF in the above infections in dogs and to investigate its association with MCHC, other routine hematological and biochemical parameters and dyslipidemia.

MATERIALS AND METHODS

Study design and population: Based on clinical and laboratory findings, 81 dogs were divided into groups according to diagnosis: babesiosis, dirofilariosis, parvovirosis, pyometra, distemper and healthy dogs.

Babesia spp. infection was diagnosed in 29 dogs (17 males, 12 females, average age: 23 months) when dogs had fever, anorexia, depression, pale mucous membranes, tachycardia, dyspnea, jaundice and/or hemoglobinuria, and large *Babesia* forms in the blood smear; follow-up confirmed that the dogs recovered after treatment with imidocarb-dipropionate (6.6mg/kg).

Dirofilaria immitis infection at stage II/III was diagnosed in 22 dogs (19 males, 3 females, average age: 80 months) when exercise intolerance, chronic cough and progressive weight loss were consistent with a positive rapid antigen (Canine Heartworm, BioNote, Korea) and positive modified Knott test.

Parvovirosis was diagnosed in 9 puppies (4 males, 5 females, average age: 5.5 months) when dogs had fever, anorexia, depression, vomiting, diarrhea, and positive Canine Parvovirus Antigen test (BioNote, Korea).

Six female dogs (average age: 85 months) were diagnosed with pyometra based on fever, anorexia, depression, vaginal discharge, polyuria, polydipsia and ultrasound-detectable uterine dilation with anechoic to hyperechoic fluid.

Canine distemper was suspected in 7 dogs (3 males, 4 females, average age: 15 months) with fever, depression, and purulent discharge from the eye and nose, and confirmed by a positive rapid antigen test (Canine Distemper, BioNote, Korea).

Eight dogs without clinical signs of disease (5 males, 3 females, average age: 83 months) were included in the healthy group.

The exclusion criteria were: 1) reticulocytosis and polychromasia in the blood smear, 2) immune-mediated hemolytic anemia detected by saline agglutination test (SAT) and 3) any comorbidity.

The study was conducted at the Faculty of Veterinary Medicine, University of Belgrade, Serbia, between March 2018 and November 2019 and was approved by an institutional ethics committee (numbers: 323-07-03455/2015-05/3 and 323-07-04497/2019-05).

Hematological and biochemical analyses: The venous blood was collected using 20-G needle in K_2EDTA and Clot Activator BD Vacutainer tubes by experienced technician. A complete blood count was obtained on Abacus Junior Vet (Diatron, Vienna, Austria); blood smear and reticulocyte count were performed within 1 hour of sample collection. The SAT was performed using one drop of EDTA blood and four drops of saline at room temperature. Erythrocyte true agglutination was observed microscopically. Serum biochemical profiles included total protein, albumin, glucose, urea, creatinine, cholesterol, triglycerides, phosphorus concentrations, ALT, AST and ALP activity on Technicon RA-XT (Bayer, Dublin, Ireland) using commercial reagents (Elitech Clinical Systems, France).

Serum lipoprotein agarose electrophoresis: Agarose strips were prepared and analyzed as described by Milanović *et al.* (2019). LDL and VLDL bands were combined and the fraction of triglyceride-rich lipoproteins (TRL) was calculated, whereas HDL1 and HDL2 were summed in one HDL band to increase the accuracy of interpretation.

Osmotic fragility test (OFT): OFT was performed as previously described by Tritschler *et al.* (2016). Samples were tested within 24 hours of collection. Briefly, 15µl of EDTA whole blood was mixed with a series of 2mL phosphate-buffered NaCl dilutions, concentrations of 0.85, 0.8, 0.75, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.25, and 0% NaCl. After incubation at room temperature for 30 minutes, samples were centrifuged (2000*g*/10 minutes), and optical densities of the supernatants were measured at 540 nm. The percentage of hemolysis in each tube was calculated, and the mean osmotic fragility was determined from the obtained lysis curve. The concentration of NaCl at which 50% of the erythrocytes were lysed was designated as the mean corpuscular fragility (MCF).

Statistical analysis: Descriptive statistics, Kruskal-Wallis test with post-hoc analysis by Conover, Spearman's rank correlation, multiple and logistic regression were used for statistical analysis using MedCalc® software.

