Serum Intestinal Fatty Acid-Binding Protein and Calprotectin Concentrations to Assess Clinical Severity and Prognosis of Canine Parvovirus Enteritis [1]

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Abstract

This study was conducted to assess the usefulness of serum intestinal fatty acid-binding protein (IFABP) and calprotectin (CALP) concentrations in comparison with other biomarkers [total leucocyte counts (TLC), C-reactive protein (CRP) and procalcitonin (PCT)] in predicting the clinical severity and prognosis of Canine Parvoviral (CPV) enteritis. Ten healthy dogs (CON group) and 40 dogs with natural CPV enteritis (INF group) were used. The INF group was also divided into survivor and non-survivor. Blood samples were collected twice in the INF group and once in the CON group. The clinical health score (CHS) was calculated for each patient by scoring certain clinical findings. Serum CRP and IFABP, and plasma PCT concentrations of the INF group at hospital admission (0 h) were significantly higher than in the CON group. Compared to the survivor subgroup, mean serum PCT and IFABP concentrations in the non-survivor subgroup were significantly higher at both 0 h and after initiation of treatment (24 h), while the mean TLC was significantly lower at 24 h. The correlation between CHS and serum IFABP (r=0.501; P=0.000) was stronger than other biomarkers evaluated. Based on the sensitivity and specificity from the Receiver Operating Characteristic curve analysis, TLC (24 h) and serum IFABP (0 h) serve as the most valuable biomarkers among the parameters in this study to predict the prognosis of CPV enteritis.

Keywords: Canine parvovirus enteritis, Biomarker, Intestinal fatty acid-binding protein, Calprotectin

Kanin Parvoviral Enterit'in Klinik Şiddeti ve Prognozunu Değerlendirmede Serum İntestinal Yağ Asidi Bağlayıcı Protein ve Kalprotektin Konsantrasyonları

Öz

Bu çalışma, köpeklerde Parvoviral (CPV) enteritin klinik şiddetini ve prognozunu öngörmede serum intestinal yağ asidi bağlayıcı protein (IFABP) ve kalprotektin (CALP) konsantrasyonlarının kullanılabilirliğinin, diğer belirteçlerle [toplam lökosit sayıları (TLC), C-reaktif protein (CRP) ve prokalsitonin (PCT)] karşılaştırmalı olarak değerlendirmek için gerçekleştirildi. On sağlıklı köpek (CON grubu) ve 40 doğal CPV enteritisli (INF grubu) köpek kullanıldı. INF grubu da hayatta kalan (survivor) ve hayatta kalmayan (non-survivor) olarak ikiye ayrıldı. INF grubundan iki ve CON grubundan bir kez kan örnekleri alındı. Belirli klinik bulgular puanlanarak her hasta için klinik sağlık skoru (CHS) hesaplandı. Hastaneye yatışta (0. saat) INF grubunun serum CRP, IFABP ve plazma PCT konsantrasyonları CON grubundan anlamlı derecede yüksekti. Survivor altgrubu ile karşılaştırıldığında, non-survivor altgrubunun ortalama serum PCT ve IFABP konsantrasyonları, hem 0. saatte hem de tedavi başlangıcından 24 saat sonra (24. saat) önemli ölçüde yüksekti, ortalama TLC ise 24. saatte önemli ölçüde düşüktü. CHS ile serum IFABP (r=0.501; P=0.000) arasındaki korelasyon, değerlendirilen diğer biyobelirteçlerden daha güçlüydü. Alıcı İşletim Karakteristiği eğrisi analizinden elde edilen duyarlılık ve özgüllüğe dayalı olarak, TLC (24. saat) ve serum IFABP (0. saat), CPV enteritinin prognozunu tahmin etmek için bu çalışmanın parametreleri arasında en değerli biyobelirteçler olarak hizmet ederler.

Anahtar sözcükler: Kanin parvoviral enterit, Biyobelirteç, Intestinal yağ asidi bağlayıcı protein, Kalprotektin

INTRODUCTION

Canine parvovirus (CPV) enteritis is a common acute viral disease that mainly affects dogs younger than six months [1]. In infected puppies, the tissues most affected by CPV are intestinal epithelium, lymphoid tissue, and bone marrow [2,3]. The virus reaches the intestinal mucosa through the bloodstream, replicating in the germinal epithelium of the intestinal crypts [2]. It induces severe and extensive necrosis of the epithelial cells, loss of intestinal glands, and villous atrophy or collapse [4,5]. All of these contribute to disrupting the gastrointestinal mucosal barrier, which allows Gram-negative and anaerobic bacteria translocation from the intestinal lumen [6]. Thus, intestinal injuries caused by CPV result in hemorrhagic enteritis, systemic inflammatory response syndrome (SIRS), sepsis and endotoxemia [2,6]. Furthermore, the CPV leads to the destruction of leukocyte precursors in the bone marrow. Along with the destruction or collapse of the thymic cortex, this condition results in a significant decrease in total leukocyte count (TLC) in infected animals [1,7]. Insufficient immunity, combined with SIRS and sepsis, puts affected animals at high risk of developing septic shock, multiple organ failure, and even death if left untreated [7]. In this context, the mortality rate can reach 20-48% in puppies despite the aggressive treatment protocol [2,8]. Considering the high morbidity (up to 100%) and mortality rates of CPV enteritis in puppies, it is crucial to determine the prognosis of the disease. So, there is a clear need for biomarkers that are compatible with the complex pathophysiology of CPV and reflect the disorders of the affected patient.

