# Neutrophil Myeloperoxidase Index in Dogs with Babesiosis

**by**

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**MMedVet (Med)**

**in the Faculty of Veterinary Science**

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**SUPERVISOR: Prof Amelia Goddard**

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| PROJECT TITLE | **Neutrophil myeloperoxidase index in dogs with babesiosis** |
| PROJECT NUMBER | **REC041-18** |
| RESEARCHER/PRINCIPAL INVESTIGATOR | **Anri Celliers** |

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28 September 2011

Prof JP Schoeman
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Dear Prof Schoeman

**V055-11 : Haemostatic changes in canine babesiosis, caused by Babesia rossi, and its association with outcome (*A Goddard)***

The application for ethical approval, dated 25 August 2011 was approved by the Animal Use and Committee at its meeting held on 26 September 2011.

Kind regards

E Mostert

**Elmarie Mostert**

**AUCC Coordinator**

Copy Prof A Goddard

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| RESEARCHER/PRINCIPAL INVESTIGATOR | **Dr. Y Rautenbach** |

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| ANIMAL SPECIES | **Dogs/Canine** | |
| NUMBER OF ANIMALS | **45** | |
| Approval period to use animals for research/testing purposes | | **December 2013-December 2014** |
| SUPERVISOR | **Prof. A Leisewitz** | |

*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

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# Summary

**Myeloperoxidase index in dogs with babesiosis**

Celliers, A. University of Pretoria, 2018

Babesiosis caused by the more virulent tick-borne haemoprotozoan, *Babesia rossi*, results in a marked systemic inflammatory host response in dogs. Neutrophils are part of the innate immune system and myeloperoxidase is the predominant component of the neutrophil lysosomal protein in azurophilic granules. This enzyme plays a crucial role in the process of destruction of microbes by neutrophils. Neutrophil myeloperoxidase index (MPXI) is a reflection of the intracellular myeloperoxidase content in circulating neutrophils.

The aims of this study were to: (a) compare MPXI in dogs with babesiosis with healthy control dogs, using the ADVIA 2120; (b) compare MPXI in dogs that died from babesiosis with dogs that survived and controls; and (c) correlate the MPXI results with (i) total band and segmented neutrophil count and (ii) various cytokine concentrations.

Data for 140 dogs, naturally infected with *B. rossi*, and 20 healthy control dogs were retrospectively evaluated. Approval was obtained from the University of Pretoria's Animal Ethics committee, as well as the Faculty Research Committee. Owner consent was obtained for enrolment of each case. MPXI was generated on an automated cell counter, ADVIA 2120, and various cytokine concentrations, including interleukin-2 (IL-2), IL-6, IL-8, IL-10, IL-18, granulocyte-macrophage colony stimulating factor (GM-CSF) and monocyte chemo-attractant protein-1 (MCP-1), were determined using a canine-specific multiplex immunoassay (MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel CCYTO-90K-07, Millipore, Billerica, USA).

The mortality rate of the *Babesia*-infected dogs was 11% (15/140). MPXI was significantly higher in the *Babesia*-infected dogs ($P$ = 0.033), as well as the *Babesia*-infected non-survivors ($P$ = 0.011), compared to the controls. For the *Babesia*-infected group a significant positive correlation was found between MPXI and IL-10 ($r$ = 0.211, $P$ = 0.039), and a significant negative correlation between MPXI and IL-8 ($r$ = -0.350, $P$ < 0.001). For the

dogs that died, significant positive correlations were found between MPXI and IL-2 ($r$ = 0.616, $P$ = 0.033), IL-6 ($r$ = 0.615, $P$ = 0033), IL-18 ($r$ = 0.613, $P$ = 0.034), GM-CSF ($r$ = 0.630, $P$ = 0.028) and MCP-1 ($r$ = 0.713, $P$ = 0.009). In dogs that survived, a significant negative correlation was found between MPXI and IL-8 ($r$ = -0.363, $P$ = 0.001).

The higher MPXI value in *Babesia*-infected dogs and especially *Babesia*-infected non-survivors, in conjunction with cytokines, could indicate an increased inflammatory response, as is expected in *B. rossi*-infections. The potential of MPXI as a novel marker of inflammation and prognosis in *Babesia rossi*-infected dogs, warrants further exploration.

**Key terms:**
ADVIA 2120, *Babesia rossi*, babesiosis, cytokines, dog, immune system, inflammatory, interleukins, malaria, myeloperoxidase, myeloperoxidase index, neutrophils.

# List of abbreviations

| | |
|---|---|
| ACTH | adrenocorticotrophic hormone |
| APC | antigen presenting cell |
| ARDS | acute respiratory distress syndrome |
| ARF | acute renal failure |
| CBC | complete blood count |
| CD | cluster of differentiation |
| DC | dendritic cell |
| DIC | disseminated intravascular coagulation |
| G-CSF | granulocyte colony stimulating factor |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| $H_2O_2$ | hydrogen peroxide |
| HOCl | hypochlorous acid |
| IL | interleukin |
| IMHA | immune mediated haemolytic anaemia |
| INFγ | interferon gamma |
| IQR | interquartile range |
| MCP-1 | monocyte chemo-attractant protein-1 |
| MHC | major histocompatibility complex |
| MODS | multiple organ dysfunction syndrome |
| MPO | myeloperoxidase |
| MPXI | myeloperoxidase index |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NET | neutrophil extracellular trap |
| NK | natural killer |
| PAMP | pathogen-associated molecular pattern |
| PRR | pattern-recognition receptor |
| RLB-PCR | reverse line blot polymerase chain reaction |
| ROS | reactive oxygen species |
| SIRS | systemic inflammatory response syndrome |
| Th1 | T-helper 1 |
| TNFα | tumour necrosis factor alpha |

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# Chapter 1: Introduction

*Babesia rossi* causes the most virulent form of babesiosis in dogs in South Africa. Several indices have been investigated in the past in an attempt to prognosticate these cases.[1-7] There have also been several studies looking into the host response against babesiosis and comparing this response with that seen in falciparum malaria in humans.[8-10]

Myeloperoxidase index (MPXI) is a marker of intracellular myeloperoxidase (MPO) content in neutrophils. Multiple human studies have evaluated the use of MPXI as a marker of inflammation in different diseases. It has also been considered as a potential prognostic indicator in several disease conditions. The ADVIA 2120, an automated haematology analyser, uses intracellular MPO content and cell volume to determine the automated differential leukocyte count. Qualitative changes for neutrophils can be determined through calculation of the MPXI. The ADVIA generates a MPXI value for each complete blood count done.

To the author's knowledge, MPXI has not yet been investigated in dogs suffering from babesiosis. Studies in veterinary medicine evaluating MPO and MPXI in horses and dogs have provided a platform for future research into the use of these indices as supplementary diagnostic and prognostic modalities. This research was undertaken in an attempt to answer several questions relating to the usefulness of the generated MPXI value in *B. rossi*-infected dogs. Will MPXI differ between *Babesia*-infected and healthy dogs? Will it be different between healthy dogs and those that succumb to the disease, as well as those that recover? Will there be a correlation between MPXI and the severity of the inflammatory response seen?

If shown to be useful, MPXI might be a conveniently available tool to broaden the understanding of the host response seen in dogs suffering from babesiosis and possibly serve a role in the prognostication of these cases.