RESULTS

Hematological and biochemical analyses: Among dogs with babesiosis and parvovirosis, MCF did not differ, whereas in dogs with dirofilariosis, pyometra, and distemper, MCF was higher than in healthy animals (*P*=0.012) (Fig. 1). In dogs with babesiosis, serum was slightly hemolyzed (pink-tinged) after centrifugation, due to the presence of *in vivo* hemolysis. Routine hematologic analysis revealed mild normocytic-normochromic anemia in all dogs infected with large *Babesia* forms, half of the puppies with parvovirosis, females with pyometra and in two-thirds of the dogs with canine distemper (Fig. 2A-D). In contrast, dogs with dirofilariosis did not exhibit anemia (Fig. 2A-D). On the basis of MCHC, hemolysis was detected in all of the examined samples (Fig. 2F). Dogs with babesiosis had severe thrombocytopenia (Fig. 2G), whereas all dogs with dirofilariosis and most dogs with parvovirosis and distemper had mild thrombocytopenia (Fig. 2G). In females with pyometra, the platelet count was within the reference range (Fig. 2G). Half of the dogs with babesiosis had mild leukopenia, one-third of the dogs with dirofilariosis had mild leukocytosis, all dogs with pyometra had severe leukocytosis and there was no change in leukocyte count in dogs with distemper (Fig. 2H).

The main biochemical changes observed were high AST activity in dogs with babesiosis (Fig. 3A), high ALP activity in all groups of dogs except those with dirofilariosis (Fig. 3B) and high urea concentration in dogs with babesiosis, dirofilariosis and canine distemper (Fig. 3C). Serum total cholesterol and triglyceride concentrations were within reference intervals and did not differ between infected and healthy dogs (data not shown).

Analysis of lipoprotein fractions showed that all dogs had similar levels of TRL (Fig. 4A). In contrast, dogs with babesiosis, pyometra, and canine distemper had lower HDL levels (*P*< 0.001) than healthy controls (Fig. 4B). In addition, dogs with dirofilariosis and parvovirosis had higher HDL levels than all other groups except the healthy dog group (Fig. 4B). Only in dogs with babesiosis and pyometra TRL/HDL ratio differed from the one in the healthy controls, whereas in dogs with dirofilariosis it was also different from that of babesiosis and pyometra (Fig. 4C).

Correlation analyses and multivariate logistic regression: In healthy dogs, MCF was positively correlated with age and glucose and albumin concentrations, whereas in babesiosis it was correlated with ALP activity. In dirofilariosis, MCF correlated negatively with urea and creatinine concentrations and in pyometra, it correlated negatively with total leukocytes, phosphates, and MCHC (Table 1). MCF and TRL, HDL, or TRL/HDL did not correlate in specific groups nor in the entire dog population studied. Using logistic regression analysis, we identified laboratory markers that might be indicative for selected diseases (Table 2). Some of these markers were negatively correlated with MCF in dirofilariosis and pyometra and positively correlated in babesiosis.

Table 1: Correlations of MCF with hematological and biochemical parameters in selected diseases.

	Spearman's coefficient of rank correlation (rho)	Significance level (P value)
Healthy dogs (N=8)		
Age	0.862	0.006
Glucose	0.826	0.011
Babesiosis (N=27)		
ALP	0.415	0.035
Dirofilariosis (N=19)		
Urea	-0.488	0.034
Creatinine	-0.524	0.021
Pyometra (N=6)		
WBC	-0.820	0.046
Phosphorus	-0.820	0.046
MCHC	-0.820	0.046

Table 2: Multivariate logistic regression analysis with the altered values of laboratory parameters as dependent variables.