In CPV enteritis, one of the factors affecting the clinical severity and prognosis of the disease in the patient is systemic inflammation [9,10]. Therefore, the biomarkers of inflammation are of clinical interest. C- reactive protein (CRP) is considered as a positive major acute-phase protein in dogs and one of the most widely used biomarkers for inflammation [10]. Several researchers [9,11] have revealed a significantly higher serum CRP concentration in dogs with CPV in comparison to healthy dogs. It was also demonstrated that CRP is a potent predictor of mortality in dogs with CPV enteritis [9]. On the other hand, it did not prove to be a good predictor of the outcome when used alone [10].

Procalcitonin (PCT), the precursor of calcitonin, is another valuable biomarker of sepsis [12,13]. In both human and veterinary medicine, PCT is released in response to microbial toxins and specific pro-inflammatory mediators, and its concentrations in serum rise early after exposure to an infectious stimulus [12-15]. Although the condition of serum PCT concentration in CPV enteritis was evaluated in one study [11], to the best of our knowledge, no study assessed the prognostic role of PCT in this disease.

Calprotectin (CALP) belongs to calgranulin protein family, calcium-binding cytosolic proteins mainly present in neutrophils, monocytes, macrophages, and epithelial and endothelial cells [16,17]. Concentrations of CALP rise at the site of local inflammation following its release from activated and dying cells [18,19]. As a relatively low molecular-weight protein (36.5 kDa), it diffuses freely from inflamed tissues into circulation and can be measured in serum/plasma [18,19]. Evidence for these mechanisms has prompted studies dealing with CALP in inflammatory disease in both human and veterinary medicine [16,17]. Significant increases in serum CALP concentration were described in dogs with inflammatory diseases such as inflammatory bowel disease, systemic inflammatory response syndrome, and sepsis [19,20]. The releasing mechanism of CALP and promising results in some studies [17,19,20] indicate that CALP is a potential marker of inflammation in dogs as well as in humans.

Plasma/serum CRP, PCT, and CALP concentrations are inflammatory biomarkers, and these parameters are not tissue/organ-specific [9,10,12,13,18]. It is known that the most radical method used in determining intestinal damage is a histopathological examination from tissue biopsy taken endoscopically. However, endoscopic examination is not considered practical, especially in cases like CPV enteritis that have an acute onset and require urgent intervention. In the last years, Intestinal Fatty Acid Binding Protein (IFABP) is considered as a non-invasive biomarker to evaluate the loss of intestinal wall integrity and dys-function of the intestinal barrier in human medicine [21-23]. IFABP is an intracellular protein specifically secreted in abundance in the epithelial cells of the mucosal layer of intestinal tissue [21-23]. The presence of IFABP, especially in the mature epithelium of intestinal villi, facilitates leakage from enterocytes into the circulation when intestinal mucosal damage occurs [21-23]. Recently, two promising studies [8,24] on serum IFABP concentrations for prognostic value in dogs with CPV enteritis have been published.

The current study aims to assess the usefulness of serum IFABP and CALP concentrations in comparison with other inflammatory markers (TLC, CRP and PCT) in predicting the clinical severity and prognosis of the disease in dogs with CPV enteritis.

MATERIAL AND METHODS

Ethical Statement

All study procedures were reviewed and approved by the Animal Research Ethics Committee of the Aydin Adnan Menderes University, under protocol number 64583101/2018/133.

Animals

In this prospective study, a total of 50 dogs, including the healthy control (CON) group (n=10) and the CPV infected (INF) group (n=40), were evaluated. Dogs in

the INF group have been subdivided into two groups as survivors and non-survivors within 7 days of initiation of treatment *(Fig. 1)*. The INF group dogs were presented to the Aydin Adnan Menderes University Veterinary Teaching Hospital between January 2019 to February 2020 with the complaint of prominent clinical symptoms of CPV enteritis such as weakness, reduced appetite, vomiting, diarrhea/hemorrhagic diarrhea, dehydration. The dogs in the INF group were of various breeds (21 mixed, 6 Golden Retrievers, 5 German Shepherd, 3 Rottweiler, 2 English Cocker Spaniel, 2 Yorkshire Terrier, and 1 Pug), sex (14 males and 26 females), and ages (between two and six months). The dogs in this group were not vaccinated against CPV. The dogs in the CON group were selected similarly to the INF group in terms of breed (6 mixed, 2 Golden Retrievers, 1 German Shepherd, and 1 Yorkshire Terriers), sex (4 males and 6 females), age (between 2 and 6 months) and vaccination status (unvaccinated). The dogs in this group were found to be healthy according to the clinical, haematological and faecal examinations (CPV, *Isospora* spp. and *Giardia* spp.). The diagnosis in CPV enteritis suspected dogs was confirmed by determining the CPV antigen in faeces with a rapid test kit (Catalog No: E-AD-C023; Canine Parvovirus Antigen Lateral Flow Assay Kit, Elabscience Biotechnology Inc, USA). The sensitivity of 98.8%, specificity of 98.5%, and accuracy values of 98.65% for the detection of CPV antigen in this test kit are specified by the manufacturer. The faecal samples of study dogs were also examined microscopically twice, at admission to the hospital (0 h) and the 24 h of the treatment, for *Isospora* oocysts by hyperosmolar sugar flotation method and *Giardia* trophozoites by zinc sulphate flotation method. The dogs whose faecal samples were found negative in both parasitological examinations were included in the study.

Clinical Examinations

A clinical examination was performed on each dog in the INF group at admission and then daily until they heal or death. Certain clinical (body temperature and heart and respiratory rates) and haematological findings were used to evaluate the general health status of the INF dogs and to determine the presence of SIRS in the patients. In this way, dogs were evaluated according to the SIRS criteria reported by Hauptman et al.[25] on admission and were defined as SIRS positive (+) or SIRS negative (-). Furthermore, appetite, and severity and character of diarrhea, vomiting, depression, dehydration, exhaustion, and the physical aspect of faeces in the INF group were noted. Dogs in the INF group were examined once a day at 0 h (pre-treatment) and 24 h initiation of treatment. Each of the above-mentioned symptoms was scored from 0 to 3 or 4, the maximum score being 20 for death [26]. Thus, clinical health scores (CHS) for each patient were calculated at 0 and 24 h using the score assignment scheme for clinical symptoms reported by Martin et al.[26].

