Oxidative burst and phagocytic activity of phagocytes in canine parvoviral enteritis

by

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Submitted in partial fulfilment of the requirements for the degree MMedVet (Clinical Laboratory Diagnostics), Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria

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Ethics

The author, Kelly du Preez, has obtained applicable research ethics approvals for the work described in this dissertation. These approvals were granted by the Research Ethics Committee of the Faculty of Veterinary Science (REC039-18) and Animal Ethics Committee of the University of Pretoria (V048-19).

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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PROJECT TITLE	**Oxidative burst and phagocytic activities of neutrophils in canine parvoviral enteritis.**
PROJECT NUMBER	**REC039-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Kelly du Preez**

DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

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Animal Ethics Committee

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PROJECT NUMBER	**V048-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. K du Preez**

STUDENT NUMBER (where applicable)	**U_27177450**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SPESIES/SAMPLES	***Canis lupus familiaris* (Domesticated)**
NUMBER OF ANIMALS	**56 *without* concurrent infection.** ***A maximum sample size of 70 is approved***
Approval period to use animals for research/testing purposes	**June 2018 - June 2019**
SUPERVISOR	**Prof. A Goddard**

KINDLY NOTE:

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APPROVED (* Condition)	Date 25 June 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

Condition: The number of animals tested, but not recruited, should also be reported to the committee

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Abbreviations

ARDS – Acquired respiratory distress syndrome
C5a – Complement factor 5a
CARS – Compensatory anti-*inflammatory* response syndrome
CBC – Complete blood count
CD – Cluster of differentiation
CHOP – Combination chemotherapy of cyclophosphamide, doxorubicin, vincristine and prednisone
CI – Confidence interval
CPE – Canine parvoviral enteritis
CPV-2 – Canine parvovirus type 2
CRP – C-reactive protein
CX3CR1 – CX3C chemokine receptor 1
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme-linked immunosorbent assay
FeLV – Feline leukaemia virus
FITC – Fluorescein isothiocyanate
FS – Forward scatter
GM-CSF - Granulocyte macrophage colony stimulating factor
IL – Interleukin
MFI – Mean fluorescence intensity
MODS – Multiple organ dysfunction syndrome
MPO – Myeloperoxidase
$NADP^+$ – Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH – Nicotinamide adenine dinucleotide phosphate oxidase (reduced form)
NETs – Neutrophil extracellular traps
ops. – Opsonised
PKC – Protein kinase C
PMA – Phorbol-12-myristate-13-acetate
rhG-CSF – Recombinant human granulocyte colony stimulating factor
rhGM-CSF – Recombinant human granulocyte macrophage colony stimulating factor
ROS – Reactive oxygen species
RT – Room temperature
SIRS – Systemic inflammatory response syndrome

SS – Side scatter

TEM – Transmission electron microscopy

TNF- α – Tumour necrosis factor α

Summary

Canine parvoviral enteritis (CPE) is a serious disease characterised by systemic inflammation and immunosuppression. The function of circulating phagocytes (neutrophils and monocytes) in affected dogs has not been fully investigated. The aim of this study was to characterise the functional capacity of canine phagocytes in CPE by determining their oxidative burst and phagocytic activities using flow cytometry. Blood was collected from 28 dogs with CPE and 11 healthy, age-matched, control dogs. Oxidative burst activity was assessed by stimulating phagocytes with opsonised *Escherichia coli* or phorbol 12-myristate 13-acetate (PMA) and measuring the percentage of phagocytes producing reactive oxygen species and the magnitude of this production. Similarly, phagocytosis was measured by incubating phagocytes with opsonised *E. coli* and measuring the percentage of phagocytes containing *E. coli* and the number of bacteria per cell. Complete blood counts and serum C-reactive protein (CRP) concentrations were also determined. Serum CRP concentration was negatively and positively correlated with segmented and band neutrophil concentrations, respectively. Overall, no differences in phagocyte function were found between the dogs with CPE and healthy control dogs. However, infected dogs with neutropenia or circulating band neutrophils had decreased PMA-stimulated oxidative burst activity compared to healthy controls. Additionally, CPE dogs with neutropenia or circulating band neutrophils showed decreased PMA- and *E. coli*-stimulated oxidative burst activity and decreased phagocytosis of *E. coli* compared to CPE dogs without neutropenia or band neutrophils. In conclusion, there is evidence that canine phagocytes have decreased oxidative burst and phagocytic activity in neutropenic CPE dogs and CPE dogs with circulating band neutrophils.

Chapter 1 Introduction

Background

Canine parvoviral enteritis (CPE) is a potentially fatal viral disease affecting young dogs with naïve immune systems.[1,2] Canine parvovirus-2 (CPV-2) infects rapidly dividing cells of the gastrointestinal tract and lymphohaematopoietic system, causing gastrointestinal and immunosuppressive disease.[2,3] Consequently, both systemic inflammatory response syndrome (SIRS) and sepsis are common complications in CPE.[1,4]

Blood phagocytes (neutrophils and monocytes) play an important role in preventing sepsis through a number of functions, including phagocytosis and production of reactive oxygen species (ROS).[5] Phagocytosis is triggered by the activation of surface pattern recognition receptors, resulting in internalisation of antigens within phagosomes.[6] These antigens are subsequently destroyed within the phagosome through oxygen-dependent (ROS production) and oxygen-independent mechanisms (antimicrobial peptides and enzymes).[7,8] This process of ROS production is known as oxidative or respiratory burst and is not only triggered by phagocytosed particulate stimuli (e.g. opsonised bacteria) but also by soluble stimuli (e.g. chemotactic peptides).[7] If these functions are impaired or if circulating phagocyte numbers are decreased, the risk of sepsis increases.[9,10]

While neutropenia and monocytopenia are well described in CPE,[1,2] evaluation of phagocyte function in affected dogs is limited. To the author's knowledge, only one unpublished study in dogs infected with CPV-2 demonstrated decreased neutrophil oxidative burst activity.[11] Decreased phagocyte and neutrophil oxidative burst activities have been reported in dogs with critical illness and sepsis.[12,13] In human medicine, phagocyte dysfunction has been described in septic children[14] and adults[15,16] as well as in animal models of sepsis.[17,18] Phagocyte dysfunction is considered a negative prognostic indicator[16] and may be progressive as the severity of sepsis increases.[15] Similarly, significant phenotypic and functional alterations of phagocytes have been described in SIRS, resulting in an increase or decrease in oxidative burst and phagocytic activities.[19]

Aims and objectives

We aimed to determine whether phagocyte dysfunction was present in dogs with CPE. Furthermore, if phagocyte dysfunction was present, we aimed to characterise this altered function.

We fulfilled this aim using flow cytometry to measure and compare the oxidative burst and phagocytic activities of the phagocytes of dogs with CPE to apparently healthy, age-matched, control dogs.

Hypothesis

We hypothesised that phagocyte dysfunction will be present in dogs with CPE compared to apparently healthy, age-matched control dogs. Specifically, dogs with CPE will have decreased oxidative burst and phagocytic activities.

Benefits

We anticipated that this study would provide additional information on the immune response in dogs suffering from CPE. In broader terms, this study would provide information regarding phagocyte function in critical illness and systemic inflammation, using CPE as a model. This information may assist future researchers in the design of novel, targeted therapies for use in CPE and canine critical illness and systemic inflammation. Lastly, this study would fulfil (in part) the requirements of the principal investigator's MMedVet (Clinical Laboratory Diagnostics) degree.

Chapter 2 Literature Review

Pathogenesis of canine parvoviral enteritis

Canine parvoviral enteritis is a common viral disease caused by CPV-2, a variant of *Carnivore protoparvovirus 1*.[3,20] Dogs without passive immunity (failure of passive transfer or waning maternal antibody titres) or acquired immunity (insufficient immunisation through vaccination) to CPV-2 are at risk of developing CPE.[1,21] For this reason, dogs under six months of age are most commonly affected.[1]

Once transmitted through ingestion, CPV-2 causes viraemia before infecting the rapidly dividing cells of the body, most importantly the cells of the lymphohaematopoietic system and the gastrointestinal tract.[22,23] Gastrointestinal disease manifests as vomiting, diarrhoea, hyporexia or anorexia, protein-energy malnutrition and weight loss.[1,24] Infection of the lymphohaematopoietic system results in leukopenia due to neutropenia, monocytopenia and lymphopenia, which contributes to the immunosuppressive state present in affected dogs.[2,3] Additionally, CPE often causes systemic inflammation, particularly in severely affected dogs. Previous studies have demonstrated increased positive acute phase proteins, namely serum C-reactive protein (CRP), ceruloplasmin and haptoglobin in dogs with CPE.[4,25] Increased circulating tumour necrosis factor α (TNF-α), a pro-inflammatory cytokine, and circulating endotoxins have also been demonstrated in dogs affected by CPE.[26,27] Leukopenia, increased serum CRP and increased TNF-α are all considered to be negative prognostic indicators in CPE.[2,4]

Ultimately, the combination of bacterial translocation through a compromised gastrointestinal mucosa and immunosuppression render affected dogs susceptible to sepsis.[2,26,28] Systemic inflammatory response syndrome is also common in CPE. In several studies conducted in dogs with CPE, a third to more than half of affected dogs met the criteria for diagnosis of SIRS.[1,4,29,30] Unsurprisingly, both sepsis and SIRS have been associated with increased risk of mortality in dogs affected by CPE.[1,26,31]

Functions of phagocytes

Blood phagocytes (neutrophils and monocytes) are vital, as part of the innate immune system, for the non-specific host defence against infectious agents.[32] For this reason,

phagocyte function in an important factor affecting immune system competency.[32] In brief, phagocytes are recruited to sites of inflammation by chemotaxis in response to released chemoattractants.[33] Once within the tissue, activated phagocytes are responsible for the production and release of pro-inflammatory cytokines and, through degranulation, neutrophils release cytotoxic and antimicrobial proteins.[33,34] As a result of their cytoskeletal structure, phagocytes are capable of internalising microorganisms into a phagocytic vacuole or phagosome.[5,35,36] Intracytoplasmic phagosome maturation and fusion with lysosomes results in the formation of a phagolysosome.[5,36] Within the phagolysosome, oxygen-dependent and oxygen-independent mechanisms are used to complete microbial killing.[36,37] Where oxygen-independent microbial killing relies on antimicrobial peptides and enzymes, oxygen-dependent mechanisms make use of the production of ROS in a process called oxidative or respiratory burst.[7,37] The oxidative burst reaction (Figure 1) occurs when nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme composed of five subunits, catalyses the formation of superoxide and hydrogen peroxide.[7,33] Myeloperoxidase (MPO) is another important enzyme in ROS production due to its role in the formation of hypochlorous (from hydrogen peroxide) and hypothiocyanous acid.[38,39]

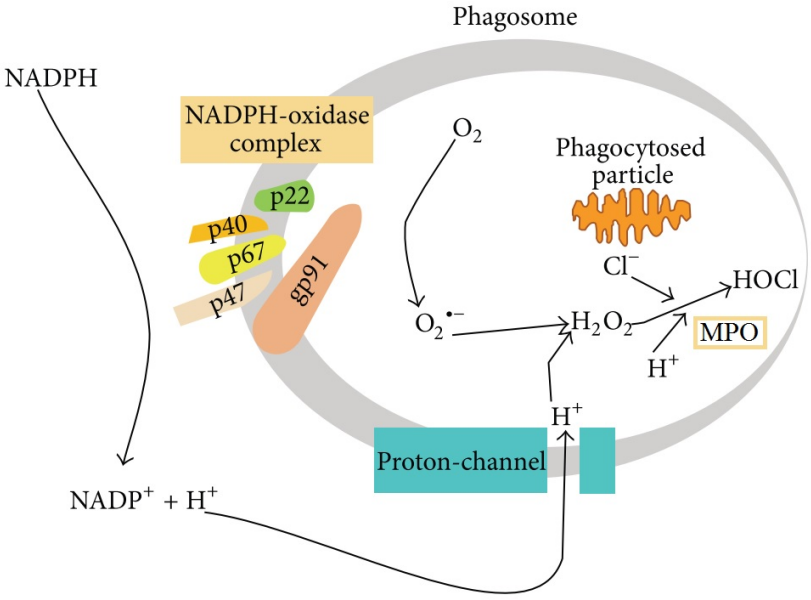

Figure 1 Oxidative burst reaction depicting the production of reactive oxygen species (ROS) through the enzymatic reactions of NADPH oxidase and MPO. The five subunits of NADPH oxidase, namely $p22^{phox}$, $p40^{phox}$, $p67^{phox}$, $p47^{phox}$ and $gp91^{phox}$, are shown in association with the phagosome membrane. $NADP^+$ – nicotinamide adenine dinucleotide phosphate; NADPH – reduced form of $NAPH^+$; O_2 – oxygen; $O_2^{\bullet-}$ – superoxide free radical; H^+ - hydrogen ion; H_2O_2 – hydrogen peroxide; Cl^- – chloride ion; MPO – myeloperoxidase; HOCl – hypochlorous acid. Adapted from Al Maruf, A and O'Brien, P – Respiratory burst initiated by NADPH oxidase.[40]

