Evaluation of serum C-reactive protein levels as a predictor of outcome in puppies infected with parvovirus

by

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Submitted to the Faculty of Veterinary Science, University of Pretoria, in partial fulfilment of the requirements for the degree MMedVet (Med)

Pretoria, October 2012

"Start by doing what is necessary, then what is possible, and suddenly you are doing the impossible."

St Francis of Assisi

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Acknowledgements

I would like to thank the following people for their help and assistance without which this project would not have been possible:

Dr Mirinda van Schoor, my promoter for her support and constant encouragement that made this project possible.

Prof. Amelia Goddard, my co-prompter for her advice and guidance that was invaluable to this project.

Prof. Mads Kjelgaard-Hansen for his valuable contribution and insight into this project.

Prof. Peter Thompson for the statistical analysis.

Mrs Cheryl Booth and the technicians in the laboratory for performing the CRP analyses.

Mrs Erna van Wilpe and the technicians at the Electron Microscopy Unit for performing the faecal EM's.

Sr Mandy Albertyn and Sr Sarina Myburgh for their help with sample collections.

The staff and students working in the outpatients department for alerting me when parvovirus was diagnosed and infected puppies were admitted to the isolation unit.

And lastly, but most importantly, my husband, John, not only for his support and for accompanying me to the parvo ward over weekends and late nights to collect samples, but for believing in me and encouraging me to follow my dreams.

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Abbreviations

APP	Acute phase protein
AGP	Alpha-1-acid glycoprotein
AUC	Area under the curve
CPV	Canine parvovirus
CPV – 2	Canine parvovirus types 2
CRP	C-reactive protein
EDTA	Ethylenediamene tetra-acetic acid (anti-coagulant)
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FPV	Feline panleukopaenia virus
HP	Haptoglobin
HR	Hazard ratio
IL	Interleukin
IQR	Interquartile range
Kg	Kilogram
MAP	Major acute phase protein
OR	Odds ratio
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
ROC	Receiver-operating characteristic
RPLA	Reversed passive latex agglutination test
SIRS	Systemic inflammatory response syndrome
SRID	Single radial immunodiffusion

TIA	Turbidometric immunoassay
TR-IFMA	Time- resolved immunofluorometric assay
TSP	Total serum proteins

Summary

Serum C-reactive protein measurements as a predictor of outcome in puppies infected with parvovirus

McClure V, University of Pretoria 2012.

Canine Parvovirus remains a leading cause of enteritis in dogs in South Africa and many other countries despite the wide availability of effective vaccines. The virus does not affect all dogs equally and the course of the disease depends on the age, immune status and breed of the puppies as well as the viral dose, route of exposure and the virulence of the strain. Although aggressive supportive treatment can be successful, the treatment and convalescent periods may be prolonged and consequently expensive and the mortality rate relatively high, causing many clients to forego treatment and elect for euthanasia of their pet.

Acute phase proteins (APP) are proteins that change in concentration by at least 25% in animals subjected to external or internal inflammatory challenges, such as infection, inflammation or surgical trauma. Increased concentrations are associated with poor outcome in certain diseases. C-reactive protein (CRP) is the most sensitive APP in dogs. Its normal physiological concentration is low but increases rapidly with inflammation or tissue destruction. Due to the fact that CRP has a relatively short half life in serum (6-8 hours) and a high response in diseased animals, it can be used as a valid measure of a systemic response to an initiating stimulus at the time of blood sampling. By taking serial measurements, objective information about the extent of the

ongoing lesions in the patient can be obtained and therefore may be used as a prognostic indicator.

The objective of this prospective observational study was to evaluate the association of serum CRP concentrations in puppies suffering from canine parvoviral enteritis with morbidity and mortality, and to determine the usefulness of CRP to predict duration of hospitalisation time.

Seventy-nine client owned puppies naturally infected with canine parvovirus were included. Parvovirus infection was diagnosed on electron microscopic examination of faeces from the puppies.

CRP was measured using an automated human C-Reactive Protein Turbidimetric Immunoassay (TIA), which has been validated for use in dogs.

Serum CRP measurements were performed at admission, twice daily for the first 48 hours, then once daily until death or discharge.

There was a positive association between odds of mortality and CRP concentration on admission, as well as 12 and 24 hours after admission (P=0.04,P=0.005 and P=0.003, respectively). Survival time was negatively associated with CRP concentration at 12 and 24 hours after admission (P=0.002and P=0.001, respectively). Among the survivors, length of hospitalisation was positively associated with CRP concentration at 12, 24 and 36 hours after admission (P=0.012, P=0.001 and P=0.002, respectively). Utility for CRP concentration to correctly differentiate between survivors and non-survivors at 24 hours after admission had a sensitivity and specificity of 78.7% and 86.7% respectively.

Although serum CRP concentration is associated with outcome in puppies infected with canine parvovirus, when used alone it did not prove to be a good predictor of survival.

Chapter 1: Literature review

1.1 Canine parvoviral enteritis

Canine parvovirus (CPV) first emerged in the mid-1970s as a new enteric pathogen of dogs[1-3] and by the end of 1983, it had been reported in 50 countries around the world[4]. It has remained a leading cause of enteritis in dogs despite the wide availability of effective vaccines[5]. Parvoviruses are small, non-enveloped, single-stranded DNA viruses that replicate using various components of the host cell DNA replication complex and since they cannot induce mitosis, only replicate in actively dividing cells[6,7]. These viruses are hardy, persisting for long periods of time in the environment (5-7 months), and are ubiquitous[7,8]. The virus is better known as canine parvovirus type 2 (CPV-2) because it was the second parvovirus described in dogs[9]. The specific ancestral strain of virus that gave rise to CPV has not been identified but it has been reported that CPV has evolved from either feline panleukopaenia virus (FPV) or one of the closely related viruses of wild carnivores. In 1979 a new strain of CPV-2 emerged and was designated CPV-2a. This virus then mutated and formed a new strain, CPV-2b which emerged in 1984. Around the year 2000 another strain, CPV-2c was identified. Since then it has been detected in many regions of the world[6]. To the author's knowledge, CPV-2c has not yet been isolated in South Africa. In a study done on 125 dogs infected with parvovirus from Southern Africa between1995 – 1998, CPV-2a was isolated from 39 dogs and CPV-2b was isolated from 86 dogs, confirming that the predominant strain found in Southern Africa was CPV-2b. This differed from the situation in Europe and Japan

during the same period where CPV-2a was the most prevalent type[10]. There appears to be a distinct seasonality for CPV infection, with peak incidences during the summer months[11]. The disease is most commonly seen in puppies from 6 weeks to 6 months of age[12,13]. In susceptible canine populations, parvovirus infection most often occurs as a severe systemic and even life threatening illness[12]. The characteristic symptoms of CPV enteritis appear within 5-7 days after infection and include haemorrhagic diarrhoea, vomiting, loss of appetite, decreased mentation, severe dehydration, sudden collapse and death. The progression of the enteritis is accompanied with virus excretion in the faeces[14].

1.2 Risk factors

Predisposing factors for parvovirus infection in puppies include lack of protective immunity, unsanitary and overcrowded environments, diet changes, weaning and enteric parasites[7,15]. A study published in 2004 found that certain breeds, including rottweilers, doberman pinschers, American pit bull terriers, Labrador retrievers and German shepherd dogs, are at an increased risk for severe CPV enteritis[7]. In another study Staffordshire terriers and Alaskan sledge dogs were also found to be at greater risk of contracting CPV[16]. The reason for the increased susceptibility in some breeds is not known, but when disease incidence is higher in pure breed dogs compared to cross breeds a genetic component for susceptibility should be considered[4]. Dobermans and rottweilers have a common ancestry which could indicate common genetically determined factor/s. It has also been suggested that

rottweilers may have a hereditary immunodeficiency but there is not enough data to support these suggestions[17].

1.3 Pathogenesis of CPV

The two clinical syndromes described in dogs infected with CPV include enteritis and myocardial failure[8,15]. Myocardial failure occurs in neonatal puppies infected in utero or shortly after birth and is rarely seen today due to vaccination protocols that promote maternal antibody production in young puppies[15]. Infection with parvovirus is acquired by the faecal-oral route of transmission. Viral replication occurs in the oropharynx and local lymphoid tissue in the first 2 days of infection. By the 3rd to 5th day viraemia is marked, with CPV preferentially targeting tissue with rapid turnover[7]. The cells most affected are the intestinal epithelium, lymphoid tissue and bone marrow due to the rapid turnover of cells in these tissues. Conditions that increase cell turnover, e.g. diet change and change in bacterial flora due to weaning or concurrent endoparasitaemia or corona virus infection, favour viral replication and increase the severity of the resulting lesions and clinical disease[7,8]. Extensive destruction of lymphoblasts in lymphatic tissue and myeloblasts in bone marrow lead to a lymphopenia and in severe cases a panleukopenia[8,18]. In the intestinal tract, parvovirus replication kills cells of the germinal epithelium of the intestinal crypts, leading to epithelial necrosis, villus atropy and collapse of the intestinal epithelium, loss of absorptive capacity and the development of haemorrhagic diarrhoea and vomiting[7,8].

