PAPER

# Inflammatory and oxidative biomarkers of disease severity in dogs with parvoviral enteritis

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**Objectives:** To study changes in serum C-reactive protein, haptoglobin, ceruloplasmin and albumin concentration, total anti-oxidant capacity and paraoxonase-1 and butyrylcholinesterase activity in dogs with parvoviral enteritis of different degrees of clinical severity.

**Methods:** Prospective study of 9 healthy and 43 dogs with parvoviral enteritis that were classified into mildly, moderately and affected groups.

**Results:** Dogs with parvoviral enteritis had a significant increase in C-reactive protein compared with healthy dogs, with an increase of higher magnitude in animals with more severe clinical signs. All dogs with parvoviral enteritis had a significant increase in haptoglobin concentration compared with healthy dogs, but with no difference according to disease severity. There was a decrease in paraoxonase-1 activity in parvoviral enteritis.

**Clinical Significance:** Major increases of C-reactive protein concentrations in dogs with parvoviral enteritis are a marker of disease severity. In addition, higher values for anti-oxidants in severe cases compared with mild and moderate cases suggest a possible compensatory anti-oxidant mechanism.

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## INTRODUCTION

Parvoviral enteritis (PVE) is a viral disease that most commonly affects young, unvaccinated dogs younger than 6 months (Macintire & Smith-Carr 1997). After ingestion, the virus attacks rapidly dividing cells in the intestinal tract and bone marrow, causing severe vomiting, diarrhoea, fever, rapid dehydration, lethargy and decreased activity. Mucosal intestinal injuries can give rise to secondary bacterial invasion from the intestines to blood and organs, resulting in a systemic inflammatory response syndrome (SIRS) and therefore sepsis (Turk *et al.* 1990, Mohr *et al.* 2003).

The biomarkers of inflammation and oxidative stress are of clinical interest in PVE, as it is known that changes in both inflammatory and anti-oxidant status occur in this disease (Panda *et al.* 2009, Kocaturk *et al.* 2010). With regard to SIRS, both endothelial cells and neutrophils are activated in such a way as to release oxygen-derived free radicals (Cowley *et al.* 1996). It appears that these oxy-radicals play a role in causing or propagating SIRS in life-threatening conditions, and that the imbalance in the redox state reflects both oxidative stress and tissue damage (Alonso de Vega *et al.* 2002).

Acute phase proteins (APPs) are considered to be the most sensitive markers of inflammation (Ceron *et al.* 2005). In dogs, they have been used to assess the clinical severity of some inflammatory diseases such as pancreatitis (Mansfield *et al.* 2008) or pneumonia (Yamamoto *et al.* 1994). In canine PVE, APPs have been used as prognostic markers (Kocaturk *et al.* 2010) and clinical scores have been correlated with serum amyloid A and α-1 glycoprotein (Yule *et al.* 1997). To the authors' knowledge, there is no data available concerning the use of other APPs such as C-reactive protein (CRP), haptoglobin (Hp), ceruloplasmin (Cp) or albumin, in determining the severity of this disease.

A state of oxidative stress with increased concentrations of lipid peroxides occurs in dogs with PVE (Panda *et al.* 2009). Total anti-oxidant capacity (TAC) is being widely used in the assessment of oxidative stress, as it is easy and cheap to measure. It also reflects the global anti-oxidant ability of the organism and the combined



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**Table 1. Score assigned to each of the three different clinical signs and leukocyte count in dogs with parvovirosis**

| Scores | Faecal consistency | Depression | Dehydration | Leukocyte count/μL |
|---|---|---|---|---|
| 0 | Normal faeces and well formed | Normal | Normal eyes and bright | 5·50 to 12·50 |
| 1 | Pasty faeces | Mild depression | Mild dehydration, skin tent less than 3 seconds | >4·50 to <5·50 or >12·50 to <15·00 |
| 2 | Semi-liquid faeces | Moderate depression | Moderate dehydration, skin tent 3 to 10 seconds | 3·500 to <4·500 or >15·00 to <17·50 |
| 3 | Watery faeces | Severe depression | Unable to stand, severe dehydration, skin tent greater than 10 seconds | <3·50 or >17·50 |

effects of the different anti-oxidants of the body; without the need to measure each anti-oxidant separately (Miller *et al.* 1993).

