Circulating markers of endothelial activation in canine parvoviral enteritis

By

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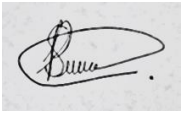
Signature of student: .....  ……

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ETHICS STATEMENT

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval (V092-15).

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

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Research Ethics Committee

PROJECT TITLE	**Markers of endothelial injury and inflammation in canine parvoviral enteritis.**
PROJECT NUMBER	**REC089-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Sune Pretorius**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Paolo Pazzi**

APPROVED	Date **06 November 2018**
CHAIRMAN: UP Research Ethics Committee	Signature

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Animal Ethics Committee

PROJECT TITLE	**Markers of endothelial injury and inflammation in caning parvo viral enteritis**
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RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. S Pretorius**

STUDENT NUMBER (where applicable)	**U_10348001**
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ANIMAL SPECIES	**Client owned domestic dogs**
NUMBER OF SAMPLES	**55**
Approval period to use animals for research/testing purposes	**October 2018 – October 2019**
SUPERVISOR	**Dr. P Pazzi**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 October 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

For my son Adam-Daniel Barnard

‘Remember to look up at the stars,

and not down at your feet.

Try to make sense of what you see

and wonder what makes the universe exist.

Be curious.’

-Steven Hawking

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My loving husband, family and son who has supported and encouraged me all the way.

List of abbreviations

CAMs	Cell adhesion molecules
CBC	Complete blood count
CPV	Canine parvovirus
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
HGF	Hepatocyte growth factor
HMGB-1	High mobility group box-1 protein
HRP	Horseradish Peroxidase
ICAM-1	Intercellular adhesive molecule-1
IL-1 β	Interleukin-1 Beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IQR	Interquartile range
Nf- κB	Nuclear factor- κB
OVAH	Onderstepoort Veterinary Academic Hospital
SIRS	Systemic inflammatory response syndrome
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular adhesive molecule-1

Figures and Tables

Summary

Canine parvovirus (CPV) is a debilitating disease affecting young wild and domestic canines. Canine parvovirus attacks rapidly dividing cells including intestinal epithelium, lymphoid tissue and bone marrow. The most common clinical manifestations of CPV infection are severe gastrointestinal signs and immunosuppression. During CPV, endotoxins are released from the compromised gastrointestinal tract. Increased inflammatory cytokines have been described in previous studies. The endotoxins, raised inflammatory cytokines and the severe immunosuppression stimulates the systemic inflammatory response causing widespread peripheral vasodilation, capillary permeability is increased, cardiac function depression and the coagulation cascade is activated. The state of the endothelium in CPV has not been investigated.

Markers of endothelial activation including intercellular adhesive molecule-1 (ICAM-1), vascular adhesive molecule-1 (VCAM-1) and high mobility group box-1 protein (HMGB-1) give insight into the state of vascular endothelium during disease. These markers of endothelial activation have been investigated in inflammatory diseases in humans and animals.

In this study, we aimed to investigate the concentration of circulating markers of endothelial activation to gain insight into the state of vascular endothelium during CPV. Thirty dogs naturally infected with CPV were used in the study and compared to ten age-matched control dogs.

A significant lower median value for ICAM-1 was found in dogs with CPV compared to control dogs. No significant difference was seen for VCAM-1 or HMGB-1. This indicates that despite the systemic inflammation and raised cytokine levels seen in dogs with CPV there seems to be a lack of endothelial activation based on the circulating levels of the markers of endothelial activation.

The significance of these findings warrants further investigation.

CHAPTER 1

Literature review

1.1. Canine parvoviral enteritis

Canine parvovirus (CPV) is characterised by a small non-enveloped, single-stranded deoxyribonucleic acid (DNA) virus and is an important cause of disease in young domestic and wild canines.[1] In 1967 canine parvovirus type-1 was first described as being responsible for disease causing gastrointestinal and respiratory signs in dogs.[2] In 1978 a new strain of parvovirus was recognized and named CPV-2.[3] Mutations of the virus have led to the recognition of CPV-2a and CPV-2b of which these two are the most common species of parvovirus worldwide.[4,5] In 2000 CPV-2c was reported in Italy.[6] The disease has a pronounced seasonality with most cases in South Africa occurring during the summer months.[7,8]

A lack of protective immunity, unsanitary and overcrowded environments, diet changes, weaning and enteric parasites are factors that predisposes puppies to parvoviral infection.[9,10] Additionally, certain breeds like Rottweilers, Doberman Pinschers, American pit bull terriers, Labrador retrievers, German shepherds, Staffordshire terriers and Alaskan sled dogs are more prone to develop severe CPV enteritis.[8,9] The reason for the increased susceptibility in some breeds is not known but there seems to be an increase in susceptibility in pure breed dogs compared to their cross bred counterparts.[11]

Transmission occurs directly via the faecal-oral route or indirectly via faecal-contaminated fomites.[1,8,12]

In the first two days of infection the virus replication occurs in the oropharynx and local lymphoid tissue.[3] By day three to five marked viraemia is present.[3] Canine parvovirus replicates primarily in rapidly dividing tissues including intestinal epithelium, lymphoid tissue and bone marrow. The viral replication, cell destruction and thus the severity of the lesions and clinical disease is determined by the rate at which the cells divide.[13,14] Lymphopenia and in some cases panleukopenia, is the result of the destruction of lymphoblasts in lymphatic tissue and myeloblasts in bone marrow.[13,12]

Replication of the virus occurs in the crypt cells of epithelium layer in the intestinal tract and results in epithelial necrosis, villus atrophy and collapse of the epithelium. The end result being haemorrhagic diarrhoea and vomiting due to the loss of absorptive capacity of the epithelium.[7,15]

Not all dogs are equally affected and disease severity depends on age, immune status, breed, viral dose and route of infection.[3]

Severe gastrointestinal signs and immunosuppression is the most common clinical manifestations of CPV infection. Initially dogs with parvoviral infection show non-specific clinical signs like depression, lethargy and pyrexia that progresses to vomiting and mucoid to haemorrhagic diarrhoea, marked by abdominal pain which is caused by the acute gastroenteritis.[5,10] A clinical scoring system has been developed to

describe the state of the patient at admission. The clinical score used in this study was adapted from previous studies and included the following parameters; habitus, appetite, vomiting, faecal consistency, mucous membranes colour, capillary refill time, total white cell count, lymphocyte count, C-reactive protein (CRP) and albumin. [3,16,27] Anaemia develops due to gastrointestinal haemorrhage.[8,18] Dehydration and hypovolaemic shock often develop due to the large amount of fluid loss through the gastrointestinal tract. Dogs present with clinical signs that include depression, cool extremities, hypotension, prolonged capillary refill time, tachycardia, poor pulse quality and hypothermia.[9] Dogs may also suffer from lymphopenia and leukopenia due to the destruction of bone marrow precursor cells and lymphatic tissue.[13,16,17]

Hypoalbuminaemia and hypogammaglobulinaemia develop as a result of intestinal haemorrhage.[20] Due to the destruction of the gastrointestinal epithelium, dogs with CPV have an increased risk of bacterial translocation and subsequent coliform septicaemia. Systemic inflammatory response syndrome (SIRS) can develop due to septicaemia which can progress to septic shock and ultimately death.[9,21]

Endotoxins released from the compromised gastrointestinal tract, inflammatory cytokines and the severe immunosuppression, stimulates the systemic inflammatory response causing an increase in capillary permeability, widespread peripheral vasodilation, depressed cardiac function and activation of the coagulation cascade.[20,21]

Dogs suffering from CPV are critically ill and the adrenal gland responds accordingly, thus they have a significantly in increase of cortisol levels when compared to healthy control dogs.[22,23] The cortisol levels in dogs suffering from CPV have positive correlation with SIRS and mortality rate in CPV.[22]

Currently there is no published research on the circulating markers of endothelial activation in CPV enteritis. There is a rise in inflammatory cytokines and evidence of systemic inflammation in CPV and we suspect that the concentration of markers of endothelial activation could shed light into the disease proses.[9,21] We do know that dogs suffering from CPV are severely immunocompromised and further investigation into the concentrations of circulating markers of endothelial activation could help explain the disease processes.[19,22] Low concentration of circulating markers of endothelial activation would explain poor transmigration of leukocytes especially neutrophils and lymphocytes in the CVP infected dog.

Canine parvovirus can be suspected in unvaccinated or incompletely vaccinated dogs that show signs associated with parvovirus, including vomiting, haemorrhagic diarrhoea, lethargy, pyrexia and dehydration.[13] Definitive diagnosis can be made with the faeces of infected dogs using one of the following tests; detection of CPV using electron microscopy (EM), faecal enzyme-linked immunosorbent

assay (ELISA), isolation of the virus, haemagglutination of faeces, latex agglutination, counter immunoelectrophoresis, polymerase chain reaction (PCR) and immunochromatography.[9,15,24,25] Blood serology from symptomatic dogs and necropsy with histopathology typical for CPV can also be used.[9,15,24,25] Virus isolation in animal cell culture and faecal haemagglutination is considered the golden standard for the diagnosis.[26]

Faecal antigen ELISA tests are used for diagnosis of clinical cases of acute CPV.[8] However, false positive results can occur if the test is done 3–10 days after the vaccination with a modified live CPV vaccine. False negative results can occur due to the test antigen binding with serum-neutralising antibodies in the diarrhoea or when the virus is no longer being shed through the faeces.[9,10,13]

Currently there is no antigen-specific treatment for dogs with CPV. Treatment is supportive and symptomatic. The survival rate is very low for untreated dogs at 9% but can be as high as 90% in dogs hospitalised in facilities that provide ongoing and intensive care.[3]

It remains essential to develop a therapy at lowest cost possible that decreases the severity of the clinical signs and thus the need for hospitalization and improve survival rates.[27] This study will broaden our knowledge of the effect of circulating markers of endothelial activation and the subsequent transmigration of leukocytes in the disease process of CPV.

1.1.2 Markers of endothelial damage

Vascular endothelium lines all blood vessels from the heart to the smallest capillaries.[28] Vascular endothelium is extremely adaptable and has the ability to adjust cell numbers and cell arrangements to meet the local requirements.[28]

Vascular endothelium is a complex organ that plays a pivotal role in haemostasis. It reacts to various environmental stimuli by remaining in a constant balance between activation and inactivation.[29] Endothelial cells are important in maintaining haemostasis, fibrinolysis and regulating vascular tone as well as providing oxygen and nutrients to surrounding tissue.[30,31] Endothelium is also integral to the inflammatory response, and inflammation results in endothelial activation through regulating inflammatory cells.[30,31]

Systemic inflammation

Inflammation involves a sequence of biochemical and cellular changes.[32,33] The mechanism of inflammation consists of vasodilation, exudation, emigration of cells and chemotaxis.[32] The most profound expression of inflammation is the hike in body temperature and the acute-phase reaction which stimulates the liver to produce acute-phase proteins including CRP.[32] At the location of inflammation increased blood flow and an influx and accumulation of effector cells are seen. Initially an influx of non-specific phagocytic neutrophils are seen.[32] This is followed by an influx of macrophages, monocytes and T and B lymphocytes. These cells perform a regulated antigen specific response and subsequently produce specific inflammatory cytokines.[32] The inflammatory cells are also responsible for the production of cell adhesion molecules (CAMs).[32] During the early stages of inflammation endothelial cells are stimulated to express adhesion molecule receptors which facilitate transendothelial migration of circulating leukocytes.