1.4 Clinical manifestations of CPV infection

CPV does not affect all dogs equally with the course of the disease depending on the age, immune status and breed of the puppies as well as the viral dose, route of exposure and the virulence of the strain[19]. Puppies with CPV myocarditis are often found dead or succumb within 24 hours after the appearance of clinical signs, which include dyspnoea, crying and retching. Usually all puppies in a litter are affected. As stated previously, this is rarely seen nowadays[16]. The most common manifestations of CPV infection are severe gastrointestinal upset and immunosuppression. Typical signs include vomiting and a mucoid to haemorrhagic diarrhoea[7,19] with marked abdominal pain that can be caused by the acute gastroenteritis or intestinal intussusception[9]. These puppies may also have significantly depressed leukocyte counts with a severe, transient lymphopenia[9]. A less clinically apparent, more global systemic inflammatory response syndrome (SIRS) can occur in many cases[7]. In a study done in 2010, it was reported that the chance of non survival were higher in parvovirus infected puppies that had evidence of systemic inflammatory response syndrome at the time of admission[20]. The systemic inflammatory response is thought to be due to bacterial translocation from the damaged intestinal tract leading to coliform septicaemia[21]. *Escherichia coli* was recovered from the lungs or liver of 90% of puppies that died due to parvovirus infection and pathological findings compatible with acute respiratory distress syndrome were also found[22], providing evidence that bacterial translocation and coliform septicaemia is an important factor in the pathogenesis of CPV infection. Endotoxin is a potent stimulus for the inflammatory response through activation of

cytokine-mediated procoagulant effect on endothelial cells[7,21,23]. Dogs with CPV have a high prevalence of clinical thrombosis or phlebitis and laboratory evidence exists of hypercoagulability without disseminated intravascular coagulopathy[23]. The inflammatory response initiated by the viral disease and associated endotoxaemia cause an increase in fibrinogen concentrations. This increase, in association with vascular stasis, activation of coagulation, and vascular injury may be a risk factor for thrombosis and contribute to the hypercoagulable state in these dogs[23].

1.5 Diagnosis of CPV

Definitive diagnostic tests include detection of CPV in the faeces of affected dogs using electron microscopy, virus isolation, faecal haemagglutination, latex agglutination, counterimmunoelectrophoresis, immunochromatography and polymerase chain reaction (PCR), serology and necropsy with histopathology[7,15,24,25].

Faecal enzyme-linked immunosorbent assay (ELISA) antigen tests are available for in hospital testing for acute CPV[16,i]. False positive results may occur 3-10 days after vaccination with a modified live CPV vaccine as all the modified live canine parvovirus vaccines are shed in the faeces[8]. False negative results may also occur due to binding of test antigen with serum neutralizing antibodies in bloody diarrhoea or cessation of faecal viral shedding[7,15,26].

1.6 Treatment of CPV

To date no agent-specific treatment has proven effective for CPV enteritis therefore treatment remains symptomatic and supportive. The survival of acute CPV cases is largely dependent on the intensive treatment given when the puppy is hospitalised[18]. Survival of infected puppies ranges from 9% if left untreated to more than 90% in those treated in tertiary veterinary facilities[20]. The mortality rate for puppies admitted and treated at the Onderstepoort Veterinary Academic Hospital (OVAH) is between 16 and 19%[27,28]. Most puppies require admission and aggressive fluid therapy with both crystalloids and colloids. Hypoglycaemia is common in these patients and needs to be corrected and monitored along with any electrolyte disturbances. Antimicrobials, anthelmintics and analgesics also form an important part of the treatment plan[9]. Antiemetic drugs should be used to control the vomiting as these puppies are usually vomiting profusely which leads to hypovolaemia and electrolyte imbalances[29]. Early enteral nutritional support is also needed as it has been shown to be associated with more rapid clinical improvement[30]. The treatment and convalescent periods may be prolonged and consequently expensive and the mortality rate relatively high, causing many clients to forego treatment and elect for euthanasia of their pets[7,31]. In human patients with severe sepsis, mortality rates may exceed 30% despite advanced supportive care[32]. This is similar to the findings in this study (20% mortality) with parvovirus treatment. There is a distinct need for therapies that decrease disease severity and duration of hospitalisation, improve

survival and reduce treatment costs[30]. Often clients are faced with situations where they have already spent a considerable amount of money on treatments for their puppy but the puppy's condition does not seem to be improving. This is often a very emotional time for the owner and they invariably look to the clinician to help them make decisions on whether to continue or withdraw treatment and euthanise their pet. There is a need for affordable objective estimates of outcome for veterinary patients with critical illness that can assist clinicians, and therefore owners, in identifying patients who will benefit from intensive care treatment or specific therapies as well as those for whom treatment will not improve chances of survival[33]. Several studies have investigated different parameters as prognostic markers for CPV, which include endocrine[27], haematological[28], and biochemical parameters[20,34].

1.7 Acute phase proteins

Acute phase proteins (APP) are a group of blood proteins that change in concentration by at least 25% in animals subjected to external or internal inflammatory challenges, such as infection, inflammation or surgical trauma[35,36]. The acute phase response is induced by protein hormones called cytokines acting as messengers between the local site of injury and the hepatocytes synthesising the APP. These pro-inflammatory cytokines are secreted from the activated leukocytes stimulated by bacterial toxins or in response to local tissue injury[37]. The APPs consist of 'negative' (albumin and transferin) and 'positive' (haptoglobin, C-reactive protein, serum ceruloplasmin, fibrinogen, alpha-1-acid-glycoprotein, and serum

amyloid A) proteins that show a decrease and increase in levels respectively, in response to challenge[36]. These proteins have been used as biomarkers of inflammation, infection and trauma for decades in human medicine. They are used in diagnosis, prognosis, monitoring response to therapy as well as in general health screening[38,39]. In animals there are variations between species in the proteins which respond during the acute phase reaction, as well as in the type of response[38,40]. (Table 1.)

Table 1. The major and moderate acute phase proteins (APP) that respond to inflammatory stimuli in a number of common domestic animal species.[38]

Species	Major APP	Moderate APP
Cat	SAA	AGP , Hp
Dog	CRP, SAA	Hp, AGP
Horse	SAA	Hp
Cow	Hp, SAA	AGP
Pig	CRP, MAP, SAA	Hp

SAA, serum amyloid A; CRP, C-reactive protein; Hp, Haptoglobin; MAP, Major acute phase protein; AGP, α_1 acid glycoprotein.

1.8 C-reactive protein

Serum C-reactive protein (CRP), a major APP in people and dogs, has been investigated as a prognostic indicator of survival in people with severe sepsis[32]. It is the APP that is most frequently requested in medical laboratories[38]. CRP is also the most sensitive APP in dogs[41]. CRP is synthesised in the liver and released into the blood stream after the hepatocytes are stimulated by pro-inflammatory cytokines especially interleukin-6 (IL6)[42,43]. Because CRP is not stored, there is a low risk of inappropriate release. The normal physiological concentration of CRP is low, but increases rapidly with the onset of acute inflammation or tissue destruction. Changes in CRP during the course of disease seems to reflect the underlying disease progression[44,ii]. Due to the fact that CRP has a relatively short half life in serum (6-8 hours) and a high response in diseased animals, it can be used as a valid measure of a systemic response to an initiating stimulus at the time of blood sampling. By taking serial measurements, objective information about the extent of the ongoing lesions in the patient can be obtained and therefore may be used as a prognostic indicator[37]. CRP has been shown to increase in a number of different diseases in animals[34,41,45-57]. (Table 2 lists the diseases in canines where CRP has been known to increase.) Several research reports have identified serum CRP as a useful acute phase marker in the dog[44]. In the study done by Jergens *et al* in 2003, the serum concentrations of CRP were found to be significantly increased in dogs with canine inflammatory bowel disease with an activity index score of greater than or equal to 5 (mild disease activity or greater) compared to the control group[49]. These same dogs showed a significant decrease in CRP values after successful

medical therapy leading to the conclusion that CRP values can be used for laboratory evaluation of the effect of therapy in these dogs. All the dogs inoculated with *Ehrlichia canis* in a study done by Shimada *et al.* showed an increase in serum CRP concentrations between 4 and 16 days and peaked at 15 to 42 days after exposure[55]. This was similar to the levels of antibodies against *E. canis* in the serum. A study by Martinez-Subiela *et al* reported that the sensitivity of CRP to detect the symptomatic dog was 93% in dogs with Leishmaniasis and 82% for asymptomatic dogs[57]. Measurement of admission CRP concentration in puppies infected with parvovirus has been show to be higher in those that did not survive compared to those that did[34].

