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Alterations in serum thiol-disulfide homeostasis and ischemia-modified albumin concentrations in clinical canine parvoviral enteritis

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Abstract: Monitoring biomarkers related to inflammation and oxidative stress is critical in dogs because parvovirus causes both inflammatory and antioxidant alterations. The aim of this study was to investigate inflammatory and antioxidant changes caused by canine parvoviral enteritis to better understand the oxidative stress process related to this disease. Thus, the total thiol, native thiol, disulfide, and ischemia-modified albumin levels of Canine parvovirus infected symptomatic puppies and healthy puppies were examined. Using the results of complete blood counts, the blood serum thiol-disulfide homeostasis and ischemia modified albumin levels of the puppies with Canine parvoviral enteritis (n = 65) and the healthy puppies (n = 34) were compared. Canine parvoviral enteritis and control groups showed a statistically significant difference in thiol disulfide levels ($p < 0.01$), while no significant difference was observed in ischemia modified albumin levels between the two groups. As a result of this study, a picture contradictory to the literature information was discovered; it is believed that integrating research on oxidative stress at various stages of disease progression, including the early stage, clinical period and recovery processes may provide more information about the dynamics of oxidative stress during disease progression.

Key words: Canine parvoviral enteritis, ischemia modified albumin, oxidative stress, thiol-disulfide homeostasis

1. Introduction

Canine parvoviral enteritis (CPVE) is a globally prevalent disease characterized by high morbidity and mortality rates. It impacts the gastrointestinal tract, bone marrow, lymphoid tissues, and cardiac myocytes in puppies [1–3]. While the disease may progress asymptomatically, infection symptoms can range from severe and/or hemorrhagic gastroenteritis to marked leukopenia, dehydration, vomiting, lethargy, tachycardia, myocarditis, and death. Canine parvoviral enteritis has the potential to infect dogs of all ages, although it poses a higher risk to puppies under 6 months old and unvaccinated dogs [3–5]. The most common clinical manifestations are protein loss, bacterial sepsis, villous atrophy, bloody diarrhea, and vomiting caused by intestinal damage [3,6].

Although CPVE infection can be diagnosed on the basis of clinical findings, laboratory evaluation plays a very important role in distinguishing between this disease and other infections that cause bloody diarrhea in puppies [5]. While there are polymerase chain reaction (PCR) tests available with high sensitivity and specificity for diagnosing this disease, the most common method for initial rapid diagnosis is the use of inexpensive and reliable rapid test kits for fecal antigenic diagnostics with moderate/low specificity compared to PCR [7–10]. The most consistent hematologic findings in puppies affected by CPVE infection are leukocytic and erythrocytic abnormalities [6]. Since the virus attacks actively proliferating bone marrow, the thymus and other lymphoid tissue cells, infection decreases the total leukocyte counts and causes leukoneutropenia and immunosuppression [3,11,12].

Oxidative stress is defined as the imbalance between an organism's production rate of reactive oxygen and nitrogen species (ROS/RNS) and its ability to balance, counteract and maintain homeostasis through antioxidant defenses [13,14]. Reactive oxygen species (ROS) are oxygen-containing, chemically reactive products of aerobic metabolism. Reactive oxygen species are reactive substances such as oxygen radicals and superoxide anions, hydroxyl radicals, and hydrogen peroxide [15]. Maintaining antioxidant capacity is important for cellular homeostasis in living organisms. Antioxidants

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prevent the formation of ROS or damage caused by them and contribute to detoxification [16]. There are several methods utilizing different molecules that can measure the levels of different oxidants in serum or plasma to examine oxidative stress. However, these measurements can be time-consuming, costly, and require complex techniques, while only providing information about the specific molecule being measured [17].