# Chapter 2: Literature review

## 2.1 Canine babesiosis

More than a hundred different *Babesia* spp. have been described and it is one of the most frequently encountered infections in the world, affecting a wide range of hosts including domestic animals, humans and wildlife.[11-14] There are six different *Babesia* spp. currently known to cause infection in dogs.[12,14,15] Three large *Babesia* spp. including *B. canis*, *B. rossi* and *B. vogeli* and three small *Babesia* spp. including *B. gibsoni*, *B. annae* and *B. conradae*, have been described in dogs.[16-18] *B. rossi* and *B. vogeli* are the most common species found in Africa. *Babesia rossi* is transmitted by *Haemophysalis elliptica* ticks and is considered the most pathogenic of the canine babesias, whereas *B. vogeli*, transmitted by *Rhipicephalus sanguineus*, usually cause milder symptoms.[3,12,14,19]

Recently, particular attention has been paid to this disease as an emerging zoonosis, especially since the numbers of immunocompromised people worldwide have been increasing.[13,20,21] *Babesia microti*, *B. duncani*, *B. venatorum* and *B. divergens* are the main species responsible for human infections, of which *B. microti*-like species have been detected in dogs in Spain.[11,13,20,22,23] Globally, *Babesia* spp. have a significant economic impact due to the high rates of morbidity and mortality associated with the infection. It has also been compared to human malaria infection for decades and its model potential has been postulated.[8-10,24] *Babesia*, like *Plasmodium*, belongs to the apicomplexan group of haemoparasites and shares a similar structure.[11,12] Babesiosis in humans results in haemolytic anaemia, acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS).[11] Immune suppression, caused either by concurrent disease or exogenous corticosteroids treatment, resulting in weakened cell-mediated immunity, has been linked to more severe clinical signs in humans.[20,25] A septic shock-like syndrome can develop in both human babesiosis and malaria infections.[26] Both conditions cause multiple organ pathology which can lead to MODS even after parasite clearance.[8,26]

Traditionally, the clinical disease in dogs caused by *B. rossi* has been classified along the same lines as falciparum malaria in humans, namely as uncomplicated or complicated

disease, depending on haemodynamic stability.[3,12,27-29] In uncomplicated cases, the clinical signs can be ascribed to haemolytic anaemia, whereas in complicated cases, clinical presentation cannot be credited to haemolysis and anaemia alone. Complicated disease has been reported to rather be related to the host's immune response to the infection, resulting in obvious parenchymatous organ dysfunction and/or failure.[3] Complicated cases have an estimated 15% mortality rate and the conditions associated with this presentation include ARDS, acute kidney injury (AKI), cerebral babesiosis, hepatopathy, disseminated intravascular coagulation (DIC), acute pancreatitis, immune mediated haemolytic anaemia (IMHA), haemoconcentration, hypoglycaemia, acid-base disturbances (including lactacidosis) and MODS.[1,3,12,27,30] Marked systemic inflammation is thought to be the predominant contributor to the pathophysiology of the above mentioned complications.[27] In studies done on *B. canis*, cases with a low parasitaemia on peripheral blood counts, did not ensure an uncomplicated disease course, but if the parasitaemia was above 1%, it did predict the progression to complicated babesiosis.[31] Higher venous and capillary parasitaemias were significantly associated with mortality in dogs infected with *B. rossi*.[29]

Several prognostic indicators can be used to decide whether a patient needs to be hospitalized or can be treated on an outpatient basis. These include high capillary or venous parasitaemia, a collapsed presentation, hypoglycaemia, hyperlactaemia, coagulopathy and specific organ dysfunction or failure (especially the lung, brain and kidney). Elevated cortisol and adrenocorticotrophic hormone (ACTH) concentrations and decreased total thyroxine (T4) are endocrine predictors of a poor outcome.[1,27,30,32,33] The involvement of the kidneys, brain, lungs or haemoconcentration in the face of severe haemolysis carry the worst prognosis.[34]

Treatment is largely symptomatic, with diminazene aceturate or imidocarb dipropionate being the mainstay antibabesials.[14,34,35] The main supportive therapy includes intravenous fluids and packed red blood cell transfusions.

## 2.2 The immune response to haemoprotozoan parasites

It has been suggested that there are four major pathophysiological events that occur during infection with a haemoprotozoan parasite: 1) the release of pro-inflammatory mediators initiated by the parasite, 2) adherence of parasitised erythrocytes to the vascular endothelium, 3) anaemia and hypoxia due to haemolysis and 4) the removal of infected erythrocytes by macrophages in the spleen.[36] The immune system that is responsible for these events can broadly be classified into the innate and adaptive immune responses.[37,38]

The innate immune system is the non-specific, rapid, germ-line encoded arm of the immune response to pathogens.[39] No immunological memory develops after a microbial encounter and the immune response to pathogens thus remains unchanged regardless of subsequent exposure.[40] The innate response consists of soluble components such as complement, leukotrienes, kinins and prostaglandins, and phagocytic or cytotoxic cells (i.e. neutrophils, eosinophils, basophils, mast cells, monocytes/macrophages, dendritic cells and natural killer cells).[41] In contrast, the adaptive immune system has very specific antigen recognition although not germ-line encoded and therefore does not show predetermined antigen receptor recognition.[39] The adaptive immune response needs three to five days to allow for clonal expansion in response to infection.[39] Lymphocytes are the cells involved in the adaptive response and confer the characteristics of specificity, pathogen recognition diversity and immunological memory to the adaptive immune system.[41]

The innate immune system allows for broad-based, immediate immunity to swiftly control the replication of pathogens and in so doing grants the adaptive immune system time to undergo clonal expansion.[39] Cells of the innate system are genetically predestined to recognize a limited number of highly preserved pathogen-associated molecular patterns (PAMPs) that are presented only by microbial pathogens, through pathogen-recognition receptors (PRRs). These PAMPs are not produced by the host, they are usually vital for microbe pathogenicity or survival and they are similar between entire groups of pathogens.[39] Macrophages, neutrophils and dendritic cells are phagocytes that display a diversity of these PRRs.[41] Receptors for complement and antibodies are present on macrophages and neutrophils, that facilitate phagocytosis of microbes coated in complement or antibiodies.[40]

The adaptive response cannot react to a pathogen unless it has first been recognized by the innate immune system.[39]

Studies have suggested that monocytes and neutrophils are actively involved in the innate response to *Babesia bovis*.[42] Neutrophils showed increased phagocytic ability in the peripheral circulation at peak parasitaemia concentrations, but phagocytosis by antigen presenting cells (APCs) is ultimately required to link the innate and adaptive immunity.[42] Immunity against intraerythrocytic organisms such as *Babesia* and malaria parasites is Type I (Th1) dependent and require both innate and adaptive responses, which include humoral and cellular means, to work in concert to limit the level of parasitaemia and facilitate parasite clearance.[43-47] The cytokines produced by cells of the innate immune system influence the direction of the adaptive immune response, causing these two systems to be intimately connected.[47] The main effector cytokines involved in the Type I immune response are: interferon (IFN)-γ, IL-17, IL-21 and IL-22.[46] They target macrophages, neutrophils, cytotoxic T lymphocytes and B lymphocytes as ultimate effector cells.[46] *Babesia* antigens become incorporated into erythrocyte membranes after invasion, which primes the erythrocyte for opsonisation by antibodies for removal via phagocytosis.[46] Phagocytic cells and cytotoxic lymphocytes recognize these antigen-antibody complexes and cause destruction via an antibody-dependent cell-mediated mechanism.[46] Protozoa, including *Babesia* spp., can evade the immune system by inducing immune suppression and varying the surface antigens that are expressed.[46] Immune reactions to blood-borne pathogens are usually instigated in the spleen.[40]

Cytokines are transient proteins with various different structures and receptors. They can act systemically or locally and affect a multitude of cell types. Cytokines are redundant in their biological roles and are dangerous in high concentrations and therefore carefully regulated.[46] They act as mediators of inflammation and immune reactions and are important in maintaining the balance between disease and health.[37,48] Their roles differ depending on different disease conditions and species.[48] The role of specific cytokines are dependent on the overall cytokine environment and immediate host tissue factors. More significant information can therefore be obtained from measuring multiple cytokines rather than a single cytokine, in different disease conditions.[48] No single assay can currently distinguish

between the stages of SIRS, sepsis and MODS, but the investigation of cytokine profiles in different diseases have contributed to the understanding of these conditions.[48]