There are two serum enzymes, butyrylcholinesterase (BChE) and paraoxonase-1 (PON1), that have recently generated interest in dogs as they are related to inflammation and oxidative status. A drop in activity of the two enzymes (BChE and PON1) has been observed in experimentally induced endotoxaemia in dogs (Tvarijonaviciute *et al.* 2012a) and in naturally occurring sepsis in humans and experimental animals (Novak *et al.* 2010). Both enzymes appear to be functionally related and involved in oxidative stress, as BChE is inherently protected from oxidative stress by PON1 (Ofek *et al.* 2007).

The clinical presentation of the severity of canine PVE can vary from mild to severe. There are different clinical severity scales, mostly based on clinical signs and physical examination findings (Yule *et al.* 1997, Panda *et al.* 2009, Markovich *et al.* 2012). However, serum biomarkers have not yet been included in these scales. The inclusion of objective and simple tools to assess the severity of PVE and to differentiate between moderate, mild and severe forms of the disease may be of help in clinical situations. The objective of this work was to evaluate the changes in inflammatory biomarkers (CRP, Hp, Cp and albumin), an oxidative biomarker (TAC), and two biomarkers that are related both to inflammation and oxidative stress, BChE and PON1, in dogs with PVE of different clinical degrees of severity. High-density lipoprotein (HDL)-cholesterol was also measured, as PON1 is an HDL-associated protein.

## MATERIALS AND METHODS

### Case material

Forty-three dogs admitted with a history of vomiting and haemorrhagic diarrhoea were included. These animals have been used for a previous study in which APPs were evaluated as prognostic markers in dogs with PVE, and were of various breeds (16 mixed breed, 11 German shepherd dogs, 10 Anatolian shepherd dogs, 5 Rottweilers, 1 pointer), sex (20 males and 23 females) and ages (between 2 and 6 months) (Kocaturk *et al.* 2010).

In all dogs, based on the history and the results of clinical and haematological examinations, PVE was suspected and then confirmed by positive Snap CPV antigen tests (Antigen Rapid CPV Kit, Animal genetics, Inc., Korea) in faecal samples, taken at first admission.

In addition, a group of nine healthy dogs (five males and four females) was used as a control group. They were between 2 and 6 months (4·8±0·7 months) of age, of various weights (5·2±0·9 kg) and breeds. They had presented for routine check-ups and had normal physical examination and haematological test results. The use of control animals was approved by the University Ethical Committee. Owner consent was obtained for all the dogs included in the study.

### Grouping

Dogs were grouped following the protocol used by Panda *et al.* (2009). This consisted of assigning to each animal a score based on the severity of three clinical signs, dehydration, depression and faecal consistency, with a modification based on the total leukocyte count as a marker of inflammation (Yilmaz & Senturk 2007) (Table 1). The total clinical score was recorded for each animal and used to classify the dogs into four groups: healthy, mildly, moderately or severely affected (Fig 1).

### Sample collection and measurements

Clinical (body temperature and heart and respiratory rates) and haematological parameters (complete blood cell count) were recorded for each of the animals. Venous blood samples were collected into tubes with EDTA (Vacutest EDTA K3, BD, 3 mL, Hema & Tube Tic. Ltd. Sti., Ankara-Turkey) for a complete blood cell count (Celldyn 3500, Abbot) and without anticoagulants (Vacutest, BD, 10 ml, Hema & Tube Tic. Ltd. Sti., Ankara-Turkey) for biochemical analysis before starting treatment. Tubes were centrifuged after clot retraction and the serum frozen and stored at -20°C until the time of analysis.

The serum CRP concentration was measured using a human immunoturbidimetric assay (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan's Mills, Co. Clare, Ireland) that showed a correlation of 0·98 with a specific canine ELISA assay (Tridelta Phase range canine CRP kit, Tridelta Development Ltd., Bray, Ireland) which had been previously validated for use in dogs (Martinez- Subiela & Ceron 2005). Hp concentration was measured by a commercially available colorimetric method (Tridelta Phase range haptoglobin kit, Tridelta Development Ltd, Bray, Ireland) that had been previously validated for use in dogs (Martinez-Subiela & Ceron 2005). The serum concentration of Cp was measured by a spectrophotometric method based on the in vitro oxidase activity of Cp with phenylenediamine, validated for use in canine samples (Ceron & Martinez-Subiela 2004). The serum albumin concentration was measured using a commercially available bromocresol green reagent (Albumin OSR 6102 Olympus Life and Material Science Europe).