The process of reversing thiol oxidation inside proteins is referred to as dynamic thiol-disulfide homeostasis (TDH). Thiol disulfide homeostasis is a measurement of the balance between thiols and disulfides within an organism. Thiols are chemical compounds distinguished by sulfhydryl groups composed of sulfur and hydrogen atoms. Disulfides, on the other hand, constitute a crucial category of covalent bonds that are redox-reactive and formed between two thiol groups. The equilibrium between thiols and disulfides, represented by TDH, is a significant parameter linked to numerous biochemical processes. It has been correlated with various disorders of both known and unknown causes and is influenced by conditions that lead to increased oxidative stress [17–20]. Thiols play a crucial role as antioxidants by eliminating ROS through both enzymatic and nonenzymatic mechanisms [21]. Various methods have existed for measuring thiol levels for many years. Recently, a novel automated technique devised by Erel and Neselioglu [19] has been employed for quantifying disulfide levels. In this approach, disulfide levels are assessed in conjunction with native thiol, and the combined result of these measurements is presented as the total thiol level. The adoption of this method has contributed to an improved comprehension of TDH in organisms [17–19,22].

Given that CPVE induces both inflammatory and antioxidant alterations, there is clinical significance in monitoring biomarkers related to inflammation and oxidative stress [23]. Studies have shown that oxidative stress markers increase and antioxidant defense efficiency decreases in dogs with CPVE. Recognizing this phenomenon, along with the potential for disseminated intravascular coagulation complications, becomes essential from a prognostic standpoint when treating these cases [24].

Ischemia arises when there is an imbalance between the supply and demand of blood flow in the heart [25]. Ischemia-modified albumin (IMA) is a chemical derivative of serum albumin whose N-terminal end is modified due to exposure to ROS secondary to oxidative stress and/or ischemia [26]. Endothelial or extracellular hypoxia were shown to be possible causes of elevated IMA. Elevated IMA values have also been associated with many other conditions where troponin elevation may also occur, such as cardiac ischemia, acute stroke, pulmonary embolism, multiple trauma, terminal renal disease, and vascular and nonvascular surgery [25,27,28].

The aim of this study was to examine the levels of TDH and IMA in symptomatic CPVE-infected puppies and compare them with those in healthy puppies, aiming to gain a deeper insight into the oxidative stress mechanism.

2. Materials and methods

2.1. Ethical statement

This study was carried out in compliance with the guidelines established by the Council of Europe Directive 2010/63/EU regarding the welfare of animals used for scientific research, as well as the applicable Turkish laws (Law No. 5199, Regulation No. 28141, dated 12/13/2011). Ethical approval for the study (approval number: 52, 12/24/2021) was granted by the Kırıkkale University Animal Experiments Local Ethics Committee.

2.2. Animal material

The material of the study consisted of infected and healthy young dogs in the age range affected by the disease, which were brought to Kırıkkale University Veterinary Faculty Education Research and Practice Animal Hospital with complaints of disease or for general examination. Variables such as the breed, sex, and body weight were not factors in the sample selection. The selected dogs were separated into 2 groups according to whether they had parvoviral enteritis CPVEg (n = 65) or were healthy CTg (n = 34).

2.3. Blood and stool samples

Blood samples from dogs were collected in anticoagulant tubes containing ethylenediaminetetraacetic acid (EDTA) for complete blood counts and gel separator tubes without anticoagulant to obtain serum. In order to diagnose animals infected with CPVE, fecal samples were collected using a rectal swab and subjected to immunochromatographic rapid test kits (Bionote, Korea) for the identification of antigens.

Complete blood counts were determined immediately with a fully automatic blood counting device (Abacus Junior Vet5, Diatron, Hungary). Blood samples in gel separator tubes were allowed to stand undisturbed for 30 min, after that, they were centrifuged at 3000 rpm for 10 min to separate the serum. These serum samples were then stored at –80 °C until total thiol (TT), native thiol (NT), disulfide (D), and IMA analyses.

2.3.1. Thiol disulfide homeostasis and IMA assays

The serum levels of NT, TT, D, and the % D/NT ratio were measured using the innovative approach by Erel and Neselioğlu [19]. Ischemia-modified albumin levels were determined in absorbance units using the method developed by Bar-Or et al. [29].