IL-2 is a pro-inflammatory cytokine produced by activated Th1 helper T cells. It activates macrophages, natural killer (NK) cells, promotes B cell expansion, controls the survival and death of T regulatory (Treg) cells and further promotes Th1 activation.[46] Therefore, IL-2 promotes the Type I immunity that is necessary in the immunological control of protozoal infections. IL-6 is a key mediator in septic shock and the acute-phase reaction. Its production by T cells, macrophages and mast cells is triggered by IL-1 and TNF-α release in response to severe infections and tissue damage. It is also involved in the Type I response to protozoal infections and contributes to the activation of neutrophils, macrophages, cytotoxic T cells and B cells. IL-6 is a pyrogenic cytokine and most likely responsible for the fever reaction seen in babesiosis.[46] IL-18 is a pro-inflammatory cytokine produced by macrophages as part of the body's innate immune response. It stimulates the production of IFN-γ by T cells, IL-2 and GM-CSF, and increases NK cell activity.[46,49,50] In several human studies IL-18 was found to be markedly elevated in sepsis compared to healthy individuals.[49,51] It was also elevated as part of the host defense against the blood-borne stages of malaria.[50] GM-CSF together with IFN-γ, enhances the production of reactive nitrogen species (RNS) by M1 macrophages. This allows macrophages to kill protozoa, but at the expense of host tissue damage. GM-CSF is an important regulator of granulocyte and macrophage populations for all steps of their maturation.[52] As part of its pro-inflammatory effect, GM-CSF causes an increase in granulopoiesis of neutrophils in the bone marrow, affecting the maturation of these cells. It is speculated that GM-CSF might play a major part in the local activation, recruitment and survival of neutrophils and macrophages.[52] MCP-1 is produced by activated neutrophils and endothelial cells in response to IL-6.[46] It serves as a pro-inflammatory cytokine tasked with the recruitment of monocytes from the bone marrow.[53-55] It was found to be increased in critically ill dogs, with the highest concentrations seen in septic dogs.[54]

In human falciparum malaria, high concentrations of IL-6, IL-8, IL-10, MCP-1, tumour necrosis factor (TNF)-α, IFN-γ and IL-18 have been associated with more severe and complicated disease.[56-58] In an experimental *B. gibsoni* infection study, increases in GM-CSF, IL-2, IL-6, IL-10 and IL-18 were seen more than two weeks after infection, with IL-8

only increasing in one of the two experimental dogs. The increases in cytokines coincided with detectable parasitaemia.[59] In dogs with naturally occurring *B. rossi* infections, significant increases in GM-CSF, IL-2, IL-6 and IL-18 were seen in dogs with clinical disease duration less than 48 hours compared to dogs with clinical disease duration more than 48 hours. Concentrations of IL-6 and MCP-1 were significantly higher in non-survivors compared to survivors. Interleukin-8 concentrations were significantly lower, while IL-10 and MCP-1 were higher in the infected dogs when compared to the healthy controls.[4] In dogs infected with *B. canis*, significant time-course (between day one and day seven) variation were shown within uncomplicated cases for IL-10, IL-8 and GM-CSF. IL-10 and IL-8 differed significantly over time in complicated cases, with IL-8 showing an increase between the different time points and IL-10 a decrease. IL-8 was also significantly lower in healthy dogs when compared to uncomplicated and complicated infected dogs. MCP-1 showed significant time-course increases between healthy and complicated diseased dogs. There was also significant differences between uncomplicated and complicated cases on day one and seven, with MCP-1 being higher in complicated infections and higher on day one overall. MCP-1 could therefore differentiate between complicated and uncomplicated cases on day one.[58]

## 2.3 Neutrophil myeloperoxidase and myeloperoxidase index

Neutrophils form a major part of the innate immune system, because they express a broad range of PRRs and can therefore respond to wide distribution of PAMPs, by producing pro-inflammatory cytokines.[46] Granulocyte colony-stimulating factor (G-CSF) regulates the production of neutrophils by stem cells in the bone marrow. The rate of production is matched with the rate of neutrophil apoptosis. Macrophages produce IL-23 when phagocytosing apoptotic neutrophils. IL-23 in turn stimulates the production of IL-17 by lymphocytes and IL-17 stimulates the production of G-CSF.[46] Neutrophils are tasked with recognition, phagocytosis and/or neutrophilic extracellular trap formation (NETs) and destruction of microbes.[60] The myeloperoxidase-system plays a fundamental role in this process.[61] Myeloperoxidase (MPO) is a major component of neutrophils with a dry matter concentration of more than 5% and can be used as a marker for neutrophil activation.[62] It is also contained in smaller quantities in monocytes and as a different form in eosinophils.[61,63]

MPO is a haem protein synthesised during myeloid development and represents the predominant component of the neutrophil lysosomal protein in primary, azurophilic granules.[61] After recognition and attachment of the microbe to the neutrophil membrane, the membrane invaginates around the microbe to form a phagosomal vesicle.[60] The phagosome then fuses with the cellular lysosome to form a phagolysosome.[60] Membrane-bound nicotine adenine dinucleotide phosphate (NADPH) oxidase becomes activated during this process (respiratory burst) and hydrogen peroxide ($H_2O_2$) is formed within the phagolysosome.[61] Halides (particularly chloride) present in the phagolysosome are oxidized by MPO in the presence of $H_2O_2$ to form hypochlorous acid (HOCl).[61,64] The HOCl formed is crucial to the microbicidal function of neutrophils.[61] The MPO-system is effective against an array of microbes (including protozoa), bacterial toxins, mammalian cells and cell mediators.[61] It can also have a stimulatory role with regards to mast cell secretion and platelet and protease activation.[65,66] A high localized concentration of MPO is needed for NET formation, which is essential in limiting systemic infections and the manipulation of the host response to fungal agents.[67,68] NETosis is particularly useful when microbes are too large for phagocytosis and for trapping and killing protozoa such as *Leishmania* and *Eimeria*.[46] Oxidative stress and tissue toxicity can be induced by exposure of the regional tissues or circulation to the MPO-system.[69] It can therefore also result in the oxidation of haemoglobin to methaemoglobin within *Babesia*-infected and non-infected erythrocytes, facilitating clearance by the spleen.[42] Ten percent of MPO can be found extracellulary after neutrophil degranulation.[69] MPO also upregulates the surface expression of CD11b on neutrophils needed for adherence to the endothelium before extravasation.[70]

The ADVIA 2120, an automated haematology analyser, uses intracellular MPO content and cell volume to determine the automated differential leukocyte count.[71,72] Quantitative changes in neutrophil MPO staining can be determined through calculation of the myeloperoxidase index (MPXI), as well as by observing the staining intensity (peroxidase activity, *x*-axis) and light scatter (cell size, *y*-axis), that determine the location of cells on the generated peroxidase scattergram.[71,72] Cluster analysis software gates these cell populations based on the above mentioned variables.[73] MPXI is a unitless value calculated by the ADVIA, based on the deviation of the mean *x*-axis value for the gated neutrophil population, from a stored representative population.[73] The MPXI is a reflection of the intracellular MPO content and has been used as an indicator of neutrophil activation.[74,75] The MPO content is

influenced by the age, toxicity and degranulation of the neutrophil.[74] It is important to note that MPO is predominantly produced in promyelocytes and mature neutrophils only function to store MPO.[76] The MPXI thus seems to be regulated by a balance between MPO production in promyelocytes in the bone marrow and consumption due to inflammation with respiratory burst generation.[76,77] Lower MPXI values are reported to indicate neutrophil activation and thus systemic inflammation.[75] In MPXI measurements in children, negative values indicate that the tested group of cells contain less peroxidase than the ideal population and a positive value indicate a higher MPO content than the ideal population.[78]

Studies in humans have investigated the usefulness of MPO and MPXI as an additional diagnostic tool in certain conditions. Most of the studies indicate that changes in MPXI levels differ in specific illnesses and infections. MPXI has been reported not to be useful in differentiating between acute complicated and acute non-complicated appendicitis in humans, but was increased in non-tuberculous non-septic bacterial infections, acute and chronic myeloid leukaemia, megaloblastic anaemia, megaloblastosis and essential thrombocythaemia.[76,79-82] MPXI was found to be higher in sepsis than non-infectious SIRS in one study, while another study showed MPXI to be decreased with severe bacterial sepsis, remain unchanged with viral infections and increased with other bacterial infections.[76,83] Interestingly, serum MPO was elevated in patients with parasitic infections such as onchocerciasis, bancroftian filariasis, intestinal schistosomiasis and malaria.[84] In murine studies, the intraperitoneal injection of β-hematin, a crystal that is architecturally and biologically identical to the malarial pigment, haemozoin, led to elevated MPO quantities in peritoneal exudate.[62] This indicated intraperitoneal neutrophil influx and activation in response to β-hematin, which could possibly be a contributory factor to the pathophysiology of severe malaria infections.[62] MPXI was increased in mild cases of arteriosclerosis obliterans and decreased when these patients developed ischaemic heart disease.[77] The MPXI for each individual seems to be stable and therefore small changes within that patient could give significant insight into disease progression.[77] MPO can be used as an indicator of neutrophil activation and plaque instability in patients with coronary artery disease.[85] During chemotherapy treatment, MPXI increased in the prenadir stage and decreased at the nadir and may therefore have value in predicting the onset of neutropenia.[86] Children showed age- and gender-related differences in neutrophil MPO concentrations with MPXI being lower in males and during the first month of age.[78]