Parameter	Condition	OR	95% CI	*P*-value
WBC>17x10^9/L	*Pyometra*	120.00	9.17 – 1569.68	<0.001
	Dirofilariosis	11.08	2.00 – 61.47	0.006
WBC<6x10^9/L	*Babesiosis*	29.71	3.54 – 249.73	0.002
PLT<200x10^9/L	*Babesiosis*	62.50	4.71 – 829.30	0.002
	Dirofilariosis	14.00	1.30 – 150.89	0.030
Urea>10 mmol/L	*Dirofilariosis*	11.00	1.82 – 66.37	0.009
AST>60 U/L	*Babesiosis*	19.38	2.26 – 166.51	0.007
ALP>114 U/L	*Babesiosis*	15.75	4.44 – 55.93	<0.001
	Parvovirosis	22.50	2.20 – 229.45	0.009

DISCUSSION

The level of RBCOF was elevated in dogs with dirofilariosis, pyometra, and distemper. Surprisingly, RBCOF in dogs with acute babesiosis and parvovirosis did not differ from that in healthy control animals. The first consideration that illuminates these results is the different course of dirofilariosis, pyometra, and distemper compared with babesiosis and parvovirosis. Dirofilariosis is a chronic disease, whereas pyometra and distemper may be subacute and have more profound effects on RBCOF than acute diseases such as babesiosis and parvovirosis, because of the longer time period over which the insult can act. Babesiosis in Europe is usually acute/peracute disease but could be chronic as well. The hypothesis that increased MCF could be established as a marker of disease duration warrants further investigation.

HDL fraction was lower in dogs with babesiosis, pyometra, and distemper than in healthy dogs, but it did not correlate with RBCOF in the group or in the overall population. For example, elevated MCF was shown to be typical of dirofilariosis, which is characterized by chronic inflammation and no change in HDL, and distemper and pyometra, which are characterized by acute inflammation and low HDL. Thus, it could be concluded that changes in HDL do not affect MCF and that changes in TRL are more important for erythrocyte membrane stability, as shown by Behling-Kelly and Collins-Cronkright (2014). Also, TRL are more tightly associated with increased cholesterol content of RBC membranes and increased RBCOF than total cholesterol (Behling-Kelly and Collins-Cronkright, 2014).

Our results showed that MCF increases with age in healthy dogs. It seems that different studies do not provide conclusive results regarding the effect of age on RBCOF and often report contradictory results, even for the same species (Paraiso *et al.,* 2017; Igbokwe, 2018; Kabakçi *et al.,* 2022). However, recent studies in cats are consistent with our findings and show that both aging and OF are

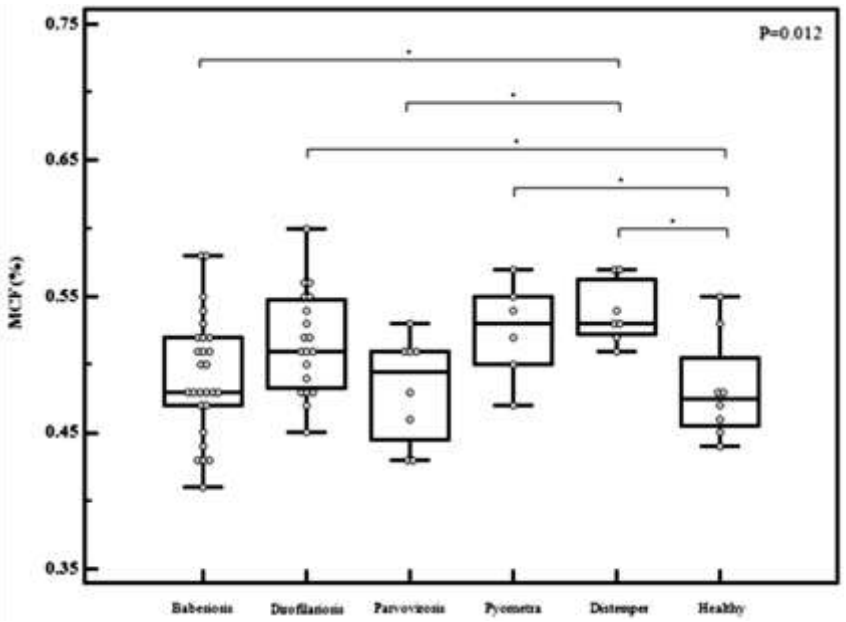

Fig. 1: Box and whisker plot of MCF values for healthy dogs, dogs with babesiosis, dirofilariosis, parvovirosis, pyometra, and distemper infection. Boxes represent the interquartile ranges from the 25th to the 75th percentile; solid horizontal lines through the boxes represent the mean values, and the minimum and maximum values are represented by the capped vertical bars.