2.4. Statistical analysis

GraphPad PRISM software was used to conduct the statistical analysis and significance was defined as $p < 0.01$. After identifying outliers in the data, the Shapiro-

Wilk test was used to test the normality of the distribution. Descriptive statistics were reported as the mean and standard deviation. Group comparisons were made using the Mann-Whitney U test.

3. Results

Compared to the CPVEg, which had 33 (51.77%) female and 32 (49.23%) male dogs, the healthy group CTg had 19 (55.88%) female and 15 (44.12%) male dogs. The mean age of the dogs was 3.1 ± 1.27 months in the CTg, and it was 3.21 ± 1.32 months in the CPVEg. There were no statistically significant differences between the groups with respect to age and sex. Table 1 displays the canine subjects' white blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU), red blood cell (RBC), hematocrit (HCT), and platelet (PLT) values. When the data from the complete blood counts were compared between the groups, it was discovered that while the lymphocyte numbers showed a statistical difference ($p < 0.0001$), WBC, RBC, HCT, and PLT did not.

The serum NT, TT, D levels and ratios, and IMA levels of the dogs in the CPVEg and CTg are presented in Table 2. Figure 1 and Table 3 show the results of a correlation between TDH parameters, IMA, and WBC values in dogs with parvoviral enteritis. It was determined that there is no relationship between this data in either direction.

Table 4 and Figure 2 demonstrate the ROC Curve Analysis results for TDH parameters, IMA, and WBC values. The results of all TDH parameters and WBC counts were found to be statistically significant; however, the IMA values ($p = 0.1695$) did not differ.

4. Discussion

It is well-known that CPVE infection in dogs causes noticeably different hemogram results, serum biochemical values, various inflammation mediator levels, and oxidative stress markers [30–32]. In this study, it was presumed that

Table 1. The white blood cell, red blood cell, hematocrit, and platelet values of the dogs.

Parameters	CPVEg				CTg				
	n	Median	Mean	Std. Dev.	n	Median	Mean	Std. Dev.	p
WBC (10^9 cells/L)	65	7.14	8.299	6.234	34	10.76	10.87	2.257	0.0989
LYM (10^9 cells/L)		0.7	0.9198	0.7107		2.925	3.116	2.1	<0.0001*
MON (10^9 cells/L)		0.255	0.3604	0.3152		0.565	0.5175	0.2172	0.0354
NEU (10^9 cells/L)		5.22	6.662	5.707		6.65	6.667	1.499	0.4095
RBC (10^{12} cells/L)		5.97	5.9	1.441		4.835	5.172	0.8609	0.0793
HCT %		35.44	5.496	5.998		29.36	31.83	5.393	0.1784
PLT (10^9 cells/L)		328	352.7	150.7		322	344.3	122.4	0.8088

CPVE: Parvoviral Enteritis Group, CT: Control Group; WBC: White Blood Cell, LYM: Lymphocyte, MON: Monocyte, NEU: Neutrophil, RBC: Red Blood Cell, HCT: Hematocrit, PLT: Platelet *: Denote difference between groups.

Table 2. TDH parameters and IMA levels of the dogs in the CPVEg and CTg.

TDH Parameters	CPVEg				CTg				
	n	Median	Mean	Std. Dev.	n	Median	Mean	Std. Dev.	p
NT (µmol/L)	65	269.1	284.9	112.9	34	177.4	206.7	136.7	0.0016*
TT (µmol/L)		289	315	112.4		232	252.8	129.5	0.0091*
D (µmol/L)		13.58	15.03	9.635		23.49	23.03	8.033	<0.0001
D/NT (%)		4.3	6.448	5.98		13.34	24.13	29.29	<0.0001
D/TT (%)		3.96	5.301	3.982		10.53	12.95	9.265	<0.0001
NT/TT (%)		92.09	89.4	7.966		78.95	74.1	18.53	<0.0001
IMA (ABSU)		0.72	0.7151	0.2466		0.755	0.7918	0.2367	0.1695

CPVE: Parvoviral Enteritis Group, CT: Control Group; NT: Native Thiol, TT: Total Thiol, D: Disulfide, IMA: Ischemia Modified Albumin, *: Denotes ($p < 0.01$).