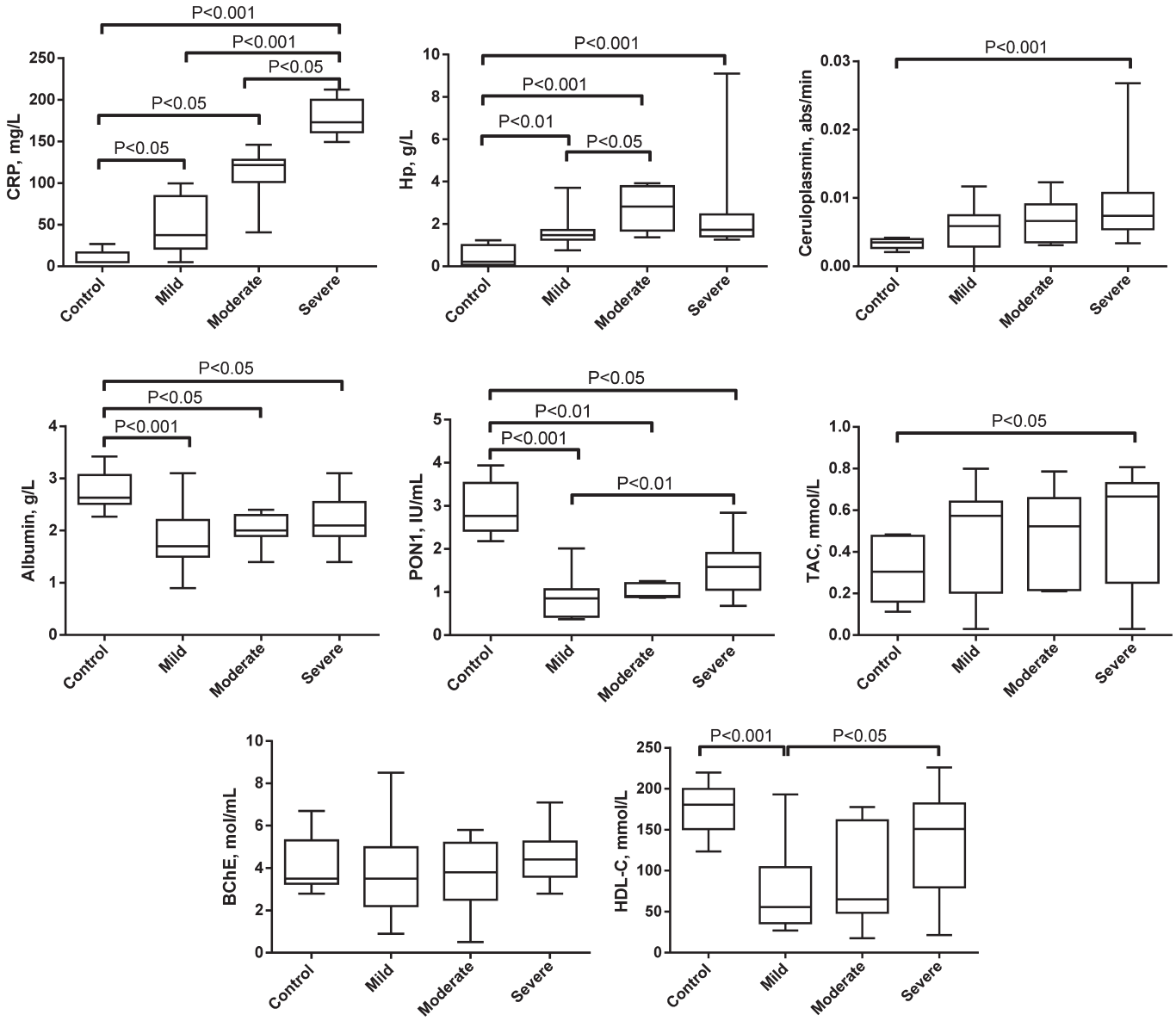


**FIG 1. C-reactive protein (CRP), haptoglobin (Hp), ceruloplasmin, albumin, paraoxonase-1 (PON1), total anti-oxidant capacity (TAC), butyrylcholinesterase (BChE) and high-density lipoprotein (HDL)-cholesterol (HDL-C) concentrations in healthy dogs (control) and groups of dogs with parvovirus of different clinical degrees of severity. The boxes depict median (horizontal line) and interquartile range (top and bottom of box), the whiskers show the range**

Serum TAC was measured by the method proposed by Erel *et al.* (2004). The serum PON1 activity was determined using 4-nitrophenyl acetate as substrate following a previously validated method (Tvarijonaviciute *et al.* 2012b). Serum BChE activity was measured by a previously reported method (Tecles *et al.* 2000). Serum HDL-cholesterol concentration was measured by a spectrophotometric method (Tvarijonaviciute *et al.* 2012a).