When diagnosing a dog with SIRS a comprehensive diagnostic approach is followed. First the body temperature, heart rate and respiratory rate is measured and compared to the SIRS criteria.[33] This is followed by a complete blood count, biochemistry and a coagulation test. Common findings in the haemogram include anaemia, lymphopenia, leukopenia and thrombocytopenia.[33] Biochemical abnormalities usually include hypoalbuminemia, hypoglycaemia and hypocalcaemia.

Dogs suffering from CPV are predisposed to developing SIRS and sepsis. The increased risk to develop sepsis is due to intestinal cellular destruction and disruption to the gastrointestinal mucosal barrier which predisposes the dog to bacterial translocation and subsequent bacteraemia.[33] These dogs have impaired immunity that increases the risk of secondary infections.[33] Dogs with CPV have marked leukopenia which further increases the risk for developing sepsis.

Endothelial cell activation

Inflammation and haemostasis are known to have common pathophysiologic processes.[31] Inflammation and resultant endothelial damage cause a loss of the structural and functional integrity of the endothelium, which hampers endothelial capacity to maintain haemostasis, further promoting activation of haemostasis, inflammation and vasoconstriction.[29] Haemostatic abnormalities including anaemia, hepercoagubility and alterations in the inflammatory response have been documented in dogs with CPV.[3,20,21,34]

Endothelial dysfunction is a contributing factor in the pathogenesis of a wide number of serious diseases including sepsis, babesiosis, rheumatoid arthritis, congestive heart failure, chronic kidney disease, arteriosclerosis and cancer pathogenisis.[35,36] Endothelial activation is defined as an increase in the expression and release of adhesion molecules.[36] In CPV we suspect endothelial activation as a result of increased concentrations of inflammatory cytokines as described in previous studies.[35,36] Endothelial dysfunction leads to an increase in the permeability of the microvasculature as is seen in the severe gastrointestinal bleeding in dogs with CPV.[36] Before monocytes and leukocytes can migrate into tissues they need to attach to endothelium. The attachment to endothelium, as well as migration into tissues, is facilitated by complimentary adhesion molecules on polymorphonuclear cells and endothelium.[36] The CVP infected dog suffers from severe widespread inflammation and previous studies have shown that the CVP infected dog has raised cytokine levels.[37] We suspect that the concentration of circulating markers of endothelial activation is a contributing factor for the wide spread inflammation seen.[3,34] Different endothelial cell surface molecules have an influence on the degree of adhesion of circulating blood cells and the balance between pro- and anti-coagulant activities.[29] Soluble cell surface molecules shed from activated endothelial cells are specific to endothelial cells and have been studied in only three papers of canine diseases including babesiosis and obesity.[29] In human studies markers of endothelial activation have been used to shed light on the pathophysiology of congestive heart failure and arteriosclerosis.[35] Increased levels of markers of endothelial activation have an association with coronary artery dysfunction in humans.[35] This is of relevance to our study since it has been shown that dogs suffering from babesiosis and obesity and humans with heart disease and arteriosclerosis are all in a state of systemic inflammation.

Intercellular adhesive molecule-1

Intercellular adhesive molecule-1 (ICAM-1) is a cell surface glycoprotein expressed on vascular endothelial cells and is responsible for the firm attachment of leukocytes to endothelium and the

subsequent transendothelial migration.[38] Intercellular adhesive molecule-1 is released upon cytokine stimulation. The main cytokines responsible for release are interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and high mobility group box-1 (HMGB-1).[39,40,41,42] It has been shown that ICAM-1 can be detected in circulation 1 hour after TNF-α stimulation, it reaches peak concentrations 12-16 hours after TNF-α stimulation and has a half-life of 1 hour.[43] During activation, endothelial ICAM-1 interacts with beta-2-integrins on leukocytes resulting in firm adhesion.[44] Nuclear factor- κB (NF- κB) is the main control over TNF-α stimulation of ICAM-1 expression.[41] There is a parallel correlation between ICAM-1 concentration and NF- κB , if NF- κB is suppressed the concentration of ICAM-1 is decreased.[41] Intercellular adhesive molecule-1 is a good indicator of endothelial activation as it is released exclusively from the endothelial cell surface. The trans-endothelial migration regulated by ICAM-1 is one of the causes of leukopenia in diseased animals.[38]

Both ICAM-1 and VCAM-1 can be used to assess endothelial activation function. Circulating intercellular adhesive molecule-1 has been investigated in dogs with *Babesiosis canis* and has been found to be a valuable indicator of endothelial activation. [31] Dogs with babesiosis also had increased concentrations of ICAM-1 at presentation compared to day six after treatment.[31] Compared to healthy dogs, circulating ICAM-1 concentration was significantly increased before therapy and remained high for three days after therapy in dogs with babesiosis.[31]

Vascular adhesive molecule-1

Vascular adhesive molecule-1 (VCAM-1) is part of the immunoglobulin super family that is responsible for adhesion of lymphocytes, monocytes, eosinophils, and basophils to the endothelium and the transmigration of these leukocytes to inflammatory sites.[44] Vascular adhesive molecule-1 activates signals in endothelial cells like Rac1 and calcium fluxes through which trans-endothelial migration is regulated.[45] Cytokines responsible for the release of VCAM-1 include IL-1β, interleukin-4 (IL-4), TNF-α and HMGB-1 as well as lipopolysaccharides.[40,44] Interleukin-4 and TNF-α work synergistically to stimulate VCAM-1, a study exploring the regulation of VCAM-1 found that VCAM-1 reaches peak concentrations at 8 hours and has a half-life of 48 hours after stimulation with IL-4 and TNF-α.[46] A study conducted by S Sahinduran et. al. 2016 showed that dogs with CPV had significantly higher cytokines namely TNF-α, Il-1, interleukin-6 (IL-6) and CRP when compared to healthy control dogs at admission and during treatment.[37] Vascular adhesive molecule-1 has been found to be expressed exclusively on endothelial cells making it a good indicator of endothelial activation.[31] During endotoxaemia the intensity of expression of VCAM-1 increases dramatically on a cellular level and circulating concentrations of VCAM-

1 increase.[44] A study that focused on the anti-inflammatory effect of hepatocyte growth factor (HGF) found that HGF suppresses vascular endothelial growth factor which in turn prevents the activation of NF- κB thus blocking the NF- κB pathway responsible for ICAM-1 and VCAM-1 expression.[47]In a babesiosis study, circulating VCAM-1 was found to be increased on admission when compared to healthy dogs. This study also found that dogs with complicated babesiosis had higher levels of circulating VCAM-1 compared to uncomplicated cases.[31]

High mobility group box-1 protein

High mobility group box-1 protein is a conserved component of nuclei and is known as a non-histone nuclear protein that functions as a DNA binding protein.[48,49] High mobility group box-1 protein is a nuclear factor and a secreted protein.[48] In cells, it is a chromatin binding factor that helps form the shape of DNA and facilitates the protein structure of specific DNA sites.[50,51] In circulation, HMGB-1 has a high affinity to bind with the RAGE receptor, a receptor for advanced glycation end-products, resulting in an increased severity of inflammation.[52] High mobility group box-1 protein is passively released by necrotic cells and actively secreted by endothelial cells, macrophages and monocytes in response to endotoxins, TNF-α and IL-1β.[53] High mobility group box-1 protein induces a pro-inflammatory change by increasing the release of cytokines and chemokines from monocytes, as well as up-regulating endothelial surface receptors and secretion of soluble pro-inflammatory mediators.[53] It is well known that HMGB-1 is a late mediator of inflammation causing organ dysfunction and increasing fatalities in patients with sepsis.[54] Following acute traumatic events HMGB-1 can increase up to 30-fold within the first hour. Peak levels of HMGB-1 is reached 2-6 hours post injury in humans with haemorrhagic shock.[54] This is the contrary to what has been described for human patients with sepsis who only reach peak levels days after admission.[55] To our knowledge there has been no studies conducted in animals to describe the kinetics of HMGB-1. In human studies it has been found that HMGB-1 is a late mediator of endotoxic shock, up regulates ICAM-1 and VCAM-1 in the microvasculature and increases the expression of RAGE receptors.[53] Numerous studies have been conducted on therapies aimed at blocking the detrimental effect that HMGB-1 can have in the inflammatory process. It was found that blocking the NF- κB pathway causes suppression of endothelial HMGB-1 release.[53]

High mobility group box-1 protein has been studied as a marker of endothelial function in canine babesiosis and obesity.[31,39] Concentrations of circulating HMGB-1 were increased in dogs with babesiosis at admission compared to healthy dogs.[31] Dogs with babesiosis also had increased concentrations HMGB-1 at presentation compared to day six after treatment.[31] Dogs with complicated babesiosis had

higher concentrations of circulating HMGB-1 admission compared to the sixth day.[31] In natural overweight and obese dogs significantly elevated IL-6 and CRP values were measured but there were no significant difference in ICAM-1 and HMGB-1 concentrations contrary to what has been described in humans.[39,56,57]

C-reactive protein

During systemic inflammation the liver is stimulated by the pro-inflammatory cytokines including IL-1 to produce and release the major acute phase protein, CRP.[58,59,60] C-reactive protein is classified as a major acute phase protein in dogs since it rises by 100 to a 1000 fold in the face of systemic inflammation.[60] The magnitude of the rise in CRP reflects the magnitude of inflammation and CRP significantly decreases during effective treatment.[58] C-reactive protein is important during the early defence of the immune system by binding pathogens and damaged cells.[58] C-reactive protein also plays an important role in activating the complement system, increasing the release of cytokines and regulating the inflammatory processes.[61] C-reactive protein serves as a sensitive indicator of the presence and extent of inflammation and provides an indication of effective treatment with reducing concentrations.[3]
C-reactive protein has been investigated as a predictor of outcome in CPV dogs.[3] In a study conducted by McClure et. al. 2013 they found that a high CRP concentration at admission, 12 and 24 hours later were positively associated with death.[3] This study however showed that CRP alone was not a good predictor of outcome in the CPV dog.[3]

In summary, various biomarkers have been investigated in the CPV patient.[10] It is known that the CPV patient suffers from altered hemostasis.[3] This is thought to be due to the endotoxin and cytokine effect on the endothelium.[20,21] Based on the elevation in CRP it is also known that the CPV patient suffers from wide spread systemic inflammation.[3] It is known that the CVP infected dog has significantly raised cortisol levels and from human research it is known that raised cortisol levels inhibit the expression of NF- κB, IL-1β, Il-6 and TNF-α.[22,23,62] Currently there is no research on the state of endothelial activation and its possible role in the disease pathogenesis in CPV. In this study we aim to investigate the state of endothelial activation in CPV. The results may provide meaningful insight into the level of endothelial activation, subsequent leukocyte transmigration, disease pathogenesis and possible future related therapy.

CHAPTER 2

OBJECTIVES

1. Determine the effect of CPV enteritis on specific markers of endothelial activation.
2. Identify correlations between markers of endothelial activation, clinical score and inflammatory markers namely, CRP and albumin, and clinical score in CPV enteritis.

CHAPTER 3

RESEARCH HYPOTHESIS

H0: The markers of endothelial activation are not elevated in CPV enteritis.

H1: The markers of endothelial activation are elevated in CPV enteritis.

H0: There are no positive correlations between markers of endothelial activation, clinical score and inflammatory markers in CPV enteritis.

H1: There are positive correlations between markers of endothelial activation, clinical score and inflammatory markers in CPV enteritis.

CHAPTER 4

MATERIALS AND METHODS

4.1 Study design

A prospective, cross sectional, clinical, observational study was performed on ethylenediaminetetraacetic acid (EDTA) plasma and serum samples collected from 30 dogs presented for treatment of CPV enteritis, diagnosed by positive faecal CPV ELISA test and confirmed with faecal EM. The samples were compared to 10 healthy age-matched control dogs.