Table 2. Canine diseases in which an increase in CRP has been described.

Organ system	Disease
Gastrointestinal	Intestinal obstruction[45] Inflammatory bowel disease[49] Acute pancreatitis[53] Bacterial enteritis[45]
Cardiovascular	Chronic valvular disease[50]
Musculoskeletal	Rheumatoid arthritis[45] Polyarthritis[45]
Infectious	*E. coli* endotoxaemia[45] Babesiosis[45,75] *Ehrlichia canis*[55,56] *Bordetella bronchiseptica*[45] Leishmaniosis [45,57] Leptospirosis[45] Parvovirus infection[34,45] Trypanosomiasis[45] Pyometra[54] Pneumonia[45]
Immune mediated	Autoimmune haemolytic anaemia[51, 77] Steroid responsive meningitis arteritis[46]
Neoplasia	Lymphoma[52]
Other	Surgical trauma [41,48]

1.9 Determination of CRP concentrations

Canine CRP can be measured in serum[44], whole blood[58], body cavity effusions[59] and saliva[60]. Although obtaining saliva samples is advantageous as it is non-invasive, there are many factors that can influence CRP concentrations in saliva. The rate of saliva flow can lead to dilution of CRP and peridontitis or gingivitis increases CRP levels in saliva[60]. CRP values in serum show no diurnal variations[61], are unaffected by eating[62] and have no sex predilections[63]. The measurements have been shown not to be affected by administered glucocorticoids[64]. Viral vaccinations have also been proven not to influence the CRP concentrations[65]. Haemolysis, lipaemia and hyperbilirubinaemia cause small changes in CRP results that are unlikely to be clinically relevant[66].

A variety of assay methods to measure canine CRP concentrations have been described and validated for use in dogs, making the measurement of serum CRP accessible and affordable. These methods include electroimmunoassay[67], ELISA[68], time-resolved immunofluorometric assay (TR-IFMA)[58], single radial immunodiffusion (SRID)[63], turbidimetric immunoassay (TIA)[44] and recently the human CRP near patient slide reversed passive latex agglutination test (RPLA)[69]. A commercially available, automated, turbidimetric immunoassay for human serum CRP determination has been validated for use in canines. Heterologous determination of canine CRP with anti-human CRP antibodies usually fails due to a lack of cross-reactivity[67], but reports on sufficient cross-reactivity between anti-human CRP antibodies and canine CRP for analytical purposes exist[44]. Appropriate control is

therefore essential when using this assay, i.e. using canine specific CRP for calibration and running internal quality control controls[70].

Normal ranges for CRP concentrations have been determined by different researchers for healthy adult dogs. It was found that in acute inflammation, CRP production was lower in younger dogs (3 months old or less) than in adult dogs[65]. Therefore, the determined normal ranges for adult canines should be used with caution in very young puppies.

The attention focused on CRP reflects in part the fact that it is an exceptionally stable analyte in serum or plasma and that immunoassays for CRP are robust, well standardised, reproducible and readily available[62]. It provides better sensitivity compared to other traditional inflammatory markers such as leukocytosis and neutrophilia[71] and can serve as a prognostic tool[37].

Chapter 2: Study Objectives

2.1 Hypothesis

The estimated costs for CPV infected puppies admitted to a veterinary hospital are usually high and, due to the added guarded prognosis, some owners may consider euthanasia due to financial constraints. An affordable, effective method for monitoring the disease progression and outcome early on in the treatment will enable the owners to make an informed decision about their pet and will enable the clinician to adjust treatment more effectively by monitoring the patients' response to treatment.

We hypothesised that serial determination of serum CRP concentration in puppies infected with CPV can be used as a predictor of mortality and that serial measurements of CRP would be useful to predict duration of hospitalisation time.

2.2 Objectives of this study

- To investigate whether serial serum CRP determination can be used as a biomarker in CPV infection to monitor progression and outcome of the disease.
- To determine the usefulness of CRP to predict duration of hospitalisation time.

2.3 Benefits arising from the study

CPV is a disease of global economic importance. Treatment can be prolonged and often costly to the owner with the outcome being varied. If prognostication regarding outcome and duration of hospitalisation can be determined early in the disease course, informed decisions can be made by the clinician and owner regarding the management of the patient or whether humane euthanasia should be considered. Previous studies have evaluated leukocyte count and cortisol levels as monitoring tools. Both were found to be useful for predicting progression of the diseases but could be affected by other factors. Leukocyte count does not always reflect the true state of the disease due to bone marrow suppression and destruction of lymphoid tissue by the virus, and cortisol levels may be affected by the time of day when the cortisol is measured and is not always readily available or affordable. CRP is the most sensitive acute phase protein in canines. It is easy to measure, and its levels respond quickly to changes in disease status making it an effective and affordable prognosticating tool.

The research conducted serves as a partial fulfillment of the investigator's MMedVet(Med) degree.

Chapter 3: Materials and Methods

3.1 Model system

This project was a prospective, observational study which included client-owned puppies that were naturally infected with CPV and admitted to the isolation ward of the OVAH, Faculty of Veterinary Science, University of Pretoria, South Africa over a period of 11 months (November 2008 – September 2009).

3.2 Experimental design

This study was approved by the Animal Use and Care Committee and the Research Committee of the University of Pretoria (protocol number V046-08). Eighty five (85) client owned puppies that were presented to the outpatients clinic between November 2008 and September 2009, with a provisional diagnosis of acute parvoviral enteritis, that were admitted to the isolation unit were considered for the study. Owner consent was obtained for the puppies being admitted. **(Appendix A)**

3.2.1 Inclusion criteria

- Aged between 6 weeks and 18 months.
- Body weight ≥ 3kg; any breed and either sex.
- Exhibit clinical signs typical for parvovirus infection, such as decreased mentation, vomiting, haemorrhagic diarrhoea, anorexia and dehydration.

- Admitted to the OVAH isolation ward due to the severity of their clinical signs as decided by the clinician on duty at outpatients, and should not have received any treatment prior to admission.
- Peripheral blood smear had to be negative for blood-borne parasites (examined by the duty clinician under a light microscope.)
- A faecal wet preparation examined under a light microscope by the primary investigator had to be negative for visible *Giardia* parasites.
- Diagnosed as CPV positive, corona virus negative and canine distemper virus negative from faecal electron microscopy within 24 hours of being admitted.
- Free of any obvious other inflammatory processes that could falsely elevate CRP.

Owners of the dogs that were to be included in the study were informed of the nature of the study (**Appendix B)** and each was requested to sign a consent form before the puppies were included in the study. **(Appendix A).**

3.3 Experimental procedure

At admission, prior to any treatment, each puppy was clinically examined and the data was captured on a form **(Appendix C).** A clinical examination was performed on each

patient daily until discharge or death and the data captured on a new form **(Appendix D)**. This data was collected by the primary investigator. At admission a peripheral blood smear was made from blood collected from the anterior edge of the ear and examined by the duty clinician.

A faecal sample was also collected at admission. The sampling method depended on the size of the puppy. A lubricated 1 ml syringe was inserted into the rectum and faeces aspirated in the smaller puppies, while digital collection (lubricated gloved finger) was used in larger puppies. Some of the faeces were used to perform a faecal flotation and faecal wet preparation, which was examined under a light microscope by the duty clinician. The rest of the faeces were refrigerated immediately after collection and submitted within 12 hours of collection to the Electron Microscopy (EM) unit of the Department of Anatomy & Physiology for examination by transmission electron microscopy, with the request to be examined for the presence of parvo, distemper and corona virus particles. Over weekends and public holidays, puppies being admitted to the isolation ward had a parvo ELISA point- of- care test performed (Antigen Rapid CPV Ag test kit)[i] on their faeces and were admitted into the study conditionally if the test was positive until an EM examination could be requested to confirm the diagnosis and to exclude distemper and corona viruses.