### Statistical analysis

Statistical analysis was performed using routine descriptive statistical procedures and software (Graph Pad Prism, Version 5; SPSS 15.0, SPSS Inc Chicago, IL). Gender differences were tested by means of a chi-squared test. The results of all analytes were evaluated for approximate normality of distribution by using the D'Agostino & Pearson omnibus normality test statistics. Given that the majority of the datasets were not normally distributed, non-parametric tests were chosen for all analyses. The Kruskal-Wallis test followed by Dunn's multiple comparison test was used to compare values of the analytes between the groups. Correlations between variables were estimated using the Spearman correlation coefficient. *P* values <0·05 were considered significant for two-sided analysis.

## RESULTS

### General variables

There were no statistically significant differences in terms of age, body weight and gender between the groups. When the severity of the clinical signs was evaluated in the dogs with PVE, 15 dogs

**Table 2. Grouping of dogs based on the clinical and haematological scores**

| Groups | Total clinical score | Severity of the case | Number of dogs in each group |
|---|---|---|---|
| I | 0 | Healthy (controls) | 9 |
| II | 1 to 4 | Mild | 15 |
| III | 5 to 8 | Moderate | 7 |
| IV | 9 to 12 | Severe | 21 |
| Total | | | 52 |

were included in the mildly affected, 7 in the moderately affected and 21 in the severely affected groups (Table 2).

### Biochemical analysis

The values for APPs (CRP, Hp, Cp and albumin), TAC, PON1, BChE and HDL-cholesterol in healthy dogs and dogs with PVE in the three groups based on the severity of the clinical signs are presented in Fig 1.

All groups of dogs with PVE had a significant increase in serum CRP concentration compared with healthy dogs. The increase in CRP was significantly higher in magnitude when the dogs had more severe clinical signs. All dogs with parvovirus in the different groups also had a significantly higher Hp concentration compared with healthy dogs. Dogs with moderate clinical signs had a significant increase in Hp compared with the group showing mild clinical signs. However, no statistical differences in Hp were found between the severe and moderate groups. Although there was a tendency for Cp to increase in dogs with PVE, a significant increase was only found in dogs with severe clinical signs compared with the control group. All groups of dogs with PVE had a significant decrease in albumin concentration compared with the healthy dogs, and no significant differences in albumin values were found between the different groups in terms of severity.

TAC showed a tendency to increase in dogs with PVE compared with healthy dogs, but significantly higher values were found only in dogs with severe clinical signs. A significant decrease was found in PON1 activity in all the different groups of PVE dogs and in HDL-cholesterol concentration in the mildly affected dogs when compared with healthy dogs. Dogs with severe clinical signs had significantly higher PON1 activity and HDL-cholesterol concentration than those with mild clinical signs. No significant changes were found in BChE activity between PVE and healthy dogs.

Significant positive correlations were found between CRP and Hp (r=0·463; P=0·001), Cp (r=0·433; P=0·002) and TAC (r=0·367; P=0·009). TAC was positively correlated with Cp (r=0·345; P=0·017) and BChE (r=0·377; P=0·009). PON1 was positively correlated with albumin (r=0·770; P<0·001) and HDL-cholesterol (r=0·637; P<0·001) and negatively with Hp (r=-0·465; P=0·001).

## DISCUSSION

In this study, serum CRP concentrations showed increases of a higher magnitude when the dogs with PVE had more severe clinical signs. PVE can produce sepsis as a result of secondary bacterial infection and then endotoxaemia from endotoxin release into the peripheral circulation from invading Gram-negative bacteria in dogs (Mohr *et al.* 2003). One of the main causes of major increases in CRP is bacterial infection, whereas viral infections usually produce minor, if any, increases in CRP (Gruys *et al.* 2005). Therefore it could be postulated that the increase in CRP in PVE could be related to the magnitude of secondary bacterial infection associated with this disease. This would be in line with previous results with regard to higher CRP in PVE dogs that had died, as sepsis is the most common cause of mortality in dogs requiring intensive care (Kocaturk *et al.* 2010).

Serum Hp and Cp concentrations, although correlated with CRP, showed increases of a lesser magnitude, a fact in keeping with their role as moderate APPs (Ceron *et al.* 2005). In the case of Cp, increases were only significant in severe cases. In addition to inflammation, a compensatory mechanism against oxidative stress could be implied from the Cp increase as it is considered as an anti-oxidant compound (La Rubia *et al.* 2013), in this study being correlated with TAC.