4.2 Study Population

Inclusion criteria

STUDY GROUP

Client-owned dogs presenting at the Onderstepoort Veterinary Academic Hospital (OVAH) were considered for inclusion in the study if the dogs met the following criteria:

- Older than 6 weeks, younger than 12 months and of any sex.
- Weighs 3 kg or more.
- Free of blood borne parasites on peripheral blood smear (Appendix H).
- Demonstrates clinical signs of parvoviral enteritis such as depression, anorexia, vomiting and diarrhoea.

A preliminary diagnosis of parvoviral enteritis was initially based on a positive faecal CPV ELISA test (IDEXX SNAP Parvo Test, Netherlands). Confirmation of diagnosis was achieved through the detection of CPV on EM.

All owners of dogs fulfilling the above criteria were requested to sign a consent form (Appendix E) to include their dog in the study.

CONTROL GROUP

Client-owned dogs presenting at the OVAH for regular vaccination/deworming or sterilization/castration, and complying with the established criteria listed below were considered as control dogs for the study.

Inclusion criteria:

- Older than 6 weeks, younger than 12 months and of any sex.
- Weighs 3kg or more.
- Free of blood borne parasites on peripheral blood smear (Appendix H).

- No other dogs on the same property affected by parvoviral enteritis or related signs in the past 4 weeks.

All owners of dogs fulfilling the above criteria were requested to sign a consent form (Appendix B) to include their dog in the study.

Exclusion criteria

STUDY GROUP

- Dogs treated for CPV enteritis by another veterinarian before presentation.
- Any signs of inflammation/infection not associated with CPV (e.g. trauma).
- Dogs diagnosed with canine distemper virus on transmission EM.

CONTROL GROUP

- Dogs with a history of illness including, but not exclusively, vomiting, diarrhoea or any other abnormality in the preceding 14 days.
- Any abnormality on clinical examination including, but not exclusively, signs of inflammation/infection.
- Dogs diagnosed with CPV or canine distemper virus on transmission EM.

4.3 Sample collection and experimental procedures

GROUP 1 - Dogs with canine parvoviral enteritis

Client owned dogs presenting to the OVAH with signs suggestive of parvoviral enteritis were used for the study. On presentation, a history and clinical examination form (Appendix G) was completed. A peripheral blood smear and faecal floatation was performed (Appendix H). A CPV ELISA (IDEXX SNAP Parvo Test, Netherlands) was performed on dogs at presentation. Faecal samples were stored and CPV confirmed using EM (Appendix H).

The faecal sample was refrigerated at 4°C once collected and was submitted to the Faculty of Veterinary Science Electron Microscopy Unit for faecal EM within 72 hours of collection (Appendix H). Dogs diagnosed with CPV were treated on an outpatient or in hospital basis according to the standard treatment protocol of the OVAH (Appendix K).

The clinical parameters of each dog were recorded before blood collection or the placement of an intravenous catheter (Appendix G). The clinical scoring system used in this study is an adaption from previous studies, and although the scoring system has not been validated, it takes into account known prognostic markers such as white blood cell count, lymphocyte count, CRP and albumin concentrations in addition to clinical markers of disease severity used daily in the clinical assessment of critically ill

dogs.[3,16,27] The clinical severity score was calculated using the data from the clinical examination, complete blood count (CBC), CRP and albumin concentrations (Appendix G).

At presentation, before any medical intervention, atraumatic blood collection was performed via the jugular vein, with a 21-gauge needle, and minimal haemostasis. Blood was collected in the following order; at least 3mL whole blood in a 4mL Vacutainer Braun Serum Tubes (Beckton Dickinson Vacutainer Systems, UK) and 3mL whole blood in a 4mL EDTA tube (Beckton Dickinson Vacutainer Systems UK) filled to 75% capacity (Appendix J).

The blood collection was less than 0.5% of body weight to ensure that the volume of blood collected was in accordance with the animal use-and care committee of the Faculty of Veterinary Science specifications for blood collection.

The EDTA sample (minimum 1100 uL) was used to perform the CBC, then centrifuged at 1000 × g for 15 min, within 2h of collection to separate plasma. The plasma was aliquoted into 0.5mL cryovials and stored at −80 °C until analysis could be performed (Appendix J). The EDTA plasma was used to measure the concentrations of ICAM-1, VCAM-1 and HMGB-1.

After clotting, the serum sample was centrifuged at 1000 × g for 10 min, within 2h of collection to separate serum. The serum samples were aliquoted into 0.5mL cryovials and used to measure CRP and albumin (Appendix J). The analysis was performed within 2h of collection.

All samples (plasma, serum and EDTA pellet) were stored at -80°C (Forma Scientific -86°C freezer) and forms part of a biobank for future studies. Previous studies showed stability of soluble cell adhesion molecules and endothelial markers after long-time storage at −80 °C.[63,64]

All samples were thawed from frozen and given the appropriate amount of time to be thawed properly. All samples underwent one freeze thaw cycle before analysis.

GROUP 2 – Healthy control dogs

Dogs that met the inclusion criteria underwent the following examinations and tests (Appendix C):

- Clinical examination.
- Peripheral blood smear examination (Appendix H).
- Faecal floatation (Appendix H).

Blood samples were collected before any treatment was given (vaccination/deworming) or procedure performed (sterilization/castration). Blood was collected once off, stored and analysed in batches in the same manner as specified for the dogs with CPV enteritis in the study group by the Clinical Pathology Laboratory.

4.4 Assay methodologies

- **Complete blood count**

An EDTA blood sample was used to perform a CBC on an Advia 2120i (Siemens, Germany). Manual differential leukocyte counts were also performed by an experienced haematology technologist at the Clinical Pathology Laboratory.

- **Endothelial marker evaluation**

Sandwich enzyme immunoassays were performed for the endothelial markers ICAM-1, VCAM-1 and HMGB-1. The microplates provided were pre-coated with specific antibodies to ICAM-1, VCAM-1 and HMGB-1. Standards and prepared study samples were added to the appropriate wells with biotin-conjugated antibodies specific to the respective endothelial markers. The microplates were incubated after the addition of Avidin conjugated to Horseradish Peroxidase (HRP) to each well. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to the wells triggering the colour change dependent on the concentration of the ICAM-1, VCAM-1 and HMGB-1. The enzyme-substrate reaction was terminated by adding sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450nm ± 10nm (Thermo Scientific Multiskan™ FC Microplate Photometer, ThermoFischer Scientific) for all ELISAs. The concentrations of the ICAM-1, VCAM-1 and HMGB-1 were determined by comparing the optical density of the samples to the standard curves created for each ELISA. The intra-assay coefficient of variance was less than 10% and the inter-assay coefficient of variance less than 12% for all three immunoassays.

- **Intercellular adhesion molecule-1**

The EDTA plasma was used to determine the ICAM-1 concentrations. A canine-specific ICAM-1 ELISA (USCN Life Science, Wuhan, China) was used. The analysis includes the plate preparation and assay procedure and was performed according to the manufacturer's ELISA protocol. The detection range of this kit is 1.56-1000ng/mL. The ELISA was performed by the Veterinary Population Management Laboratory of the University of Pretoria.

- **Vascular adhesive molecule-1**

The EDTA plasma was used to perform the assay. A canine-specific VCAM-1 ELISA kit (Biotang Source International, Camarillo, USA) was used to perform the assay. The analysis includes the plate preparation and assay procedure and was performed according to the manufacturer's ELISA protocol. Samples were diluted at 1:4. The detection range of this kit is 3.12-200ng/mL. The concentration read

from the standard curve was be multiplied by the dilution factor for VCAM-1. The ELISA was performed by the Veterinary Population Management Laboratory of the University of Pretoria.

- **High mobility group box-1 protein**

EDTA plasma was used to perform the assay. A canine-specific HMGB-1 ELISA kit (USCN Life Science, Wuhan, China) was used to perform the assay. The analysis includes the plate preparation and assay procedure and was performed according to the manufacturer's ELISA protocol. Samples were diluted at 1:500. The detection range of this kit is 6.25-400pg/mL. The concentration read from the standard curve was be multiplied by the dilution factor for HMGB-1. The ELISA was performed by the Veterinary Population Management Laboratory of the University of Pretoria.

Pro-inflammatory cytokines and chemokines

- **C-reactive protein**

Serum samples were used to measure CRP concentrations using the Gentian canine CRP immunoassay (Roche Diagnostics, Indianapolis, USA) on the Cobra Integra 400 plus analyser at the Clinical Pathology Laboratory. A value greater than 10mg/L was considered abnormal.

- **Albumin**

Serum blood sample was used to measure albumin on the automated Cobra Integra 400 plus chemisty analyser at the Clinical Pathology Laboratory.

4.5 Observations

- History, signalment, clinical data and clinical severity score (Appendix C and G).
- Haematology.
- ICAM-1, VCAM-1 and HMGB-1 concentrations.
- CRP and albumin concentrations.

4.6 Data capture and Statistical analysis

History, signalment and other clinical data (Appendix C and G) was recorded manually on the appropriate forms in the patient's file and then on the OVAH computerized administration system (UVIS). The ICAM-1, VCAM-1 and HMGB-1, CRP and albumin concentrations were recorded on a Microsoft® Excel spread sheet.

Statistical analysis was performed using SPSS Statistics 25.0 software. The data was checked for normality using the Shapiro-Wilk and the Kolmogorov-Smirnov test. The Mann-Whitney *U* test was used to compare all continuous variables between the parvovirus group and the controls. Association between markers of endothelial activation, clinical score and the inflammatory markers CRP and

albumin, and clinical score in the CPV group was evaluated using Spearman's Rank correlation coefficient. A p value < 0.05 was considered significant.

CHAPTER 5

Results

A total of 30 dogs were included in the study. No dogs were excluded. All dogs diagnosed with CPV enteritis had clinical signs consistent with CPV enteritis, and were confirmed CPV positive based on a CPV antigen ELISA SNAP test and faecal EM.

Twelve breeds were included in the CPV group (Table 1), with 21 male dogs and 9 female dogs and median age of 4 months (Interquartile range (IQR): 3-6). Ten control dogs were used in the study. Seven breeds were represented (Table 1), with 6 male dogs and 4 females and a median age of 4 months (IQR: 3-6). There was no significant difference in breed (p=0.320), sex (p=0.563) or age (p=0.974) between the CPV and controls groups.

According to the owners, the duration of illness ranged from 1 to 5 days with the median being 3 days. Seven control dogs presented for routine vaccination and three dogs presented for castration/ sterilisation. The control dogs were all healthy based on history, clinical examination and blood smear evaluation.

Clinical score and hematology

The clinical score (Table 2) was calculated using the data from the clinical examination, CBC, CRP and albumin concentrations. The clinical score for the CPV group (median: 19; IQR: 17 – 23) was significantly lower than for the control group (median: 34; IQR: 34 – 34; p<0.001.).

The white cell count (Table 3) of the CPV group was significantly lower (median: 5.89; IQR: 3.19-7.85) than the control group (median: 12.43; IQR: 10.22-14.01; p=0.001. Fig 1). Segmented neutrophils were significantly lower in the CPV group (median: 4.14; IQR: 2.1 – 6.24) compared to the control group (median: 6.96; IQR: 5.57-7.87; p=0.034. Fig 2). The CPV group had significantly lower lymphocytes (median: 0.67; IQR: 0.41-0.97) compared to the control group (median: 4.1; IQR: 1.99-5.25; p<0.001. Fig 3). The CPV group showed a significantly lower value for monocytes (median: 0.32; IQR: 0.20-0.55) compared to the control group (median: 0.81; IQR: 0.68-1.08; p=0.001. Fig 4). The CPV group showed a significantly lower value for eosinophils (median: 0.05; IQR: 0.00-0.16) compared to the control group (median: 0.42; IQR: 0.21-0.79; p<0.001).