All blood samples were collected from the jugular vein with 22G vacutainer needles[iii], unless the puppy was less than 5kg in which case the sample was collected with a syringe and an appropriate size needle. A 2 ml serum sample was collected from all the

puppies in 3 ml Vacutainer Brand Serum Tubes[iv] at admission, prior to any treatment, and then twice daily (approximately 8 to 12 hours apart) for the first 48 hours. Follow up samples were collected before 10h00 each morning and no later than 19h00 in the afternoon, with at least 8 hours between collections. The first follow up samples were collected no sooner than 4 hours after admission, depending on the next scheduled collection time (10h00 or 19h00). After 48 hours, daily samples were collected until death or discharge. These samples were collected during the morning collection period. A further 1.5 ml of blood was collected daily in 3 ml EDTA Vacutainer Brand Tubes[iv]. A portion of the serum and EDTA samples were made available for the daily routine blood assays (glucose and potassium, microhaematocrit and total plasma proteins [TPP]). Serum samples were left to clot at room temperature and then centrifuged at 4000 rpm (2100 g) for 10 minutes. A minimum of 100 µL of serum was required for the CRP analysis. The remaining serum was stored at -80°C until it could be analysed as a batch, in order to avoid inter-assay variability. All of the puppies received the standard treatment for parvovirus infection as set out by the OVAH. This includes intravenous fluid therapy, electrolyte replacement, antibiotic, antiemetic, anti-ulcer and prokinetic treatment, deworming, enteral feeding and blood or plasma transfusions if needed. **(Appendix E)**

The duty clinician decided when a puppy could be discharged from the hospital in consultation with the primary investigator. If a decision was made to euthanise a patient it was discussed with the primary investigator. If a puppy was euthanised due to financial constraints the case was excluded from the study. Data regarding the outcome of the hospitalisation was recorded. **(Appendix F)**

3.4 Observations/analytical procedures

The following data was collected at the time of the clinical examination **(Appendix C and F):**

- Signalment: (Age, sex and breed)
- Vaccination status
- Number of days ill before admission
- Mentation status
- Percentage dehydration
- Capillary refill time and mucosa colour
- Temperature
- Pulse rate, quality and rhythm
- Respiration rate and type
- Lung sounds
- Abdominal palpation (monitor intestinal consistency, content and abnormalities including intussusception)
- Blood smear evaluation
- Microhaematocrit and total serum proteins (TSP)
- Faecal flotation and wet preparation
- Faecal appearance
- Blood glucose levels
- Serum potassium levels

The patients were monitored daily (**Appendix D**) and treated accordingly.

The faecal flotation was performed using a disposable flotation kit.[v] For the faecal wet preparation a small amount of faeces was mixed with 2 – 3 drops of saline and covered with a cover slip. The sample was examined under a light microscope (10×) for gross movement of protozoa. The packed cell volume (PCV) was obtained by filling a microhaematocrit tube with heparinised blood and centrifuging it at 10000 rpm (7826g) for 5 minutes. The PCV percentage was determined using a microhaematocrit reader. The plasma of the microhaematocrit tube was used to read the TPP by means of a refractometer.

Blood glucose was measured from a drop of heparinised blood on a hand held glucometer[vi] . The serum potassium was measured using a dry-chemistry analyser[vii]. The CRP concentrations were measured in batches of 100 samples to minimise analytical variations. The serum samples were stored at -80°C in the Clinical Pathology laboratory at the OVAH until sufficient numbers had been collected. Serum CRP concentrations remain stable in serum stored at -20˚C for up to a year in a controlled laboratory setting and up to 34 months in a research setting[72]. Due to this delay in analysis the primary investigator was blinded to the results to prevent any bias. The CRP was measured using an automated human C-Reactive Protein Turbidimetric Immunoassay[viii,ix] (TIA), which has been validated for use in dogs[44,73]. The assay was calibrated with commercially available purified canine CRP[x] to ensure species-specific measurement of CRP with the heterologous assay[69]. The intra-assay coefficient of variation for the CRP method, using immunoturbidimetry, was calculated as 0.1 (10%). The detection range for the assay was 5.1-163.3 mg/L. Samples that were higher on the initial run were manually diluted out to 1:3 or 1:5 at the discretion of the laboratory

technician. Internal controls were routinely run with the batch of samples. Samples were analysed as a batch and all outliers were immediately re-analysed to confirm results.

3.5 Statistical analysis

Data was analysed using Stata 11.1 statistical software[xi]. CRP concentrations were log-transformed to achieve normality therefore geometric means are presented. The means were compared between survivors and non-survivors at each time point using Student's *t*-test. The associations of CRP concentrations and relative changes in CRP concentrations with outcome (survivors vs. non-survivors) were determined using multiple logistic regression, adjusting for age, weight and sex. Separate models were used for CRP concentrations on admission, at 12, 24 and 36 hours after admission, and for changes in CRP concentration from admission to 12 and 24 hours after admission. The association between CRP concentrations at the above time points and survival time was estimated using Cox proportional hazards regression, adjusting for age, weight and sex. For those dogs that survived, the association between CRP concentrations and length of hospitalisation was estimated in a similar manner. Fit of the logistic regression models was assessed using the Hosmer-Lemeshow goodness-of-fit test and the proportional hazards assumption of the Cox regression models was assessed using Schoenfeld residuals. The clinical value of CRP to predict mortality was evaluated by means of receiver-operating characteristic (ROC) curves, with a true positive defined as a dog that died and a true negative as a dog that survived. Areas under the curve (AUC)

were calculated and compared, and sensitivity, specificity, Youden index (J = sensitivity+specificity–1) and likelihood ratios were calculated for all possible cut-off values. A significance level of P=0.05 was used throughout.

Chapter 4: Results

4.1 Signalment

Eighty-five puppies, infected with parvovirus, were admitted to the trial of which 79 met the inclusion criteria. The puppies were of various breeds with 31 intact females and 48 intact males. Their ages ranged between 1 and 18 months. There was no statistical difference between the age, mass and sex of the puppies that survived compared to those the non-survivors (table 3).

Table 3. Comparison of age, sex and mass between survivors and non-survivors

Variable	Survivors	Non-survivors	*P*-value*
n	63	16	-
Age (months),median [IQR]	4 [2, 6]	5 [3, 6]	0.352
Mass (kg), median [IQR]	5.8 [4.2, 10.4]	8.3 [4.4, 12.3]	0.267
Mass (lb), median [IQR]	12.8 [9.3, 22.9]	18.3 [9.7, 27.1]	0.267
Sex	37 males 26 females	11 males 5 females	0.572

* Age and mass: Wilcoxon rank-sum test: Fisher's exact test

4.2 CRP concentrations

Sixteen puppies suffering from CPV enteritis died (mortality rate of 20%), with a median hospitalisation time of 3 days after admission (range 1 to 6 days) in the non-survivors. The serum CRP concentrations at admission, 12, 24, 36, and 48 hours and then every

24 hours up to 168 hours are shown in Figure 1. Geometric mean CRP concentrations for survivors on admission, 12 and 24 hours after admission were 100.6 mg/L, 81.3 mg/L and 67.6mg/L, and for non-survivors 146.3mg/L, 140.1 mg/L and 116.1 mg/L, respectively.

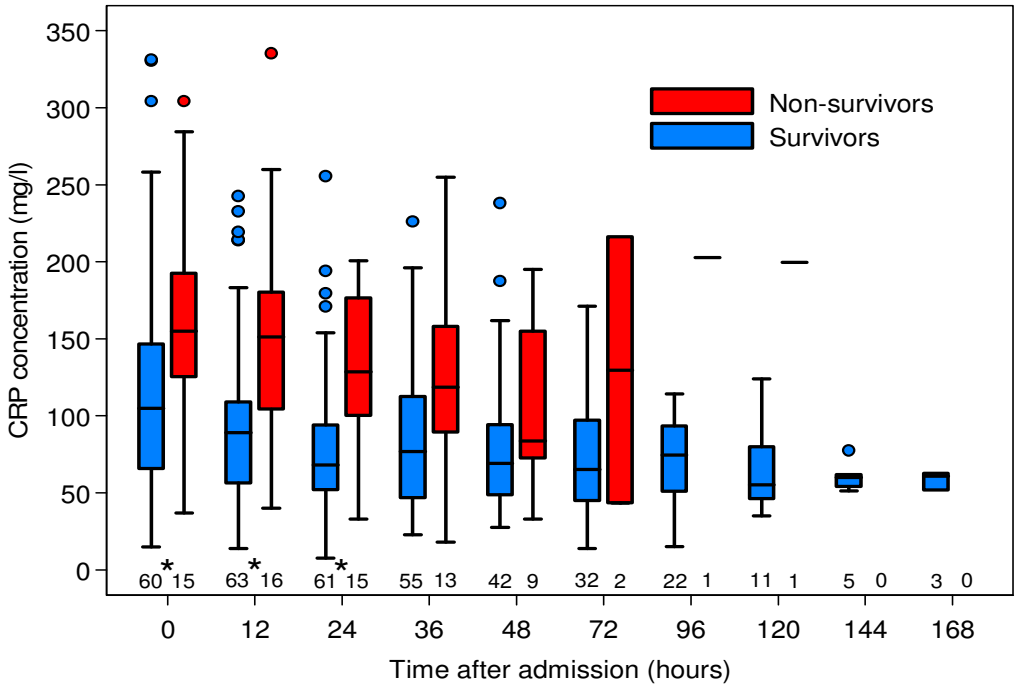

Fig 1. Box plot of CRP concentrations (mg/L) at various time intervals in CPV enteritis. The box incorporates the middle 50% of the observations with the line inside the box as the median. The whiskers extend to the smallest and largest observations that are less than 1.5 times removed from the interquartile range (IQR). Outliers, values that are more than 1.5 times removed from the IQR, are plotted separately as circles. Note: Numbers above the x-axis indicate *n* for each group and an asterisk (*) indicates that the mean CRP concentration was significantly different between survivors and non-survivors ($P< 0.05$).