Studies in veterinary medicine evaluating MPO and MPXI in horses and dogs have provided a platform for future research into the use of these indices as supplementary diagnostic and prognostic modalities. During experimentally-induced endotoxaemia in horses, MPXI was reported to be decreased on average six days after the onset of clinical signs. This could imply that endotoxaemia affects mainly the MPXI in neutrophil precursors and not mature cells.[72] In horses, a low MPXI in the face of a normal leukocyte count has been suggested to be a useful indicator of systemic inflammation or sepsis, or might suggest a developing change in neutrophil counts.[87] Mortality in horses with systemic inflammation is correlated with low MPXI values, especially when the MPXI remains unchanged 24 hours after therapeutic intervention.[87] MPXI was increased in septic foals with concurrent neutropenia compared to sick foals secondary to non-septic causes (hypoxic-ischaemic encephalopathy, prematurity, inguinal hernia, failure of passive immune transfer).[88] In canine monocytic ehrlichiosis, the MPXI was decreased 14 and 21 days post-infection. This finding was contributed to the continual neutropenia observed with ehrlichiosis, or defects in the maturation process of neutrophils resulting in lower MPO content.[89]

MPO deficiency (MPOD) can be inherited, or more commonly, acquired.[61,71] MPXI is routinely used in humans to detected MPOD during complete blood count analysis.[90] Humans with MPOD are more prone to haematopoietic neoplasms, severe infectious conditions and the adverse effects of cytotoxic drugs.[91] It has been described in dogs with lead toxicity, *Hepatozoon canis* infection and inherited ceroid-lipofuscinosis (Springer Spaniels and English Setters).[92-94] Diseases causing severe leukocyte consumption such as parvovirus enteritis, sepsis, pyometra, pyothorax, pneumonia and pancreatic abscesses, can lead to acquired MPOD in dogs.[71] These changes in MPXI seemed to be transient, further supporting the acquired nature of MPOD in most disease conditions.[71] Significantly lower MPXI were seen in parvovirus infected dogs compared to dogs suffering from other gastro-intestinal or inflammatory diseases.[71]

# Chapter 3: Methodology

## 3.1 Objectives of this study

a) To compare MPXI in dogs with *B. rossi* infection with healthy control dogs using the ADVIA 2120.
b) To compare MPXI in dogs that died from *B. rossi* infection with dogs that survived and controls.
c) To correlate the MPXI results with: (a) total leukocyte count, as well as band and segmented neutrophil count; and (b) various pro-inflammatory and immune modulating cytokine concentrations; in dogs suffering from *B. rossi*.

## 3.2 Hypotheses

Three alternative hypotheses were proposed:

a) The MPXI in dogs with *B. rossi* infection will be significantly different compared to healthy controls.
b) The MPXI in dogs that died from *B. rossi* infection will be significantly different compared to dogs that survived and control dogs.
c) There will be a positive correlation between MPXI and markers of inflammation in dogs suffering from *B. rossi* infection.

## 3.3 Benefits of this study

Babesiosis occurs worldwide and is an emerging zoonosis, with symptoms very similar to human malaria caused by *P. falciparum.* The disease has been proposed as a potential model for some aspects of human malaria. This study could potentially broaden our understanding of the host response to haemoprotozoal disease and might aid in the management and prognostication of these cases.

This research serves as a partial fulfilment of the MMedVet (Med) degree of the main author, whose name appears on the title page of this dissertation.

## 3.4 Study design

This was a retrospective observational study that evaluated the records of the MPXI in dogs with babesiosis and healthy control dogs, generated using multispecies software and the canine species setting on the ADVIA 2120 (Siemens, Munich, Germany) at the Clinical Pathology laboratory, Onderstepoort Veterinary Academic Hospital (OVAH). The study was approved by the Faculty Research Ethics committee (REC041-18).

## 3.5 Study population

Data generated by the ADVIA 2120 on two study population cohorts were evaluated: 96 *Babesia*-infected dogs and 15 healthy control dogs, collected between October 2011 to April 2013 as part of the first study cohort, as well as 44 *Babesia*-infected dogs and five healthy control dogs, collected during January to December 2014 as part of the second study cohort. Both studies were approved by the University of Pretoria's Animal Ethics committee (V055-11 and V091-13, respectively). Owner consent was acquired for enrolment of all cases in both studies.

Inclusion criteria:

- dogs of either breed or gender
- more than 12 weeks of age
- weight more than 3 kg
- demonstrable parasitaemia on blood smear and positive for *B. rossi* on RLB-PCR

Exclusion criteria:

- RLB-PCR confirmation of co-infection with *B. vogeli* or *Ehrlichia canis*
- euthanansia for reasons other than a poor prognosis

- evident coexisting inflammatory or infectious, cardiac, neoplastic or traumatic conditions
- treatment with anti-inflammatory therapy at, or within 4 weeks prior to presentation

Patients received the standard therapy for canine babesiosis, which involved treatment with diminazene aceturate (Berenil RTU 0.07 g/mL, Intervet, Kempton Park, South Africa) at 3.5 mg/kg and transfusion with packed red blood cells and/or intravenous fluids therapy as indicated. If complications were encountered they were treated as deemed appropriate by the attending clinician. Outcome was noted as short-term survival (i.e. until discharge from hospital), or death/euthanasia due to poor prognosis.

The controls included 20 healthy, client-owned dogs admitted for blood donation and routine ovariohysterectomy or castration. The control dogs were regarded to be healthy based on history, a complete physical examination, peripheral blood smear evaluation, complete blood count (CBC), complete serum biochemistry profile (total serum proteins, albumin, globulin, alanine aminotransferase, alkaline phosphatase, basal bile acids, total bilirubin, urea, creatinine, sodium, potassium, chloride and ionized calcium) as well as RLB-PCR assay to exclude co-infection with other haemoparasites. Control samples were collected during the same study period as *Babesia*-infected cases.

The study population was grouped into *Babesia*-infected and healthy control dogs. In the *Babesia*-infected group, dogs were divided based on outcome (non-survivors and survivors).

## 3.6 Experimental procedures

Blood was collected from the jugular vein in EDTA and serum vacutainer tubes at presentation, prior to any treatment, for haematologic analysis on the ADVIA 2120 and complete serum biochemical profile (total serum proteins, albumin, globulin, alanine aminotransferase, alkaline phosphatase, basal bile acids, total bilirubin, urea, creatinine, sodium, potassium, chloride and ionized calcium). Blood smear evaluation was performed on all dogs by experienced haematology technologists and a 100-cell differential leukocyte count was performed. The generated MPXI peroxidase plots were evaluated for each sample

by an experienced clinical pathologist. Blood was also collected in the first study cohort (96 *Babesia*-infected and 15 healthy control dogs) in serum vacutainer tubes for determination of various cytokine concentrations (IL-2, IL-6, IL-8, IL-10, IL-18, GM-CSF and MCP-1). The cytokine concentrations were determined using a validated commercially available canine-specific multiplex immunoassay (MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel CCYTO-90K-07, Millipore, Billerica, USA).[48] The CBC and MPXI determination was performed within 30-60 minutes of blood collection. Serum for cytokine analysis was stored at -80°C for batch analysis.

## 3.7 Data management and analysis

All laboratory data obtained from this study were captured into a spreadsheet program, (Microsoft Excel®, Microsoft Corporation, Redmond, WA, USA). Haematological variables (specifically MPXI, total leukocyte count (WBC), band neutrophil count and segmented neutrophil count) and cytokine variables were used for statistical analysis.