Sample Collection and Measurements

Faecal samples were collected from the rectum to plastic containers for parasitological examinations in the INF group at 0 and 24 h and once in the CON group. For clinical examination, blood sampling and laboratory measurements in the INF group were performed twice at hospital admission (0 h), at the 24 h initiation of treatment, and once for dogs in the CON group.

Blood samples were taken from *V. cephalica antebrachii* into tubes with lithium heparin (Vacutainer, Beckton, Dickenson) and serum separation tubes (Vacutainer, Beckton, Dickenson). Complete blood cell count has been performed with the automated blood cell counter (Abacus Junior Vet 5; Diatron MI Zrt., Hungary) using samples with lithium heparin within 30 min after blood collection. The remaining blood samples with lithium heparin were centrifuged at 2000 x g for 10 min to obtain plasma, and these samples were stored at -20°C until PCT concentrations were measured. Blood samples in serum separation tubes were centrifuged after clot retraction at 2000 x g for 10 min to obtain sera. CRP measurement from serum samples was performed with a point of care device (EUROLyser, Salzburg, Austria) using solo cCRP tests, according to the instructions provided by the manufacturer. Subsequently, serum samples were stored at -20°C until IFABP and CALP measurements. The plasma PCT (#MBS7606532, MyBioSource, Inc., USA) and, serum IFABP (#MBS2605533, MyBioSource, Inc., USA) and CALP (#CSB-EQ013485DO, Cusabio Biotech Co., China) concentrations were measured by using commercially
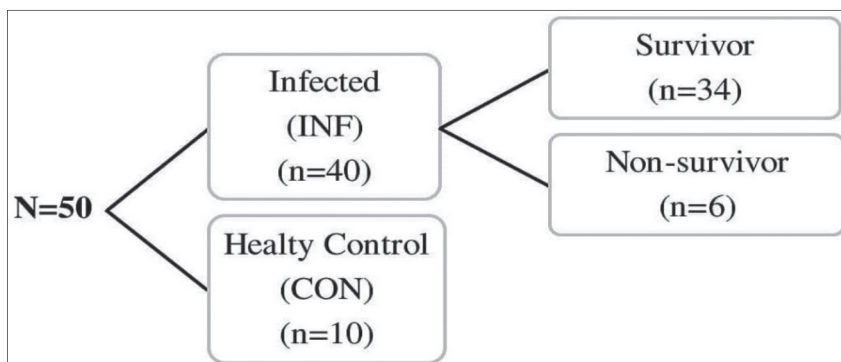

Fig 1. Grouping of dogs evaluated in this study and the number of dogs in each group

available canine-specific enzyme-linked immunosorbent assay kits following the manufacturer's instructions.

Treatment Protocol

Dogs infected with CPV were hospitalized for at least 24 h in separate cages, although hospitalization times varied depending on the severity of the disease. The discharged dogs have been admitted to our hospital for examinations and treatments twice a day until they recovered.

In general, the treatment protocol consisted of fluid therapy, antibiotic therapy, gastrointestinal support, nutritional support, supportive care, and the efficacy of the treatment was monitored. The treatment protocol used in this study was adapted from Prittie [2], Goddard and Leisewitz [27], and Judge [28]. The degree of dehydration, the presence of hypovolemia or hypovolemic shock were evaluated with a comprehensive clinical examination. The dose and rate of initial therapy varied with the patient. The first goal of fluid therapy was to correct the intravascular volume deficit. For patients displaying shock or hemodynamic compromise symptoms, initial fluid resuscitation was begun with rapid intravenous administration (7-12 mL/kg IV over 10 min) of a Lactated Ringers Solution. This procedure was repeated until signs of hemodynamic compromise were no longer present. When significant hemodynamic improvement is not achieved within 30 min of fluid therapy, the bolus of synthetic colloid hydroxyethyl starch was administered at a dose of 3-5 mL/kg, given intravenously over 10 min to prolong the effectiveness of crystalloid therapy. Lactated ringer and potassium chloride (20 mEq/L) added 5% dextrose solutions were used in the dehydrated dogs but not in shock and the maintenance fluid treatment of dogs in shock. The hydration deficit was calculated according to the degree of dehydration. This fluid volume is administered to the patient over 8-24 h and the patients' daily fluid requirement (60 mL/kg/24 h). The β-lactamase resistant penicillin (amoxicillin-clavulanate, 12.5 mg/kg SC every 12 h, at least 5 d) was used as an antimicrobial. In addition, metronidazole (10 mg/kg IV every 12 h, at least 3 d) was combined with this antibiotic in SIRS+ dogs. Butorphanol (0.1 mg/kg IV) was administered in dogs in need of pain relief. Maropitant citrate at a concentration of 1 mg/kg (SC every 24 h) was administered for 3-5 days until vomiting had ceased. Nil per os was ordered for an initial 6-h period following admission to the hospital, especially for dogs with severe vomiting and diarrhea. After suppressed vomiting, it was gradually switched to food with an easily digestible carbohydrate and a lean protein source.

The clinical examinations and treatment plans of the dogs with CPV were made by the same clinician (CDA) in order to prevent any changes that may occur due to the discretion of the clinician.

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, USA) and MedCalc 19.1.3 (MedCalc Software bvba, Ostend, Belgium). The distributions of all parameters were checked with the Shapiro–Wilk test. Except CALP, all parameters showed normal distribution, and CALP was not distributed normally despite log transformation. Means, standard error of means (SEM), medians, and interquartile ranges (IQR) for each evaluated parameter were calculated using descriptive statistics. Parameters were analyzed using parametric (TLC, CRP, PCT and IFABP) and non-parametric (CALP) tests under consideration of their distributions.