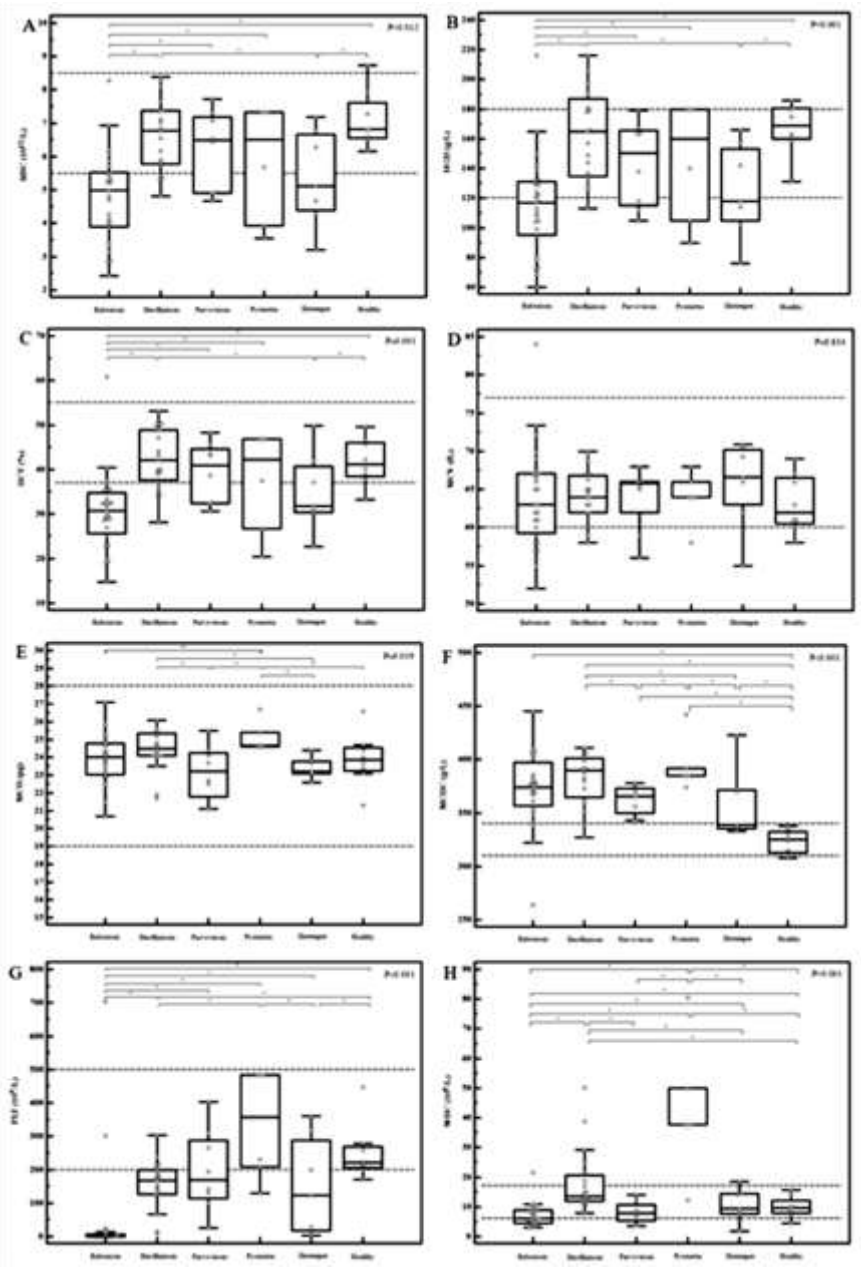

Fig. 2: Box and whisker plot of hematologic parameters (A-H) for healthy dogs, dogs with babesiosis, dirofilariosis, parvovirosis, pyometra, and distemper infection. The boxes represent the interquartile ranges from the 25th to the 75th percentile; solid horizontal lines through the boxes represent the mean values, and the minimum and maximum values are represented by the capped vertical bars.