## 3.8 Statistical considerations

Statistical analysis was performed using a commercial software package (SPSS Statistics version 24 ®IBM, New York, USA). The Shapiro Wilk test was used to assess data for normality. The data was found to be not normally distributed and therefore non-parametric statistical analysis, such as Kruskal Wallis and Mann-Whitney *U* was used to determine significance across groups; the groups being infected vs control and non-survivors vs survivors vs controls. Gender proportions between groups were assessed using the Chi square test. Correlation between MPXI and cytokine concentrations, as well as MPXI, and selected leukocyte counts (WBC count, band and mature neutrophil counts) were determined using the Spearman's rank correlation coefficient. The level of significance was set at $P < 0.05$. Data was represented as median and interquartile range (IQR) values.

# Chapter 4: Results

## 4.1 Study population characteristics

Data from 140 client-owned dogs naturally infected with *B. rossi* and 20 healthy control dogs were included. Of the 140 *Babesia*-infected dogs, 15 died (11%) and 125 survived (89%). There were significant differences in age and weight between the groups. The median age of the *Babesia*-infected dogs (20 months; range: 2 - 144) and the survivors (20 months; range: 3 - 144) was significantly lower ($P = 0.005$ for both), than the healthy control dogs (46 months; range: 3 - 84). There was no significant age difference between the dogs that died and the survivors or healthy control dogs. The median weight of the *Babesia*-infected dogs (14.9 kg; range: 2.6 - 65.0) and the survivors (14.4 kg; range: 2.6 -65.0), was significantly lower ($P = 0.004$ and $P = 0.003$, respectively) than the healthy control dogs (29.0 kg; range: 8.0 - 65.0). There was no significant weight difference between the dogs that died and the survivors or healthy control dogs. The ratio of male:female for each group was as follows: healthy control dogs (7:13), survivors (83:43) and non-survivors (10:5), with a significant difference found between groups ($P = 0.028$). Cytokine concentrations were evaluated in 96/140 infected dogs and 15/20 healthy control dogs.

## 4.2 Comparison of MPXI, WBC- and neutrophil counts between dogs with babesiosis (survivors and non-survivors) and healthy control dogs

Table 1 contains a summary of the MPXI and leukocyte data, including the median and the IQR. The median MPXI was significantly higher in the *Babesia*-infected dogs (19.2) compared to the healthy control dogs (16.8; $P = 0.033$) (Fig. 1). The median MPXI was also significantly higher in the non-survivors (22.8) compared to the healthy control dogs (16.8; $P = 0.011$) (Fig. 2). There were no significant differences between *Babesia*-infected dogs that survived and the healthy control dogs ($P = 0.053$), as well as between the survivors and non-survivors ($P = 0.111$). The median WBC- and segmented neutrophil counts were significantly lower in the *Babesia*-infected dogs ($P = 0.001$ and $P = 0.006$, respectively), and the band neutrophil count significantly higher ($P < 0.001$) compared to the healthy

control dogs. For the dogs that survived the WBC- and segmented neutrophil counts were significantly lower ($P < 0.001$ and $P = 0.002$, respectively), and the band neutrophil count significantly higher ($P < 0.001$) compared to the healthy control dogs. For the dogs that died only the band neutrophil count was significantly higher ($P < 0.001$) compared to the healthy control dogs. Both the segmented- and band neutrophil counts were significantly higher in the non-survivors compared to the survivors ($P = 0.049$ and $P < 0.001$, respectively).

**Table 1:** Descriptive statistics for selected leukocyte counts, MPXI and cytokine concentrations in dogs with babesiosis and healthy controls

| **Variable** | **Unit** | **Controls**<br>Median (IQR) | ***Babesia*-infected**<br>Median (IQR) | **Survivors**<br>Median (IQR) | **Non-survivors**<br>Median (IQR) |
|---|---|---|---|---|---|
| WBC count | $\times 10^9$/L | 9.9 (8.1-12.1) | 6.2 (4.8-9.8) | 6.1 (4.7-9.4) | 14.4 (5.0-21.6) |
| Seg Neut count | $\times 10^9$/L | 5.8 (5.0-7.5) | 4.0 (2.8-6.2) | 4.0 (2.8-5.8) | 9.2 (3.4-15.1) |
| Band Neut count | $\times 10^9$/L | 0 (0-0.1) | 0.3 (0.2-0.8) | 0.3 (0.1-0.6) | 1.0 (0.5-1.9) |
| MPXI | | 16.8 (12.8-19.7) | 19.2 (14.9-22.8) | 19.0 (14.7-21.8) | 22.8 (17.7-26.1) |
| IL-2 | pg/mL | 19.0 (12.2-227.3) | 28.2 (3.4-107.9) | 27.5 (3.4-100.7) | 40.8 (3.4-314.3) |
| IL-6 | pg/mL | 43.6 (23.4-348.3) | 92.9 (37.1-497.8) | 84.1 (35.7-329.9) | 643.9 (77.1-1097.4) |
| IL-8 | pg/mL | 3316.7 (1528.1-4886.1) | 1066.3 (342.1-2883.5) | 1066.3 (318.0-3209.2) | 1171.7 (780.1-2447.8) |
| IL-10 | pg/mL | 44.6 (8.4-280.9) | 620.3 (319.5-1126.1) | 607.5 (308.7-1043.5) | 849.3 (339.6-2035.1) |
| IL-18 | pg/mL | 43.6 (24.8-420.1) | 52.3 (30.6-179.1) | 52.3 (31.4-168.1) | 65.1 (20.7-610.0) |
| GM-CSF | pg/mL | 21.8 (9.1-166.3) | 29.3 (11.1-130.1) | 29.2 (13.5-123.5) | 45.8 (9.1-419.9) |
| MCP-1 | pg/mL | 211.7 (115.0-275.3) | 544.2 (370.2-813.3) | 527.6 (361.7-741.2) | 921.7 (577.5-1356.2) |