The results of the multiple logistic regression models are summarized in Table 4. Higher CRP concentrations on admission, 12 and 24 hours after admission were associated with increased odds of mortality (*P*=0.04, *P*=0.005, *P*=0.003, respectively). However, there was no significant association between mortality and change in CRP concentration between admission and 12 hours (*P*=0.33), admission and 24 hours (*P*=0.62) and 12 and 24 hours (*P*=0.99).

Table 4. Summary of results of multiple logistic regression models of association between $\log_2$ CRP concentration and mortality in hospitalised puppies with CPV infection. Each odds ratio (*OR*) estimates the effect of a twofold increase in CRP concentration on the odds of mortality, adjusted for age, weight and sex.

Predictor	Odds ratio	95% CI (*OR*)[xii]	*P*-value
CRP on admission	2.34	1.04, 5.27	0.041
CRP 12 h after admission	4.28	1.57, 11.7	0.005
CRP 24 h after admission	4.93	1.71, 14.2	0.003
CRP 36 h after admission	1.89	0.86, 4.17	0.115
Change in CRP 0 to 12 h	1.78	0.56, 5.64	0.327
Change in CRP 0 to 24 h	1.20	0.59, 2.43	0.619
Change in CRP 12 to 24 h	1.00	0.32, 3.10	0.993

The results of the Cox proportional hazards regression models are shown in Table 5. In the models of survival time, rate of mortality was positively associated with CRP

concentration at 12 hours (*P*=0.002) and 24 hours (*P*=0.001), indicating that higher CRP concentrations were associated with shorter survival times. In the models of hospitalisation time, rate of discharge from the hospital was negatively associated with CRP concentration at 12 hours (*P*=0.012), 24 hours (*P*=0.001) and 36 hours (*P*=0.002), indicating that higher CRP concentrations were associated with lower discharge rate, i.e. longer hospitalisation time.

Table 5. Summary of results of Cox proportional hazards regression models of association between $\log_2$ CRP concentration and survival and hospitalization times in hospitalized puppies with CPV infection. Each hazard ratio (*HR*) estimates the effect of a twofold increase in CRP concentration, adjusted for age, weight and sex.

	Predictor	**Hazard ratio**	**95%CI (*HR*)**[xiii]	***P*-value**
Survival time (event = death)				
	CRP on admission	1.82	0.91, 3.64	0.089
	CRP 12 h after admission	3.61	1.59, 8.20	0.002
	CRP 24 h after admission	4.90	1.88, 12.8	0.001
	CRP 36 h after admission	1.61	0.76, 3.44	0.217
Hospitalisation time (event = discharge)				
	CRP on admission	0.84	0.65, 1.09	0.198
	CRP 12 h after admission	0.63	0.44, 0.90	0.012
	CRP 24 h after admission	0.53	0.36, 0.78	0.001
	CRP 36 h after admission	0.53	0.36, 0.79	0.002

Evaluation of the ROC curves (Fig. 2) showed that there were no statistically significant differences between the AUC of the four curves, however, the model in which CRP concentration had the greatest ability to discriminate between survivors and non-survivors was at 24 hours after admission (AUC=0.79; 95% CI: 0.68, 0.87).

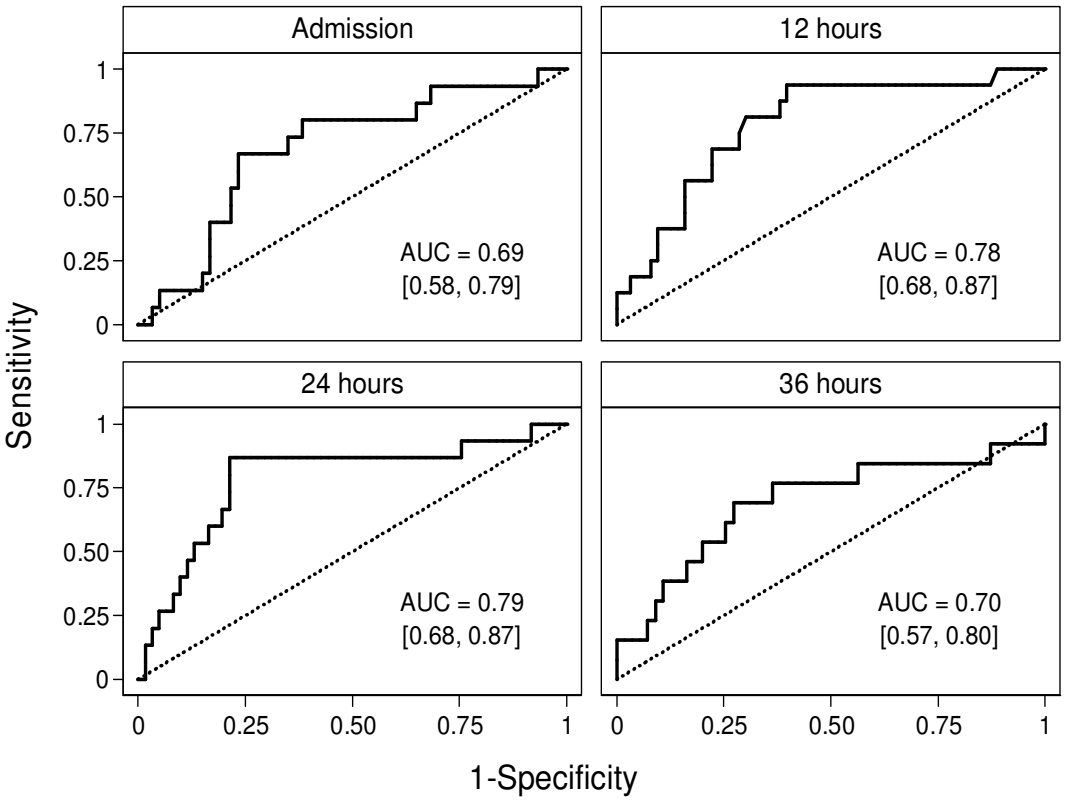

Fig 2. Receiver operating characteristic (ROC) curves for prediction of mortality in puppies with CPV infection using CRP concentration at admission and at 12, 24 and 36 hours after admission. AUC = area under the ROC curve; figures in square brackets are 95% confidence limits for the AUC.

Evaluation of cut-off values for the various time points are shown in Figure 3. Using CRP concentration at 24 hours after admission as a predictor of mortality, the Youden index was maximized at a cut-off of 97.3 mg/L, giving a sensitivity and specificity of 86.7% and 78.7%, respectively; a positive likelihood ratio of 4.1, and positive and negative predictive values of 50% and 96%, respectively (at the 20% mortality rate in this study). Greater specificity could be achieved by using higher cut-off values to reduce false positives: at cut-off values >140 mg/L, specificity was >90%. However, due to the low sensitivity at these cut-offs, there were also very few true positives, therefore positive predictive value remained around 50% and positive likelihood ratio also did not increase. At both 12 and 24 hours after admission, negative predictive value (i.e. prediction of survival) remained greater than 95% for cut-off values up to about 100 mg/L.

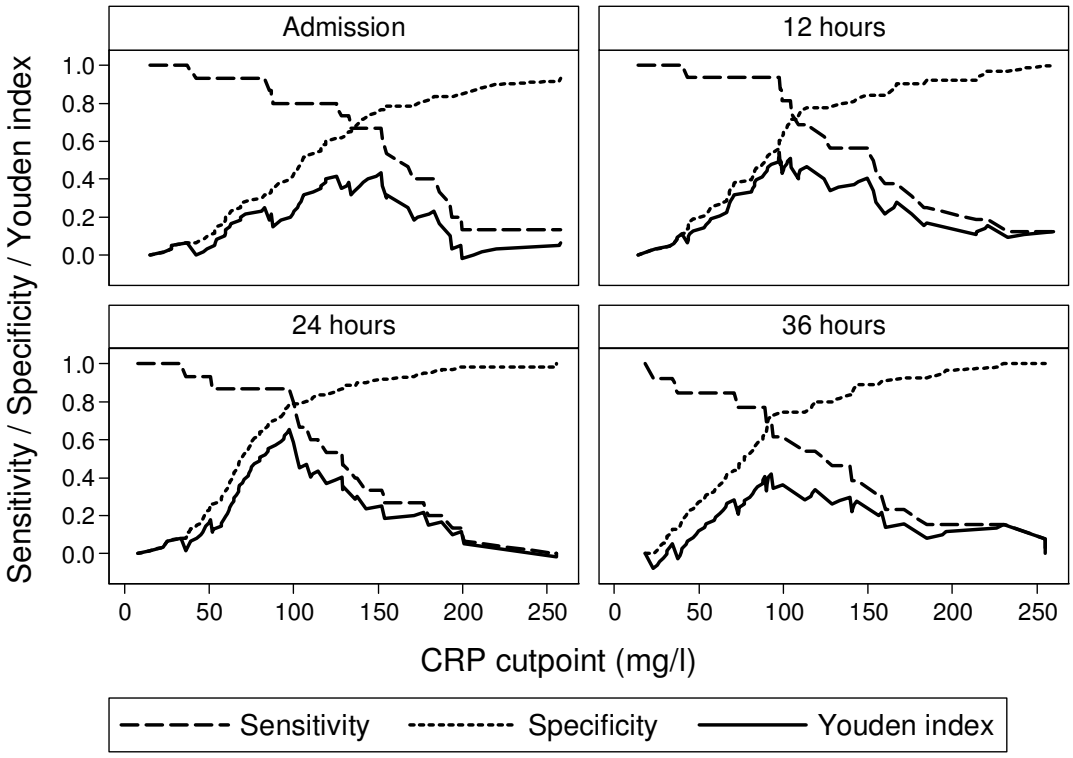

Fig 3. Sensitivity, specificity and Youden index for using CRP concentration at admission and at 12, 24 and 36 hours after admission to predict mortality in puppies with CPV infection. A true positive was defined as a dog that died due to CPV infection.Youden index = sensitivity+specificity–1.