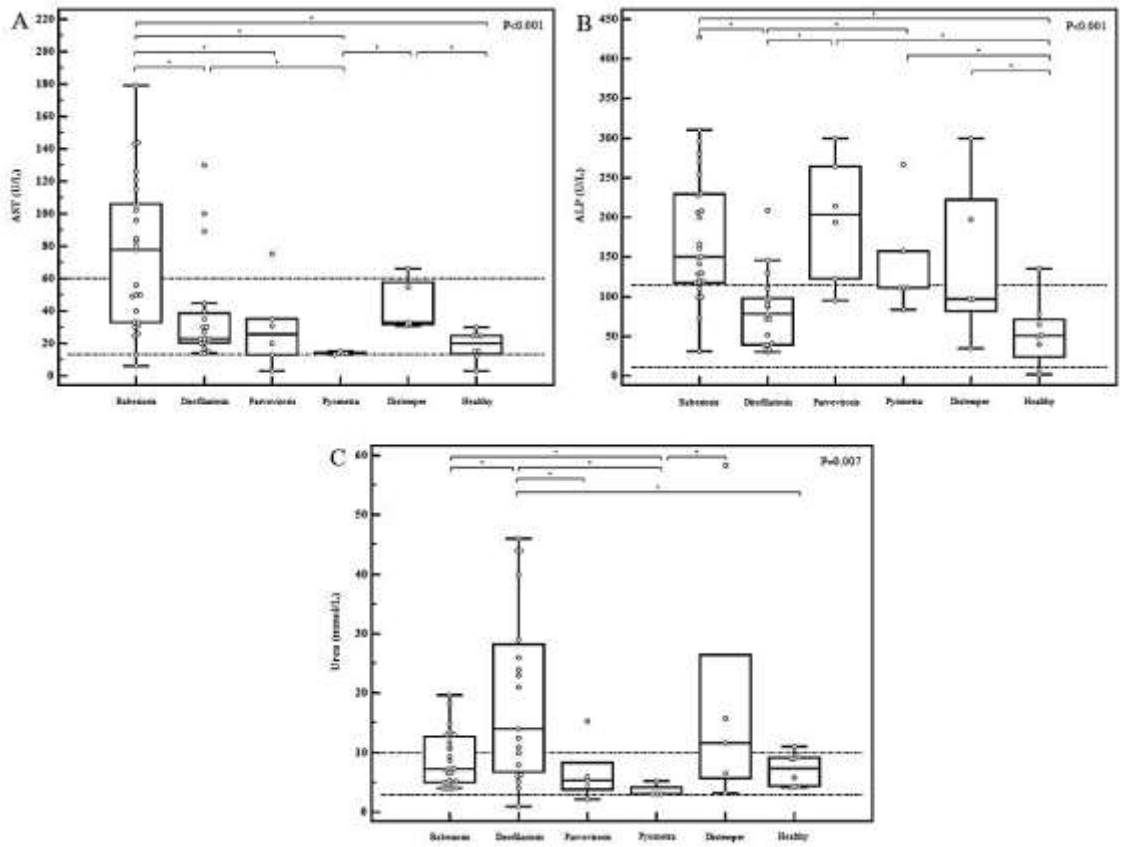

Fig. 3: Box and whisker plot of the most significant biochemical changes (A-C) in healthy dogs, dogs with babesiosis, dirofilariosis, parvovirosis, pyometra, and distemper infection. The boxes represent the interquartile ranges from the 25th to the 75th percentile; solid horizontal lines through the boxes represent the mean values, and the minimum and maximum values are represented by the capped vertical bars.