Firstly, the above-mentioned parameters in the CON group were compared separately with 0 h and 24 h of the INF group by the independent sample t-test or the Mann-Whitney U test. Then, the INF group was divided into two subgroups as survivor and non-survivor. For the parameters evaluated, the intergroup differences between these three groups (CON, survivor and non-survivor) were assessed for each sampling time using one-way analysis of variance with post-hoc Tukey or Kruskal-Wallis tests. Additionally, dependent samples t-test or Wilcoxon Signed Rank test was used to evaluate the differences between 0 and 24 h for the INF group and each subgroup (survivor and non-survivor). The Pearson correlation coefficients *(r)* were calculated for the correlations between CHS and TLC, CRP, PCT, and IFABP. The Spearman correlation coefficient (rho) was calculated for the correlation between CHS and CALP. The strength of the linear relationship was assessed using the coefficient of determination reported by Chan [29].

The prognostic cut-off values, the area under the curve (AUC), P-value, standard error, sensitivity (%), specificity (%) for the best differentiation between survivors and non-survivors were analyzed by receiver operating characteristic (ROC) curve analysis for each parameter in both sampling times. A P-value <0.05 was considered statistically significant for all analyses.

The sample size was estimated for ROC analysis by Med-Calc 19.1.3, with an AUC value of 0.8 or 0.85, an α error of 0.05, a power of 0.8 and a prevalence of non-survivors of 20%.

RESULTS

The most common clinical findings of dogs in the INF group were inappetence-anorexia (97.5%), haemorrhagic diarrhea (72.5%), non-haemorrhagic diarrhea (25%), and vomiting (70%). Also, 65% of dogs (26 cases) in the INF group were evaluated according to the SIRS criteria by Hauptman et al.[25] were found to be SIRS positive (+). Of the 40 dogs in the INF group, 34 recovered (survivor group), and 6 died (non-survivor group) within 7 days of starting treatment. Thus, the mortality of dogs infected with CPV in this study was noted as 15%. The mean survival time of the non-survivor dogs was 3.33±0.61 days from the initiation of the treatment. In addition, 5 of the 6

Table 1. *Description of parameters in the healthy CON group (once) and the survivor and non-survivor groups (0 h and 24 h)*

Marker	Groups	0 h	24 h	P [2]
CHS	CON	-		
	survivor	10.60±0.83[a]	8.48±0.72[a]	0.000
	non-survivor	17.33±0.71[b]	15.83±1.32[b]	0.151
	P [1]	0.000	0.000	
TLC (10^9 cells/L)	CON	12.88±0.75[ab]		
	survivor	17.07±1.74	16.95±2.50[a]	0.941
	non-survivor	12.28±3.58	4.57±1.79[b]	0.022
	P [1]	0.213	0.034	
CRP (mg/L)	CON	13.95±1.68[a]		
	survivor	74.32±7.66[b]	60.68±2.69[b]	0.076
	non-survivor	75.69±3.48[b]	65.05±4.83[b]	0.104
	P [1]	0.000	0.000	
PCT (pg/mL)	CON	29.54±4.01[a]		
	survivor	59.30±8.69[a]	45.10±7.38[a]	0.006
	non-survivor	141±50.87[b]	113.39±37.63[b]	0.323
	P [1]	0.002	0.002	
CALP (mg/L)	CON	1.94(1.23-3.02)		
	survivor	2.87(1.25-4.01)	2.21 (1.47-4.35)	0.216
	non-survivor	6.3(1.81-9.56)	5.82 (1.80-16.56)	0.753
	P [1]	0.135	0.255	
IFABP (ng/mL)	CON	2.93±0.29[a]		
	survivor	5.44±0.35[b]	4.15±0.22[b]	0.000
	non-survivor	7.41±0.86[c]	5.50±0.76[c]	0.017
	P [1]	0.000	0.001	

*The values have been expressed as mean ± standard error of means for CHS, TLC, CRP, PCT and IFABP, and median (interquartile ranges) for CALP. P[1] refers to the significance at the same time points between groups. The different letters (a, b, c) indicate the statistical significances (P< 0.05) among the groups. P[2] expresses the significance of the change in time within the same group. **CON:** healthy control group; **CHS:** clinical health score; **TLC:** total leucocyte count; **CRP:** C- reactive protein; **PCT:** procalcitonin; **CALP:** calprotectin; **IFABP:** intestinal fatty acid-binding protein*

dogs in the non-survivor subgroup were determined to be SIRS + dogs.

The mean CHS of the non-survivor group was significantly (P=0.000) higher than the survivor group both at 0 h and 24 h *(Table 1)*. The mean CHS of survivors was decreased significantly (P=0.000) at 24 h after initiation treatment whereas the values between 0 h and 24 h did not differ significantly in the non-survivor group.

There was no statistically significant difference in TLC in dogs with CPV enteritis at 0 h and 24 h compared to the healthy control dogs *(Fig. 2-A)*. However, the mean TLC of the non-survivor group at 24 h (4.57±1.79) decreased dramatically compared to 0 h (12.28±3.58). Thus, the mean TLC of the non-survivor group was found to be significantly (P=0.011) lower than the survivor group at the 24 h of treatment *(Table 1)*.

Serum CRP concentrations were significantly higher (P=0.000) in dogs with CPV enteritis at 0 h and 24 h than in the healthy CON dogs *(Fig. 2-B)*. However, there was no significant difference in CRP concentrations between survivor and non-survivor groups at both sampling times *(Table 1)*.