Chapter 5: Discussion

This study demonstrated that higher serum CRP concentrations at 12 and 24 hours after admission were associated with shorter survival times in puppies suffering from CPV enteritis. The results also demonstrated that survivors that had higher serum CRP concentrations at 12 and 24 hours after admission had longer hospitalisation periods. A CRP cut-off value of 97.3 mg/L at 24 hours after admission had a sensitivity of 86.7% and a specificity of 78.7% to predict survival based on the ROC analysis and Youden index. The AUC of the ROC at 24 hours was 0.79, which is regarded as a moderately accurate diagnostic test; a highly accurate test should have an AUC >0.90[74]. A significant association was not found when investigating the change in CRP concentrations between the survivors and non-survivors.

There was a significant association between serum CRP concentrations and survival in CVP infected puppies. At admission, 12 and 24 hours after admission, increased serum CRP concentrations were negatively associated with survival. This is to be expected because the half-life of CRP is constant under all conditions, so that the sole determinant of serum concentration is the synthesis rate which directly reflects the intensity of the pathological process that is causing the production of CRP[62]. The rate of lymphoid and intestinal cell turnover appears to be the main factor determining the severity of the parvovirus infection. The higher the turnover rate the greater the virus replication and cell destruction[9]. The viral infection leads to the stimulation of the immune response which results in the release of cytokines. The cell destruction also results in the release of inflammatory cytokines. All these cytokines are responsible for

the stimulation of the production of CRP from the liver. When the stimulus is removed the circulating CRP concentration decreases rapidly[62]. The most notable feature of CRP is the rapidity of its appearance and increase in the serum following an acute stimulus[67]. The increase in canine CRP concentration is more rapid than in humans. Increased concentrations are first detected 4 hours after a stimulus and increase dramatically to peak at about 24 hours as opposed to CRP concentration in humans where an increase is detected after 6 hours with a peak at around 48 hours[41,62]. Due to the rapid changes in plasma CRP concentration frequent blood collection was indicated for this study. The lack of significance between survivors and non survivors when evaluating changes in CRP over time was surprising because of the short half life of CRP, however a similar finding was found in a study evaluating CRP in canine babesiosis[75]. This could be due to the severe tissue damage and systemic inflammation present in CPV enteritis that may persist for an extended period.

The prognostic value of serum APP was evaluated in dogs with CPV at admission in a different study[34]. Serum CRP, ceruloplasmin and haptoglobin concentrations were reported to be higher in dogs with CPV than control dogs but the magnitude of the increase in CRP concentration was higher than that for the other APPs. CRP concentrations at admission were higher in puppies that died compared to those that survived[34]. These findings correlate with the results of our study. The above mentioned study also reported that an admission CRP value of 92.4mg/L had a sensitivity and specificity of 91% and 61% respectively to differentiate survivors from non survivors[34]. This differs from the results of our study where a similar cut of value of 97.3 mg/L for CRP was determined, but at the 24 hour interval rather than at admission with a

sensitivity and specificity of 86.7 and 78.7% respectively. The difference could be due to the fact that the first study measured CRP on admission only.

Various studies have evaluated other parameters that can be used as predictors of outcome in CPV infected puppies. One study reported that an accurate prognosis could be obtained for puppies suffering from CPV enteritis at 24 hours after admission by evaluating blood leukocyte changes[28]. Another study reported that CPV infected puppies that were lymphopenic and hypoalbuminaemic at admission were hospitalised for longer periods compared to puppies without these abnormalities[20]. Endocrine predictors of mortality in puppies suffering from CPV enteritis, including serum cortisol and thyroxine concentrations, found that high serum cortisol and low thyroxine concentrations at 24 and 48 hours after admission were associated with death[27]. Yilmaz and Senturk found that serum total cholesterol and high-density lipoprotein cholesterol levels decreased in puppies infected with CPV and these parameters could be used as an index of the severity of the CPV enteritis[76]. Measuring CRP concentration has some advantages over these parameters. In response to inflammation, CRP increases more rapidly compared to changes in white blood cell counts and because CRP is not subjected to bone marrow response or fluctuations due to extravasation, it is a more stable parameter to monitor throughout the disease process[45]. Cortisol and thyroxine can be more expensive and difficult to measure compared to CRP, depending on the laboratory facilities available. However, despite the significant negative association between increased serum CRP concentrations and survival in this study, the ROC analysis demonstrated that the discriminative ability of CRP alone in predicting survival was only moderately accurate. This is in accordance with several other clinical studies

where CRP as a standalone parameter does not correlate to outcome[42,51,77-79]. A limitation of the present study was that the investigators had no control over when in the course of the disease process puppies were brought to the clinic. This could have affected the CRP results at admission as well as patient outcome. Modifications of the standard supportive treatment by different attending clinicians may have had an effect on the results, but this is unlikely as the treatment is not agent specific and does not affect the pathogenesis of the disease.

Chapter 6: Conclusion

Prognostication remains a challenging topic. In the past 20 years, much research has been done to find ways of improving the precision and accuracy of clinicians' estimates, but we are still not able to propose any of the existing tools as the ideal one to be recommended for widespread use[77] therefore using CRP alone to make decisions about therapy or euthanasia is not practical, but serial measurements of serum CRP may prove useful to monitor the progression of the disease and adapt treatment accordingly. Future studies are warranted to evaluate serial CRP in conjunction with other parameters proven to be of value in the prognostication in CPV. These parameters could include leukocyte count, serum biochemistry and endocrine profiles.

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APPENDICES

University of Pretoria

APPENDIX A

Consent form for Parvovirus enteritis CRP trial
Approved protocol number:……..
Case number:……
Client number:……..

I,(Full name)……
Hereby give permission for the dog under my care, (Dogs name)……..
(Breed)…….(colour)……
(Sex)…….(age)……
to participate in the clinical study on C-reactive proteins in parvovirus at the Onderstepoort Veterinary Academic Hospital.

The trial has been explained to me and I understand that the study will in no way harm my dog and that the costs of the additional test will be borne by the trial fund. I will only be liable for costs pertaining to the treatment that would in any event be required by my dog.

Signed at: Onderstepoort on the …….day of
(month)……(year)……

Signature owner/ authorised person…….
Home tel:……..
Cell:……..
Work tel:……...

Thank you for allowing your pet to be entered into this study.

University of Pretoria

Toestemmingsvorm vir parvovirus enteritis CRP studie

Protokol nommer:…….
Geval nommer:…….
Klient nommer:……

Ek,(Volle naam en van) ……
gee hiermee toestemming dat my hond (naam)……..……..
(ras)…….(kleur)……..……
(geslag)……(ouderdom)……..

mag deelneem aan die studie op C-reaktiewe proteïne in parvovirus, by die Onderstepoort Veterinêre Akademiese Hospitaal.

Die doel van die projek is aan my verduidelik, en ek verstaan dat die studie nie my hond op enige manier skade sal aandoen nie, en die koste van die addisionele toetse deur die projekfonds gedra sal word. Ek verstaan dat ek aanspreeklik is vir die koste van standaard behandeling en diagnostiese toetse wat in elk geval deur my hond benodig sou word.

Geteken te Onderstepoort op die …….dag van
(maand)……(jaar)……

Handtekening van eienaar / gemagtigde persoon……..
Huis tel:……..
Sel:……..
Werk tel:……..

Dankie vir u bereidwilligheid om u dier te laat deelneem aan hierdie studie.

University of Pretoria

APPENDIX B

Client information sheet

From the history, clinical examination and tests done to date, it seems that your dog has contracted an infectious viral disease called canine parvovirus or "cat flu". This virus causes severe damage to the intestinal tract resulting in intestinal bleeding, decreased food absorption, vomiting, diarrhoea and fever. The virus also causes suppression of the bone marrow, which leads to a decrease in white blood cells and in turn decreases the dog's ability to fight infections. Unfortunately there is no antivirus treatment that exists at this time so we have to treat the dogs symptomatically.