Haemoglobin concentration, red cell count, haematocrit and platelet count showed no significant difference between the CPV and control groups.

Markers of endothelial activation

The CPV group showed a significantly lower value for ICAM-1 (median: 5.86; IQR: 4.28-8.27) compared to the control group (median: 8.02; IQR: 6.93-10.33; p=0.008. Fig 5). The CPV group showed no

significant difference for VCAM-1 (median: 459.5; IQR: 363.0-505.0) compared to the control group (median: 410; IQR: 363.0-452.0; p=0.267). The CPV group showed no significant difference for HMGB-1 (median: 91.5; IQR: 84.0-101.0) compared to the control group (median: 86.5; IQR: 80.0-87.0; p=0.150).

Inflammatory markers

The CPV group showed a significantly higher value for CRP (median: 134; IQR:85.0-195.0) compared to the control group (median: 1; IQR:0.0-7.0; p<0.001 Fig 6).

The CPV group showed a significantly lower value for albumin (median: 29.21; IQR:25.90-31.09) compared to the control group (median: 33.34; IQR:30.84-35.40; p=0.003).

C*orrelation analysis*

ICAM-1 showed no correlation with any other variable measured.

There was significant negative correlation between VCAM-1 and band neutrophils (r_s=-0.369, P=0.045). There was a significant positive correlation between VCAM-1 and clinical score (r_s=0.384, P=0.036), white cell count (r_s=0.582, P=0.001) and segmented neutrophils (r_s=0.612, P<0.001).

There was significant positive correlation between clinical score and lymphocytes (r_s=0.676, P>0.001) and segmented neutrophils (r_s=0.572, P=0.001) and a significant negative correlation between clinical score and band neutrophils (r_s=-0.451, P=0.012).

Table 1: Signalment of the study population		
	Control group	**CPV group**
Age *(months) Median* (IQR)	4 (3-6)	4 (3-6)
Sex		
Male	6	21
Female	4	9
Breed		
Mixed breed	2	9
Boerboel		1
American Pitbull Terrier		6
Jack Russel Terrier		1
Labrador Retriever	1	6
Golden Retriever	2	
Bullterrier	1	
Pekingese	1	1
Siberian Husky	2	1
Saint Bernard		1
Yorkshire Terrier		1
Dachshund	1	1
Rhodesian Ridgeback		1
Fox terrier		1

Table 2: Clinical severity score of CVP infected dogs

New Parvo Number	Age (Months)		Habitus (1-4)	Appetite (1-4)	Vomition (1-4)	Faecal consistency (1-4)	Mucous membranes (1-3)	CRT (1-3)	Total WBC (1-3)	Lymphocyte count (1-3)	Serum CRP (1-3)	Albumin (1-3)	TOTAL CLINICAL SCORE (/34)	CRP	Albumin (1-3)	Albumin
1	6		2	1	2	1	2	2	1	1	2	3	12	82.4	3	30.98
2	6		3	1	2	3	2	3	2	1	1	3	17	125	3	29.61
3	4.5		3	1	3	3	1	3	2	3	1	3	17	142	3	30.99
4	4		3	1	4	2	3	2	1	1	1	3	18	155	3	31.18
5	3		3	1	1	2	2	2	3	1	2	3	15	59	3	32.23
6	3		3	1	2	3	2	3	3	3	1	3	19	122	3	29.21
7	6		3	1	2	2	2	2	2	1	2	3	15	82.4	3	30.98
8	2		3	1	2	3	3	3	2	3	2	3	19	91	3	24.57
9	4		2	1	1	1	3	3	2	1	1	3	14	197	3	32.07
10	3		3	1	3	2	2	2	3	1	2	3	17	82.4	3	30.98
11	4		2	1	1	1	2	3	3	3	1	3	20	191	3	23.4
12	6		3	1	4	1	2	3	3	1	1	3	20	195	3	29.1
13	10		3	1	2	1	2	2	1	1	1	3	17	211	3	27.9
14	9		2	1	2	1	2	2	1	1	1	3	16	204	3	26.1
15	3		3	1	2	2	2	2	2	1	1	3	19	220	3	29.7
16	4		3	1	3	4	3	3	3	1	1	3	25	126	3	32.1
17	3		4	1	2	2	3	3	3	1	1	3	23	191	3	30.8
18	4		2	1	3	1	2	2	1	1	1	3	17	181	3	25.1
19	2		3	2	3	2	3	3	3	3	2	3	27	99	3	27.5
20	3		3	1	3	2	3	3	2	1	2	3	23	91	3	25.7
21	12		4	1	2	1	3	3	1	1	1	3	20	290	3	31.5
22	2		3	3	2	2	3	3	3	1	2	2	24	85	2	20.6
23	4		4	1	3	3	3	3	3	3	2	3	28	57	3	35.9
24	3		3	1	2	1	3	1	3	1	1	3	19	195	3	24.4
25	4		4	1	3	3	3	3	3	3	2	3	28	82	3	29.9
26	6		3	1	4	1	2	3	1	1	1	3	20	209	3	27.9
27	3		3	1	1	1	3	2	3	1	1	3	19	423	3	34.6
28	6		2	1	2	1	3	2	1	1	1	2	13	150	2	17.94
29	4		3	1	4	4	3	3	2	1	2	3	28	86	3	27
30	4		3	1	3	1	2	3	1	3	2	3	22	70	3	30.1

CRP C-reactive protein

CRT Capillary refil time

Table 3: Summary of data				
Variable	***Group***	***Median***	***Interquartile range***	***P value***
White cell count (x 10^9/L)	Control Parvovirus	12.43 6.11	10.22 – 14.01 3.30 – 9.91	0.001
Segmented neutrophil count (x 10^9/L)	Control Parvovirus	6.97 4.14	5.57 – 7.87 2.10 – 6.24	0.031
Lymphocyte count (x 10^9/L)	Control Parvovirus	4.1 0.67	1.99 – 5.25 0.41 – 1.18	<0.001
Monocyte count (x 10^9/L)	Control Parvovirus	0.81 0.32	0.68 – 1.08 0.20 – 0.57	0.001
Haemoglobin (x 10^9/L)	Control Parvovirus	135.5 130	119-138 114-156	0.901
Red Cell Count (x 10^9/L)	Control Parvovirus	5.67 5.99	5.31-6.33 5.3-6.96	0.390
Haematocrit (x 10^9/L)	Control Parvovirus	0.4 0.38	0.37-0.41 0.36-0.45	0.913
Platelet count (x 10^9/L)	Control Parvovirus	416 340	292-506 258-386	0.07
ICAM-1 (ng/mL)	Control Parvovirus	8 5.90	6.90 – 10.30 4.30 – 8.30	0.008
VCAM-1 (ng/mL)	Control Parvovirus	410.0 459.5	363.0-452.0 363.0-505.0	0.267
HMGB-1 (ng/mL)	Control Parvovirus	86.5 91.5	80.0-87.0 84.0-101.0	0.150
CRP (mg/L)	Control Parvovirus	1 150	0 – 7 91 – 196	<0.001
Albumin (g/L)	Control Parvovirus	33.34 29.21	30.84-35.40 25.90-31.09	0.003

ICAM-1 Intercellular adhesive molecule-1

VCAM-1 Vascular adhesive molecule-1

HMGB-1 High mobility group box-1 protein

CRP C-reactive protein

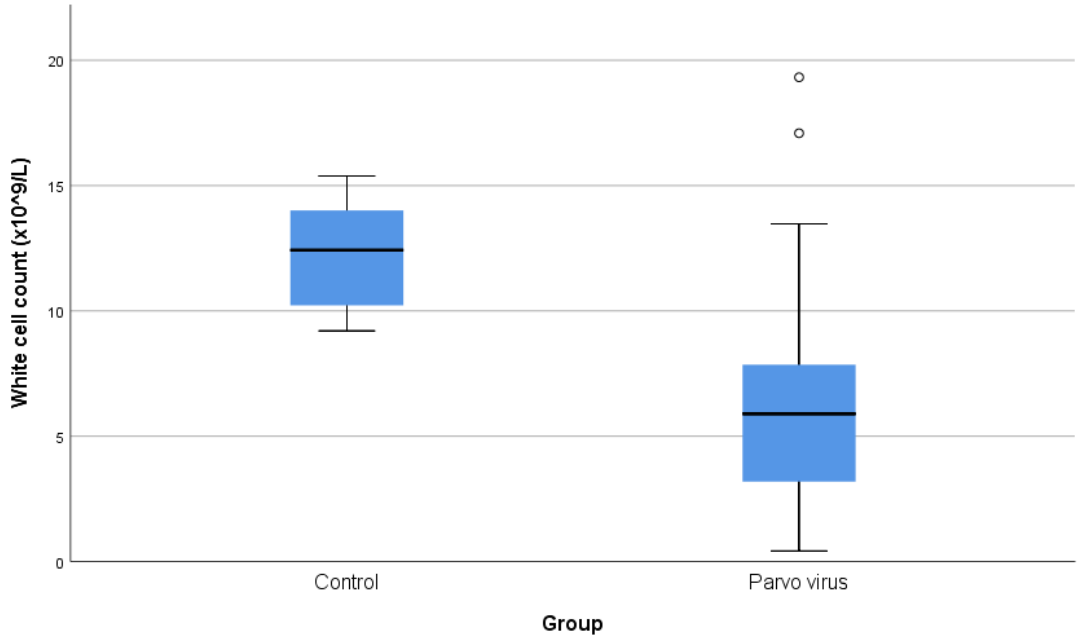

Figure 1. Box plot graph for White Cell Count (x 10^9/L) for the control (0) and CPV (1) groups. The white cell count was significantly lower in the CPV group compared to controls (p=0.001). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

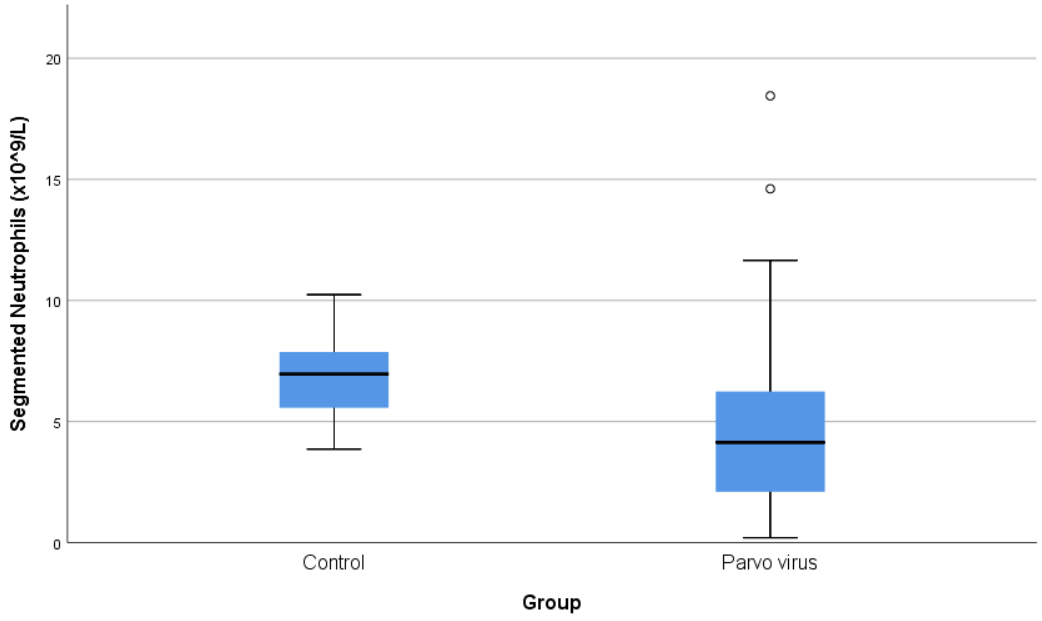

Figure 2. Box plot graph for segmented neutrophils count (x 10^9/L) for the control (0) and CPV (1) groups. The segmented neutrophils count was significantly lower in the CPV group compared to controls (p=0.034). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