An additional mechanism of microbial killing, unique to neutrophils, is the formation of neutrophil extracellular traps (NETs).[41,42] These net-like structures consist of DNA and proteins that form once the neutrophil ruptures, binding microorganims.[41,42] These structures have been described in dogs.[43]

Tests of phagocyte function

There are several available tests that can be used to evaluate the various functions of neutrophils and monocytes. These tests are typically divided into classical assays and flow cytometric assays.[44]

Classical assays

Classical functional assays are used to evaluate individual functions; thus, a panel of tests is required to obtain a complete evaluation of neutrophil and/or monocyte function. When using classical assays, separation of the neutrophils and/or monocytes is necessary.[45] This can be achieved by osmotic lysis of other cells,[49] sedimentation of erythrocytes and mononuclear cells using dextran,[46] gradient centrifugation,[49] cold-aggregation,[47] and/or the use of antibody-coated magnetic beads.[45,47]

Chemotaxis and chemokinesis, adherence, particle uptake and killing can all be evaluated using these types of assays. Chemotaxis and chemokinesis assays are performed by adding a chemoattractant to a population of neutrophils or monocytes and assessing their movement through a migration chamber.[48-50] Adherence assays are performed by incubating neutrophils or monocytes with an inorganic[51] or organic[52,53] substance and determining the percentage of adhered cells. Particle uptake assays assess the ability of the neutrophils or monocytes to phagocytose various types of particles (organic[46,54] or inorganic[50,55]) which are often fluorescence labelled and, subsequently, measured by a fluorescence plate reader. Lastly, killing assays assess the ability of the phagocyte to kill microbes, either by reducing the activity of bacteria incubated with the phagocytes[56] or through the production of ROS, measured by chemiluminescence or fluorescence plate readers.[57]

Cytokine production by phagocytes can also be measured and has been performed in several studies in humans and veterinary species. Whole blood is mixed with a variety of activating factors and the concentration of various important cytokines (e.g. TNF-α,[18,58,59]

interleukin(IL)-6,[58,60,61] IL-8,[58] IL-10[60,61]) are measured using a canine-specific multiplex bead-based assay[60,61] or enzyme-linked immunosorbent assay (ELISA).[18,58,62] The concentration of various neutrophil and monocyte adhesion molecules can also be determined with the use of ELISA[62] and immunofluorescence.[63] Lastly, apoptosis and cell viability can be determined by staining the cells with trypan-blue[60] or a mixture of acridine orange and ethidium bromide,[64] both of which only stain non-viable cells.

Flow cytometry

Flow cytometric assessments of phagocyte function have largely replaced the classical assays due to their convenience.[44] Light from a laser is passed through a sample of whole blood resulting in forward and side light scattering.[12,59] Based on their light scatter properties, the populations of cells within the sample are separated and identified based on their size (determined by forward scatter) and internal granularity or complexity (determined by side scatter).[65] This information is reported onto a two dimensional scatterplot and/or histogram where the population of interest may be gated (i.e. demarcated from the other cells in the sample) (Figure 2).[12,65] Cells may be further identified and separated using antibody markers and fluorochromes, the choice of which are determined by the availability, cross-reactivity and type of laser used.[65] The properties of the population of interest may then be evaluated depending on the type of markers or kits used to process them.[65] Thus, cell separation, which is time consuming and may introduce pre-analytical variation, is not always necessary for flow cytometric assays.[44,66]

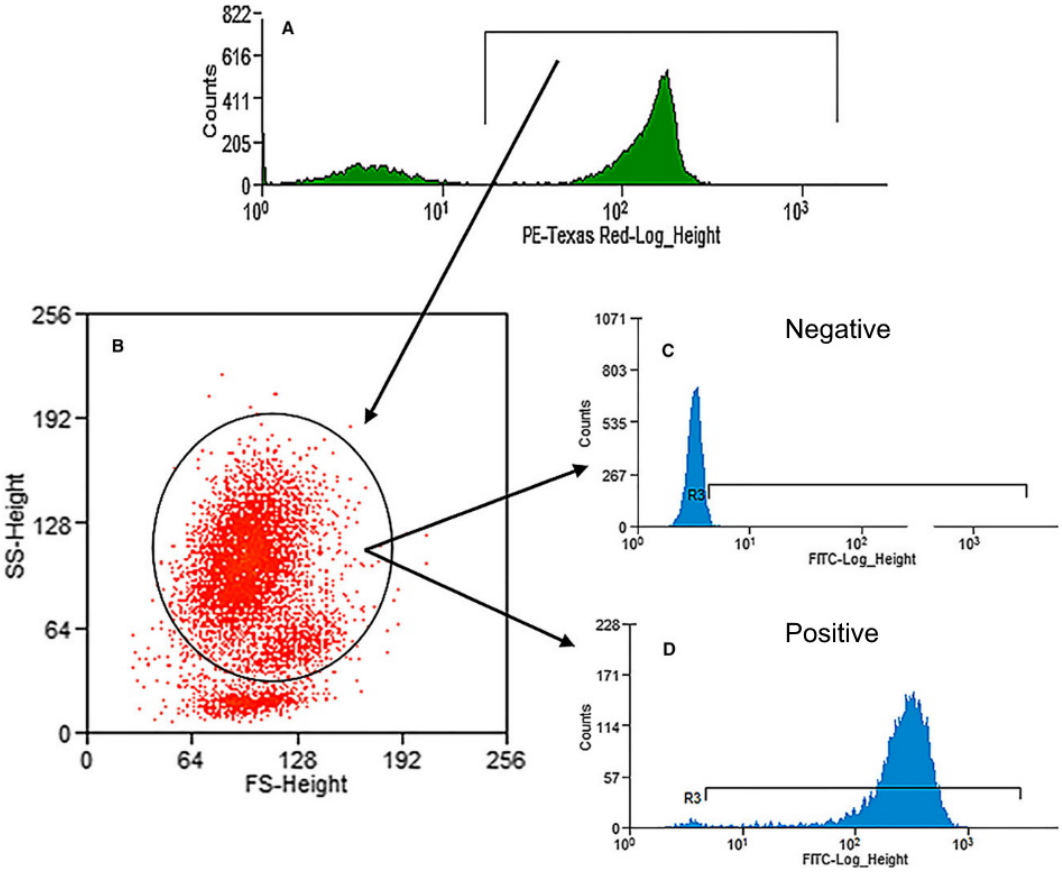

Figure 2 Example of a gating strategy using a scatterplot and histograms to evaluate the oxidative burst and phagocytic activities in a population of phagocytes (i.e. neutrophils and monocytes). (A) Histogram used to identify viable cells (green peak on the right as indicated by the black square bracket). (B) Scatterplot with a black circle indicating the phagocytes (population of interest) – the larger red population represents neutrophils and the smaller red population to the bottom right of the circle represents monocytes. (C) Histogram of the negative control sample indicating the population of fluorescence negative cells (outside the black bracket) and a population of less than 3% fluorescence positive cells (within the black bracket). (D) Histogram of a patient sample indicating a population of fluorescence positive cells (inside the black bracket) – in this case the majority of cells are fluorescence positive. SS – side scatter; FS – forward scatter; FITC - fluorescein isothiocyanate. Adapted from Hoffman et al. – Example of the gating scheme for phagocytosis and respiratory burst function.[12]

Numerous phagocyte functions, including phagocytic activity,[12,67,68] oxidative burst activity,[12,67,68] migration,[69,70] cell surface receptors,[10,71] apoptosis,[72] and cytokine production[36] have been evaluated using flow cytometry in both humans and animals. Many such function studies prioritise assessment of oxidative burst and phagocytic activities of the neutrophils and/or monocytes when evaluating a certain disease or condition.

Oxidative burst and phagocytic activity studies

Humans

Numerous studies evaluating phagocyte oxidative burst and/or phagocytic activities have been conducted in human medicine. These studies encompass a wide range of diseases and conditions such as immunosuppressive disease (e.g. human immunodeficiency virus infection[71]), neoplasia (e.g. multiple myeloma,[73] leukaemia[74]), physical injury (e.g. severe burn wounds,[75] minor surgery[68]), cerebrovascular accidents,[70,76] and recurrent bacterial infections.[77]

Amongst these studies, a major area of investigation has focussed on the function of neutrophils and monocytes in SIRS and sepsis. In these pathological inflammatory states, both decreased oxidative burst[15,78-80] and/or phagocytic[14-16,79-81] activities and increased oxidative burst[82] and/or phagocytic[14,82,83] activities have been reported in children and adults. These differing functional alterations measured in the phagocytes in SIRS and sepsis can be explained by the progressive response of the host to inflammation.[14,84] In early sepsis and SIRS, pro-inflammatory cytokines (e.g. IL-8, IL-15) predominate[85,86] and receptors important for phagocyte function (e.g. Cluster of Differentiation [CD] 64) are upregulated.[87] As the inflammation persists and increases in severity, immune modulating cytokines (e.g. IL-10), which are known to decrease phagocyte function,[88,89] increase in concentration and receptors are internalised or down-regulated (e.g. CD64, CX3CR1, C5a receptor).[16,18,90] This process is known as compensatory anti-inflammatory response syndrome (CARS) and when this response predominates, the patient enters a state of immunoparalysis.[88] The resultant phagocyte dysfunction has been demonstrated in patients with acquired respiratory distress syndrome (ARDS),[79] severe sepsis,[15] and endotoxin immunotolerance.[78]

Dogs

As with human medicine, there is a large body of literature describing oxidative burst and phagocytic activities in canine phagocytes in various conditions, diseases and therapeutic regimens. A significant proportion of these investigations made use of the Phagotest and Phagoburst kits (Glycotope Biotechnology) for flow cytometry.[59-61,91,92] Most studies report neutrophil function only, however, in those studies using the Phagotest and Phagoburst kits, only a small proportion of authors published forward-scatter vs side-scatter plots to

illustrate their gating strategy.[12,59,93,94] Based on the author's experience with these kits and based on discussions with technicians using these kits in human laboratories (B Strybos, personal communication), the distinction between the neutrophil and monocyte populations is inconsistent between various individuals and stimulants (Figure 3). Thus, some of the studies using the above-mentioned kits may have been investigating the function of the phagocyte population (i.e. neutrophil and monocytes), rather than the neutrophil population alone.