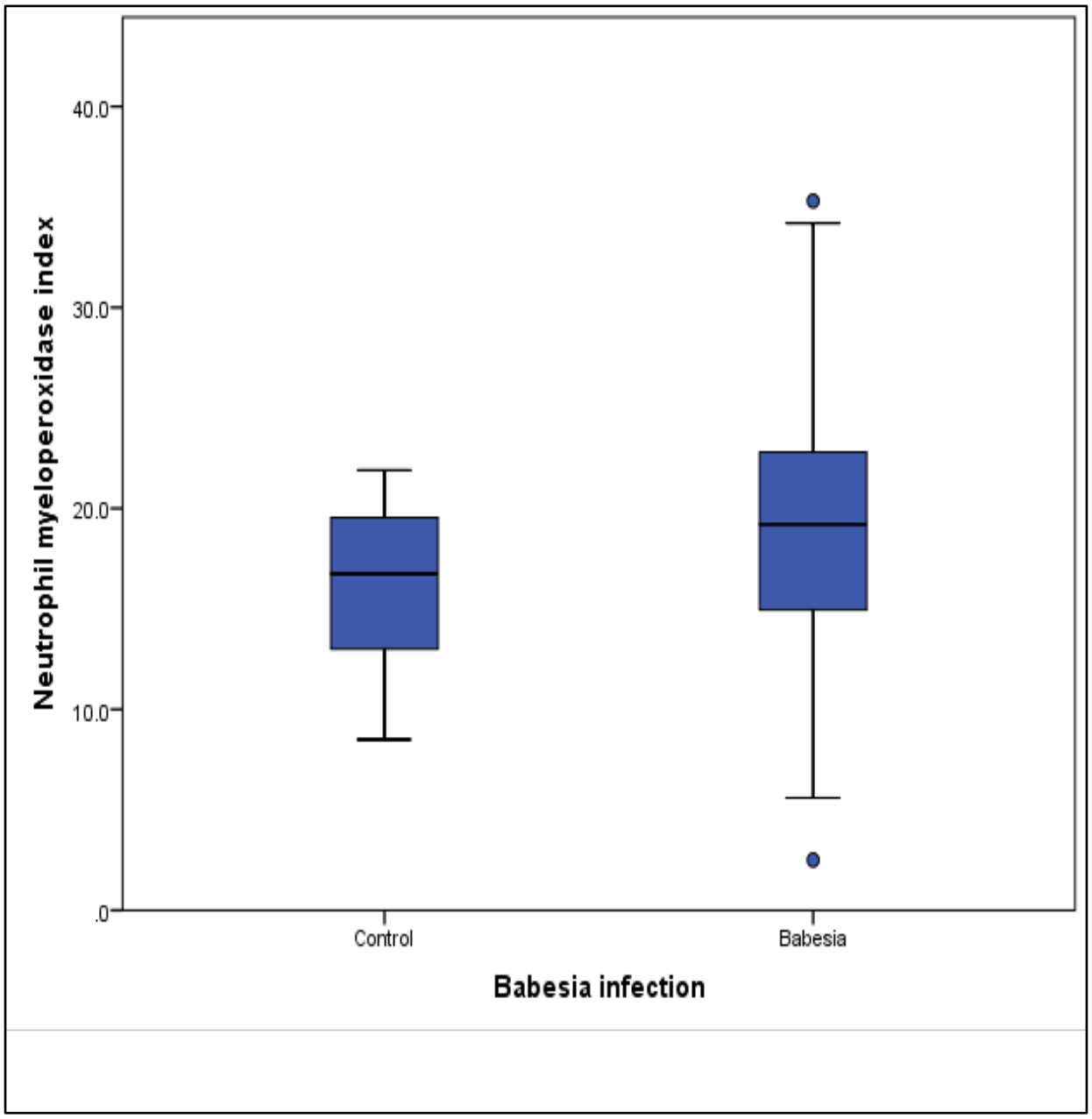


*Figure 1*: *Box plot of the admission MPXI of the Babesia-infected group compared to the healthy control group.* The graph displays as horizontal lines the 10$^{th}$, 25$^{th}$, 50$^{th}$, 75$^{th}$ and 90$^{th}$ percentiles of the MPXI. All values below the 10$^{th}$ percentile and above the 90$^{th}$ percentile are plotted separately as dots.

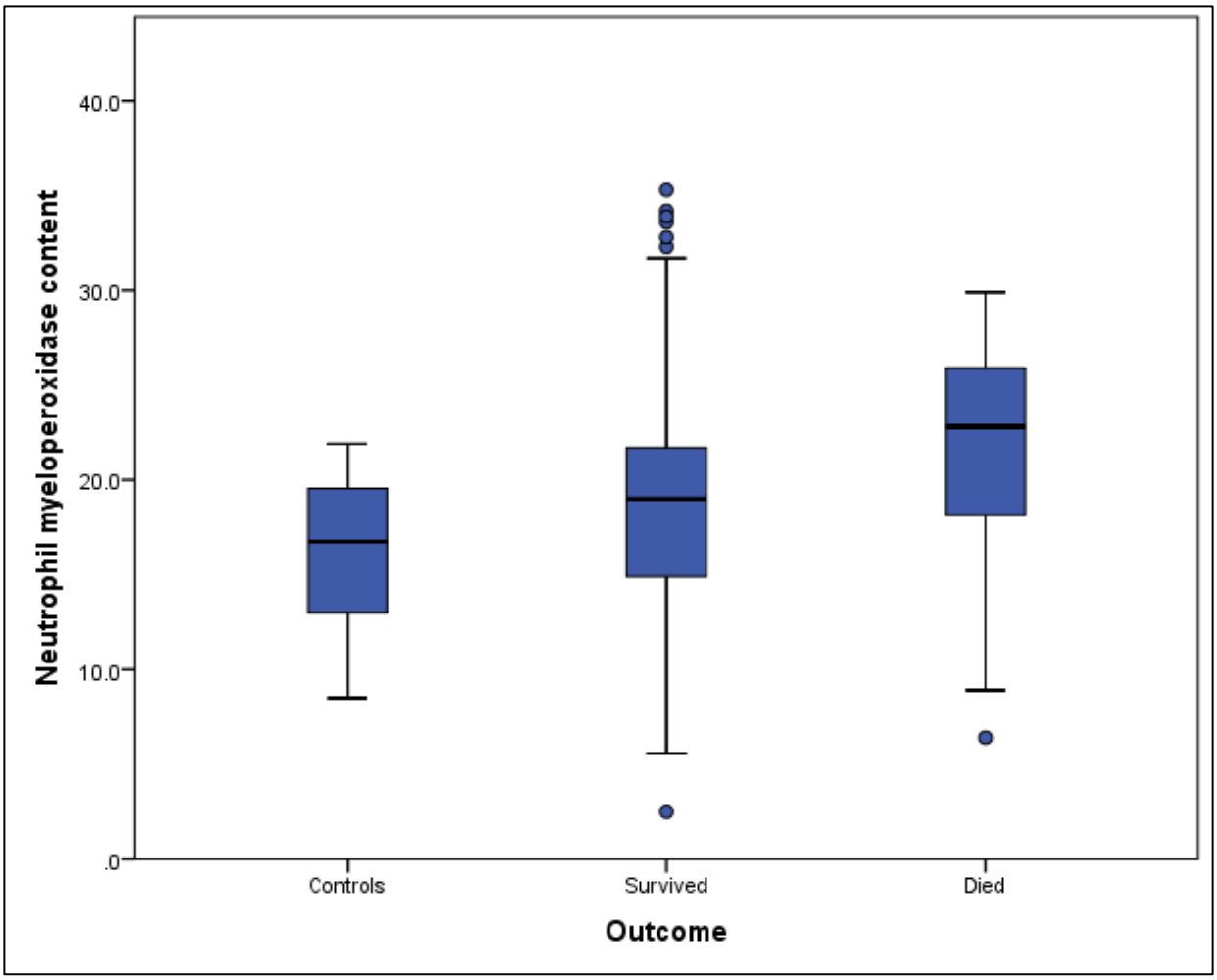


*Figure 2*: *Box plot of the admission MPXI of the Babesia-infected survivors and non-survivors compared to the healthy control group.* The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the MPXI. All values below the 10th percentile and above the 90th percentile are plotted separately as dots.

## 4.3 Correlation of the MPXI results with selected leukocyte counts and various pro-inflammatory and immune modulating cytokine concentrations in dogs suffering from *B. rossi*.

Table 1 contains a summary of all the data, including the median and the IQR for each variable. No significant correlations were found in any group (controls, survivors and non-survivors) between MPXI and leukocyte count. Similarly, no significant correlations were found between MPXI and the segmented or band neutrophil counts.

For the *Babesia*-infected group a significant positive correlation was found between MPXI and IL-10 ($r$ = 0.211, $P$ = 0.039), and a significant negative correlation between MPXI and IL-8 ($r$ = -0.350, $P < 0.001$). For the dogs that died, significant positive correlations were found between MPXI and IL-2 ($r$ = 0.616, $P$ = 0.033), IL-6 ($r$ = 0.615, $P$ = 0033), IL-18 ($r$ = 0.613, $P$ = 0.034), GM-CSF ($r$ = 0.630, $P$ = 0.028) and MCP-1 ($r$ = 0.713, $P$ = 0.009). In dogs that survived, a significant negative correlation was found between MPXI and IL-8 ($r$ = -0.363, $P$ = 0.001).

# Chapter 5: Discussion

## 5.1 General discussion

This is the first report on the use of MPXI, as determined by the ADVIA 2120, as a marker of disease severity in dogs with babesiosis. The findings suggest that a higher MPXI in infected dogs, especially non-survivors, is associated with a more severe inflammatory response in dogs with *B. rossi* infection.