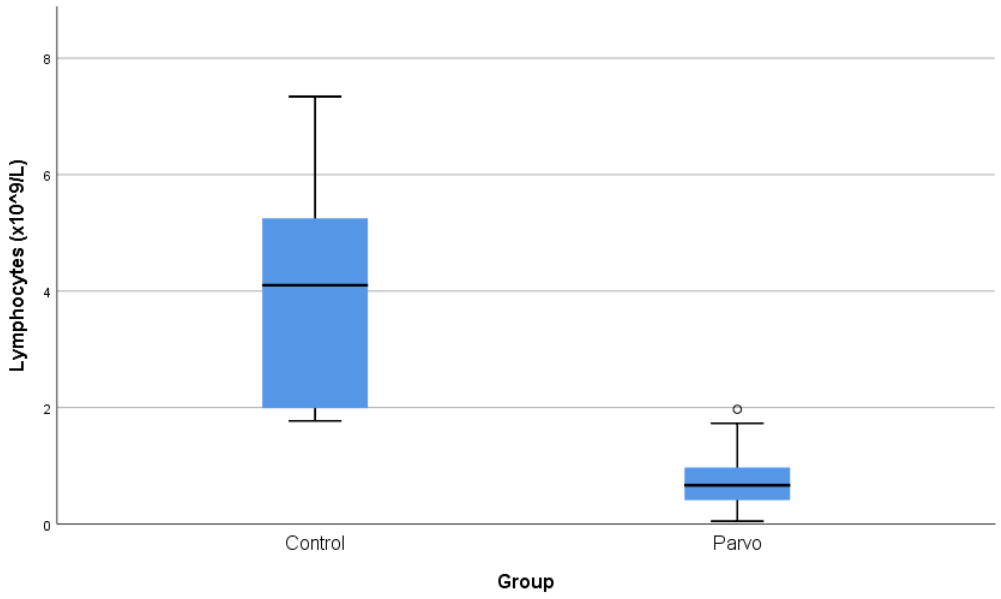

Figure 3. Box plot graph for lymphocyte count (x 10^9/L) for the control (0) and CPV (1) groups. The lymphocyte count was significantly lower in the CPV group compared to controls (p<0.001). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

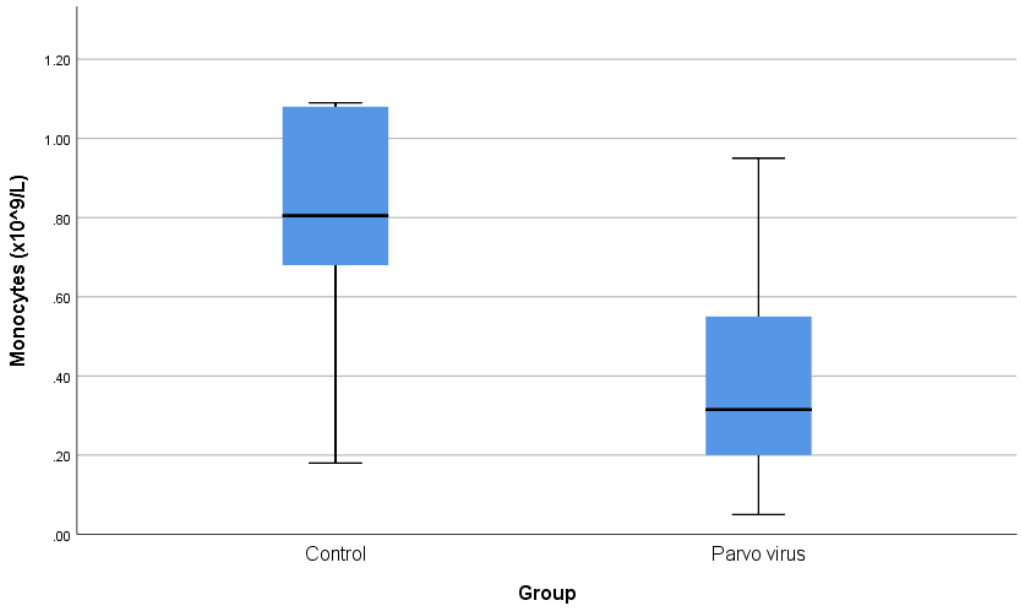

Figure 4. Box plot graph for monocytes count (x 10^9/L) for the control (0) and CPV (1) groups. The monocyte count was significantly lower in the CPV group compared to controls (p=0.001). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

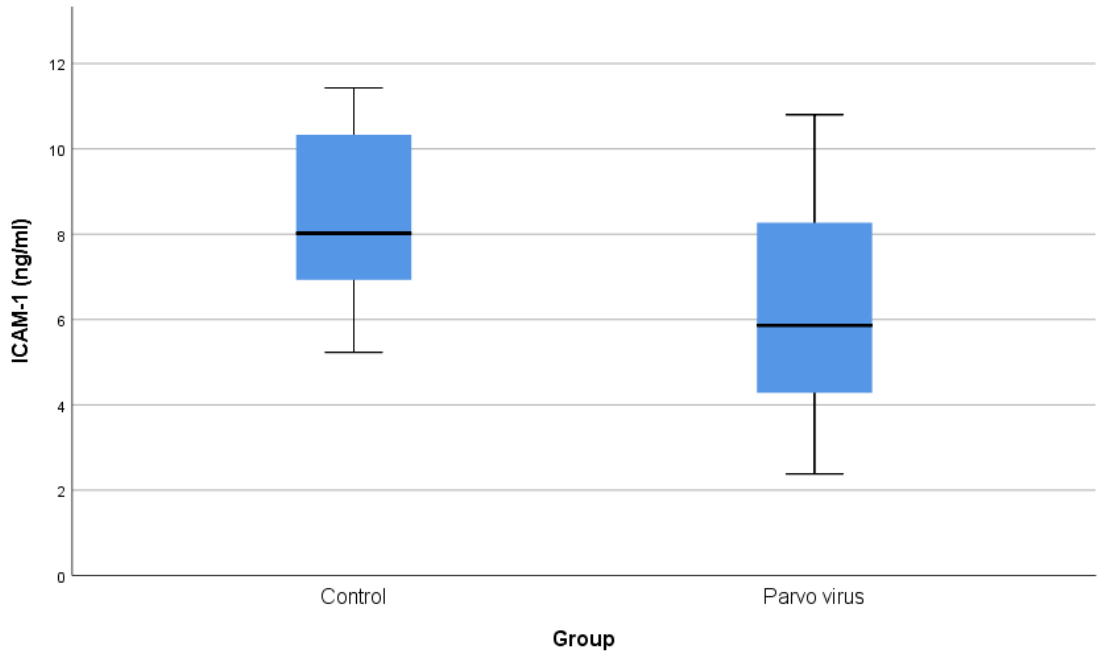

Figure 5. Box plot graph for ICAM-1 concentration (ng/mL) for the control (0) and CPV (1) groups. The ICAM-1 was significantly lower in the CPV group compared to controls (p=0.008). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

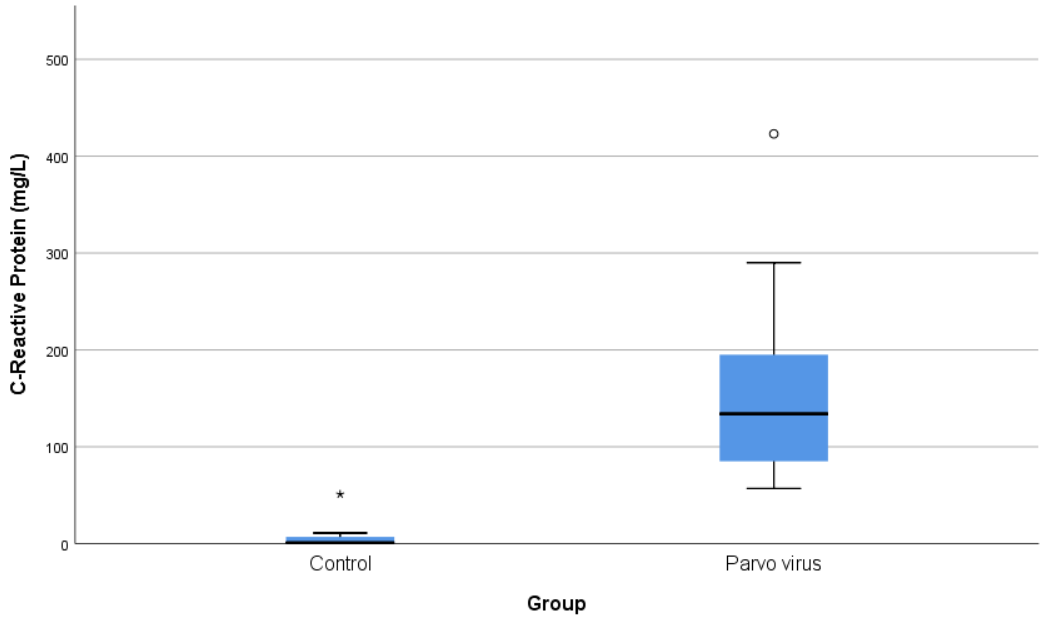

Figure 6. Box plot graph for CRP concentration (mg/L) for the control (0) and CPV (1) groups. The CRP concentration was significantly higher in the CPV group compared to controls (p<0.001). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median.

CHAPTER 6

Discussion

To our knowledge this is the first study to assess concentrations of circulating markers of endothelial activation in the CPV dog. A significant difference was found in this study with regards to the CPV group and the control group for ICAM-1, no significant difference was however found between the CPV and control group for VCAM-1 and HMGB-1. Leukopenia and raised CRP results were found in the CPV group compared to the control group as has been described in previous studies.[3,8,13,16,17] The study identified a strong correlation between VCAM-1 and segmented neutrophils.