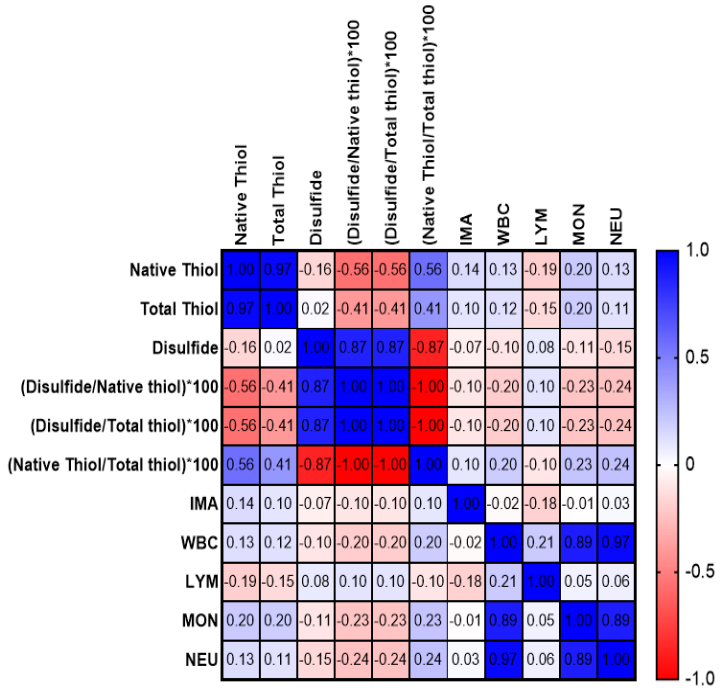

Figure 1. Spearman correlation coefficients graph for variables in the CPVE group.

Table 3. Spearman correlation coefficients graph for variables in the CPVE group.

	NT	TT	D	(D/NT)*100	(D/TT)*100	(NT/TT)*100	IMA	WBC	LYM	MON	NEU
NT r	1.000	0.972	–0.162	**–0.563**	**–0.563**	**0.564**	0.139	0.126	–0.191	0.205	0.135
TT r		1.000	0.021	**–0.408**	**–0.408**	**0.408**	0.101	0.117	–0.148	0.195	0.113
D r			1.000	**0.869**	**0.869**	**–0.869**	–0.066	–0.102	0.082	–0.105	–0.153
(D/NT)*100 r				1.000	**1.000**	**–1.000**	–0.099	–0.199	0.104	–0.228	–0.243
(D/TT)*100 r					1.000	**–1.000**	–0.099	–0.199	0.104	–0.228	–0.243
(NT/TT)*100 r						1.000	0.098	0.198	–0.103	0.227	0.243
IMA r							1.000	–0.022	–0.181	–0.008	0.027
WBC r								1.000	0.208	0.889	0.968
LYM r									1.000	0.052	0.061
MON r										1.000	0.886
NEU r											1.000

Table 4. ROC curve analysis of TDH, CBC parameters, and IMA levels.

Parameters	**Area Under Curve**	**Cut-off**	**Sensitivity (%)**	**Specificity (%)**	**p value**
NT (µmol/L)	0.6914	>238.7	66.15	73.53	0.0018*
TT (µmol/L)	0.6593	>268	66.15	67.65	0.0095*
D (µmol/L)	0.762	<20.23	78.46	64.71	<0.0001
D/NT (%)	0.786	<11.82	93.55	56.67	<0.0001
D/TT (%)	0.7941	<9.555	90.63	61.76	<0.0001
NT/TT (%)	0.7941	>80.89	90.63	61.76	<0.0001
IMA (ABSU)	0.5846	<0.6150	40	82.35	0.1682
WBC (10^9 cells/L)	0.6541	<8.305	58.49	91.67	0.0975
LYM (10^9 cells/L)	0.8639	<0.9650	63.27	91.67	<0.0001
MON (10^9 cells/L)	0.6958	<0.2500	50	91.67	0.0363
NEU (10^9 cells/L)	0.5778	<5.225	50.94	91.67	0.4026