The median MPXI was significantly higher in *B. rossi*-infected dogs, specifically the non-survivors, compared to healthy control dogs. *Babesia rossi* is the predominant tick-borne parasite infecting dogs in South Africa and is the most virulent of the dog *Babesia* species.[34,95] The mortality rate of 11% observed in this study was similar to previously described mortality rates.[34,95] This disease has been found to be highly inflammatory with frequently encountered complications due to the exuberant host pro-inflammatory response.[4,27,30,96] It is thought that a large part of the disease pathogenesis is due to the host inflammatory reaction to the infection, rather than the parasite itself.[97] Since babesiosis can be classified as a protozoal septic condition, it's expected to display shared pathophysiological features with malaria, sepsis and SIRS due to other causes.[3,98,99] Highly inflammatory conditions can trigger increased granulopoiesis in the bone marrow, leading to toxic neutrophil changes. The severity of the toxic changes is often correlated with the disease severity.[73] It is reported that neutrophils with toxic changes, due to accelerated maturation, contain an increased amount of MPO.[80,87,100] With local or systemic inflammation and resultant neutrophil activation, MPO can be expected to increase, with a concomitant increase in MPXI.[76] When neutrophils are stimulated to degranulate, often due to a severe inflammatory stimulus, MPO levels will decrease since it is consumed in the respiratory burst reaction.[76]

In humans, MPXI has been investigated as a marker of inflammation.[76,85] One study reported that MPXI was higher in sepsis than in SIRS due to non-infectious causes, but in most other studies it was found to be decreased in sepsis and severe inflammatory reactions.[76,83,84] Studies in horses showed that neutropenic septicaemic foals had an

increased MPXI.[88] In contrast, other studies have shown decreased MPXI with sepsis and systemic inflammation in horses.[72,87] Dogs with severe inflammation and sepsis due to viral or bacterial causes, showed a decrease in neutrophil MPXI and acquired MPOD.[71]

The increased MPXI in the *Babesia*-infected dogs in our study can therefore be secondary to the established inflammatory response present in this disease. Considering that the marked pro-inflammatory host response reported in *B. rossi* infection has been correlated with mortality, it was surprising, based on the current literature on MPXI changes in severe inflammation and sepsis, that the non-surviving dogs had the highest MPXI. In *B. rossi*-infections, MODS is a frequent cause of death.[4,14,27] One of the causes of lethal tissue damage is the excessive release of neutrophil reactive oxygen species (ROS) during the respiratory burst.[46] Therefore, the expected change would have been a decreased MPXI in non-survivors, due to widespread neutrophil degranulation and respiratory burst in an upregulated response to the *Babesia* parasite.

Earlier research on neutrophil function in *B. bovis* reported that neutrophils displayed increased phagocytic activity, but reduced respiratory burst during peak parasitaemias.[42] No explanation was offered for these findings. Two more recent studies, looking at immune function in septic and critically ill dogs, found that neutrophil function was impaired.[101,102] One study reported that both respiratory burst ability and cytokine production (TNF-α, IL-6 and IL-10) were reduced in these cases.[102] It was hypothesized that there could be dysfunction in the NADPH oxidase complex, which is the enzyme tasked with catalysing one of the initial reactions in the respiratory burst complex.[102] A concept called sepsis-induced immunosuppression or immunoparalysis, similar to what has been portrayed in humans, was used to describe this phenomenon.[101] Immunoparalysis imparts a negative prognosis on affected animals.[101] One study that investigated the lymphocyte phenotypes in dogs with *B. rossi* infection reported that the percentage of T lymphocytes and specifically helper T lymphocytes were reduced in complicated cases, concluding that functional immune suppression or immunoparalysis was most likely the underlying cause.[103]

Both a hypo- and hyper-inflammatory immune response can thus be detrimental to the host.[104] It is therefore possible that the significantly higher MPXI present in the non-survivors, compared to the healthy controls, were not only due to an excessive inflammatory

response, but also secondary to impaired neutrophil respiratory burst function, resulting in unutilised intracellular MPO store and a poorer prognosis in these cases. No significant difference was present between the survivors and the healthy control dogs.

No significant correlation was found between neutrophil MPXI and leukocyte, segmented neutrophil or band neutrophil counts. This was an unexpected finding since increased MPXI has been associated with a neutrophilia, especially a left shift neutrophilia in acute inflammation.[73] However, one study in dogs with various disease conditions, did report that MPXI did not correlate well with band neutrophilia. This was thought to be due to variation in neutrophil activation and behaviour for the various conditions, which limited the extent of change identified within the peroxidase indicators.[73] Furthermore, toxic changes found in immature neutrophils can be apparent before changes in the leukogram are detected. In horses and humans, MPXI was found to change independently of the different leukocyte types.[72,76,105] In babesiosis, a neutropenia or neutrophilia has been described depending on the sampling time-point during the disease process. Neutropaenia is often encountered initially before antibabesial treatment, with the suspected cause to be increased neutrophil-endothelial interaction.[2] Nearly 50% of *B. rossi* cases in one study had neutropaenia at presentation.[106] In another study, investigating complicated babesiosis cases, only 10% showed leukopaenia and 60% had leukocytosis.[27] Similar changes could be seen in our study. Nevertheless, MPXI seems to be more dependent on the balance between granulopoiesis, with increased MPO production in response to inflammation and degranulation.[76]

A strong positive correlation was found between MPXI and various pro-inflammatory cytokines, including IL-2, IL-6, IL-18, GM-CSF and MCP-1, in the dogs that died. A previous study by our group, using the same dataset as the current study, showed significantly higher IL-6 and MCP-1 concentrations in dogs that died.[4] It also found that IL-2, IL-6, IL-18 and GM-CSF were significantly higher in the acute stages of *B. rossi* infection.[4] Based on the earlier discussion, an increase in MPXI can be in response to inflammation or possibly due to immunoparalysis, secondary to critical disease, and thus a positive correlation with the above mentioned pro-inflammatory cytokines is expected. The increases in IL-6 and MCP-1 in non-survivors might also then explain why MPXI values were the highest in the non-survivor group.

A negative correlation was observed between MPXI and IL-8 in the *Babesia*-infected dogs that survived. IL-8 is responsible for the attraction and activation of neutrophils to undergo respiratory burst.[46] It is therefore an important moderator of neutrophil function and plays an essential role in the development of acute inflammation.[107,108] IL-8 can also inhibit neutrophil accumulation at inflammatory sites by inhibiting the adhesion of neutrophils to endothelium.[108] In human malaria studies, IL-8 was found to be positively correlated to the degree of parasitaemia and disease severity upon presentation.[108] Similar results were seen in *B. canis*, where serum concentrations of IL-8 were found to be increased.[58] This is in contrast with results in *B. rossi*-infected dogs, where IL-8 had been reported to be significantly lower compared to healthy controls, with no associations found with disease outcome.[4] The results of our study were interesting since it would be expected to see a positive correlation between MPXI and IL-8, especially in the acute stages of the disease. Higher MPXI in our study was correlated with lower IL-8 in survivors. A similar finding was reported in humans with falciparum malaria where IL-8 was found to be significantly lower in septic patients that survived, compared to the non-survivors.[108] At low IL-8 concentrations, stimulation of the respiratory burst reaction might not take place, resulting in higher neutrophil MPO reserves and a resultant increased MPXI, as observed in this study. This could probably have an immunosuppressive effect or a bystander-tissue protective effect, depending on the location of inflammation and the stage of the disease process.[62] It is also possible that this can result in an ineffectual pro-inflammatory response, especially in the acute phase, that can lead to a poorer prognosis.

A significant positive correlation was found between MPXI and IL-10 in *Babesia*-infected dogs in this study. IL-10 is produced by M2 macrophages and functions to suppress macrophages, Th1 and Th2 cytokine responses, NK cells and dendritic cells, and increases the expression of Treg cells. IL-10 therefore acts as an immune-modulating, anti-inflammatory cytokine by inhibiting both the innate and adaptive immune response.[46] Production of IL-10 seems essential in preventing an excessive inflammatory host response and thereby improving survival.[109] Previously, IL-10 was found to be significantly increased in *B. rossi*-infected dogs compared to control dogs, but no significant difference was seen between survivors and non-survivors.[4] IL-10 was also increased in dogs experimentally infected with *B. gibsoni*.[59] The positive correlation seen between MPXI and IL-10 is speculated to be due to two reasons. During severe sepsis, neutrophils can potentially undergo a phenotypic alteration enabling them to also produce IL-10.[110] Early host

neutrophil response is protective against sepsis, while later reactions can contribute to the pathological process.[110] IL-10 is not only produced by neutrophils, but also functions to inhibit them.[110,111] An increase in neutrophil activity during sepsis could therefore result in an increased MPXI and also an increased production in IL-10. One of the ways IL-10 inhibits neutrophil function is by directly preventing respiratory burst by inhibiting NADPH oxidase.[111] The inhibition of respiratory burst, leads to an increase in neutrophilic MPO stores and a resultant MPXI value. A positive correlation between MPXI and IL-10 as seen in this study, is thus expected.