While the mean plasma PCT concentration of the INF group at 0 h was found to be statistically higher than the CON group, no significant difference between the two groups in the mean PCT concentrations at 24 h *(Fig. 2-C)*. In addition, the plasma PCT concentrations of the non-survivor group were statistically higher than both the survivor and CON groups at both sampling times. Furthermore, PCT concentrations of the survivor subgroup decreased at 24 h of the treatment compared to the 0 h (pre-treatment). In contrast, the PCT concentrations did not change over time in the non-survivor group *(Table 1)*. In terms of serum CALP concentrations, both the 0 and 24 h values in the INF group were not statistically different from the CON group *(Fig. 2-D)*. Although the CALP concentrations of the non-survivor group were numerically higher than those of the survivor and CON groups at both 0 and 24 h, these elevations did not reach statistical significance *(Table 1)*.
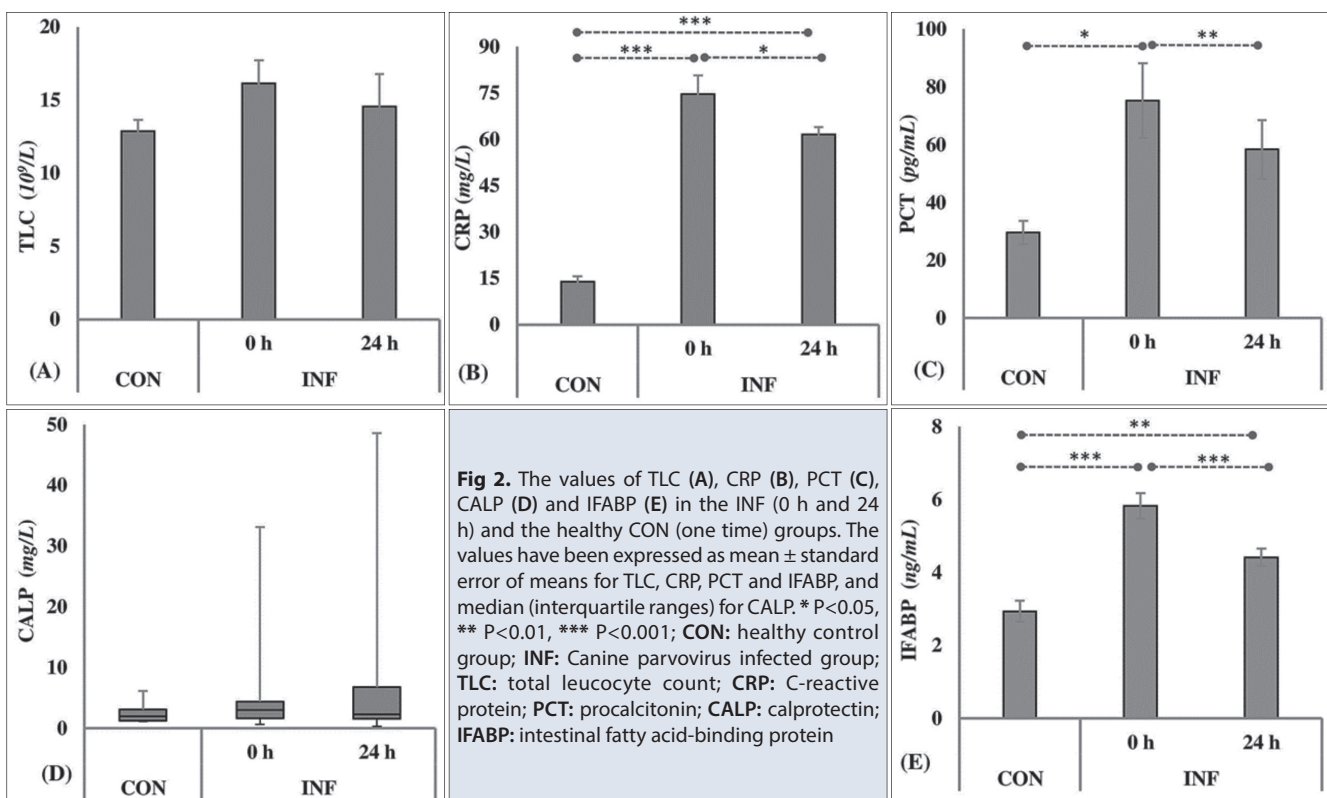
Fig 2. The values of TLC (**A**), CRP (**B**), PCT (**C**), CALP (**D**) and IFABP (**E**) in the INF (0 h and 24 h) and the healthy CON (one time) groups. The values have been expressed as mean ± standard error of means for TLC, CRP, PCT and IFABP, and median (interquartile ranges) for CALP. * P<0.05, ** P<0.01, *** P<0.001; **CON:** healthy control group; **INF:** Canine parvovirus infected group; **TLC:** total leucocyte count; **CRP:** C-reactive protein; **PCT:** procalcitonin; **CALP:** calprotectin; **IFABP:** intestinal fatty acid-binding protein

Table 2. *Correlation coefficients (r/rho) and significance (P) levels between CHS and biomarkers in CPV-infected dogs*

Parameters		Correlation Coefficients	P-Value
CHS	TLC	r = -0.195	P = 0.130
	CRP	r = 0.375	P = 0.003
	PCT	r = 0.271	P = 0.033
	CALP	rho = 0.389	P = 0.002
	IFABP	r = 0.501	P = 0.000

CHS: *clinical health score;* **TLC:** *total leucocyte count;* **CRP:** *C- reactive protein;* **PCT:** *procalcitonin;* **CALP:** *calprotectin;* **IFABP:** *intestinal fatty acid-binding protein*

Serum IFABP concentrations of the INF group on both sampling days were significantly higher than that of the CON group *(Fig. 2-E)*. The mean IFABP concentrations of the non-survivor, survivor, and CON groups were significantly different from each other at both the 0 and 24 h of the study. Also, IFABP concentrations of the non-survivor group were significantly higher than the survivor and CON groups on both days. Besides, time-dependent changes of mean serum IFABP concentration within the group were found to be significant for both the survivor (P=0.000) and non-survivor (P=0.017) groups *(Table 1)*.