NT: Native Thiol, TT: Total Thiol, D: Disulfide, IMA: Ischemia-Modified Albumin, ABSU: Absorbance Units, WBC: White Blood Cell, LYM: Lymphocyte, MON: Monocyte, NEU: Neutrophil.

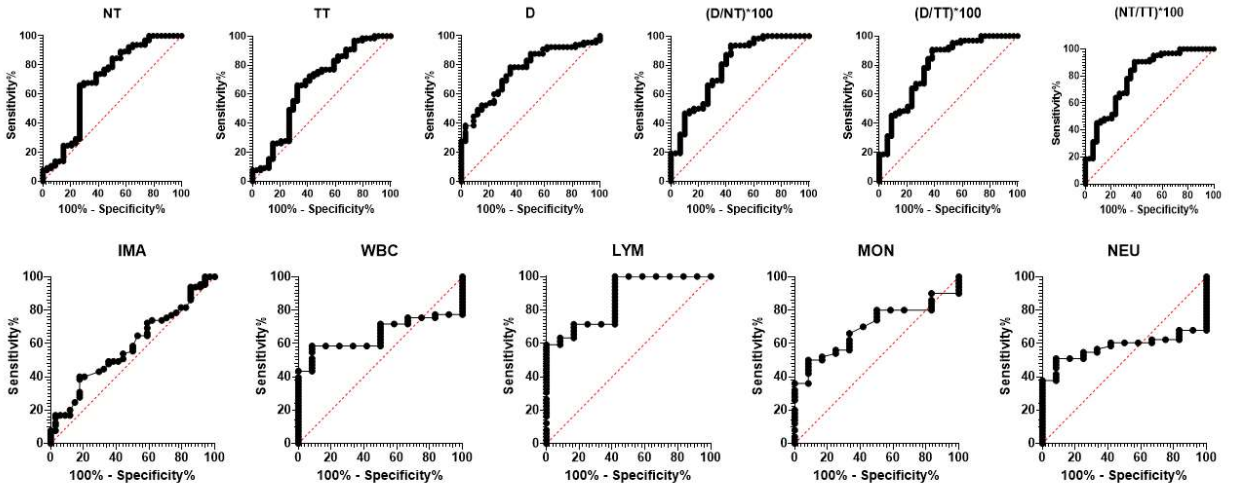

Figure 2. ROC curve analysis of TDH, CBC parameters, and IMA levels.

all infected dogs were affected by the infection to varying extents and despite complete blood count results showed significant leukopenia in some of the dogs in the CPVE group, the WBC numbers of these dogs did not differ from those of the CT group to a statistically significant extent ($p = 0.0989$). Elsayed et al. [6] reported that hemogram results revealed a significant decrease in granulocyte counts of the CPVE group. While the total leukocyte counts in the CPVE group were lower than those in the CT group in the current trial, the difference was not statistically significant ($p = 0.0989$). The only statistically significant difference between the CPVE and CT groups was in lymphocyte counts ($p < 0.0001$). It was hypothesized that the decline in leukogram parameters in CPVE-infected dogs, especially in the granulocyte and total leukocyte counts, may have been due to the increased leukocyte recruitment due to the inflamed intestine [33]. In its simplest definition, oxidative stress is the increase in ROS to an extent that exceeds the body's antioxidant defense capacity, which can cause damage to cells [34,35]. Prior studies have shown that there is a relationship between increased ROS and various pathologies [36]. Elevated oxidative stress is known to occur in metabolic [37,38] and neoplastic [39,40] afflictions, as well as bacterial [41], parasitic [42], and viral [43,44] infective diseases. In their study, Elsayed et al. [6] reported that CPVE-infected dogs have elevated oxidative stress, as well as higher total antioxidant status (TAS), compared to healthy dogs. Previous studies have also reported that the TAS or various antioxidant enzymes are elevated rather than lowered by sarcoptic scabies and gastroenteritis, despite increased oxidative stress [43,45]. Kocatürk et al. [30] revealed that the TAS of dogs with CPVE was higher than that of healthy dogs, with the group with the most severe clinical symptoms having the greatest TAS. It is likely that elevated antioxidant levels, rather than expected decreased levels, may be attributable to antioxidants created as a result of the buffer mechanism for removing oxidative stress [30,43,45]. In this study, it was observed that the levels of NT and TT in symptomatic CPVE-infected dogs were significantly higher compared to those in the CT group. These findings lend support to the hypothesis that antioxidant parameters like NT and TT may increase as a response to counteract oxidative stress associated with the disease [46].