The clinical scoring system was used to evaluate the overall status of the patient. A significant difference between the clinical score of the CPV group was found, compared to that of the control group. The clinical scoring system used in this study have not been validated. In 1991 the measures used to establish whether a critically ill human was suffering from systemic inflammatory response syndrome was established to correlate sepsis to systemic inflammatory response syndrome.[65] The parameters that was used included body temperature, heart rate, respiratory rate and white cell count. In 2001 a new scoring system was developed in order to stage septic animals.[66] The system uses the acronym PIRO which stands for predisposition, infection, response and organ dysfunction.[66]A study was conducted in 2020 to establish the prognostic usefulness of the new scoring system using CPV infected dogs as a model for sepsis.[33] The study conducted by Alves et. al. 2020 found that the SIRS system was more specific when including mucous membrane colour and capillary refill time.[33] The study also found that determining and using a classification system for suspected sepsis in the canine patient was possible.[33] The clinical score used in this study was adapted from previous studies and included these parameters; habitus, appetite, vomiting, faecal consistency, mucous membrane colour, capillary refill time, total white cell count, lymphocyte count, CRP as well as albumin. The objectively measured parameters used included total white cell count, lymphocyte count, CRP as well as albumin have been used in previous studies and have shown to be related to disease severity, outcome or prognosis.[23,67] The most consistent finding in dogs with CPV is leukopenia with a significant lymphopenia as seen in our results.[3,67] It has been shown that at hospital admission, non-survivors have a significantly lower total white cell count with a significant low neutrophil, band neutrophil, lymphocyte and eosinophil count compared to survivors.[68] The absence of a cytopenia has a predictive value of 100% for survival 24 hours after admission.[68] The CPV group had significant higher CRP values than the control group. This too is in line with previous studies.[3,68] It has been found that an elevated CRP concentration at 12 and 24 hours after

admission is a positive indicator of mortality in the CVP infected dog.[68] C-reactive protein is an acute phase protein that rises by 100 to 1000-fold during inflammation.[59] The release of CRP is stimulated by IL-1.[24,58,59] The substantial rise in CRP confirms that there is a widespread systemic inflammatory response during CPV. Although it has been shown that CRP is a sensitive indicator of the extent of inflammation it cannot be used alone as predictive value of mortality in CPV.[3] Hypoalbuminemia is a frequent finding in dogs suffering from CPV.[68] The loss of plasma proteins is due in combination to the protein-losing enteropathy, gastrointestinal haemorrhage and the SIRS-mediated vascular permeability.[68] During critical illness acute phase proteins are generated at the expense of albumin.[68] Thus, although the clinical scoring used for this study is not validated, it does assist in the objective determination of disease severity of dogs with CPV and the biochemical parameters measured were in line with what previous studies described.[33,65,66]

A marked leukopenia was seen. This is in line with previous studies which showed that the leukopenia exists due to suppression of haemopoietic precursor cells, the exhaustion of lymphatic tissue, the massive increase in demand from the severely inflamed gastrointestinal tract, gastrointestinal haemorrhage and sequestration of leukocytes in the gastrointestinal tract.[8,13,16,17,18] When the role of leukocytes in endothelial activation and release from the endothelium is considered, the leukopenia is likely to have played an important role in the unexpected normal or decreased circulating endothelial markers.

Our study found a significant segmented neutropenia compared to controls. This is in line with previous studies.[14] Neutrophils are the most abundant leukocyte in dog blood and is responsible for destruction of amongst others bacteria and viruses in the infected dog.[14] The severe neutropenia in CVP infected dogs is due to the viral destruction of the rapidly dividing myoblast in the bone marrow, neutrophil margination due to endotoxemia and sepsis and the massive loss through the gastro intestinal tract.[14] Neutrophils are responsible for the release of TNF-α which stimulates the release of ICAM-1, VCAM-1 and HMGB-1. [22,23,41] During significant neutropenia it can be assumed that there would be a decrease in TNF-α release which could lead to the subsequent decrease in the concentration of circulating ICAM-1, VCAM-1 and HMGB-1. Neutrophil transmigration is regulated by endothelial CAMs which in turn is released by enzymes called sheddase.[67] Neutrophil elastase is an important sheddase that is released by neutrophils and macrophages.[67] It is possible that during neutropenia there is a reduction in the release of neutrophil elastase which may result in a reduction in CAM release from endothelial cells. The

reduction in neutrophil elastase and the reduction of TNF-α release could explain the reduced concentration of markers of endothelial activation seen in our study.

As found in previous studies, a marked significant lymphocytopenia was confirmed in our CVP infected dogs compared to controls.[8,13,16,17,18] Lymphocytes are the second most abundant leukocyte in canine blood. It is essential for humoral and cell-mediated immune responses.[68] Lymphocytopenia develops due to an endogenous release of cortisol during acute infection that causes redistribution of lymphocytes and draining lymph nodes to enhance antigen contact, as a result of viral destruction and atrophy of lymphoid tissue and through the loss, sequestration or blockage of lymphocyte-rich lymph as a result of protein-losing enteropathy.[14] During inflammation lymphocytes release pro-inflammatory cytokines including TNFα, IL-1β and IL-6.[22,23,39] These cytokines in turn stimulate the release of ICAM-1, VCAM-1 and HMGB-1.[22,23,39] It can be assumed that in the face of lymphopenia the cytokines required for endothelial activation and subsequent release of endothelial adhesion molecules will be reduced, possibly contributing to the reduced CAM expression.

Monocytopenia is a common finding in CVP infected dogs and was confirmed in our results which showed a significant monocytopenia for the CPV group.[68] Monocytes form part of the mononuclear phagocytes and as macrophages are responsible for phagocytosis of foreign microorganisms and cellular debris.[16] Macrophages are responsible for secretion of inflammatory cytokines including TNF-α, IL-1β and IL-4. [22,23,41] Monocyte numbers are affected by CPV and the phagocytic ability of monocytes is also impaired by the virus.[68] A contributing factor of this impaired phagocytic ability could be the susceptibility of CVP infected dogs to secondary infections. The reduced secretion of inflammatory cytokines which can be expected in the face of monocytopenia could be a contributing factor to the low concentration of circulating endothelial markers seen in our study.

Intercellular adhesive molecule-1 was significantly lower in the CPV group compared to the control group. This is contrary to what has been described in other studies done on dogs suffering from inflammatory conditions.[31,39,69] Intercellular adhesion molecule-1 is a membrane bound glycoprotein that is induced by cytokines including IL-1β and IL-4, it is also induced by HMGB-1.[31] It is exclusively expressed on endothelial cells and is released during inflammation. Soluble ICAM-1 is released from the cell membrane and indicates systemic endothelial activation.[69] In a study conducted in dogs suffering from babesiosis it was shown that there was no significant difference in ICAM-1 on day null and day six for dogs with babesiosis when compared to healthy controls.[31] Another study conducted in dogs with babesiosis showed that ICAM-1 was increased significantly before and three days after antiparasitic

treatment when compared to the control group.[69] CRP has been shown to have a pro-inflammatory effect on the expression of endothelial adhesive molecules by activating the endothelial cells.[31] In human literature it has been shown that CRP induced chemokine secretion is a direct result of the interaction of adhesion molecules.[31] It has also been shown that the increased body temperature of dogs suffering from babesiosis has a positive correlation with ICAM-1 expression.[31] The low ICAM-1 concentration seen in our study is contradictory to the widespread inflammation seen in CPV and evidence by the significant increased CRP in this study although this has been seen before in dogs suffering from inflammatory conditions such as babesiosis.[3,31] A study conducted in dogs suffering from obesity, which has been proven to cause wide spread systemic inflammation, did not show a significant increase in ICAM-1 when compared to the control group which is the contrary to what has been described in human literature.[39] A study conducted in postpartum dairy cattle showed that cows had low ICAM-1 concentrations during postpartum metritis.[62] This study used LPS-stimulated Raw264.7 macrophages which is able to produce a rapid immune response and has the ability to release high concentrations of pro-inflammatory cytokines and inflammatory mediators. They found that cortisol significantly suppressed the mRNA expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6. It also found that NF-κB signaling was suppressed by cortisol.[62] This is of relevance to our study as CPV dogs have significantly increased cortisol levels and that would lead to the inhibition of the NF-κB induced inflammatory cytokine release especially TNF-α, IL-1β and IL-6 and the subsequent release of markers of endothelial activation including ICAM-1, VCAM-1 and HMGB-1.[22,23,41,62] Human studies have found that women suffering from breast cancer have elevated concentrations of ICAM-1 and that it has an association with tumour growth.[70] This study found that chemotherapy initially reduces the concentration of circulating ICAM-1, suspected secondary to immunosuppression or reduction of expression by the neoplasia itself or both, but after long term treatment patients showed severely elevated ICAM-1 concentrations.[70] A review study conducted in 2014 focused on soluble adhesion molecules as a marker for sepsis and looked at the potential differences between adults, children and neonates.[71] They found that ICAM-1 concentrations in neonates are comparable to the basal levels in adults and children.[71] They also showed that during sepsis all adhesion molecules were raised but the relative and absolute extent of the increase in neonates, was significantly lower when compared to adults and children.[71] This indicates that neonates are less affective at shedding adhesion molecules and that neonates do not up-regulate the expression of adhesion molecules as effectively as adults and children. This is of particular interest in our study as the population affected by CPV have a mean age of four months. It is possible that due to the fact that juvenile canines are at risk of contracting CPV these

dogs do not have the capability to shed or the capacity to up-regulate adhesion molecules expression appropriately. It is also possible that ICAM-1 was decreased in the CPV group secondary to immunosuppression as evidenced by the significant reduced white cell counts compared to the control group. The significant leukopenia could also lead to a decreased expression and release of inflammatory cytokines that stimulate the release of endothelial activation molecules which could further explain our results. Further investigation into the temporal changes of circulating ICAM-1 in acute inflammatory conditions is required.

Vascular adhesive molecule-1 and HMGB-1 has not been previously described in canine parvovirus.[3,34] Despite the widespread inflammation that dogs with canine parvovirus are known to suffer from, there was no significant difference in VCAM-1 and HMBG-1 between CPV dogs and control dogs.

Vascular adhesive molecule-1 is released exclusively by endothelial cells.[31] Vascular adhesive molecule-1 concentrations are increased by cytokine induction, specifically IL-1β, IL-6 and TNF-α.[22,23,41,62] The interaction of VCAM-1 and integrins expressed on leukocytes is responsible for the transmigration of leukocytes through the epithelium to the site of inflammation.[31] High mobility group box-1 plays a very important role in VCAM-1 expression and release.[40,44] A study conducted in dogs suffering from babesiosis found a significant increase in VCAM-1 when compared to a control group. It also found a significant increase in VCAM-1 in dogs with complicated babesiosis compared to dogs who recovered.[31] It has also been recorded that dogs with sepsis had significantly higher VCAM-1 concentrations than a control group.[31] In malaria it has been shown that VCAM-1 was responsible for increased margination and sequestration of neutrophils.[31] A study conducted in human patients showed that increased VCAM-1 was observed in all patients suffering from human immunodeficiency virus and that the increase had a strong correlation to the level of inflammation observed in the patients.[72] The study previously mentioned that focused on soluble adhesion molecules as a marker for sepsis and looked at the potential differences between adults, children and neonates found that neonates had significantly higher basal concentrations for VCAM-1 when compared to adults and children.[71] They also showed that in the face of sepsis neonates were unable to sufficiently express and release VCAM-1 when compared to adults and children.[71] This is of relevance to our study as it could be possible that young puppies infected with CPV does not have the ability or the reserves to express increased concentrations of VCAM-1. This could further indicate that due to the immature nature of the CVP infected dog they cannot elicit an appropriate immune response due to the lack of reserves and the inability to up-

regulate endothelial adhesion molecules. Cortisol concentrations are significantly increased in the CVP infected dog which may suppress subsequent release of VCAM-1.[62] We saw a strong positive correlation between VCAM-1 and leukopenia. This is of significance to our study since we suspect that in the face of leukopenia there will be a reduced capacity to release cytokines responsible for stimulating the release of markers of endothelial activation.