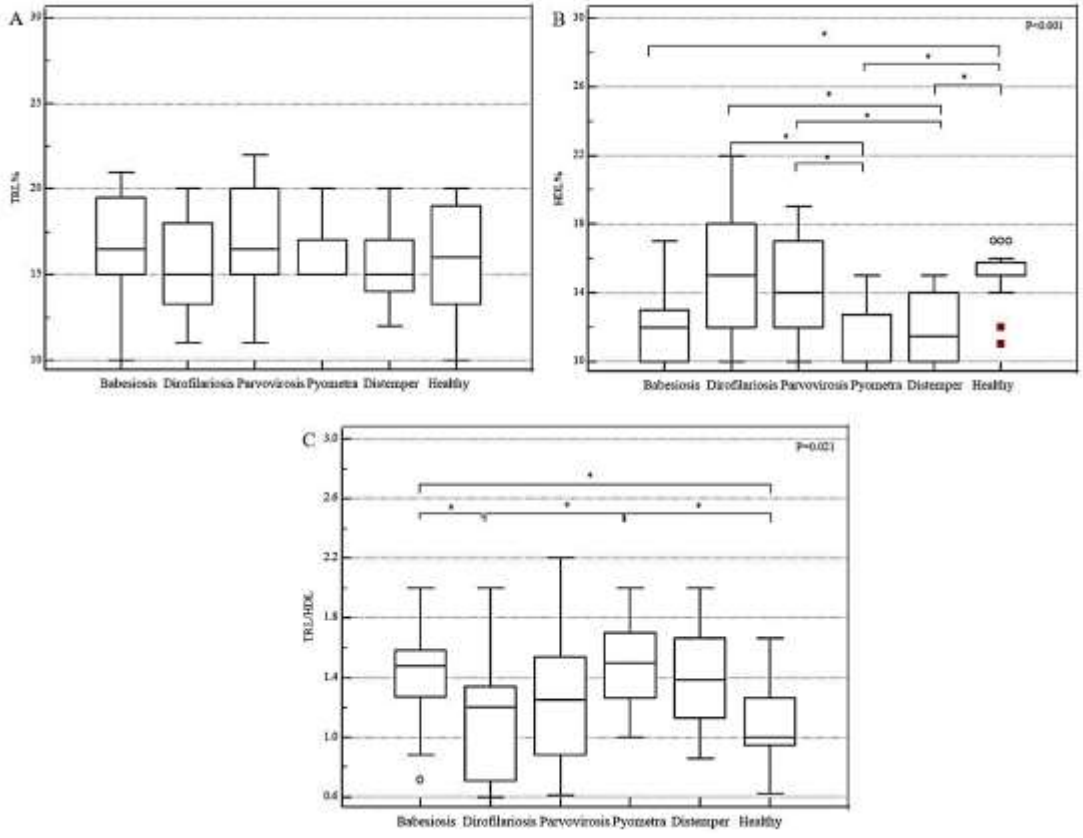

Fig. 4: Box and whisker plot of electrophoretic lipid fractions and their ratio (A-C) in healthy dogs, dogs with babesiosis, dirofilariosis, parvovirosis, pyometra, and distemper infection. The boxes represent the interquartile ranges from the 25th to the 75th percentile; solid horizontal lines through the boxes represent the mean values, and the minimum and maximum values are represented by the capped vertical bars.

associated with increased oxidative stress (Kabakçi *et al.,* 2022). This suggests that animal's age is clearly a risk factor for increased RBCOF; however, the lack of standardization of the MCF test in veterinary medicine is the reason that different studies cannot be compared, even when healthy dogs are involved (Behling-Kelly and Collins-Cronkright, 2014). Given that dogs with dirofilariosis and pyometra have almost the same average age as healthy dogs, no correlation was observed between MCF and age, probably because the factors involved in the mechanisms of the disease were predominant at that time.

The MCHC was elevated in all diseased dogs. Since, *in vivo*/*in vitro* hemolysis increases the MCHC, distinction between artifactual or pathological causes should be done (Whipple *et al.,* 2020). Given that blood sampling and analyses were standardized in both diseased and healthy animals, with no *in vivo* /*in vitro* hemolysis observed, we assume that preanalytical sample collection error is reduced to a minimum. Even so, pathological hemolysis is a relatively uncommon clinical finding (Whipple *et al.,* 2020). MCF was increased in dogs with dirofilariosis, pyometra and distemper infection, indicating that factors related to pathogenesis of underlying disease state is involved.

Interestingly, MCF, although difficult to interpret, correlated positively with glucose concentration, which was within the reference range. This could mean that even the upper range of the reference intervals for glucose concentration affects the glycation process, which can damage erythrocyte membranes.