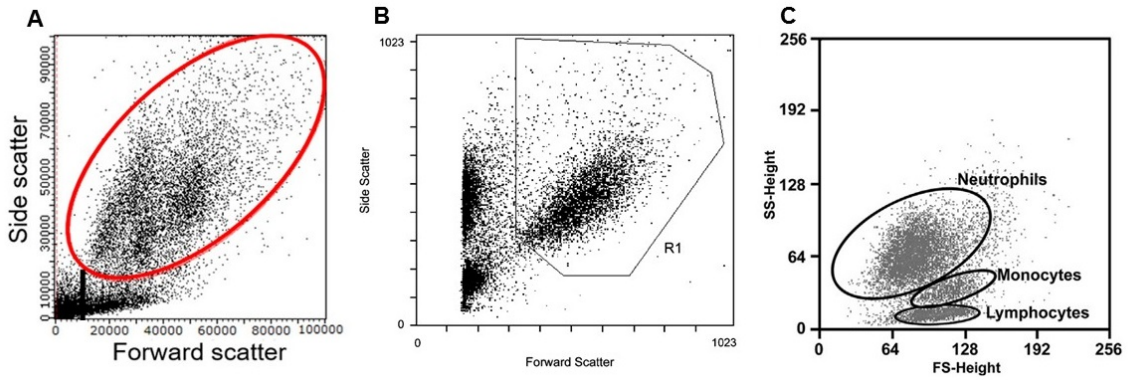

Figure 3 Scatterplots from three different publications using the Phagotest and Phagoburst kits showing inconsistent distinction between the neutrophil and monocyte populations. (A) and (B) No distinction between the neutrophil and monocyte populations is present so that one population of phagocytes (neutrophils and monocytes) are gated. (C) Clear distinction between the neutrophil and monocyte populations so that separate neutrophil and monocyte populations are gated as indicated by the labels. SS – side scatter; FS – forward scatter. Adapted from (A) Allison et al. – Gating scheme for flow cytometry of phagocytosis and oxidative burst following exposure to FITC-labelled *Escherichia coli*[93]; (B) LeBlanc et al. – Forward vs side scatter plot[59]; and (C) Woode et al. – Gating scheme for flow cytometry.[60]

Based on the literature, the most investigated infectious disease is canine leishmaniasis. Multiple studies have shown decreased oxidative burst and phagocytic activities in the phagocytes of naturally infected dogs.[95-97] This dysfunction was associated with more severe forms of disease and was attributed to prolonged phagocyte activation, increased apoptotic rates and uraemia, all of which are more prevalent in advanced leishmaniasis.[97] Similar immunosuppressive effects secondary to phagocyte dysfunction have also been described in experimental *Bartonella vinsoni* spp*. berkhoffii* infections.[98] To the author's knowledge, only one study investigating the effects of CPE on phagocyte function has been conducted.[11] Using chemiluminescence, non-surviving dogs with CPE demonstrated significantly lower neutrophil oxidative burst activity when compared to survivors.[11] This dysfunction of the neutrophils was identified as a negative prognostic indicator, independent of leukopenia and/or neutropenia.[11]

Phagocyte dysfunction has also been described in multiple chronic diseases. Dogs suffering from chronic kidney disease have decreased oxidative burst activity, likely secondary to uraemic toxins.[99] By contrast, a more recent study found that oxidative burst activity was increased in uraemic dogs – an effect which has also been reported in cats.[72,100] A likely reason for the discrepant results was provided by Cendoroglo et al., who suggested that uraemia may initially increase oxidative burst activity but, as it also induces neutrophil apoptosis, decreased oxidative burst activities are seen in the later stages of uraemia.[101] Interestingly, in studies evaluating chronic[102] and recurrent[103] deep pyoderma, no phagocyte dysfunction was found. Thus, further immunological investigation to determine the cause of susceptibility to deep pyoderma in affected dogs is warranted.[103] Phagocyte function has also been investigated in numerous neoplastic diseases. Sarcomas and carcinomas have been shown to elicit decreased oxidative burst (both) and phagocytic activity (sarcomas only) in the neutrophils of affected dogs.[59] In a follow-up study conducted *in vitro*, recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) showed therapeutic promise in that it was able to improve the sarcoma-associated dysfunction.[94] This colony stimulating factor has also been suggested as the possible cause for the markedly increased oxidative burst and phagocytic activities reported in the neoplastic neutrophils of a dog with chronic myeloid leukaemia.[104] Although rhG(M)-CSF and recombinant canine GM-CSF have been investigated in dogs with CPE, their use is controversial.[28] Several studies have shown that these treatments do not significantly improve survival and thus, they are not widely recommended for the treatment of CPE.[105-107]

Certain breeds, including Weimaraners,[108] Grey Collies,[109] and Doberman Pinchsers[110] have been linked with congenital defects of neutrophil oxidative burst activity. In all three reports, affected dogs showed recurrent infections, highlighting the importance of the oxidative burst reaction as part of the host defence. A presumed congenital defect in neutrophil phagocytosis has also been report in a Weimaraner puppy with a history of recurrent infections.[111] Lastly, a case report of two related mixed breed dogs with familial β_2-integrin-related neutrophil dysfunction (a condition similar to canine leukocyte adhesion deficiency) showed that the neutrophils of these dogs had both decreased oxidative burst and phagocytic activities.[10]

Studies in apparently healthy dogs have shown wide variations in both phagocytic and oxidative burst activities.[66] For this reason, population-based reference intervals for these variables are not recommended.[66] Additionally, it is recommended that the results of phagocyte function assays be interpreted by one examiner in order to minimise inter-assay

variation.[66] In doing so, post analytical variability may be reduced in both classical and flow cytometric function assays. Conflicting data on the effect of age on phagocyte function has been published. In one study that included a group of Labrador Retriever dogs between the ages of 4 and 11 years, age had no significant effect on neutrophil phagocytic activity.[112] This study also showed that, like age, sex did not affect phagocyte function.[112] In contrast, another study revealed an age related decrease in neutrophil phagocytosis, evident from the ages of 1 to 10 years in Beagles.[113] Therefore, based on this physiological effect, age should be taken into consideration when investigating phagocyte dysfunction (e.g. age matched control group, similar aged patients in study population). The effect of psychological stress, which is known to cause neutrophil dysfunction in humans,[114] was determined in a group of apparently healthy shelter dogs housed for period of more than 7 days.[93] The phagocytes of these dogs had decreased oxidative burst and phagocytic activities when compared to healthy age, sex and breed-matched control dogs.[93] Lastly, the effects of progesterone on the neutrophils of healthy intact bitches in oestrus have been assessed by measuring neutrophil function in various phases of the oestrus cycle.[90] Compared to the neutrophil function in the early luteal phase, there was both decreased oxidative burst and phagocytic activity in the late luteal phase of oestrus.[90] The authors suggested that this dysfunction could be a contributing factor to the increased incidence of pyometra in the late luteal phase.[90]

Finally, of all the literature available, the publications most relevant to our study are those assessing phagocyte function in inflammation, SIRS, sepsis and critical illness. While this is a major area of research in human medicine, only a small number of studies have been conducted in dogs. In one early study, acute inflammation was induced by injecting turpentine intramuscularly into a group of dogs.[115] The authors discovered that, while phagocytosis was unaffected, the microbial killing of *Staphylococcus aureus* was decreased in the neutrophils and that there was a wide variation in the neutrophil response to the bacteria.[115] Later, a study characterising phagocyte surface markers in septic and non-septic inflammation demonstrated that dogs suffering from sepsis and multiple organ dysfunction syndrome (MODS) had decreased neutrophil granularity and decreased monocyte major histocompatibility complex II expression.[116] The authors concluded that these findings likely indicate CARS in the dogs suffering from severe inflammation.[116] The results from another study that included only septic dogs elaborated on these findings.[13] The neutrophils of these dogs had decreased oxidative burst activity but increased phagocytosis in neutrophils, indicating some dysfunction secondary to sepsis.[13] While the authors were surprised by the increased phagocytic activity, they concluded that this was likely because the severity of sepsis in most of the dogs was not enough to elicit

immunoparalysis.[13] This is supported by the lower mortality rate in the cohort of included dogs (15.4%)[13] compared to previously reported mortality rates for canine sepsis (25 – 70%).[117-119] The most recent study investigating phagocyte function in critical illness included a small number of dogs suffering from various inflammatory diseases and conditions, including CPE.[12] As with previous studies, the phagocytes had decreased oxidative burst activity providing further evidence of immune dysfunction secondary to severe inflammation.[12] This study did not, however, find differences in phagocytic activity between critically ill and healthy dogs.[12]

Other species

Although many reports on phagocyte function in other species have been published, most of these studies pertain to cattle, with an emphasis on dairy cows. This is due to the high incidence of peri-parturient immunosuppression and negative energy balance in this species.[120,121] Several studies have shown that in the peri-parturient phase[122] and in early lactation,[123] the neutrophils have decreased oxidative burst and phagocytic activity, particularly in multiparous cows.[124] This can be attributed, in part, to ketosis. In ketotic states, bovine neutrophils have impaired function as shown by *in vivo*[120,121] and *in vitro*[120] studies. This altered immunity predisposes them to mastitis and other infections in early lactation.[124,125] The immunosuppressive effect of ketosis has also been observed in sheep. In two studies by the same group, ewes had decreased oxidative burst activity with mild ketosis and decreased neutrophil phagocytic activity with severe ketosis.[50,51] This effect was only seen with increased serum β-hyroxybutyrate concentrations.[50,51] Sheep suffering from experimentally-induced endotoxaemia also show decreased oxidative burst and phagocytic activities.[126]

Various studies of phagocyte function have been conducted in cats, often focussing on feline leukaemia virus (FeLV) infections, in which decreased phagocyte oxidative burst[127,128] and phagocytic[128] activities have been reported. This dysfunction of the innate immune system is a likely contributory factor to an immunosuppressive state in affected cats.[127,128] The effects of immunosuppressive doses of dexamethasone have also been studied in cats. This drug reportedly causes decreased phagocytosis and variable oxidative burst effects and should be used with caution in cats with compromised immunity.[129] In horses, phagocyte dysfunction has been described in pars pituitary intermedia dysfunction, a condition commonly complicated by secondary infections. This is likely, in part, due to the decreased oxidative burst activity that is present in affected horses.[130]

Lastly, the effects of age on phagocyte function have been extensively studied in production animals and horses. Calves show fluctuations in oxidative burst and phagocytic activities from 0-90 days of age, indicating changes associated with increasing maturity of the innate immune system and loss of passive immunity.[131] Additionally, increased phagocyte function in calves has been associated with colostrum ingestion[132] but not with plasma transfusions.[67] Similar fluctuations to those seen in calves have been described in foals up to the age of 211 days.[122] In newborn foals (less than 1 week old), the reported decrease in phagocyte function may explain their increased susceptibility to infections at this age.[133] Lambs are also at increased risk of infections in the first two months of life due to decreased neutrophil phagocytic activity and killing ability.[134] This dysfunction may be mitigated by the administration of Biolex-MB40, a commercially available prebiotic, however more studies into the immunomodulatory effects of prebiotics are needed.[135] In contrast to young ruminants and horses, no difference in phagocyte function was detected in piglets between the ages of 7 – 63 days.[136]

Conclusion

Canine parvoviral enteritis may result in both inflammation and immunosuppression.[2-4] Affected dogs often develop sepsis and/or SIRS, both of which have been associated with decreased survival.[1,26,31] Although neutropenia and monocytopenia have been well described in CPE, the function of these cells requires more investigation.[1,2]

Phagocyte function can be tested using classical or flow cytometric assays.[44] In recent years, flow cytometric assays have gained favour due to their convenience compared to classical assays.[44] With the use of these assays, phagocyte function has been well described in a variety of diseases and conditions (physiological and pathological) in both people and animals. On the surface, the results from studies in humans with sepsis and SIRS appear to be conflicting.[14,15,78-80,82,83] However, these seemly conflicting results are due the spectrum of responses mounted by the host secondary to a pathological inflammatory state.[14,84] As inflammation persists and increases in severity, anti-inflammatory responses predominate over pro-inflammatory responses, resulting in inappropriately down-regulated phagocyte function.[88]

Although phagocyte dysfunction, particularly in neutrophils, has been extensively researched in dogs and other veterinary species, there are limited reports on phagocyte function in CPE, sepsis and SIRS.[11-13] Only one previous study, to the author's knowledge,

has investigated canine neutrophil function in CPE.[11] Based on the findings of this study and others investigating canine critical illness and sepsis, we believe that more investigations are required to fully understand the pathogenesis of CPE and how the inflammation elicited by this disease affects phagocyte function.

Chapter 3 Materials and Methods

Animals and study design

This prospective, observational study was approved by the Research Ethics Committee of the Faculty of Veterinary Science (REC039-18) and Animal Ethics Committee of the University of Pretoria (V048-19). Client-owned dogs presented to the Onderstepoort Veterinary Academic Hospital were considered eligible for inclusion if they were 2-12 months of age, weighed at least 3 kgs and showed signs of gastrointestinal disease (vomiting, diarrhoea, anorexia or hyporexia). Dogs were included if a valid, rapid patient-side immunoassay[a] for faecal CPV-2 antigen tested positive and if CPV-2 viral particles were visualised on transmission electron microscopy (TEM). Dogs were excluded if TEM was negative, if co-morbidities were identified by thorough physical examination or clinicopathological data, or if there was a history of treatment or hospitalisation within a week prior to presentation.