We saw no significant difference between HMGB-1 in the CVP infected dogs and control dogs. This is contrary to what has been described in dogs suffering from babesiosis.[31] High mobility group box-1 has been shown to play a pivotal role in the pathogenesis of inflammatory and autoimmune diseases in humans.[31] It is released passively by necrotic cells and actively by macrophages and monocytes upon cytokine stimulation by IL-1β and TNF-α.[50] Once in circulation, HMGB-1 acts as a pro-inflammatory mediator further enhancing the release of IL-1β and TNF-α as well as other inflammatory cytokines.[31] As pro-inflammatory mediator it also plays a very important role in the expression and release of ICAM-1 and VCAM-1.[49] High mobility group box-1 acts as a self-regulating agent and once released it stimulates macrophages to produce more HMGB-1.[49] In a study done by Sha et. al. 2008 it was shown that the pro-inflammatory activity of HMGB-1 is blocked in the absence of IL-1β.[73] This is of relevance to our study and could explain the results that we obtained for ICAM-1 and VCAM-1 concentrations. Since macrophages actively secrete HMGB-1, the leukopenia found in parvo dogs could be a contributing factor in the low concentrations seen.[52] A study performed on dogs suffering from babesiosis found that dogs had increased HMGB-1 concentrations at admission and on day six after admission when compared to the control group.[31] High mobility group box-1 has been studied in dogs with systemic inflammatory response syndrome, lymphoma and canine prostate cancer and has been found to be significantly increased during inflammatory disease.[31] This is of significance to our study since we know that dogs suffering from CPV has wide spread systemic inflammation but it is possible that the combination of raised cortisol levels and the subsequent inhibition of the NF-κB activation pathway, leukopenia and the juvenile nature of the CVP infected dog, could result in inadequate expression and release of HMGB-1. This absence of an appropriate HMGB-1 response would in turn lead to a decrease in the expression and release of ICAM-1 and VCAM-1 which is supported by the results that we obtained.

The markers of endothelial activation indicate that there is a lack of endothelial activation in the CPV dog. This could indicate that dogs with CPV have a lack of leukocyte attachment and transmigration which is likely to further contribute to the severe immunosuppression seen in the CPV dog.

During this study, several limitations were present. The point at which each dog was presented to the hospital as well as disease severity and duration of illness could not be standardized. The sample size was small and may not have given an accurate reflection of the population as a whole, this may have affected statistical analysis. Due to financial and time constraints, a bigger sample size was simply not possible. The clinical scoring system utilized requires validation. Samples were only collected at a single time point at presentation and not followed over time. Follow-up sampling would give a better representation of endothelial activation as the disease progressed. Temporal changes in leukocyte count, cytokine concentrations and subsequent circulating endothelial concentrations was not evaluated in this study.

This study provided evidence of a lack of endothelial activation or the presence of endothelial activation dysfunction based on the concentrations of circulating markers of endothelial activation. The study found significant lower concentrations for ICAM-1 in CVP infected dogs. The effect of cortisol and the NF-κB pathway responsible for expression and release of ICAM-1, VCAM-1 and HMGB-1 should be further investigated as well as the stimulating inflammatory cytokines including TNF-α, IL-1β and IL-6. The basal levels of ICAM-1, VCAM-1 and HMGB-1 should be investigated in dogs comparing concentration differences which might exist between juvenile and adult dogs. The low level of ICAM-1 in the CVP infected dog points to reduced transmigration of leukocytes which is likely to contribute to altered immune response in the CPV infected dog. Further investigation, including temporal changes in the expression and release of endothelial markers and cytokines is warranted to provide insight into the progression of disease and possible related therapies.

CHAPTER 7

Conclusion

- This study showed no proof of endothelial activation despite the known widespread inflammation that CPV dogs suffer from.
- Further study into the effect of leukopenia on endothelial activation and release of endothelial markers of activation, as well as temporal changes of circulating endothelial expression is warranted.
- A reduction in leukocyte attachment and transmigration through the endothelium possibly occurs in dogs with CPV patient.

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APPENDIX A

Client information sheet: Healthy control dogs for Markers of endothelial injury and inflammation in canine parvoviral enteritis study

Dear Client,

Your puppy has met the selection criteria to act as a control dog in a parvoviral enteritis study. Parvoviral enteritis or "cat flu" is a debilitating disease that has a high morbidity and mortality rate. The virus causes severe damage to the intestinal tract that leads to intestinal bleeding, vomiting, diarrhoea and fever. The virus also affects the bone marrow causing suppression of white blood cells - this leads to a decrease in the patient's ability to fight infections.
In our study, we are focusing on markers of endothelial activation in parvo-affected puppies. Endothelium is the cell layer that lines blood vessels. When puppies are infected with parvovirus they have severe and widespread inflammation that affects the endothelial cells may cause them to become activated. Activated endothelium releases specific biomarkers that can lead to a number of complications in the already debilitated patient. We want to measure these biomarkers and compare the results with inflammatory markers.
This study could help us determine whether there are correlations between the rise of inflammatory markers and the rise in markers of activated endothelium.
Your dog will serve as a control animal which means that since your dog is not currently suffering from parvoviral enteritis we can measure the biomarkers as they would be in a healthy animal. We will then use the results from your dog and compare it to the results of a similar dog who is affected with parvoviral enteritis.

For our study we will need to take two blood samples and a stool sample. The volume of blood and stool we will collect will in no way harm your pet or change the procedure for which your pet is admitted for.
You will not be responsible for any costs related to the trial. You will only be liable for the cost of vaccination or ovariohysterectomy or castration as discussed with you. This study has been approved by the Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial you are welcome to contact me.

Sincerely

Dr. Brogan Atkinson & Dr. Sune Pretorius

Tel 012 529 8036

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APPENDIX B

Consent form for control dogs – Markers of endothelial injury and inflammation in canine parvoviral enteritis study.

Dear client,

Your dog has been selected to serve as a healthy control dog for a study to evaluate the markers of endothelial damage (blood vessel wall damage) in parvovirus (cat flu) infected dogs. The study requires a stool sample and two blood samples.

The volume of stool and blood we will collect will in no way harm your pet or change the procedure for which your pet is seen or was admitted for.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial you are welcome to contact me.

Sincerely

Dr. Brogan Atkinson & Dr. Sune Pretorius

Tel 012 529 8036

I, ……, hereby give permission that my dog (Dog's name)……..,

a (breed)……. (colour) ……..

(sex)……. (age) ……

may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

The study has been explained to me and I understand that this study will in no way harm my dog. Furthermore, I understand that no additional costs will be incurred by me in respect of this trial for the collection of blood and stool samples or the blood test and stool analysis required over and above the normal vaccination and deworming or ovariohysterectomy or castration costs.

Signed at..... on the ……. day of …… 20….

Signature Owner/Agent ……..

Home Tel: ……..

Work Tel: ……

Cell No: ……

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APPENDIX C

Control dog Questionnaire and Check list:

Collector: BA or SP Date of collection: ……../……../……

Habitus (1-4)	
Body condition (1-5)	
Skin	
Eyes	
Mucous membranes	
CRT	
Lymph nodes	
Thoracic auscultation	
Heart rate	
Respiratory rate	
Abdominal palpation	
Temperature	

1.	**Reason for visit to clinic**	Vaccination/deworming	Sterilization/castration
2.	**Has the dog had any previous illnesses?**	YES	NO
3.	**Has the dog had any previous visits to the vet/ hospital?**	YES	NO
4.	**Has the dog vomited or had diarrhoea in the last 14 days?**	YES	NO
5.	**Are there other pets at home?**	YES	NO
6.	**Are any other dogs on the property ill (parvo symptoms)?**	YES	NO
7.	**What diet is fed at home?**		
8.	**Is anybody in the household using antibiotics?**	YES	NO

Bloodsmear	Parasites	RBC	WBC	PLT
Faecal appearance				
Faecal float				

Serum Biochemistry and Coagulation:

	Collect 1 serum tube (minimum 3mL)
	Collect 1 EDTA tube filled to 75% capacity
	Store serum and EDTA after being centrifuged at -80°C

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Appendix D

Client information Sheet: Markers of endothelial injury and inflammation in canine parvoviral enteritis.

Dear Client,

Based on your puppy's history, clinical examination and tests performed, your puppy has contracted parvoviral entiritis or "cat flu". This means that your puppy meets our inclusion criteria for a study on endothelial markers in parvoviral enteritis.
Parvoviral enteritis is a debilitating disease that has a high morbidity and mortality rate. The virus causes severe damage to the intestinal tract that leads to intestinal bleeding, vomiting, diarrhoea and fever. The virus also affects the bone marrow causing suppression of white blood cells this leads to a decrease in the patient's ability to fight infections. Patients suffering from parvoviral should ideally be hospitalised to receive intensive supportive treatment as there is no antivirus treatment.
In our study, we are focusing on markers of endothelial activation in parvo-affected puppies. Endothelium is the cell layer that lines blood vessels. When puppies are infected with parvovirus they have severe and widespread inflammation that affects the endothelial cells causing them to become activated. Activated endothelium releases specific biomarkers that can lead to a number of complications in the already debilitated patient. We want to measure these biomarkers and compare the results with inflammatory markers.
This study could help us determine whether there are correlations between the rise of inflammatory markers and the rise in markers of activated endothelium.

For our study we will need to take two blood samples and a stool sample. The volume of blood and stool we will collect will in no way harm your pet or change the treatment he/she will receive.

This trial will in no way harm your pet and he/she will still receive the same treatment as if he/she was not involved in the trial. You will not be responsible for any costs related to the trial. You will only be liable for the cost of treatment as discussed with you. This study has been approved by the Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial you are welcome to contact me.

Sincerely
Dr. Brogan Atkinson & Dr. Sune Pretorius
Tel 012 529 8036

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APPENDIX E

Client Consent Form: In Hospital patient

Project title: Markers of endothelial injury and inflammation in canine parvoviral enteritis study.

(To be completed by the patient's owner / authorised agent)

Please encircle Yes or No where necessary

1. Has your puppy received any treatment for canine parvoviral enteritis within the last week? Yes / No
2. Has your puppy received any vaccinations? Yes / No

If yes, how many and when was the last vaccination administered?

_____

2. Have you read the information sheet on canine parvoviral enteritis? Yes / No
3. Have you had the opportunity to ask questions about the research project? Yes / No
4. Have you received satisfactory answers to your questions? Yes / No
5. Have you received enough information about this study? Yes / No
7. Please supply the name of the person to whom you have spoken:

_____

8. Do you grant consent that blood samples may be drawn from your dog? Yes / No

I, _____ (name and surname), hereby give permission that my puppy _____ (patient's name), a _____ _____ (breed of dog) may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my puppy. I also understand that I will not be liable for costs of the additional tests as part of the clinical study but that I will be liable for costs pertaining the hospitalisation and treatment of canine parvoviral enteritis and any complications that may arise as a result thereof.

Signed at Onderstepoort on the _____ day of _____ 20_____

Signature Owner/authorised agent _____

Home Tel: _____ Work: _____

Tel: _____Cell No: _____

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APPENDIX F

Client Consent Form: Outpatients

Project title: Markers of endothelial injury and inflammation in canine parvoviral enteritis study.

(To be completed by the patient's owner / authorised agent)

Please encircle Yes or No where necessary

1. Has your puppy received any treatment for canine parvoviral enteritis within the last week? Yes / No
2. Has your puppy received any vaccinations? Yes / No

If yes, how many and when was the last vaccination administered?

_____

2. Have you read the information sheet on canine parvoviral enteritis? Yes / No
3. Have you had the opportunity to ask questions about the research project? Yes / No
4. Have you received satisfactory answers to your questions? Yes / No
5. Have you received enough information about this study? Yes / No
7. Please supply the name of the person to whom you have spoken:

_____

8. Do you grant consent that blood samples may be drawn from your dog? Yes / No

I, _____ (name and surname), hereby give permission that my puppy _____ (patient's name), a _____ - _____ (breed of dog) may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my puppy. I also understand that I will not be liable for costs of the additional tests as part of the clinical study but that I will be liable for costs pertaining the hospitalisation and treatment of canine parvoviral enteritis and any complications that may arise as a result thereof.