To reveal the relationship between the clinical severity of the disease and measured parameters, correlation analyses between the CHS and these biomarkers were performed considering both 0 and 24 h values of all canine parvovirus infected dogs. As seen in *Table 2*, while the relationship between CHS and TLC was not significant (r=-0.195; P=0.130), the positive correlations were found significant between CHS and CRP (r=0.375; P=0.003), PCT (r=0.271; P=0.033), CALP (rho=0.389; P=0.002) and IFABP (r=0.501; P=0.000).

The ROC curves *(Fig. 3)* were drawn using survivor and non-survivor dogs at 0 and 24 h to determine the prognostic roles of measured parameters in dogs with CPV enteritis. Data from ROC analysis were offered in *Table 3*. In this context, the ROC analysis on 0 h for the utility of CRP and IFABP in differentiating in the INF group between the survivor and non-survivor dogs estimate an AUC of 0.733 (cut-off value of >69.05 mg/L with 83.33% sensitivity and 64% specificity) and 0.80 (cut-off value of >6.39 ng/mL with 83.3% sensitivity and 80% specificity), respectively. The ROC curve analysis also indicated that TLC and PCT were effective in distinguishing survivors from non-survivors at 24 h, with the AUCs of 0.873 (cut-off value of ≤5.04 10⁹/L with 83.33% sensitivity and 94.3% specificity) and 0.78 (cut-off value of >47.86 pg/mL with 66.7% sensitivity and 85.7% specificity), respectively. On the other hand, the CALP was not effective enough to predict mortality at both 0 h and 24 h.

Discussion

Canine parvovirus enteritis is among the most common causes of gastrointestinal emergencies in puppies [30]. Considering both its' prevalence and the poor outcomes, predicting the severity and prognosis of the disease
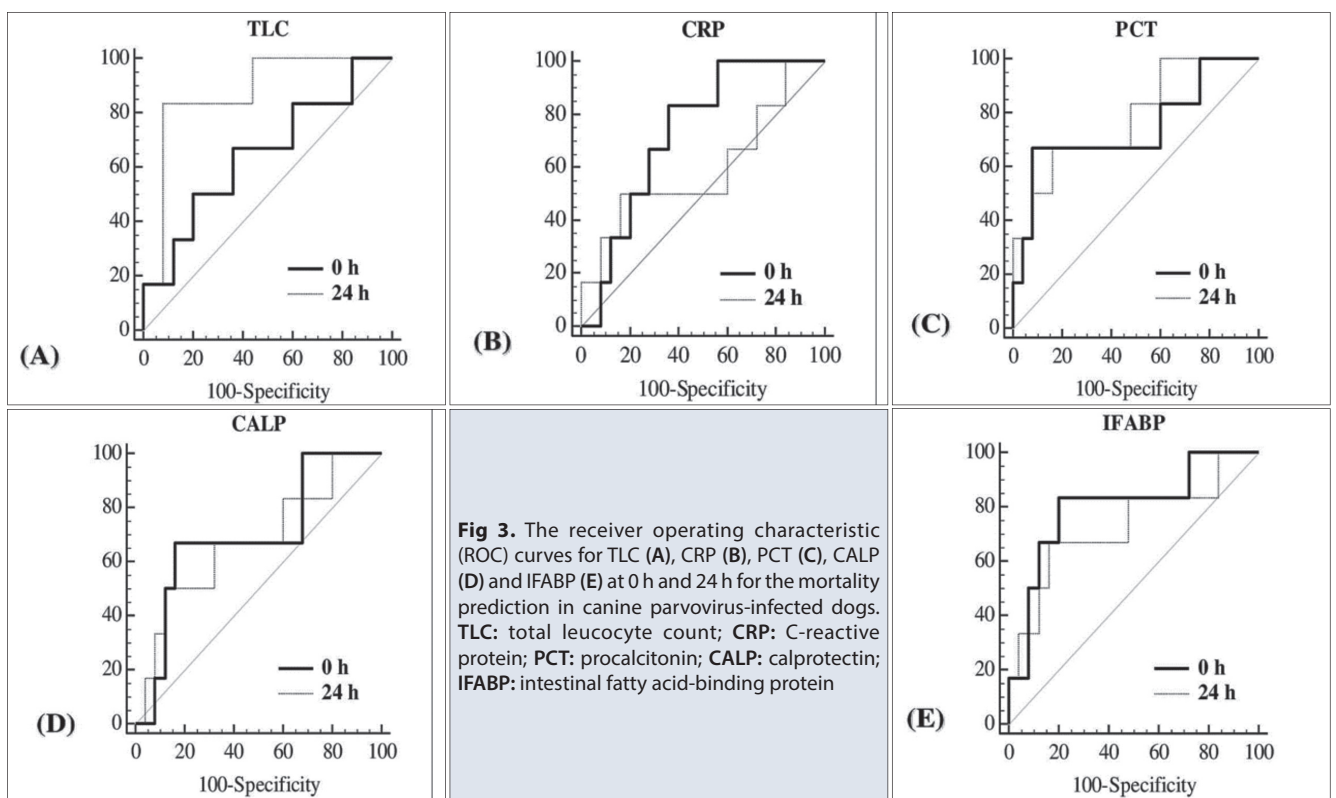
Fig 3. The receiver operating characteristic (ROC) curves for TLC **(A)**, CRP **(B)**, PCT **(C)**, CALP **(D)** and IFABP **(E)** at 0 h and 24 h for the mortality prediction in canine parvovirus-infected dogs. **TLC:** total leucocyte count; **CRP:** C-reactive protein; **PCT:** procalcitonin; **CALP:** calprotectin; **IFABP:** intestinal fatty acid-binding protein