A group of apparently healthy, staff- and client-owned dogs, presented for elective sterilisation or vaccination, were selected as controls and were age-matched to the study population. These dogs were considered healthy based on physical examination, faecal flotation and TEM, complete blood count (CBC) (including blood smear evaluation) and serum CRP concentrations. Written informed consent was obtained from all owners (Appendices 1 – 3).

Sample collection

Blood and faeces were collected once, prior to any treatment, from all dogs in the CPE and control groups. Blood was collected by jugular venipuncture directly into serum, lithium heparin and EDTA vacutainer tubes[b]. Faeces was collected rectally. The serum was harvested within 3 hours of collection and used to determine the serum CRP concentration using a validated[137] particle-enhanced immunoturbidimetric assay[c] on the Cobas Integra 400 Plus chemistry analyser.[d] This assay has a limit of quantification of 10 mg/L. The lithium heparin sample was used for flow cytometric analysis within 2 hours of collection. The EDTA

[a] IDEXX SNAP Canine Parvovirus Antigen Test Kit, IDEXX, Westbrook, Maine, United States
[b] BD Biosciences, Franklin Lakes, New Jersey, United States
[c] Gentian canine CRP, Gentian Diagnostics AS, Moss, Norway
[d] Roche, Basel, Switzerland

sample was used to perform a CBC,[e] manual blood smear evaluation and a 200-cell leukocyte differential count by experienced, registered veterinary technologists. In extremely leukopenic cases, multiple blood smears were used to complete the 200-cell leukocyte differential count. Neutropenia and neutrophilia were identified by the laboratory reference interval as segmented neutrophil concentrations of $<3 \times 10^9$/L or $>11.5 \times 10^9$/L, respectively. A band (immature) neutrophil was defined as a hyposegmented neutrophil with nuclear constrictions not exceeding half of the widest part of the nucleus.[138] Metamyelocytes, if present, were included in the band neutrophil count. The faecal sample was submitted for TEM to confirm the presence or absence of CPV-2 in the CPE and control dogs, respectively.

Oxidative burst and phagocytic activities

Oxidative burst and phagocytic activities were determined using commercially available kits.[f] Both kits have been used successfully in previous canine studies[12,59,92] and were used according to the manufacturer's instructions. Briefly, oxidative burst activity was determined, as shown in figure 4, by incubating two samples of 100 µL of precooled (0°C) heparinised whole blood, one with opsonised *E. coli* bacteria and one with PMA, at 37ºC for 10 minutes. Both the PMA and opsonised *E. coli* were included in the kit. A negative control sample was simultaneously incubated without either stimulant. The samples were subsequently incubated for 10 minutes at 37°C with 20 µL of dihydrorhodamine 123, a fluorogenic substrate, which was oxidised to rhodamine 123 by the newly produced ROS. A lysing and fixing solution (2 mL) was added to halt ROS production and, after a washing step, the samples were incubated with 200 µL of propidium iodide (DNA staining solution) to exclude non-viable cells and bacterial aggregates. The samples were protected from light and analysed by flow cytometry within 30 minutes.

Phagocytic activity was determined, as shown in figure 5, by incubating 100 µL of precooled (0°C) heparinised whole blood with fluorescein isothiocyanate (FITC)-labelled opsonised *E. coli*, which was included in the kit, at 37ºC for 10 minutes. A negative control sample with no bacteria was kept at 0°C. After incubation, the test sample was placed on ice to halt phagocytosis and a precooled quenching solution (100 µL) was added to quench the fluorescence of surface bound and free *E. coli*. After two washing steps, the samples were incubated with 2 mL of lysing and fixing solution. After another washing step, 200 µL of

[e] ADVIA 2120i haematology analyser, Siemens, Munich, Germany
[f] Phagoburst™ and Phagotest kits™, respectively; Glycotope Biotechnology GmbH, Berlin, Germany

propidium iodide was added. The samples were protected from light and analysed by flow cytometry within 60 minutes.

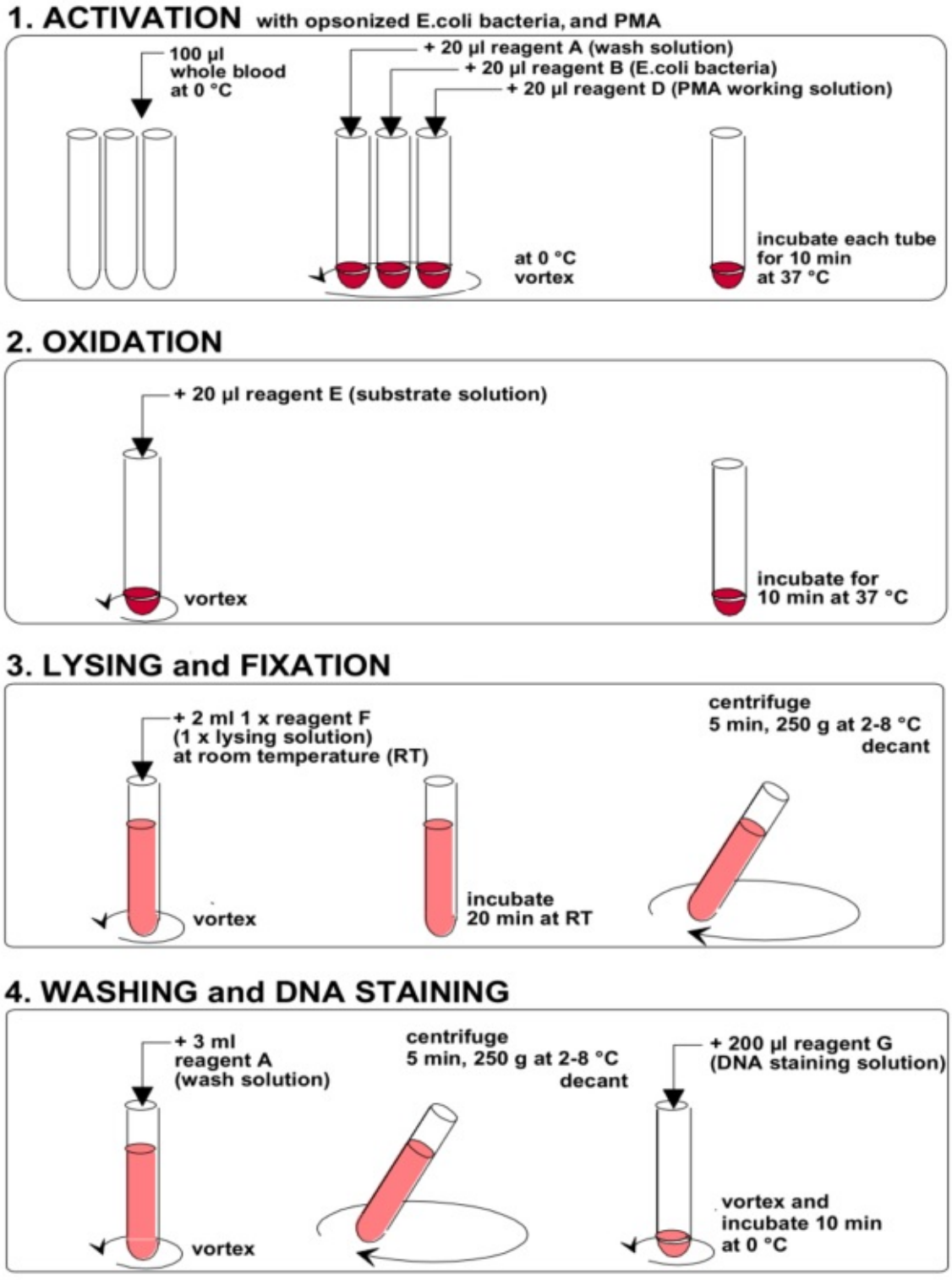

Figure 4 Steps for the preparation of three samples (one negative control sample, one stimulated with *Escherichia coli* bacteria and one stimulated with PMA) for the determination of oxidative burst activity of the phagocytes in canine blood. PMA – phorbol-12-myristate-13-acetate; RT – room temperature (22 - 25°C); DNA – deoxyribonucleic acid; *g* – unit of relative centrifugal force. Adapted from Phagoburst™ package insert – Sample preparation procedure.[139]

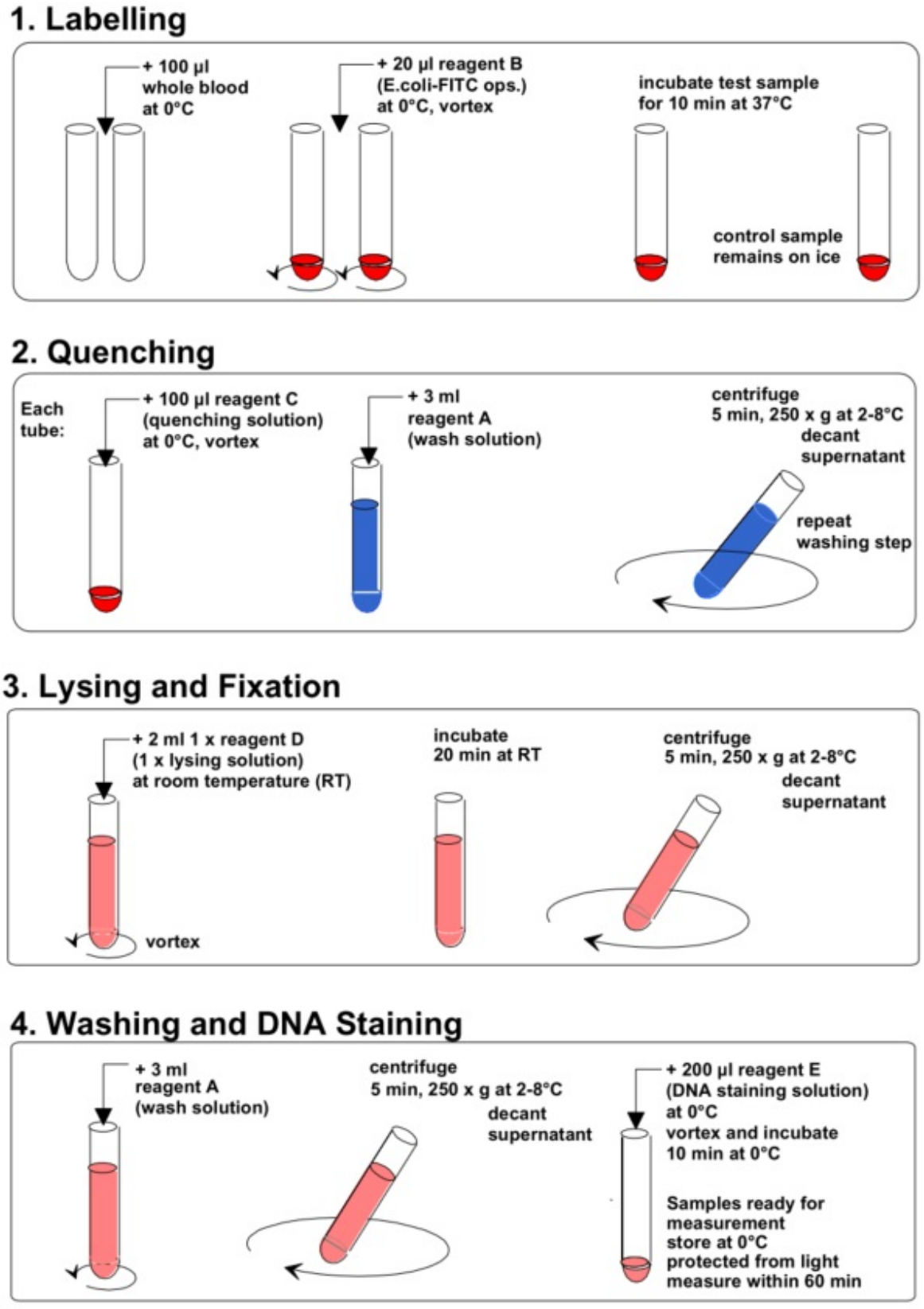

Figure 5 Steps for the preparation of two samples (one negative control sample and one stimulated with FITC-labelled opsonised *Escherichia coli* bacteria) for the determination of phagocytic activity of the phagocytes in canine blood. FITC – fluorescein isothiocyanate; ops. – opsonised; RT – room temperature (22 - 25°C); DNA – deoxyribonucleic acid; *g* – unit of relative centrifugal force. Figure from Phagotest™ package insert – Sample preparation procedure.[140]

Flow cytometric analysis

The samples were analysed using the Accuri™ C6 Plus flow cytometer[g] and Accuri™ C6 Plus Software version 1.0.23.1.[h] Using a blue laser emitting light at a wavelength of 488 nm, 10 000-15 000 viable leukocytes were acquired for each sample, using a live gate set to capture events with the same red fluorescence as a human diploid cell, thus excluding bacterial aggregates and non-viable cells. The gating strategy, as previously described,[12] was as follows: the negative control samples were used to set the gate on the blue-green histogram for the fluorescence positive population and a forward-scatter versus side-scatter plot was used to gate the phagocytes, excluding lymphocytes (Figure 6). In order to reduce the analytical variation of the assay, only the principle investigator gated the phagocyte populations, as recommended.[66] The results were reported as the percentage of fluorescence positive cells, which equates to the proportion of phagocytes containing rhodamine 123 or FITC-labelled *E. coli* as a measure of oxidative burst or phagocytic activity, respectively. The mean fluorescence intensity (MFI) was also reported and equates to the average magnitude of ROS production per cell when stimulated by *E. coli* or PMA or the average number of phagocytosed *E. coli* per cell.