Signed at Onderstepoort on the _____ day of _____ 20_____

Signature Owner/authorised agent _____

Home Tel: _____ Work:_____

Tel: _____Cell No:_____

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APPENDIX G

Clinical/Disease Scoring Assessment

Project title: Markers of endothelial injury and inflammation in canine parvoviral enteritis.

Patient F-number: _____

Date: _____

Admission (day 0) ☐

Temp: _____ **Pulse:** _____ **Respiration:** _____

Weight: _____

Encircle the applicable choice below:

Habitus	**1**	**Collapsed/ moribund**
	2	**Severe depression**
	3	**Mild-to-moderate depression**
	4	**Normal**
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**
Vomition	**1**	**Severe (≥ 6 times per 12h)**
	2	**Moderate (3-5 times per 12h)**
	3	**Mild (1-2 times per 12h)**
	4	**Absent**
Faecal consistency	**1**	**Watery diarrhoea, bloody**
	2	**Watery diarrhoea, not bloody**
	3	**Soft**
	4	**Well-formed**

Mucous membranes	**1**	**Congested**
	2	**Pale**
	3	**Normal**
CRT	**1**	**<1 second**
	2	**>2 seconds**
	3	**1–2 seconds**
Total WBC[2]	**1**	**<4 × 10^9/L**
	2	**4-6 × 10^9/L**
	3	**>6 × 10^9/L**
Lymphocyte Count[2]	**1**	**<1 × 10^9/L**
	3	**>1 × 10^9/L**
Serum CRP[32]	**1**	**>100**
	2	**30–100**
	3	**<30**
Albumin[1]	**1**	**<14 g/L**
	2	**14–23 g/L**
	3	**>23 g/ L**

This form has been adapted from Mohr *et al*[18], Goddard *et al*[2] and McClure[32])

Parvoviral enteritis research trial Dr S Pretorius

APPENDIX H

Bloodsmear Protocol

- Clip hair on the ventral surface of the ear near the caudal margin with clippers or curved scissors.
- Use a 23G hypodermic needle to gently pierce the skin or the ear near the caudal margin but away from the marginal ear vein.
- Massaged blood toward the needle hole.
- Use the first drop of blood appearing and collect it with a spreader slide and with this drop of blood make a blood smear.
- The smear will be evaluated according to standard protocol.

Faecal analysis:

- Collect faeces according to the size of the patient. In large dogs collect faeces with a well lubricated gloved finger and in small dogs use a lubricated 1 mL syringe.
- Perform a faecal floatation using the Kyron disposable faecal floatation kit and Kyron egg flotation fluid ($NaNO_3$).
- The faecal floatation and wet preparation will be evaluated according to standard protocol.

Collection of faeces for Electron microscopy

- Sampling method depends on the size of the patient:
    - Larger puppies (>8 kg): digital collection (lubricated glove finger).
    - Smaller puppies (<8 kg): lubricated 1mL syringe inserted rectally and aspiration of faeces.
        - Collect at least 0.1mL of faeces
        - The faecal sample will be refrigerated once collected and will be submitted to the Faculty of Veterinary Science Electron Microscopy Unit for faecal EM within 12 hours of collection.
        - Samples collected after hours will be refrigerated for a maximum of 72 hours.

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APPENDIX I

Data Capture Sheet

Date:

Day of study: Admission (day 0) ☐

Owner:		
Owner no:		
Species:	**Sex:**	**Age:**
Breed:		
Weight:		
Patient name:		
Patient number:		

History

Chief complaint	
Duration of illness	
When did the dog last eat?	
Treatment received after diagnosis and collection of samples	

Physical examination

Parameter	Admission			Day 1		
Mentation (1 – 4+)						
Vital Signs	T:	P:	R:	T:	P:	R:
Mucous Membranes and CRT						
Peripheral Lymph nodes						

Abdominal palpation		
Faecal colour and consistency		
Helminth ova score (1 – 4+)		

Patient Outcome

Died/recovered/euthanased?	
Date (died/recovered/euthanased)	
Time (died/euthanased)	
Days to recovery/death	

Serum tube collected and sent to Clin Path Lab ☐ Store Serum ☐

EDTA tube collected and sent to Clin Path Lab ☐ CBC performed ☐ Store EDTA ☐

Heparin tube collected and sent to Clin Path Lab ☐ Store Heparin ☐

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APPENDIX J

Check List:

1. History ☐
2. Clinical examination ☐
3. CPV ELISA performed ☐
4. Peripheral blood smear ☐
5. Faecal Floatation done ☐
6. Faecal collection done and stored to be sent for EM ☐
7. Atraumatic blood collection via jugular vein:

   Using a venepuncture and a 21G needle, collect blood in the following sequence:

   3mL whole blood in a 4mL Brain serum tube. RED ☐

   3mL whole blood in a 4mL EDTA tube. PURPLE ☐
8. After blood collection:
   - Let serum sample clot. After clotting centrifuge the serum sample at 1000g for 10 min within 2 hours of collection. ☐
   - Aliquote the serum sample into a 0.5mL cryovial and store at -80°C until analysis can be performed. ☐
   - Centrifuge the EDTA sample at 1000g for 15 min within 2 hours of collection. ☐
   - Aliquote the plasma into a 0.5mL cryovial and store at -80°C until analysis can be performed. ☐

The samples will be stored in the forma scientific freezer that is set at -86°C.

APPENDIX K

OVAH Isolation Unit Treatment Guidelines

Intravenous catheters only to be placed after collection of blood samples.

All results will be made available as soon as received:

- **CBC (Ht) and coagulation costs covered by study**
- **On admission: TSP, Alb, Na, K, Cl will be performed in the ClinPath laboratory on collected serum but is paid for by owner.**
- **Glucose can be tested using a drop of the collected blood on the handheld glucometer**

FLUID THERAPY: CRYSTALLOIDS

At admission, determine the presence of:

- Dehydration (Table 1)
- Hypoperfusion/hypovolaemia

Table 1: Subjective parameters used to estimate the degree of dehydration

Estimated degree of dehydration	**Clinical signs**
< 5%	History of vomiting or diarrhoea or other fluid loss, normal mucous membranes, unable to detect <5% on physical examination
5%	History of vomiting or diarrhoea or other fluid loss, tachy or dry mucous membranes
7%	History of vomiting or diarrhoea or other fluid loss, dry mucous membranes, increased skin tenting, tachycardia, normal pulse quality and arterial blood pressure
10%	History of vomiting or diarrhoea or other fluid loss, dry mucous membranes, increased skin tenting, tachycardia, weak pulses, hypotension
12%	History of vomiting or diarrhoea or other fluid loss, dry mucous membranes, sunken eyes, increased skin tenting, tachycardia or bradycardia, weak to absent pulses, hypotension, cold extremities, hypothermia

Type of fluid:

- Isotonic crystalloid: Ringer's lactate

Rate of fluid:

Hypovolaemia:

- 10/kg IV boluses over 15 minutes with frequent re-assessment of perfusion parameters.

- The end-point of fluid resuscitation is when normal perfusion parameters are met or up to a total volume of 80-90ml/kg.

Dehydration without shock:

- Replacement of dehydration (% dehydration x Body Weight x 10)
  Replace over 6 -24 hours (based on rate of development of dehydration)

PLUS

- Maintenance (40 – 100ml/kg/day)

PLUS

- Ongoing losses (estimated ml lost with vomit/diarrhoea + insensible losses)

Once hydrated:

- Maintenance (40 – 100ml/kg/day) **PLUS** ongoing losses or 2x maintenance requirements.

Take the size and age of the dog into consideration when deciding on the rate of fluid therapy.

FLUID THERAPY: COLLOIDS

Type of fluid:

- Hydroxyethyl starches (Voluven®)
- Gelatine-based products (Gelofusin®)

Rate of fluid:

- Initial bolus **10 – 20 ml/kg** over **20 minutes** OR **CRI: 0.5-2ml/kg/h**
- When combining colloids with crystalloids the dose of crystalloids is decreased by 50% to avoid volume overload.
- Do not exceed20ml/kg body weight per 24 hours

Cases with significant anaemia may necessitate blood (packed red blood cell) transfusions and where significant thrombocytopaenia is present, fresh whole blood will be required.

POTASSIUM SUPPLEMENTATION:

Use the following table as a guideline:

Potassium supplementation	
Serum Potassium (mmol/L)	**Potassium addition per 1000ml**
3.5 – 5.5 (normal)	20 mEq
3.0 – 3.4	30 mEq
2.5 – 2.9	40 mEq
2.0 – 2.4	60 mEq
< 2.0	80 mEq

*** NB: The maximum rate at which potassium can be infused is **0.5mg/kg/h**
*** Potassium is administered within the intravenous fluids, never by bolus injection.

GLUCOSE:

- If the glucose level is below 3 mmol/L and the patient is severely depressed or seizuring:
    - give a bolus of **0.5 - 1 ml/kg** of **50% dextrose** diluted with the same volume of sterile water
    - follow up by adding dextrose to the intravenous fluids as a 2.5% dextrose solution
- If hypoglycaemia persists, a 5% solution may be indicated.

- In normoglycaemic anorexic patients that are at risk for developing hypoglycaemia, a 1% solution may be used to maintain normoglycaemia.

Glucose Supplementation		
Amount of fluid (Ringer's lactate)	**Amount in ml of 50% dextrose to add**	
	2.5 % Solution	**5% Solution**
250 ml	12.5	25
500 ml	25	50
1000 ml	50	100

ANTIBIOTICS:

During the first 24 hours after admission, the following antibiotics are to be given to all patients:

Antibiotic	Dose	Route	Frequency
Ampicillin	20mg/kg	IV	q8h
Metronidazole	15mg/kg	IV	q12h

ANTI-EMETICS& PRO-KINETICS:

During the first 6 hours after admission, the following anti-emetic treatment is allowed:

Anti-emetic	Dose	Route	Frequency
Maropitant ** (Cerenia®)	1mg/kg	SC	q24h for up to 5 consecutive days

** Only for use in dogs older than 8weeks of age.

NUTRITION:

Enteral nutrition should be initiated as early as possible.

Resting energy requirements is calculated as follows:

- Bodyweight-linear formula (dogs >2kg and <45kg)
  [BW x 30] + 70 = kcal/24 hours

- The daily caloric requirement is divided into 9 meals.
- Eukanuba High calorie: 1 tin mixed with 50ml of water and blended provides 1.6kcal/ml.

- In tube fed animals, the feeding tube is flushed with water (5-20ml depending on patient size) at each meal prior to feeding and after feeding to avoid blocking of the tube.
- Maximum stomach capacity in debilitated dog can be estimated as 10ml/kg body weight initially.
- Patients that do not tolerate larger volumes can be fed small volumes more often even hourly. If small frequent meals are not tolerated, continuous rate infusion (CRI) feeding /trickle feeding can be attempted.
- The feeding infusion should be interrupted 8hourly and residual stomach volume determined via suction on the feeding tube to ensure that delayed gastric emptying is not present.

ANALGESIA:

In cases with severe abdominal pain, the following analgesics can be used:

Analgesic	Dose	Route	Frequency
Buprenorphine (Temgesic®)	0.02mg/kg	IV	q8-12h
Fentanyl (Fentanyl®)	3ug/kg/h	IV	CRI

ANTHELMINTICS:

An anthelmintic will only be administered after the last blood collection for TEG (REP + 215 min).

Anthelmintic	Dose	Route	Frequency
Fenbendazole (Panacur®)	50mg/kg	PO	q24h for 5days

Adapted from previous studies *Whitehead et. al*.