Total antioxidant status is a measurement of homeostasis that antioxidants maintain by neutralizing ROS produced as a result of reactions in cells, which have the potential to harm the living organism [14]. During cellular activities, disulfide bonds are formed by covalent bonding between thiols, which are highly sensitive to oxidation, due to redox reactions. Therefore, increased oxidative stress causes elevated D levels, and in order to maintain homeostasis, thiol oxidation in proteins is reversed and the thiol levels become elevated as well. Irregularities of the TDH are associated with a variety of disorders and diseases. NT, TT, and D levels have been proven to be altered by cardiological, endocrine, neurological, psychiatric, respiratory, rheumatoid, dermatological, gynecological, urological, infective, and gastrointestinal diseases [17].

Neşelioğlu et al. [47] discovered that NT, TT, and D levels decreased in human patients with ulcerative colitis. In a separate study, Terzi et al. [48] observed that calves with neonatal diarrhea exhibited lower NT and TT levels and higher D levels compared to healthy calves, suggesting this could indicate oxidative stress. Değirmençay et al.

[49] reported similar results, noting decreased NT and TT levels and elevated disulfide levels in dogs with distemper. In a study conducted with asymptomatic CPVE infected puppies [50] diagnosed using rapid diagnostic kits, results similar to those of Terzi et al. [48] and Değirmençay et al. [49] were obtained, and there was a reported decrease in the NT and TT levels within the patient group, meaning that the antioxidant status lowered, while the disulfide levels increased, and oxidative stress emerged. However, although the same disease examined by Kurtdede et al. [50] was evaluated in the present study, it was found that the NT and TT levels of the CPVEg were higher and the disulfide levels were lower compared to the healthy group. It was thought that this important difference may have been due to the fact that in the study of Kurtdede et al. [50], the patients in the previous study were asymptomatic and likely in the early stages of the disease, whereas the dogs in the current study were treated after developing all of the disease's known symptoms and had pathological damage as a result of the disease's advanced progression. In fact, a study [51] with symptomatic feline panleukopenia (FPV) patients revealed that the NT and TT levels of the patient group were lower compared to the healthy group, while the disulfide levels were reported to be higher in the patient group than in the healthy group. This result is consistent with the research conducted by Kurtdede et al. [50]. When these three studies, with very similar or identical causative agents, target species, and disease pathogenesis are considered, it is seen that the parameters of the patient groups differed from the healthy groups, but inclinations towards elevated or decreased values varied. Since oxidative stress is expected to increase as the disease progresses, but TAS increases to neutralize ROS in order to maintain homeostasis and was shown to be elevated in sick groups than in healthy groups, it is possible that this difference in the parameters varies depending on whether the patients included in the study are in the early or later stages of the disease. Thus, it is apparent that more research is needed to investigate the changes in TDH caused by diseases, as well as TDH tendencies during diseases.