[g] BD Biosciences, Franklin Lakes, New Jersey, United States
[h] BD Biosciences, Franklin Lakes, New Jersey, United States

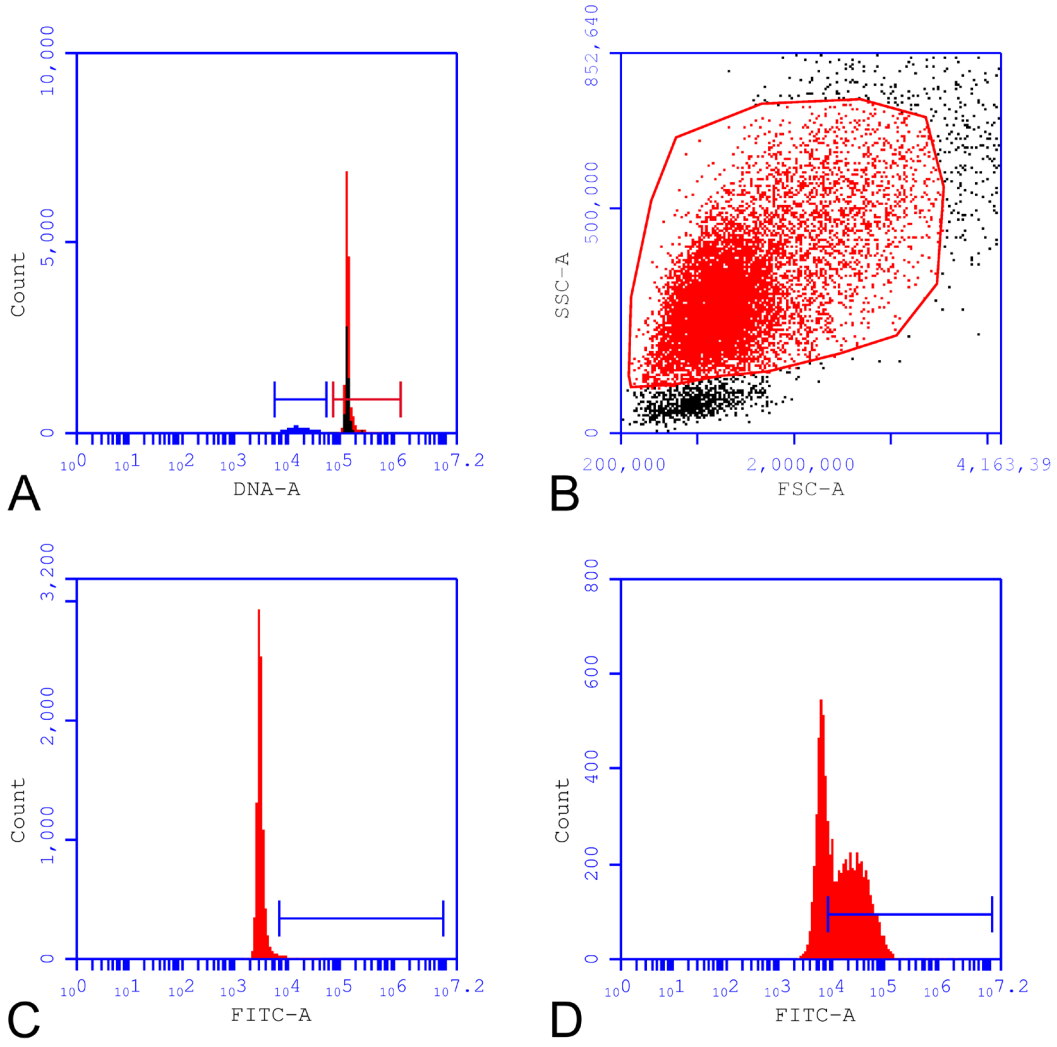

Figure 6 Gating strategy for measuring oxidative burst and phagocytic activities using flow cytometry. (A) Live gate on a histogram to exclude non-viable cells and bacterial aggregates (blue population) from viable leukocytes (red and black populations). (B) Forward (FSC) vs side scatter (SSC) plot to gate phagocytes (red population) – in this example there is no distinction between the neutrophil and monocyte populations; the lymphocytes are presented by the black population. (C) Histogram of a negative control sample with <3% fluorescence positive cells – this histogram has the same appearance for all analyses conducted. (D) Example of a histogram from a dog with CPE showing a population of majority fluorescence positive or stimulated cells (to the right of the blue gate as set by the negative control) with some fluorescence negative or unstimulated cells (to the left of the blue gate as set by the negative control) – the appearance of this histogram is dependent on the percentage of fluorescence positive cells, regardless of the stimulant used.

Data analysis

Statistical analyses were performed using MedCalc® Statistical Software version 19.5.3. Due to the small sample size (n ≤30), non-parametric analyses were selected, and summary statistics were reported as median (interquartile range). The Mann-Whitney *U* test was used to compare the data between the CPE and control dogs. Additionally, neutropenic dogs and dogs with circulating band neutrophils were each compared to the control group

and to CPE dogs without neutropenia or band neutrophils using the Mann-Whitney *U* test. Correlations between clinicopathological and flow cytometric data were determined using Spearman's coefficient of rank correlation with 95% confidence intervals (CI). For statistical analysis, a serum CRP concentration of 10 mg/L was assigned to dogs with results below the limit of quantification. Statistical significance was set at $P < 0.05$.

Chapter 4 Results

Animals

Samples were included from 28 dogs with CPE and 11 apparently healthy controls. The median age was four (3-6) months for the CPE dogs and four (2-7.5) months for the control dogs. There was no difference in age distribution between the two groups. There were 19 (68%) males and nine (32%) females in the CPE group and eight (73%) males and three (27%) females in the control group. Breeds included in the CPE group were mixed breeds (9), American Pitbull terrier (4), Labrador retriever (4), Boxer (2), Dachshund (2), Jack Russell terrier (2) and other (5). Breeds included in the control group were mixed breeds (2), Boerboel (2), Dachshund (2), and other (5).

Clinicopathological data

Compared to the control dogs, the CPE dogs had lower total leukocyte (P = 0.018), lymphocyte (P = <0.001), monocyte (P = 0.014) and eosinophil (P = <0.001) concentrations, and significantly higher band neutrophil (P = <0.001) concentrations (Table 1). Nine (32%) of the infected dogs were neutropenic and three (11%) were neutrophilic. The remaining dogs with CPE and all the control dogs had segmented neutrophil concentrations within the *laboratory* reference interval. Band neutrophils were identified on blood smear review in 18 (64%) of the dogs with CPE. No circulating band neutrophils were identified in any of the control dogs. The median percentage of band neutrophils was 5.9% (2-15.6%) and the proportion of band neutrophils never exceeded the proportion of segmented neutrophils. All neutropenic dogs with CPE had band neutrophils on blood smear review. The serum CRP concentration was higher in the infected dogs compared to healthy control dogs (P <0.001) (Table 1). Serum CRP concentration was also higher in neutropenic CPE dogs compared to non-neutropenic CPE dogs (P = 0.011), as well as in CPE dogs with circulating band neutrophils compared to those CPE dogs without (P = 0.004). Serum CRP concentration was negatively correlated with the segmented neutrophil concentration (r_s = -0.472, 95% CI = -0.719 to -0.120, P = 0.011) and positively correlated with the band neutrophil concentration (r_s = 0.678, 95% CI = 0.408 to 0.839, P <0.001).

Table 1 Median values (interquartile range) and comparison of select clinicopathological data between dogs with canine parvoviral enteritis (CPE) and control dogs.

Clinicopathological data	**Dogs with CPE**	**Control dogs**	***P* value**
Total leukocyte concentration ($\times 10^9$/L)	6.83 (3.41–10.88)	11.33 (10.32–12.90)	0.018
Segmented neutrophil concentration ($\times 10^9$/L)	4.74 (2.64–9.96)	7.18 (5.42–8.00)	0.533
Band neutrophil concentration ($\times 10^9$/L)	0.09 (0–0.24)	0 (0)	<0.001
Lymphocyte concentration ($\times 10^9$/L)	0.71 (0.39–1.13)	3.86 (2.61–4.34)	<0.001
Monocyte concentration ($\times 10^9$/L)	0.31 (0.21–0.58)	0.69 (0.63–0.74)	0.014
Eosinophil concentration ($\times 10^9$/L)	0.05 (0–0.17)	0.53 (0.20–0.79)	<0.001
Serum C-reactive protein concentration (mg/L)	181 (92.08–200.77)	10 (10–10.75)	<0.001

Flow cytometric data

In two dogs with CPE, PMA failed to stimulate oxidative burst, but opsonised *E. coli* was successful, indicating an error in the test procedure. These two failed PMA analyses were excluded from statistical analysis. Overall, there was no difference for all measures of oxidative burst and phagocytic activity between dogs with CPE and control dogs. These measures included the percentage and MFI of fluorescence positive cells when ROS production was stimulated by *E. coli* or PMA and when phagocytosis was stimulated by *E. coli*. However, infected dogs with neutropenia (P <0.001) or with circulating band neutrophils (P = 0.007), had lower percentages of positive phagocytes with PMA stimulation of ROS production compared to the healthy control dogs (Figure 7). None of the measures of phagocyte function, using *E. coli* as a stimulant for oxidative burst or phagocytosis, were different for the neutropenic CPE dogs or the CPE dogs with circulating band neutrophils compared to the healthy control dogs.