Your dog has been admitted to the isolation unit at the Onderstepoort Veterinary Academic Hospital where he/she will receive intensive treatment. Your dog will receive intravenous fluid (drips), antibiotics, medication to control the nausea and vomiting, deworming and other treatments such as blood or plasma transfusions that may be needed.

We are conducting a study, which measures the levels of an inflammatory protein called C-reactive protein in blood of dogs affected with parvo enteritis in order to monitor the progression of this disease. This may enable us to predict the outcome of the disease more accurately and thus make treatment more cost effective.

This trial will not harm your pet in any way as he/she will still receive the same treatment were he/she not involved in the trial. You will also not endure any extra costs for the trial, you will only be liable for the usual cost of treatment as discussed with you by the admitting clinician. This study has been approved by the Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for allowing your pet to be included in our study. If you have any further questions you can contact me on (012) 529-8196 or 083 232 4002 (cell)

Dr Vanessa McClure (BVSc)
Department of Companion Animal Clinical Studies

University of Pretoria

Kliente Informasie vorm

Vanuit die geskiedenis, kliniese ondersoek en laboratorium toetse sover uitgevoer op u hondjie, blyk dit asof u hondjie aan ‘n virusinfeksie lei, genaamd honde parvovirus of die sogenaamde “katgriep” virus. Hierdie virus veroorsaak skade aan die dermkanaal wat lei tot dermbloeding, swak absorpsie van voedingstowwe, braking, diarree en koors. Dit veroorsaak ook ander probleme soos ‘n verlaging in die witseltelling as gevolg van beenmurgonderdrukking wat veroorsaak dat die dier nie infeksies doeltreffend kan beveg nie. Ongelukkig is daar tans geen antivirus behandeling nie en moet ons die diere simptomaties behandel.

U hondjie word opgeneem na die isolasie-eenheid by die Onderstepoort Veterinêre Akademiese hospital waar hy/sy intensiewe behandeling sal ontvang. U hondjie sal behandel word met binne-aarse vloeistowwe (drips), antibiotika, middels wat naarheid en braking onderdruk, ontwurming, en indien nodig, bloed- of plasma oortapping.

In hierdie studie wil ons die vlakke van ‘n inflammatoriese proteïn C-reaktiewe proteïn meet in die bloed van honde met katgriep om die verloop van die siekte beter te kan monitor. Dit mag ons in staat te stel om ‘n meer akkurate voorspelling van die uitkoms te maak en dus behandeling ook meer koste effektief te maak.

Die studie sal u dier geen skade aandoen nie en hy/sy sal steeds dieselfde behandeling ontvang sou hy/sy nie in die studie ingesluit wees nie. U sal nie verantwoordelik gehou word vir die koste van die addisionele bloedtoetse nie. U sal slegs vir die koste van behandeling verantwoordelik wees soos met u bespreek is deur die dokter aan diens. Hierdie studie is goedgekeur deur die Etiese komittee van die Fakulteit Veeartsenykunde, Universiteit van Pretoria.

Dankie dat u toelaat dat u hond ingesluit kan word in die studie. Indien u enige verdere navrae het kan u gerus die klinikus aan diens vra of myself kontak [Tel: (012) 529 8196(w) of 083 232 4002 (cell)]

Dr Vanessa McClure BVSc
Department van Geselskapdiere Kliniese Studies

APPENDIX C
Clinical Examination

Vaccination status ..... Case Number: .....

Date of Admission:..... Date of examination:.....

Number of days depressed	1	2	3	>3
Number of days anorectic	1	2	3	>3
Number of days vomiting	1	2	3	>3
Vomiting episodes per day	1	2	3	>3
Description of vomitus				
Number of days diarrhoea	1	2	3	>3
Diarrhoea episodes per day	1	2	3	>3
Description of diarrhoea				
Mentation status	1+	2+	3+	4+
% Dehydrated	0-5%	5%	10%	>10%
Mucosae	Moist		dry	
	Pale	pink	congested	
Oral ulcerations	Yes		no	
Temperature				
Capillary refill time				
Pulse rate				
Pulse quality	Weak	strong	waterhammer	
Pulse rhythm	Regular		irregular	
Respiratory rate				
Depth of respiration	Normal	laboured	shallow	
Abnormal lung sounds	Yes		no	
If yes, describe				
Abdominal palpation	Tense		easily palpable	
	Painful		not painful	
	thickened gut loops		fluid filled gut loops	
	gas in intestines		intussussception	
Blood smear	Parasites		leukopaenia	
	thrombocytopaenia		reticulocytes present	
Faecal appearance				
Faecal flotation				
Faecal wet preparation				
Faecal smear	Leukocytes	erythrocytes	protozoa	
	Spirochaetes	fungi	normal flora	
Hematocrit				
TPP				
Blood glucose				
Serum potassium				

Appendix D

Daily monitoring

Patient number:

Date:

Day number: 0 (admission), 1,2,3,4,5,6,7 or 8. (Encircle choice)

Temp: Pulse: Resp: Weight:

Encircle the applicable choice under 1 – 8 below:

1) Habitus	1	Collapsed / moribund
	2	Severe depression
	3	Mild-to-moderate depression
	4	Normal
2) Appetite:	1	No interest in food
	2	Voluntarily eats small amounts of food offered
	3	Voluntarily eats moderate amounts of food offered (but not normal)
	4	Normal
3) Vomiting:	1	Severe (≥ 6 times per 12h)
	2	Moderate (3-5 times per 12h)
	3	Mild (1-2 times per 12h)
	4	Absent
4) Faecal consistency:	1	Watery diarrhoea, bloody
	2	Watery diarrhoea, not bloody
	3	Soft
	4	Well-formed
7) Mucous membranes	1	Congested
	2	Pale
	3	Normal
8) Capillary Refill Time	1	> 2 seconds
	2	< 1 second
	3	1-2 seconds

APPENDIX E

Whole blood transfusion therapy – volume to be transfused

Whole blood transfusion therapy will be performed if the patient's Ht<15%, and the amount to be transfused calculated by the following formula (KRISTENSEN, A.T., FELDMAN, B.F.1995: Blood banking and transfusion medicine, in (eds): *Textbook of Veterinary Internal Medicine,* edited by S.J Ettinger &B.F Feldman. Philadelphia: W.B. Saunders,1:347-360.)[A value of 25% will be taken for the Ht (desired).]

$$\text{Volume (ml) blood needed} = \frac{\text{Ht (desired)} - \text{Ht (patient)}}{\text{Ht (donor)}} \times 90 \times \text{Body weight (kg)}$$

Plasma volume to be transfused

Plasma transfused at 10ml/kg. (HOHENHAUS AE, 2005: Blood transfustions, component therapy and oxygen carrying solutions in *Textbook of Veterinary Internal Medicine 6th ed, Ch 127,* edited by SJ Ettinger and EC Feldman. Missouri. Elsevier Saunders, 1: 464 – 468)

APPENDIX F

Patient out come

Patient number:……

Client number:…….

EM results: Parvo:…..……..

Corona:…….

Distemper: ……

Died/ Euthanased / Recovered:…….

Days to recovery / death:……..

Complications? (Describe):……..

……..

……..

Appendix G Raw data

Puppy No	Outcome	Days to outcome	Em result	Complications	Age (months)	Weight (kg)	Sex
P1	R	5	POS		1	6.2	f
P2	R	5	POS		1	6.2	m
P3	R	5	POS		1	6	m
P4	R	4	POS		5	4.2	f
P5	R	8	NEG		6	4.2	f
P6	R	5	POS		3	9.6	f
P7	R	5	POS		4	12.6	m
P8	R	12	NEG	DISCO?	2	5	m
P9	R	3	POS		2	4.2	m
P10	R	2	POS		6	4	f
P11	R	4	POS		5	4.4	f
P12	R	6	POS		4	14.2	m
P13	R	4	POS		4	11.4	m
P14	R	3	POS		2	3.2	m
P15	R	3	POS		2	3.6	m
P16	R	2	POS		6	5.2	m
P17	E	7	POS	POLYATHRITIS?	2	6.4	f
P18	R	5	POS		14	10.7	m
P19	E	5	NEG		2	4.6	m
P20	D	3	POS		3	5.8	m
P21	E	2	POS		6	5.4	m
P22	E	4	POS		5	19.6	m
P23	R	8	POS		3	8	m
P24	R	2	POS		6	4.8	m
P25	R	5	POS		3	10.2	m
P26	R	7	POS		4	11.6	f
P27	E	4	NEG		3	4	m
P28	R	11	POS		3	11.6	m
P29	R	7	POS		2	10.4	f
P30	R	12	POS		6	11.4	f
P31	R	7	POS		1	3.6	m
P32	R	4	POS		2	3.4	m
P33	D	2	POS		2	3.7	m
P34	R	6	POS		4	19	m
P35	R	9	POS		5	14.2	m
P36	R	11	POS		2	3	m
P37	E	2	POS		3	4.2	f
P38	R	6	POS		11	7.4	f
P39	R	6	POS		3	4	f
P40	R	3	POS		12	5.8	f
P41	E	2	POS		5	10	f