Not all dogs with acute babesiosis had elevated MCF, which was an unexpected finding. One possible explanation may be that secondary anti-erythrocytic hemolytic antibodies do not develop early in the course of the disease. High ALP activity is associated with increased MCF in dogs with acute babesiosis. Furthermore, our previous studies have shown that inflammation in dogs with babesiosis is associated with oxidative stress, which is evident in the form of elevated malondialdehyde levels even 15 days after successful treatment of the disease (Spariosu *et al.,* 2021). This suggests that bilirubinemia / bilirubinuria with high ALP activity indicative of cholestasis and oxidative stress in babesiosis may have an impact on MCF, although not strong enough to increase RBCOF.

Interestingly, dogs with dirofilariosis had a high MCF. Considering that adult *D. immitis* and billions of larvae live in a dog's blood vessels for at least six months prior to blood collection (Noack *et al.,* 2021), a fine-tuned balance between proinflammatory and anti-inflammatory mechanisms that could affect MCF must be pushed to the limit, where these mechanisms are still controlled *in vivo* but are no longer operative during blood collection. In addition to possible inflammatory events affecting the MCF index, higher urea and creatinine levels also appear to have an influence, although without obvious explanation, as the correlation with MCF was inverse. Dogs from the present study infected with *D. immitis* had moderately high urea levels without any change in creatinine levels, suggesting possible dehydration without significant renal disease.

Dogs with parvovirosis were extremely young (median 5.5 months). MCF was not different from that of healthy dogs. MCHC was higher than in healthy dogs but lower than in dogs with other infections. Both results are consistent with the hypothesis that young animals have lower erythrocyte osmotic fragility, i.e., better osmotic resistance (Igbokwe, 2018).

Pyometra is usually associated with severe inflammation. Our results showed that a lower leukocyte count is positively correlated with increased MCF. This seems paradoxical, as leukocytes are the main source of oxygen free radicals that damage erythrocytes (Veenith *et al.,* 2022). However, leukocytosis in pyometra results in two ways: increased bone marrow production with the release of young, low-functioning neutrophils and decreased trans-endothelial migration that allows them to remain in blood vessels. Neither young nor mature neutrophils have optimal function, which may explain this inverse non-intuitive correlation. The other inverse correlation, relating low MCHC and high MCF, is questionable and remains to be confirmed or rejected. An interesting finding in pyometra is that low phosphorus levels are associated with higher erythrocyte osmotic fragility. In cats with hepatic lipidosis and cows with puerperal hypophosphatemia, erythrocytes are depleted of this element due to low serum phosphorus and ATP synthesis is impaired to the point of abrupt intravascular hemolysis (Adams *et al*., 1993; Grünberg *et al.,* 2015).

Dogs with canine distemper had elevated MCHC and high MCF. This could be explained, at least in part, by the fact that morbillivirus replicates in many different cell types, including red blood cells (Schultze, 2010). Although not all dogs suffered from anemia, RBCOF was strongly affected by this infection.

Interestingly, the majority of laboratory parameters which could predict selected diseases correlate with MCF, suggesting their influence on erythrocyte lifespan through different mechanisms which are still insufficiently examined.

Limitations of this study include small sample size, heterogeneous groups, and bias in parvovirus and canine distemper MCF due to the young age of the dogs.

Conclusions: Our results show that common inflammatory infectious diseases in dogs are typically characterized by some degree of hemolysis, as reflected by elevated MCHC and MCF. The surprising finding was that MCHC and MCF did not correlate. In addition, changes in HDL did not correlate with changes in MCF. In each individual disease, MCF correlated with several hematologic and biochemical parameters, often those shown to be highly associated with disease, suggesting a possible underlying mechanism of increased osmotic fragility.

Animal Welfare Statement: Dog owners signed informed consent that the residual samples and the obtained results could be used for scientific purposes. The research was approved by Ethical Committee at the Faculty of Veterinary Medicine, University of Belgrade, Serbia and based on the Serbian Law of Animal Welfare, permission was acquired from the Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (permission numbers: 323-07-03455/2015-05/3 and 323-07-04497/ 2019-05).

Declaration of Competing Interests: The authors declare that they do not have any financial or personal conflicts of interest that could bias the study.

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Authors contribution: JFA designed the study; MI^1, MI^2, KS and NA collected the samples; MI^1, MI^2, MR, KS and JFA performed the analyses; AB and MKF performed and interpreted the statistical analyses. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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