The N-terminal section of serum albumin is recognized as a binding site for transition metal ions such as cobalt, copper, and nickel [52]. Due to the impact of free radicals generated during ischemia, albumin undergoes modifications in its ability to bind transition metals, leading to the formation of a variant known as IMA (Ischemia-Modified Albumin) [53]. There are studies showing that free oxygen radicals produced especially as a result of myocardial ischemia damage the metal binding sites of albumin [29]. In a study on humans with intestinal ischemia [54], Cobalt Albumin Binding Assay (CABA) results were higher significantly in the group with ischemia. As a consequence of ischemic factors and the ensuing oxidative stress, the ability of albumin to bind cobalt was compromised due to the disruption of its N-terminal end. Consequently, the ischemic group exhibited an increased quantity of IMA, leading to elevated CABA results. In a study involving human subjects with necrotizing enterocolitis [55], it was noted that the levels of IMA in patients with enterocolitis on days 0, 3, and 7 were significantly higher than the control group ($p < 0.001$). In a study conducted on humans with inflammatory bowel disease (IBD) [56], the IMA levels were significantly elevated in the IBD group compared to the control group. ($p = 0.02$). This led to the conclusion that increased IMA levels, which serve as markers of oxidative stress in inflammatory diseases, suggest a connection between inflammation and oxidative stress in the development of IBD. In another study involving humans diagnosed with ulcerative colitis and Crohn's disease [57], both patient groups exhibited significantly elevated IMA levels in comparison to the control group. Generally, research involving human subjects with enteritis has consistently reported elevated IMA levels within the patient group.

Through a literature review, two studies in the field of veterinary medicine on IMA levels were also evaluated. In a study on neonatal diarrhea in calves [48], it was reported that the IMA levels of the group with neonatal diarrhea were higher ($p = 0.0018$) than the control, a result similar to those of studies on enteritis in humans. In another study on the IMA levels of dogs with distemper [49], similar results were found. In our study, both positive and negative correlation values were found between IMA and TDH parameters, as well as with WBC values, similar to the results of the distemper study [49]. However, none of these correlation patterns were found to be statistically significant.

When the ROC Curve Analysis data are analyzed, it is clear that all TDH parameters have high sensitivity values, with (D/NT)*100, (D/TT)*100, and (NT/TT)*100 ratios over 90%. The IMA value has a modest sensitivity (area under curve: 0.5846, $p = 0.1682$). However, Değirmençay et al. [49] found that the IMA value had a sensitivity of 72.2% and that the TDH parameters had lower sensitivity and specificity values than the current study. Looking at the ROC Curve Analysis results for white blood cells, it is clear that WBC and NEU numbers have low and statistically insignificant sensitivity values, but lymphocytes have high sensitivity values (area under curve: 0.8639, $p < 0.0001$). While total white blood cell and neutrophil amounts in the blood are expected to be very low following the diagnosis of diseases such as parvoviral enteritis, the fact that only lymphocyte numbers alter in this way is noteworthy.

In conclusion, dogs with parvoviral enteritis had lower IMA levels than controls, but this difference was not statistically significant ($p = 0.1695$), whereas NT, TT, and

D differed significantly. Both the IMA and TDH related parameters showed changes opposite of those reported by the aforementioned studies [48,49]. It is thought that the reason for this difference between the groups is due to buffering by antioxidants. Hence, it has been deduced that integrating investigations on oxidative stress across various stages of diseases, including the early phase, clinical period and recovery processes could yield greater insights into the dynamics of oxidative stress during disease progression. Such an approach may prove more valuable in comprehending the evolving nature of the disease.

Acknowledgment/disclaimers/conflict of interest
This study has not been supported by any institute or foundation. The authors declare that they have no conflict of interest.

Informed consent
The study was conducted in accordance with the standards determined by the European Council Directive 2010/63/EU on the protection of animals used for scientific purposes and the pertinent Turkish laws (Law No. 5199, Regulation No. 28141) of 13.12.2011. Additionally, prior to the study, approval from the animal experiment local ethics committee at Kırıkkale University was obtained (approval number: 52, 24 December 2021).

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