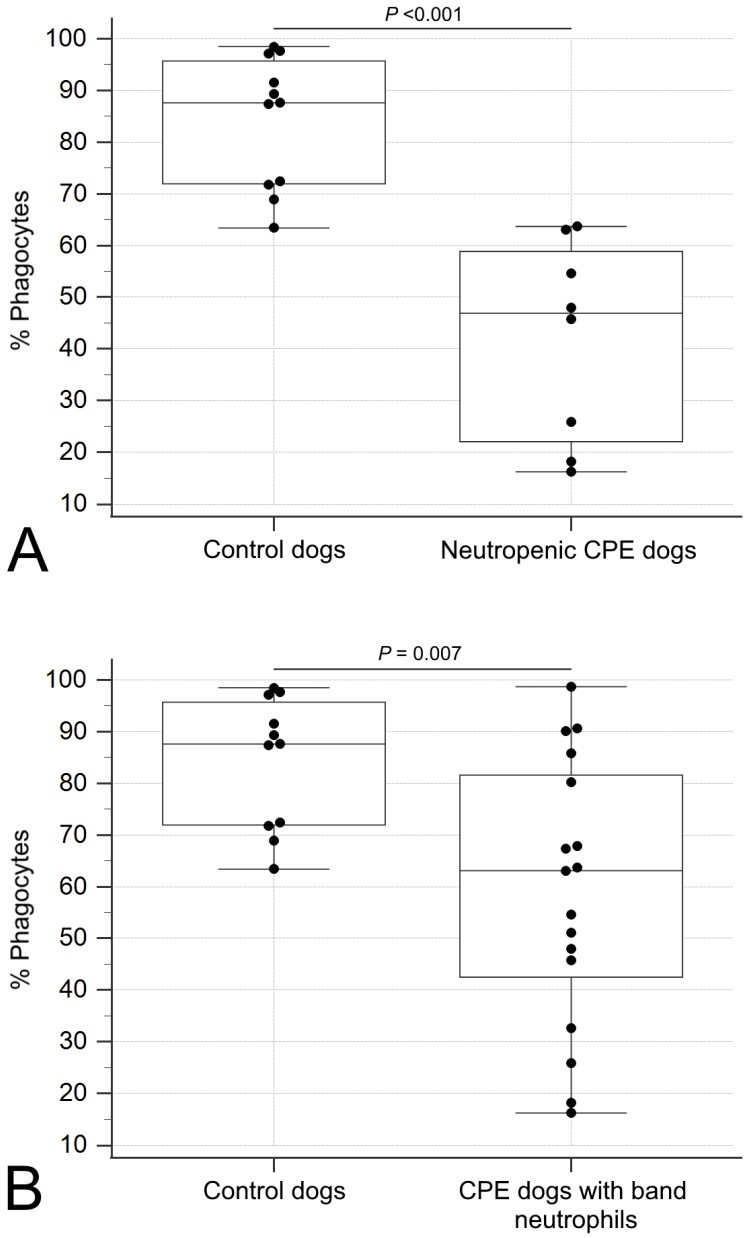

Figure 7 Box-and-whisker plots comparing the percentage of positive phagocytes undergoing PMA-stimulated oxidative burst between control dogs and dogs with CPE. (A) Control dogs and neutropenic CPE dogs. (B) Control dogs and CPE dogs with circulating band neutrophils. The box represents the IQR and the bottom and top box margins represent the 25th and 75th interquartiles, respectively; the central line represents the median; the bottom and top whiskers represent the range of the data; and the solid dots represent cases. PMA – phorbol-12-myristate-13-acetate; CPE – canine parvoviral enteritis; IQR – interquartile range.

Compared to CPE dogs without neutropenia, neutropenic CPE dogs exhibited decreased oxidative burst activity in terms of the percentage of positive phagocytes with PMA (P <0.001) and *E. coli* stimulation (P = 0.021) and the MFI (magnitude of ROS production) with PMA stimulation (P = 0.003) (Figure 8). Additionally, the percentage of cells phagocytosing

E. coli was decreased (*P* = 0.006) but the MFI (number of bacteria per cell) was increased (*P* = 0.041), in neutropenic dogs compared to non-neutropenic dogs (Figure 9).

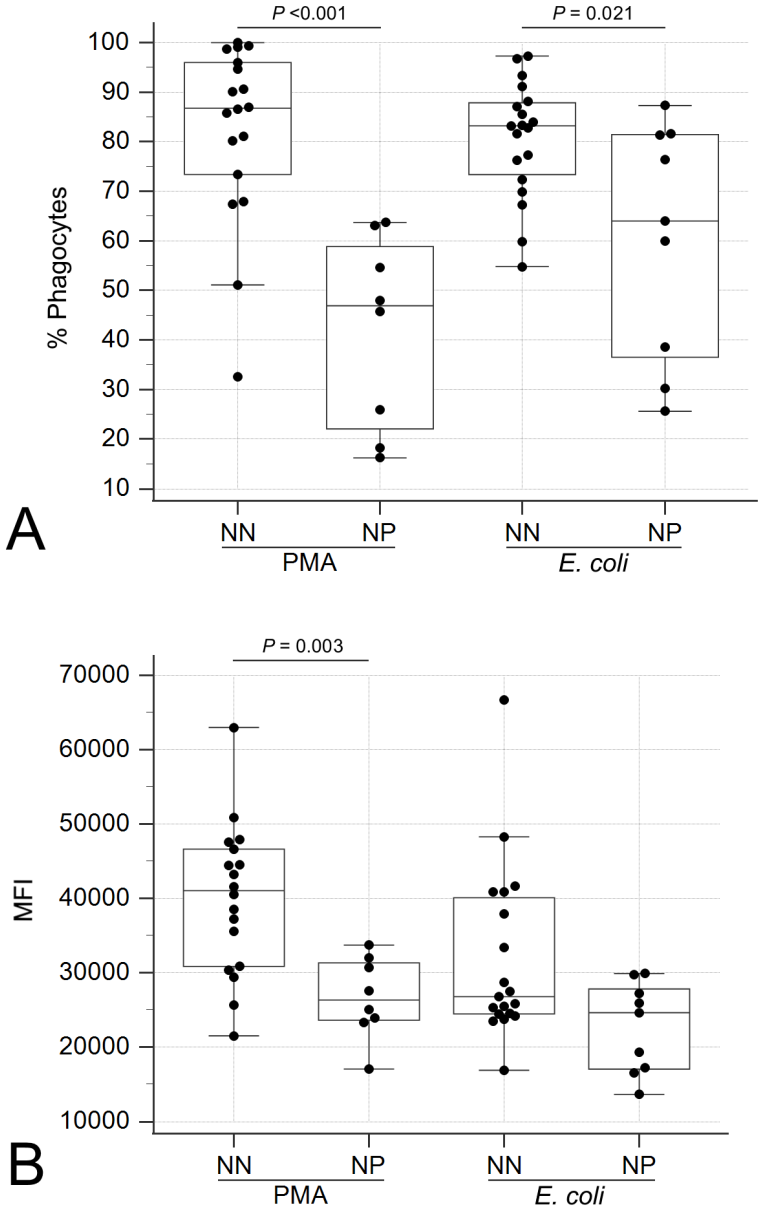

Figure 8 Box-and-whisker plots comparing oxidative burst activities between neutropenic (NP) and non-neutropenic (NN) CPE dogs. (A) Percentage of positive phagocytes undergoing oxidative burst with PMA or *Escherichia coli* stimulation. (B) MFI of positive phagocytes undergoing oxidative burst with PMA or *E. coli* stimulation. The box represents the IQR and the bottom and top box margins represent the 25th and 75th interquartiles, respectively; the central line represents the median; the bottom and top whiskers represent the range of the data; and the symbols represent cases, including outliers which are outside the whiskers. CPE – canine parvoviral enteritis; PMA – phorbol-12-myristate-13-acetate; MFI – mean fluorescence intensity; IQR – interquartile range.

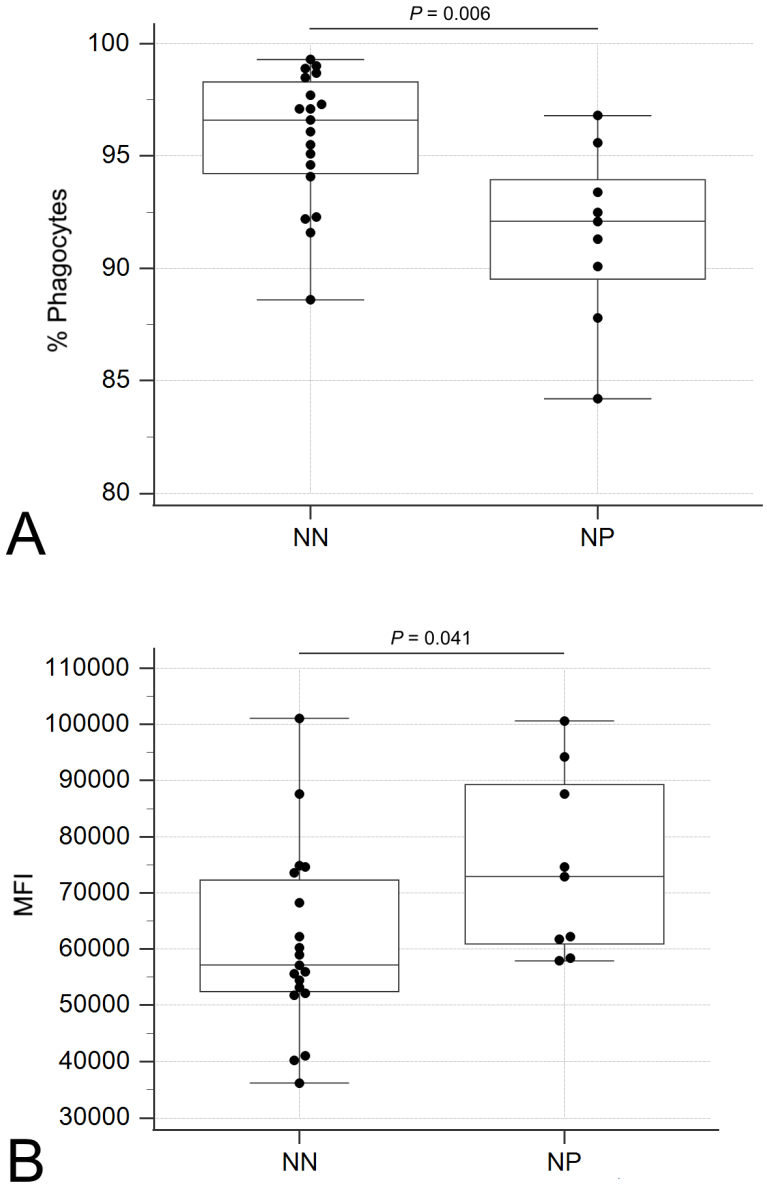

Figure 9 Box-and-whisker plots comparing phagocytosis between neutropenic (NP) and non-neutropenic (NN) CPE dogs. (A) Percentage of cells phagocytosing opsonised *Escherichia coli*. (B) MFI of cells phagocytosing opsonised *E coli*. The box represents the IQR and the bottom and top box margins represent the 25th and 75th interquartiles, respectively; the central line represents the median; the bottom and top whiskers represent the range of the data; and the symbols represent cases, including outliers which are outside the whiskers. CPE – canine parvoviral enteritis; MFI – mean fluorescence intensity; IQR – interquartile range.

Similarly, CPE dogs with circulating band neutrophils had decreased oxidative burst activity compared to those without circulating band neutrophils, as measured by the percentage of positive phagocytes with PMA (P = 0.001) and *E. coli* stimulation (P = 0.006) and the MFI with PMA stimulation (P = 0.004) (Figure 10). The percentage of cells phagocytosing *E. coli* was also lower (P = 0.005) in CPE dogs with circulating bands compared to those without (Figure 11).