Interestingly, dogs with severe PVE, despite having significantly higher values of CRP compared with dogs with moderate PVE (with a mean of 180 mg/L *versus* 125 mg/L), did not have an increase in Hp compared with moderate cases. This could be because of the presence of gastrointestinal haemorrhage that was present in all severe cases. Haemorrhage and/or haemolysis associated with haemolytic anaemia are known to decrease Hp concentrations (Buchanan *et al.* 1990, Matijatko *et al.* 2007). Clinical cases with severe inflammatory conditions in which CRP concentrations are approximately 180 mg/L or higher, are usually accompanied by values of Hp higher than 4 g/L, a fact that is also observed in experimental conditions (Tvarijonaviciute *et al.* 2012a). Thus, the presence of very high values of CRP in a PVE dog which are not accompanied by very high Hp values (>4 g/L) should raise the suspicion that, in addition to severe sepsis (reflected by a major increase of CRP), the case could be complicated by haemorrhage or haemolysis. This is an example in which divergences in the behaviour between major (such as CRP) and moderate (such as Hp) APPs can provide useful clinical information (Ceron *et al.* 2008).

In the present study, TAC did not decrease in dogs with PVE, and even then there was a tendency to increase with the severity of the disease. Increases in other anti-oxidant enzymes such as catalase and superoxide dismutase have been reported in PVE dogs. This could be because of enhanced synthesis of these anti-oxidant enzymes in moderate and severe cases of gastroenteritis as an in-built compensatory mechanism (Panda *et al.* 2009). A similar situation with increases in oxidative enzymes but no TAC decreases has been reported in other diseases in dogs such as sarcoptic mange that could be explained by an increase in the enzymatic anti-oxidant response to compensate for the predominant oxidative status (Camkerten *et al.* 2009). Serum TAC was also reported to increase in critical surgical patients with septic shock, and could be due to a defence mechanism against the disease (Pascual *et al.* 1998).

PON-1 activity decreased significantly in dogs with PVE compared with healthy dogs. Similarly, major decreases in PON1 (approximately threefold) were found in humans with sepsis (Kedage *et al.* 2010, Novak *et al.* 2010). There are several reasons for the decrease in PON1. One could be due to a rapid and marked reduction in PON1 mRNA in the liver in response to sepsis/endotoxaemia (Kedage *et al.* 2010, Fuhrman 2012). As a direct effect of inflammatory cytokines on liver PON1 expression has been described (Feingold *et al.* 1998), PON1 is considered to be a negative APP in dogs (Tvarijonaviciute *et al.* 2012a). The high correlation found in the present study between PON1 and albumin, which is a negative APP that decreases in inflammation by reducing its synthesis in the liver, could support this hypothesis. Another cause of the PON1 decrease could be the oxidative stress associated with PVE (Panda *et al.* 2009), as PON1 is known to decrease in dogs in situations of oxidative stress such as diabetes mellitus or onion intoxication (Tvarijonaviciute *et al.* 2012b).

In this study, PON1 tended to have higher values in dogs with severe disease compared to those with moderate or mild disease, although the values were lower than those found in healthy dogs. PON1 is a protein associated with HDL-cholesterol, and both demonstrated similar behaviour. Various hypotheses could be postulated to explain the higher values of PON1 in dogs with severe disease. One could be that higher values of PON1 in severe cases could be attributed to a protective anti-oxidant effect as described for TAC. The other explanation might be that PON1 is also involved in the bacterial virulence mechanism (Ozer *et al.* 2005). Therefore, in a situation of sepsis, PON1 could reflect the higher pathogenicity of bacteria. Also, dogs with more severe clinical signs tend to have a more reduced appetite, and therefore an increase in lipid mobilisation that could contribute to an increase in HDL-cholesterol and PON1.

The lack of changes in cholinesterase could reflect the equilibrium between the inflammation, as BChE decreases in inflammation (Tvarijonaviciute 2012a), and the lipid mobilisation and increase in triglycerides associated with PVE (Yilmaz and Senturk 2007) are positively correlated with cholinesterase (Tvarijonaviciute 2013).

In conclusion, dogs with severe PVE have higher CRP values than dogs with moderate and mild signs, and therefore major increases in CRP in a dog with PVE should alert veterinarians to the severity of the disease and the existence of a possible sepsis. In addition, in dogs with PVE there is a decrease in PON1 and a tendency for Cp and TAC to increase. In more severe cases, PON1 and TAC are higher than in mild and moderate cases, possibly indicating the existence of a compensatory anti-oxidant mechanism.

## Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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