P42	E	1	POS		12	10.8	f
P43	R	5	POS		4	4.6	f
P44	R	5	POS		6	4.8	m
P45	R	5	POS		4	4.2	m
P46	D	6	POS		18	16	m
P47	R	2	POS		5	4.4	f
P48	R	5	POS		2	3.4	m
P49	R	2	POS		2	8.8	m
P50	R		NEG				
P51	R	4	POS		7	4	m
P52	R	6	POS		18	6.2	m
P53	R	3	POS		2	4	f
P54	R	2	POS		5	12.8	f
P55	R	4	POS		4	4	f
P56	R	4	POS		1	5.2	m
P57	R	12	POS	Interssuseption	1	5.4	f
P58	R	5	POS		1	4.8	m
P59	R	7	POS		1	5	f
P60	R	4	POS		1	5.4	f
P61	R	1	NEG		2	7	m
P62	R	9	POS		2	6	f
P63	R	10	POS		2	8.4	m
P64	R	9	POS		2	11.2	f
P65	D	5	POS		2	13.2	f
P66	D	4	NEG	Snap Pos	3	8.6	m
P67	D	3	NEG	PM = PARVO	6	18.2	m
P68	R	2	POS		6	15.6	m
P69	R	3	POS		5	7.8	m
P70	E	2	POS		7	8	m
P71	R	4	POS		6	5.2	m
P72	E	2	POS		5	11.4	f
P73	D	4	POS		5	3.6	m
P74	R	2	POS		14	7.4	m
P75	R	1	POS		4	9.6	f
P76	R	4	POS		4	8	m
P77	R	3	POS		6	14.8	m
P78	R	3	POS		6	17.6	f
P79	R	3	POS		6	17.2	f
P80	R	3	POS		4	4	f
P81	R	4	POS		5	4.5	m
P82	R	6	POS		3	4.2	f
P83	E	5	POS	Intussuseption	4	3.4	m
P84	R	2	POS		4	3.4	m
P85	R	3	POS		9	4.1	m

R = recovered, E = Euthanased, D = Died.

Patient No	CRP admission	CRP 12h	CRP 24h	CRP 36h	CRP 48h	CRP 72h	CRP 96h	CRP 120h	CRP 144h	CRP 168h	CRP 192h	CRP 216h
P1	81.0	54.4	57.4	77.0	75.6	69.4	87.3					
P2	141.2	87.3	92.4	60.1	45.7	27.4						
P3	131.3	97.2	94.1	43.1	41.6	29.1						
P4	87.6	57.2	39.1	32.9	31.0							
P5	133.9	144.4	75.2		73.4	57.8	76.8	55.3	77.6	62.8		
P6	100.2	141.8	136.5	83.1	69.8	52.9						
P7	173.3	162.3	153.9	129.0	133.8	105.4	114.2					
P8	68.7	67.1	60.1	74.9	113.4	101.9	91.8	86.3	94.9	100.1		
P9	48.9	32.6	22.9	25.5	27.6	13.9	15.1					
P10	27.6	13.9	15.1									
P11	219.7	102.6	84.8	49.9								
P12	102.4	70.2	45.2	26.6	36.0							
P13	86.7	36.9	46.5	46.7								
P14	62.0	56.4	46.5	73.8								
P15	59.7	43.3	38.0	29.2								
P16	71.3	44.3	25.4									
P17	109.8	53.7	63.0	96.7	83.7	59.5	66.2	65.7				
P18	116.0	104.0	67.3	53.4	127.0	167.6	104.6	78.1				
P19	152.6	97.8	100.0	99.5	83.5							
P20	132.8	155.0	107.4	70.8	40.4							
P21	192.6	184.9	199.0	171.6	155.0							
P22	86.8	104.0	114.4	139.4	131.9	43.4						
P23	54.5	70.8	94.7	116.2	79.0	53.3	43.8	46.2	54.0	51.8	31.5	32.3
P24	103.1	90.6	61.7	62.4								
P25	92.4	127.6	83.1	141.1	29.7	35.7						
P26	193.6	213.9	179.6	160.5	112.9	90.5	101.8					
P27	186.4	104.6	97.3	92.9	72.5							
P28		89.2	67.2	82.0	68.6	51.8	58.3	63.2	61.8			
P29	135.4	66.2	63.3	141.8	97.2	97.9	45.0	51.8				
P30	211.4	242.6	255.5	226.0	187.4	129.3	87.2	35.1	60.1	60.9	85.2	71.8
P31	62.9	67.9	73.0	73.4	53.0	50.6	55.5					
P32	139.2	109.0	62.2	37.4								
P33	82.7	98.6	50.7									
P34		83.2	61.0	64.4	48.9	135.4	89.0	97.6				
P35	118.9	84.0	84.9	184.7	238.1	143.7	103.8	124.0				
P36	87.6	68.7	57.3	65.9	49.7							
P37	151.9	167.1	176.7									
P38	330.4	214.3	96.0	194.0	161.9	88.3	109.9	80.0				
P39	144.0	165.3	80.0	87.8	60.6	93.5	63.5	52.9				
P40	106.1	62.3	42.7	40.2								
P41	182.9	149.6	137.6	118.6								
P42	155.0	153.0										
P43	98.1	81.0	74.7	93.9	79.2							

P44	27.8	35.0	73.4	90.3	112.6	113.7						
P45	46.6	53.2	49.8	73.6	76.2							
P46	167.4	113.9	101.3	158.0	195.0	216.2	202.9	199.7				
P47		67.8	47.8	31.3								
P48	154.9	100.0		91.3	78.7	68.8						
P49	99.5	82.9	64.7									
P50	Don't do CRP											
P51	257.1	97.9	66.2	39.0	35.3	31.8						
P52	135.4	97.1	76.8	39.7	30.5							
P53	68.5	46.6	52.2	31.1								
P54	258.2	232.7	119.4	84.6	48.0							
P55	180.2	159.9	103.4	80.0	75.6	65.4						
P56	105.2	92.8	70.5	59.2	56.4	32.5	39.3					
P57	98.8	96.2	87.2	90.8	62.8	56.4	67.6	65.2	69.4	46.8	60.7	
P58	111.7	109.0	64.4	76.8	56.9	39.2	93.4					
P59	128.1	109.9	89.4	90.0	68.6	56.9	60.4	41.5	51.3			
P60	118.8	99.3	79.8	87.9	48.6	64.7	51.0					
P61	No CRP's											
P62	14.9	37.2	109.8	112.6	93.1	96.9	73.6					
P63	331.2	108.8	68	156.4	127.4	74.0	45.7					
P64	116.1	82.0	59.5	48.4	142.2	171.2	75.7					
P65	198.9	175.9	128.5	254.8	194.7							
P66	37.0	40.2	33.2	18.1	83.7							
P67	284.5	335.2	152.2	89.3								
P68	42.4	40.2	23.9	22.9								
P69	53.0	43.7	36.2	42.5								
P70		259.9	187.6	230.3								
P71	59.5	43.5	36.2	66.5	64.7							
P72	304.3	220.8	200.7	34.4								
P73	125.3	124.1	129.3	143.5	33.0							
P74	68.6	24.4	51.9									
P75	104.4	66.7	58.3									
P76	206.6	219.3	111.1	90.7	41.7							
P77	85.8	132.7	171.1	133.3								
P78	53.5	100.3	194	140.7								
P79	23.5	18.1	7.8	No reading								
P80	149.1	102.1	69.7	54.4	71.0	56.9						
P81	59.3	97.6	128.9	117.1	50.6	32.3						
P82	199.5	90.2		90.0	94.2							
P83	195.8	96.3	74.7	75.9	98.6	40.8	36.7					
P84	171.2	183.2	124.6									
P85	304.2	165.3	142.9	196.1								

[i] Antigen, Animal genetics, Inc Korea

[ii] Kjelgaard-Hansen M, Strom H, Mikkelsen LF, et al. Grading of surgical trauma by means of canine C-reactive protein measurements. Vet ClinPathol 2008;37:6 (abstract)

[iii] Precision Glide™, UK

[iv] Beckton Dickinson Vacutainer Systems, UK

[v] Kyron

[vi] Ascensia Elite™XL, Bayer

[vii] Reflovet®Plus (Roche).

[viii] Alpha Wassermann, Woerden,NL

[ix] Ace Alera Orb diagnostics, South Africa

[x] Life Diagnostics, West Chester, PA,USA

[xi] StataCorp, College Station, TX, USA

[xii] Confidence interval for the estimate of the odds ratio

[xiii] Confidence interval for the estimate of the hazard ratio