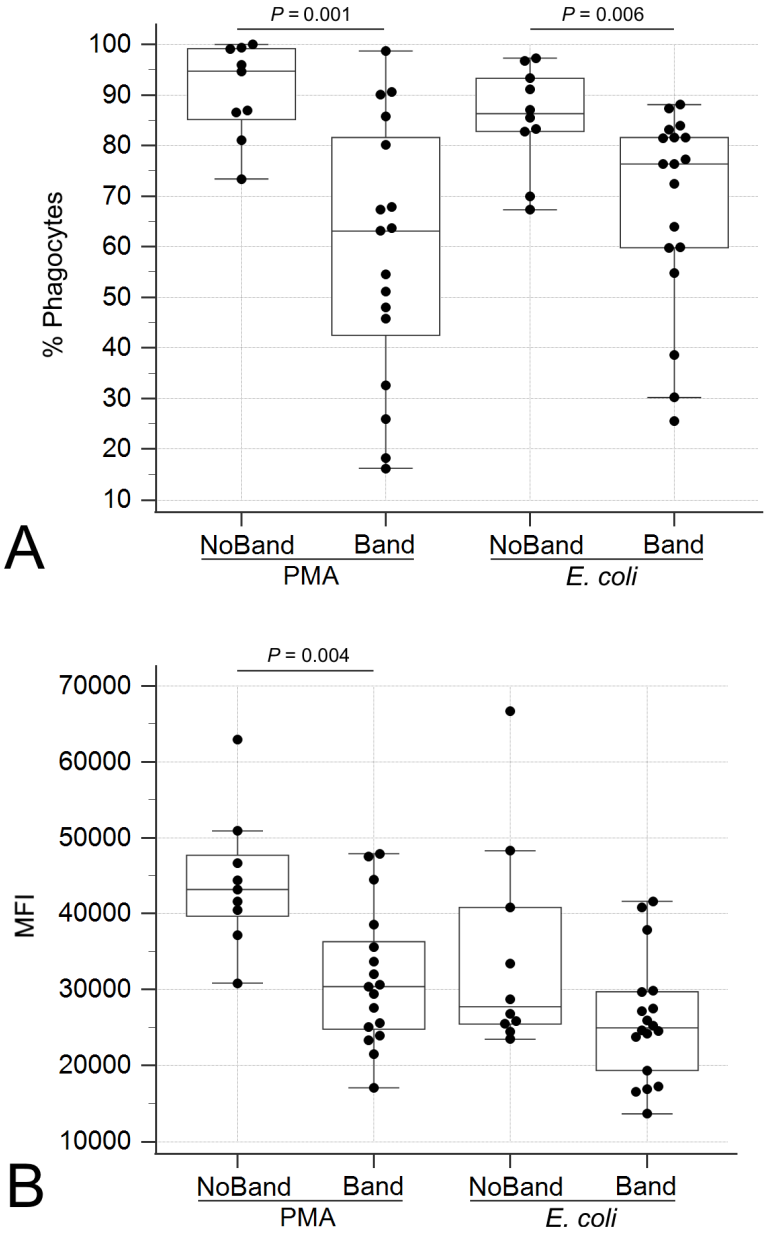

Figure 10 Box-and-whisker plots comparing oxidative burst activities between CPE dogs with circulating band neutrophils (Band) and CPE dogs without circulating band neutrophils (No Band). (A) Percentage of positive phagocytes undergoing oxidative burst with PMA or *Escherichia coli* stimulation. (B) MFI of positive phagocytes undergoing oxidative burst with PMA or *E. coli* stimulation. The box represents the IQR and the bottom and top box margins represent the 25th and 75th interquartiles, respectively; the central line represents the median; the bottom and top whiskers represent the range of the data; and the symbols represent cases, including outliers which are outside the whiskers. CPE – canine parvoviral enteritis; PMA - phorbol-12-myristate-13-acetate; MFI – mean fluorescence intensity; IQR – interquartile range.

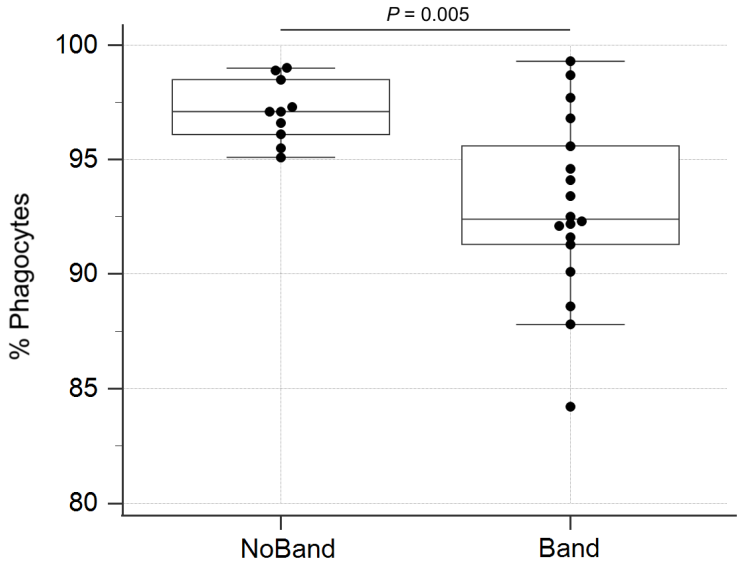

Figure 11 Box-and-whisker plot comparing the percentage of cells phagocytosing opsonised *Escherichia coli* between CPE dogs with circulating band neutrophils (Band) and CPE dogs without circulating band neutrophils (No Band). The box represents the IQR and the bottom and top box margins represent the 25th and 75th interquartiles, respectively; the central line represents the median; the bottom and top whiskers represent the range of the data; and the symbols represent cases, including outliers which are outside the whiskers. CPE – canine parvoviral enteritis, IQR – interquartile range.

Segmented neutrophil concentrations were positively correlated with the percentage and MFI of cells undergoing PMA-stimulated oxidative burst, the percentage of cells undergoing *E. coli*-stimulated oxidative burst, and the percentage of cells phagocytosing *E. coli* (Table 2). Segmented neutrophil concentrations were negatively correlated with the MFI of cells phagocytosing *E. coli* (Table 2). Band neutrophil concentrations were negatively correlated with the percentage and MFI of cells undergoing PMA-stimulated oxidative burst and the percentage of cells phagocytosing *E. coli* (Table 2). No correlations were found between segmented or band neutrophil concentrations and the MFI of cells undergoing *E.coli*-stimulated oxidative burst. Monocyte concentrations were not correlated with any measure of oxidative burst or phagocytic activity.

Table 2 Significant correlations of flow cytometric variables with segmented and band neutrophil concentrations and serum C-reactive protein (CRP) concentrations in dogs with canine parvoviral enteritis (CPE).

Flow cytometric variable	**Clinicopathological variable**	**Correlation coefficient (r_s)**	**95% CI**	**P value**
Oxidative burst activity				
Percentage of PMA-stimulated cells	Segmented neutrophils	0.876	0.739 to 0.943	<0.001
	Band neutrophils	-0.628	-0.817 to -0.318	<0.001
MFI of PMA-stimulated cells	Segmented neutrophils	0.622	0.309 to 0.813	<0.001
	Band neutrophils	-0.512	-0.752 to -0.159	0.007
	Serum CRP	-0.411	-0.689 to -0.028	0.037
Percentage of *E. coli*-stimulated cells	Segmented neutrophils	0.558	0.233 to 0.770	0.002
Phagocytosis				
Percentage of cells phagocytosing *E. coli*	Segmented neutrophils	0.532	0.199 to 0.755	0.004
	Band neutrophils	-0.439	-0.698 to -0.0786	0.020
MFI of cells phagocytosing *E. coli*	Segmented neutrophils	-0.577	-0.782 to -0.260	0.001

CI – confidence interval
MFI – mean fluorescence intensity
PMA – phorbol-12-myristate-13-acetate

Chapter 5 Discussion

This study shows that canine phagocytes have decreased oxidative burst and phagocytic activity in neutropenic CPE dogs, as well as in CPE dogs with circulating band neutrophils.

In this study, most infected dogs were young, with a median age of four months. In humans, decreased phagocytosis of *E. coli* and *Staphylococcus aureus* has been reported in healthy, premature and septic newborns compared to healthy adults.[141] Similarly, healthy lambs and foals show reduced neutrophil oxidative burst activity when stimulated by opsonised zymosan and PMA, respectively.[133,134] To the authors' knowledge, there are no published studies investigating phagocyte function in young dogs. In order to mitigate the possible effect of age, an age-matched control group was selected. Another factor that may have affected our results are congenital defects of phagocyte function. The risk that we included a dog (in either group) which such a defect is very low for two reasons. Firstly, none of the breeds in which these defects have most commonly been described were included in the study (i.e. Weimaraners,[108] Grey Collies,[109] Doberman Pinchsers[110]). Secondly, these defects are exceptionally rare with limited case reports.

The leukocyte changes seen in the dogs with CPE compared to the control dogs, most importantly the lower total leukocyte concentrations and the presence of band neutrophils, are expected based on previous studies.[1,2] The higher serum CRP concentration observed in dogs with CPE was also expected. Increased serum CRP, a major acute phase protein in dogs, is considered an indicator of systemic inflammation and disease severity in CPE and shows moderate accuracy in predicting mortality in affected dogs.[4,25] The systemic inflammation elicited by CPE results in the release of band neutrophils from the bone marrow as part of the innate immune response.[142] This need for early neutrophil release is augmented by neutropenia. Neutropenia is a common, multifactorial complication in CPE caused not only by viral effects on the bone marrow but also by marked gastrointestinal inflammation and endotoxaemia resulting in neutrophil margination, demand, consumption and loss.[1,2,22,143] Thus, the increased serum CRP concentrations in neutropenic CPE dogs and CPE dogs with circulating band neutrophils, as well as the positive correlation between serum CRP and band neutrophils and the negative correlation between serum CRP and segmented neutrophils all demonstrate the expected shifting relationship between these 3 measurands secondary to the host inflammatory response.

Surprisingly, oxidative burst and phagocytic activities of the phagocytes were not different between dogs with CPE and the control dogs. The reason for this is likely multifactorial. Firstly, the oxidative burst and phagocytic activities of neutrophils in healthy dogs varies widely between individuals,[66] which was also demonstrated in our study in both health and disease. Secondly, in humans, systemic inflammation induces functional heterogeneity between neutrophil phenotypes so that cells with differing functional capacities are simultaneously present in circulation.[144] This functional heterogeneity may also exist between circulating monocyte subpopulations in sepsis.[14] In our study, naturally infected dogs at various stages of disease and with varying ratios of circulating band and segmented neutrophils and monocytes were included, resulting in a heterogeneous group.

Nevertheless, compared to the healthy control dogs, CPE dogs with neutropenia and/or circulating band neutrophils showed decreased oxidative burst activity with PMA stimulation. Additionally, decreasing segmented neutrophil concentrations and increasing band neutrophil concentrations were correlated with decreasing PMA- and *E. coli*-stimulated oxidative burst activity. In humans with SIRS and sepsis, band neutrophils were less efficient at ROS production compared to mature neutrophils.[145] The decreased oxidative burst activity in our study may, therefore, be secondary to increased numbers of circulating band neutrophils compared to the control dogs, which had no circulating band neutrophils. However, this is only a partial explanation, as the median percentage of band neutrophils was only 5.9%, suggesting that the decreased oxidative burst activity is not solely due to the presence of band neutrophils. In dogs, two similar studies, one conducted in critically ill dogs[12] and the other in septic dogs,[13] also found a decrease in oxidative burst activity when phagocytes were stimulated by PMA and immune complexes, respectively. One study postulated that in canine critical illness, dysfunction in the protein kinase C (PKC) signalling pathway may be the reason for decreased oxidative burst activity.[12] Protein kinase C is a group of serine-threonine kinases that, when activated, results in signal transduction leading to activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce ROS.[7,146] Phorbol-12-myristate-13-acetate (PMA) is a strong stimulant of oxidative burst through activation of PKC.[146] The proposed dysfunction in the PKC signalling pathway may be due to accumulated ROS (superoxide and H_2O_2) attaching to NADPH oxidase subunits, decreasing their activity and, in turn, decreasing further ROS production.[147] Additionally, PKC is highly susceptible to oxidation and, in a highly oxidative environment created by ROS production, modifications may occur which render PKC inactive.[7,148] Lastly, endotoxin tolerance may be contributing to the decreased activity. This phenomenon, described in humans and animal models, is characterised by phagocytes that are unresponsive to subsequent endotoxic insults within as little as 30 minutes of sustained

endotoxin exposure.[78,149] Endotoxin tolerance results in a multifactorial decrease in oxidative burst activity, leading to an immunosuppressive state in the patient.[150] As endotoxaemia has been described in dogs with CPE,[26] endotoxin tolerance is possible in this study.

The decreased oxidative burst activity in neutropenic dogs and dogs with circulating band neutrophils, compared to healthy dogs, was further emphasized when these dogs were compared to the remaining dogs in the CPE group. Infected dogs with neutropenia or circulating band neutrophils had decreased PMA- and *E. coli*-stimulated oxidative burst activity as well as decreased phagocytic activity compared to those CPE dogs without neutropenia or circulating band neutrophils. This may be explained by the response of the innate immune system to systemic inflammation. Initially, pro-inflammatory cytokines predominate and receptors important for phagocytosis are upregulated.[85-87] As the inflammation persists and increases in severity, immune modulation may result in decreased phagocyte function.[15,18,150] Therefore, it is possible that the dogs without neutropenia and circulating band neutrophils had a upregulated phagocyte response, so that the difference between these dogs and those with neutropenia and circulating bands is more apparent.