Table 3. *The receiver operating characteristic (ROC) curve analysis of biomarkers at 0 h and 24 h for the mortality prediction in canine parvovirus-infected dogs*

Parameters	Hours	AUC	Standard Error	P Value	95% Confidence Interval	Sensitivity (%)	Specificity (%)	Cut off Value
TLC	0 h	0.647	0.140	0.293	0.455-0.809	66.67	64.0	≤13.27 (10⁹/L)
	24 h	0.873	0.078	< 0.001	0.705-0.965	83.3	94.3	≤ 5.04 (10⁹/L)
CRP	0 h	0.733	0.098	0.017	0.545-0.875	83.33	64	>69.05 (mg/L)
	24 h	0.600	0.156	0.521	0.409-0.771	50	84	>69.65 (mg/L)
PCT	0 h	0.740	0.141	0.088	0.552-0.880	66.67	92	>99.97 (pg/mL)
	24 h	0.780	0.114	0.014	0.595-0.908	66.7	85.7	>47.86 (pg/mL)
CALP	0 h	0.693	0.132	0.143	0.503-0.845	66.67	84	>4.103 (mg/L)
	24 h	0.673	0.137	0.207	0.482-0.830	80	88	>8.05 (mg/L)
IFABP	0 h	0.80	0.116	0.01	0.618-0.921	83.3	80.0	>6.39 (ng/mL)
	24 h	0.727	0.140	0.104	0.538-0.870	66.67	84	>5.09 (ng/mL)

AUC: area under the curve; TLC: total leucocyte count; CRP: C- reactive protein; PCT: procalcitonin; CALP: calprotectin; IFABP: intestinal fatty acid-binding protein

are essential in guiding the treatment protocol and monitorization of the patient. Therefore, the need for biomarkers compatible with the pathophysiological mechanism of the disease is obvious. Additionally, considering the outcomes of CPV enteritis, we believe that evaluating TLC, the inflammatory biomarkers and intestinal damage marker together will shed light on the pathophysiology of the disease. The findings of this

research that investigated the associations of selected markers with the severity and prognosis of CPV enteritis mainly include: (1) IFABP showed a moderate correlation (*r*=0.501; P=0.000) with the clinical severity of the disease, which was superior to other evaluated markers in determining the clinical severity of the CPV enteritis, (2) CRP and IFABP values at 0 h (hospital admission) and TLC and PCT values at 24 h (24 h of treatment) were significant for predicting poor outcomes, (3) serum CALP was not sufficiently successful in predicting neither the clinical severity nor the prognosis of CPV enteritis.

The decrease in TLC during CPV enteritis is generally the most consistent haematological finding [31,32]. This finding is widely accepted to be attributable to the destruction of hematopoietic progenitor cells of the various leukocyte types in lymphoproliferative organs, mainly in the bone marrow. Potential sepsis and endotoxemia that can cause to margination of neutrophils and pronounced loss of neutrophils through the inflamed intestinal wall are also are thought to contribute to the reduction in TLC [1,32]. The prognostic significance of total or differential leukocyte counts on admission or overtime in dogs with CPV enteritis has previously been assessed [5,31]. In this study, although there was no statistical difference in TLC between survivor and non-survivor dogs at hospital admission, TLC at the 24 h of treatment was significant in predicting the prognosis *(Fig 3-A; Table 3)*. Similarly, Goddart et al.[31] and Eregowda et al.[8] emphasize the role of TLC in determining prognosis at 24 h and 72 h of treatment, respectively. In this context, persistent leukopenia is one of the most successful findings for predicting outcomes in dogs with CPV enteritis among the evaluated parameters in the current study.

Multiple factors such as gut inflammation, cellular destruction and disruption of the gastrointestinal mucosal barrier contribute to the development of SIRS and sepsis in CPV enteritis [2,6,11]. The fact that 65% of the dogs in the INF group and, 5 of the 6 dogs who died in our study were SIRS positive (+) confirms that systemic inflammation and possible sepsis are important in the pathogenesis of this disease. Thus, CRP, PCT and CALP were evaluated in this study within the scope of inflammatory markers.

The status of CRP, a major acute-phase protein of dogs in CPV enteritis, has been addressed previously. Our results on serum CRP *(Fig. 2-B)* are similar to previous studies [9-11] which proved significant increases in serum CRP concentration in dogs with CPV enteritis. To differentiate survivors from non-survivors, a serum CRP concentration of >92.4 mg/L at the time of admission had a sensitivity and specificity of 91% and 61%, respectively [9]. A cut-off of 97.3 mg/L in CRP at 24 h after admission appeared to have greater sensitivity and specificity (86.7% and 78.7%, respectively) than did values at 0 h [10]. In this study, a sensitivity of 83.33% and a specificity of 64 % for a cut-off of 69.05 mg/L at 0 h *(Fig. 3-B)* are comparable with the values previously reported. The differences in the sensitivity and specificity of CRP between the studies may be explained that infected dogs are first evaluated at different times during their disease (some are examined soon after the onset of clinical signs, while others may be ill for a day or longer), as McClure et al.[10] pointed out.