In addition to the decreased phagocytosis of *E. coli* in CPE dogs with neutropenia or circulating band neutrophils compared to CPE dogs without neutropenia or band neutrophils, phagocytosis was correlated to neutrophil concentration. A decreasing percentage of phagocytosing cells was correlated with decreasing segmented neutrophil concentrations and increasing band neutrophil concentrations. By comparison, a study on phagocyte function in critically ill dogs[12] reported no differences in the phagocytosis of *E. coli* between critically ill and healthy control dogs. This may have been due to the small sample size and/or variability of diagnoses and disease states in the dogs included.[12] In human studies, a decreased percentage of phagocytosing cells has been described in neutrophils exposed to *S. aureus* components as a model for systemic inflammation and sepsis.[90] This dysfunction was associated with a downregulation of complement receptors on the neutrophil surface.[90] In our study, the *E. coli* used to stimulate phagocytosis were opsonised with antibodies and complement proteins, thus, a downregulation of complement receptors on the neutrophil surface may explain our findings. Another point to consider is that immature neutrophils have reduced phagocytic activity when compared to mature segmented neutrophils in healthy humans and in humans with sepsis and SIRS.[151,152] Considering, again, that the median band neutrophil percentage was only 5.9% in the CPE dogs, it is unlikely that band neutrophils in circulation are the sole cause of the decreased

percentage of phagocytosing cells. Interestingly, an increasing number of phagocytosed *E. coli* per cell was correlated with decreasing segmented neutrophil concentrations. A study investigating neutrophil function in septic dogs also reported increased neutrophil MFI when phagocytosing *E. coli*, compared to a control group.[13] This inverse correlation between phagocytosed *E. coli* and segmented neutrophil concentrations likely reflects a functional heterogeneity in the circulating phagocytes created by systemic inflammation.[14,144]

Limitations

This study was limited by the expense of the flow cytometric kits used. For this reason, our sample size was insufficiently powered to detect the presence of subtle changes in variables where there was a large degree of overlap between healthy and infected dogs. Based on the findings in this study, and considering the heterogeneity of the circulating phagocytes, future studies including a large number of severely affected CPE dogs would assist in further characterisation of the immunological dysfunction present in this disease. Although there was no effect of sex on phagocyte function in one study,[112] only adult dogs (four years of age and older) were included and, to the author's knowledge, this effect has not been investigated in younger dogs. As the control and CPE groups were not sex matched, an effect of sex on phagocyte function in this study cannot be excluded but is unlikely. Another limitation is that no neutrophil or monocyte markers or separation techniques were used to differentiate the leukocyte populations on flow cytometry and the choice was made to report on phagocyte flow cytometric results. This approach has been followed previously in a number of veterinary studies using the same kits as in our study.[12,91,93] Both neutrophils and monocytes are important cells in the innate immune system, responsible as the first line of defence in the prevention of sepsis.[6,32] For this reason, a decrease in oxidative burst and/or phagocytic activities in either or both populations will increase the risk of sepsis in dogs affected by CPE.

Chapter 6 Conclusion

In summary, there is evidence of phagocyte dysfunction in dogs suffering from CPE. This evidence includes decreased phagocyte oxidative burst activity in CPE dogs with neutropenia and circulating band neutrophils compared to healthy control dogs. Furthermore, phagocyte oxidative burst and phagocytic activities are decreased in CPE dogs with neutropenia or circulating band neutrophils compared to CPE dogs without either of these two leukocyte changes. Decreasing segmented neutrophil concentrations and increasing band neutrophil concentrations are also correlated with a decrease in several measures of oxidative burst and phagocytic activity.

Based on these results, two major conclusions can be made. First, there is evidence of innate immune system dysfunction in CPE and second, this dysfunction is associated with leukocyte changes that occur in the face of increasing systemic inflammation. These novel findings add to the body of knowledge regarding how canine phagocytes, particularly neutrophils, respond to severe inflammation. While CPE can be used as a model for SIRS and sepsis, further studies are warranted in order to fully understand the response of the canine innate immune system to these pathological inflammatory states. Additionally, further studies are warranted to determine how the dysfunction described in this research affects outcome in patients and whether known and novel therapies can reverse the dysfunction.

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Appendices

Appendix 1 – Client information sheet

DEPARTMENT OF COMPANION ANIMAL
CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Dear Client,

From the clinical examination and laboratory tests we have performed so far on your puppy, it seems most likely that he/she is suffering from a viral infection caused by canine parvovirus (also called “cat flu”/ “katgriep”). This virus causes severe damage to the lining of the intestines, resulting in bleeding and decreased absorption of nutrients. It also causes other problems such as decreased white blood cell count, leaving any affected puppies vulnerable to secondary infections. It has been advised that your puppy be admitted to the Onderstepoort Isolation unit for intensive treatment.

We are currently conducting a study on the function of the neutrophils (a type of white blood cell) in puppies with canine parvoviral enteritis. We wish to collect blood, faeces (collected rectally) and urine (collected by cystocentesis i.e. withdrawn from the bladder directly via a needle and syringe) once, before your puppy is admitted. We will then compare the results we get with that of healthy puppies. With this information we hope to learn more about this disease to, hopefully, find new treatments.

The tests necessary for this study will incur no extra costs over and above what it would have usually cost to treat your puppy. We will be paying for all the additional blood, faecal and urine tests performed on your puppy. This study has been approved by the Animal Ethics Committee and Faculty Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for allowing your puppy to be included in the study. If you have any further questions, please feel free to ask the veterinary clinician on duty, or myself.

Yours Sincerely,

Dr Kelly du Preez
Tel: +27 12 529 8491
Email: kelly.dupreez@up.ac.za

Appendix 2 – Client consent form

DEPARTMENT OF COMPANION ANIMAL
CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Project title: Oxidative burst and phagocytic activities of neutrophils in canine parvoviral enteritis

(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

The undersigned parties further agree that:

1. No compensation will be payable to the animal's owner or anybody else and that all research associated costs will be covered by the researcher(s).
2. This form would serve to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from the specified procedure by or on behalf of the animal's owner.

3. No material of any kind, including data and research findings, obtained or resulting from the procedure, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal.
4. No personal information will be disclosed but may be used unanimous in publications. As owner, it is my right to withdraw my animal/s from the trial.

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.

Signed at Onderstepoort on the _____ day of _____20_____

Signature of owner/authorised agent_____

Home Tel: _____

Work Tel: _____

Cell No: _____

Appendix 3 – Information sheet and client consent form for control dogs

DEPARTMENT OF COMPANION ANIMAL
CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Dear Client

Your puppy has been selected to serve as a healthy control for a study to that will help us to determine whether the neutrophils (a type of white blood cell) are dysfunctional in canine parvoviral enteritis ("cat flu"/ "katgriep"). For this study, blood, faeces (collected rectally) and urine (collected by cystocentesis i.e. withdrawn from the bladder directly via a needle and syringe) will be needed. The volume of blood we need to collect will in no way harm your puppy or change the procedure for which he/she was admitted for.
We appreciate your willingness to participate in this clinical study. Should you have any further questions about the trial, please feel free to contact me.

Yours Sincerely,
Dr Kelly du Preez
Clinical Assistant: Clinical Pathology at Onderstepoort Veterinary Academic Hospital
Email: kelly.dupreez@up.ac.za
Tel: +27 12 529 8491

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.

The undersigned parties further agree that:

1. No compensation will be payable to the animal's owner or anybody else and that all research associated costs will be covered by the researcher(s).

2. This form would serve to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from the specified procedure by or on behalf of the animal's owner.
3. No material of any kind, including data and research findings, obtained or resulting from the procedure, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal.
4. No personal information will be disclosed but may be used unanimous in publications. As owner it is my right to withdraw my animal/s from the trial.

Signed at Onderstepoort on the _____ day of _____20_____

Signature of owner/authorised agent_____

Home Tel: _____

Work Tel: _____

Cell No: _____

Appendix 4 – Data capture form

DEPART MENT OF COMPANION ANIMAL
CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

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Date:

Admission: ☐ Outpatient: ☐

Sticker or:		
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 ☐ Store EDTA ☐

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

Urine collected and urinalysis performed ☐ Store Urine ☐

Flow cytometry assays performed, and data captured into Excel ☐

Appendix 5 – Clinical pathology request form

Neutrophil function in canine parvoviral enteritis study

SECTION OF CLINICAL PATHOLOGY
DEPARTMENT OF COMPANION ANIMAL CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

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Provisional Diagnosis: Neutrophil function study in parvoviral enteritis					
History (esp. date symptom first seen):					
	Note only changes since the first specimen was submitted				
Clinical Examination	**Date:** Sample 1 (Admission)	**Date:**	**Date:**	**Date:**	**Date:**
Temperature					
Appetite/Water					
Habitus					
Faeces/GIT					
Urinary					
Mucous Membranes					
Hydration					
Lymph nodes					
Respiratory					
Cardio-Vascular					
Abdominal					
Skin/coat					
Musculoskeletal					
Laboratory Examinations Requested	CBC (200 cell leukocyte diff count)				
	Serum CRP				
	Albumin				
	Flow cytometry				
Was bleeding easy?	Yes / No				
Was animal excited before or during?	Yes / No				
Clinician initials MANDATORY	Dr K du Preez				
Drugs used and dosages:					

Appendix 6 – Standard operating procedure for processing of samples

SOP for sample handling and storage: neutrophil function study in parvoviral enteritis

Sample handling

1. Faeces
    - Submission of faecal sample 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.

2. Urine
    - Perform complete urinalysis and aliquot urine supernatant into 2 cryovials and store at -80ºC.

3. Serum
    - Perform the requested biochemistry panel (i.e. serum CRP and albumin) within 60 minutes of collection. If the sample is collected after hours, the serum will be aliquoted within 60 minutes and stored until analyses can be performed.
    - Aliquot the remaining serum between 2 cryovials and store at -80ºC

4. EDTA anti-coagulated blood
    - Perform automated blood counting within 60 minutes of collection. Make and stain a blood smear.
    - Aliquot the remaining whole blood between 2 cryovials and store at -80ºC

5. Lithium Heparin anti-coagulated blood
    - Perform oxidative burst and phagocytic activity assays on flow cytometer within 2 hours of collection.
    - Centrifuge the sample and aliquot the remaining plasma between 2 cryovials and store at -80ºC

All results should be archived. Samples in cryovials, once are to be stored in the -80ºC freezer in their appropriate boxes (i.e. EDTA plasma, EDTA pellet, serum, heparinised plasma)

Appendix 7 – Standard operating procedure for biosecurity measures

Measures to limit the spread of canine parvovirus (CPV-2) from the isolation unit at the Outpatients clinic in the Onderstepoort Veterinary Academic Hospital (OVAH)

1. On admission, all consumables and equipment used to diagnose and examine CPE affected patients will be discarded in the appropriate biohazard waste bins and disinfected with F10 disinfectant for a minimum of 30 minutes, respectively. All surfaces which came into contact with CPE affected dogs will also be disinfected prior to reuse.
2. The principal investigator, supervisors and co-workers will also use disposable gowns and shoe covers available in the Isolation Unit in the OVAH before contact with CPE affected dogs to limit contamination of their clothes and shoes, respectively. After contact and removal of the disposable gown, they will also spray their clothes with F10 disinfectant.
3. The principal investigator, supervisors and co-workers will wash and disinfect their hands with F10 disinfectant (thoroughly and according to the manufacturer's guidelines) after coming into contact with CPE affected dogs.
4. All samples will be collected in the Isolation Unit in the OVAH. The areas where blood (lateral neck) and urine (caudal ventral abdomen) will be collected will be aseptically prepared prior to collection. All consumables used for collection of samples will be discarded in the appropriate biohazard waste bins in the Isolation Unit.
5. Once collected, the samples will be distributed to the necessary laboratories, where appropriate biosecurity measures are in place. These samples will not come into contact with any other animals.
6. Contact with CPE affected dogs by the principal investigator, supervisors and co-workers will be limited to once incidence at presentation and will not be made again, unless assistance with discharge is required.
7. Hospitalised CPE affected patients will be kept exclusively in the Isolation Unit at the OVAH and will not be allowed to leave this Unit until discharge or death. Walks and "toilet breaks" for the patients will take place in a dedicated, fenced off area outside the Isolation Unit.
8. All cages, floors and surfaces and cleaned and disinfected daily while there are patients in the Isolation Unit.