Procalcitonin is one of the promising inflammatory markers which is relatively more recent than CRP in veterinary medicine [13-15]. In humans, increased blood PCT concentrations are used to differentiate bacterial sepsis from non-infectious systemic inflammation [12]. Similarly, the plasma PCT concentrations of dogs with sepsis were found higher than those of healthy dogs [13,14]. In this context, PCT concentrations at admission to the hospital in dogs with sepsis may predict organ dysfunction and septic shock, and serial measurement of serum PCT can provide prognostic information in dogs with sepsis [14]. Kubesy et al.[11] reported that dogs with CPV enteritis had a higher serum PCT concentration compared to healthy controls, but this did not reach statistical significance. In the present study, although the plasma PCT concentrations of dogs with CPV enteritis were higher than those of healthy CON dogs, its concentrations in survivor dogs of the INF group were not statistically different from the healthy CON group *(Table 1)*. This suggests that the clinical severity of the disease and its complications, such as sepsis, may affect plasma PCT concentration. Additionally, the plasma PCT concentrations of the survivor dogs decreased statistically at 24 h of the treatment whereas it did not significantly reduce despite the treatment in the non-survivor group *(Table 1)*. This finding can be related to the short kinetics (12-24 h) of plasma PCT. Although blood culture for determining sepsis in our study was not performed, one reason for no significant decrease in the plasma PCT despite treatment in the non-survivor group may be associated with possible sepsis caused by CPV enteritis. Moreover, this study demonstrated that plasma PCT concentrations at 24 h of dogs with CPV enteritis were moderately effective (AUC=0.78; P=0.014; *Table 3*) in predicting poor outcomes and this result is not comparable because of no previous present study.

Calprotectin is released into the circulation after the activation of neutrophil granulocytes, regulates the adhesion of leukocytes to the endothelium and extracellular matrix during the inflammatory process and protects cells against microorganisms [33]. Therefore, the utility of serum CALP concentration in evaluating systemic inflammatory conditions and sepsis in human medicine [33,34] and for detecting inflammation in dogs with inflammatory bowel disease [19] has been demonstrated. In a study conducted on dogs with sepsis and non-septic SIRS [20], serum CALP concentrations were significantly higher than that of dogs in the healthy control group. In this study, serum CALP concentrations in non-survivor subgroup of INF group were only numerically higher than those of the survivor and CON groups on both sampling days *(Table 1)*. The

result in our study is in agreement with a previous study by Thames et al.[20] in which no significant difference in serum CALP levels between survivor and non-survivor dogs with sepsis and non-septic SIRS has been proved. In addition, a weak positive correlation (rho=0.389; P<0.033) between serum CALP concentration and CHS indicated that serum CALP appeared insufficient for predicting clinical severity and prognosis in dogs with CPV enteritis. Since the information on serum CALP concentrations (e.g. serum CALP kinetics, the relation-ship between serum CALP and local-circulating neutrophil count) in dogs is quite scant, it is not easy to make a firm judgment on the results of this study. This situation may also be originated from the relatively small sample size in this study, apart from factors related to the patho-physiology of CPV enteritis and the biology of serum CALP.

It is known that IFABP is found in the cytoplasm of the enterocytes and enters the bloodstream when the integrity of the intestinal mucosa is disrupted [21-23]. Thus, its use in various diseases has been investigated to detect intestinal damage especially in humans [21-23]. On the contrary, studies on the condition of serum IFABP in various diseases are scant in veterinary medicine. Two recent studies [8,24] demonstrated that IFABP is a valuable biomarker in dogs with CPV. Eregowda et al.[8] reported that serum IFABP concentration at 72 h of treatment in CPV enteritis could serve as a reliable predictor of prognosis (AUC=0.888; P<0.003). Gulersoy et al.[24] also revealed that serum IFABP concentration could be used to predict mortality (AUC=0.787; P=0.043) at admission to the hospital (0 h) in dogs with CPV enteritis. In accordance with the finding of the above-mentioned previous studies, IFABP values at 0 h (pre-treatment; hospital admission) in this study can be considered as a reliable biomarker with high sensitivity (83.3%) and specificity (80%) in predicting mortality. Apart from the results of previous studies, serum IFABP concentration and the CHS in dogs with CPV enteritis were *moderately positively correlated (r=0.501; P=0.000)* and thus the importance of serum IFABP among the other parameters examined for determining the clinical severity of the disease was proved *(Table 2)*. A strong positive correlation between clinical signs and the extent of intestinal epithelial necrosis in CPV enteritis has been described [4]. Given the biology of IFABP, this suggests that IFABP may be a direct indicator of CPV-induced intestinal damage. However, there is a clear need for studies investigating the relationship between the histopathological grade of intestinal damage and serum IFABP concentration in dogs with CPV enteritis.

This study has some limitation factors for the results and these especially include the inability to uniformize environmental and stress factors due to its clinical nature, and the absence to evaluate Coronavirus and other possible concurrent agents that may occur between 2 to 6 months of age in dogs. Furthermore, the polymerase chain reaction analysis was not used for the diagnosis of CPV in this study. However, the diagnosis of CPV in the INF group was made by evaluating both clinical findings and the rapid test kit result, which has high sensitivity and specificity. Despite these limitations, this study provides valid information for the assessment of clinical severity and prognosis in dogs naturally infected with CPV. Additionally, a future study with a larger sample size could increase the statistical power.

In conclusion, TLC in whole blood and PCT concentration in plasma at 24 h after initiation of treatment and, serum CRP and IFABP concentrations at hospital admission (0 h) in CPV-infected dogs could be used as prognostic indicators in predicting disease outcomes. However, based on ROC curve analysis results, TLC (24 h) and serum IFABP (0 h) concentration serve as the most valuable biomarkers among the parameters in this study. Compared with other parameters evaluated, serum IFABP concentration correlated strongly with the clinical severity of the disease.

COMPETING INTERESTS

The authors declared that there is no conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHORS' CONTRIBUTIONS

Design of the study: CDA, HV and BU. Preparation of the study, management of the patients and data collection: CDA, GET, and GSEA. Performing the laboratory analysis: CDA and GSEA. Article writing, data analysis and editing: CDA, GET, HV, and BU. All authors reviewed and approved the final manuscript.

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