## 5.2 Limitations of this study

The major limitation of this study is its retrospective nature. Even though blood samples were collected at presentation, prior to treatment, the timing of sampling during the course of the disease might have differed, with some dogs presenting very early on in the disease process and others during the later stages. Based on the previous discussion, there is variation in neutrophil activation and behaviour during the *Babesia* disease course. This might have influenced the leukogram and especially the neutrophil variables. The temporal differences could also have influenced the magnitude of MPXI changes in the control population compared to the *Babesia*-infected survivors and in the infected survivors and non-survivors. It is also difficult to draw meaningful conclusions from the *Babesia*-infected non-survivor group due to its small size. Even though multispecies software is used on the ADVIA 2120, the representative population used for the calculation of MPXI is human. This could have further influenced the correlation between leukocyte parameters and MPXI values. In a study looking at the predictability of disease using MPXI on the ADVIA haematology analyzer, differences in the reference values were found between breeds.[73] In human studies MPXI variation according to age and gender was found.[78] The age, breed and gender heterogeneity of the study population could thus have affected the MPXI values generated. Although each peroxidase plot had been evaluated by an experienced clinical pathologist to confirm correct separation of the neutrophil population, myeloperoxidase deficient neutrophils could still have been misclassified as monocytes. This is due to migration of neutrophil populations with low peroxidase activity on the peroxidase

scattergram, into the monocyte cluster. This could have further influenced the MPXI values obtained.

## 5.3 Future implications

Future experimental studies to establish individual baseline values and to monitor trends in MPXI, rather than making inferences based on values taken at a single time point will be more informative and beneficial to investigate disease pathogenesis. Particular consideration should be paid to the timing of sample taking when comparing different clinical populations.[48] The development of breed-specific MPXI reference intervals will also aid in the interpretation of MPXI. MPXI does show some potential as a prognostic indicator, which could be an easily obtainable, novel addition to the current repertoire of prognostic markers in canine babesiosis.

This study has also opened up new avenues for future research that could potentially broaden our understanding of the host response to haemoprotozoal disease. The hypothesis that sepsis-induced immunoparalysis could be a contributor to death in babesiosis and be a reason for elevated MPXI seen in non-survivors, deserves further attention. Neutrophil function tests performed in *B. rossi* infections, will give valuable additional information regarding the immune reaction against this parasite.

# Chapter 6: Conclusion

This study set out to determine whether MPXI, as a potential marker of inflammation, would be different in *Babesia*-infected dogs compared to healthy controls and whether there would be correlations with other markers of inflammation. MPXI was found to be significantly higher in *Babesia*- infected dogs compared to healthy control dogs and was even higher in non-survivors. MPXI was positively correlated with the severity of the cytokine-driven pro-inflammatory host response in non-survivors and negatively correlated with IL-8 in survivors.

The higher MPXI in infected and especially non-survivors in conjunction with cytokines could indicate an increased inflammatory response, as is expected in *B. rossi*-infections. Immunoparalysis might be present in more severely affected dogs, leading to poorer outcome.

Multiple factors could have influenced MPXI in this study: the downregulation of IL-8 as seen in previous studies looking at *B. rossi* infections; the inhibition of neutrophil respiratory burst function with subsequent immunoparalysis, due to critical illness; and the pro-inflammatory process associated with the disease. Neutrophil MPO content and therefore MPXI, changes with severe inflammation and protozoal sepsis, but the relationship between neutrophilic changes, inflammation and MPXI is still not clearly understood.

The potential of MPXI as a novel marker of inflammation and prognosis in *Babesia rossi*-infected dogs, warrants further exploration.

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# Appendices

## **Appendix A**: Data collection sheet

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 24 | | Male | Infected | Survived | 4,83 | 3,14 | 0,43 | 20,3 | 53,5 | 79,5 | 532,2 | 57,0 | 27,5 | 791,4 | 32,3 |
| 2 | 24 | 14,40 | Female | Infected | Survived | 16,33 | 10,45 | 2,29 | 31,9 | 62,9 | 6186,0 | 244,1 | 66,3 | 29,2 | 314,5 | 19,8 |
| 3 | 72 | 11,00 | Male | Infected | Survived | 5,64 | 4,40 | 0,34 | 617,3 | 569,1 | 1393,2 | 437,3 | 1766,6 | 492,9 | 667,0 | 16,9 |
| 4 | 15 | 11,00 | Male | Infected | Survived | 4,11 | 3,04 | 0,08 | 3,4 | 28,1 | 125,7 | 4762,5 | 32,4 | 15,6 | 315,5 | 27,4 |
| 5 | 48 | | Female | Infected | Survived | 4,46 | 2,99 | 0,04 | 69,7 | 138,4 | 45,1 | 1167,5 | 339,1 | 133,4 | 519,8 | 31,7 |
| 6 | 36 | 22,80 | Male | Infected | Survived | 17,61 | 12,33 | 1,58 | 13,1 | 26,5 | 1310,4 | 391,5 | 29,9 | 13,9 | 348,5 | 34,2 |
| 7 | 19 | | Female | Infected | Survived | 11,09 | 5,77 | 0,22 | 12,2 | 21,8 | 687,2 | 1833,6 | 48,7 | 18,2 | 198,9 | 21,7 |
| 8 | 12 | | Male | Infected | Survived | 8,98 | 5,75 | 0,00 | 29,2 | 121,1 | 133,4 | 370,2 | 129,5 | 61,0 | 673,0 | 35,3 |
| 9 | 7 | | Male | Infected | Survived | 5,82 | 3,72 | 0,17 | 3,4 | 28,1 | 21,6 | 4756,6 | 22,3 | 9,1 | 424,1 | 32,8 |
| 10 | 24 | | Female | Infected | Survived | 4,39 | 2,33 | 0,31 | 92,4 | 128,9 | 141,1 | 1535,9 | 231,9 | 159,8 | 527,6 | 33,6 |
| 11 | 4 | 8,4 | Male | Infected | Survived | 15,22 | 9,59 | 2,28 | 22,0 | 133,7 | 5488,8 | 332,5 | 48,7 | 25,0 | 293,9 | 33,9 |
| 12 | 10 | 26,0 | Female | Infected | Survived | 6,63 | 4,77 | 0,07 | 3,4 | 29,7 | 8748,6 | 432,4 | 17,3 | 9,1 | 262,6 | 18,6 |
| 13 | 79 | 40,4 | Male | Infected | Died | 16,64 | 9,48 | 1,66 | 3,4 | 25,0 | 21,6 | 951,5 | 5,7 | 9,1 | 620,7 | 22,7 |
| 14 | 6 | 6,8 | Female | Infected | Survived | 21,06 | 17,06 | 0,42 | 3,4 | 39,3 | 3561,9 | 334,2 | 18,5 | 9,1 | 198,2 | 25,2 |
| 15 | 20 | 15,0 | Male | Infected | Survived | 6,66 | 4,33 | 0,13 | 45,2 | 171,4 | 2404,1 | 8189,7 | 169,3 | 110,4 | 613,6 | 29,4 |
| 16 | 9 | 29,8 | Male | Infected | Survived | 7,79 | 6,15 | 0,31 | 2529,7 | 9315,4 | 292,9 | 437,3 | 745,1 | 1039,9 | 1854,0 | 28,5 |
| 17 | 12 | 35,0 | Male | Infected | Survived | 6,36 | 3,24 | 0,19 | 135,9 | 268,3 | 3376,8 | 381,7 | 163,4 | 85,3 | 415,9 | 20,0 |
| 18 | 12 | 4,1 | Male | Infected | Survived | 2,90 | 1,39 | 0,17 | 34,5 | 56,6 | 3117,8 | 1134,8 | 86,2 | 19,0 | 358,5 | 17,7 |
| 19 | 53 | 33,0 | Female | Infected | Survived | 8,54 | 4,36 | 2,56 | 34,5 | 94,4 | 57,3 | 811,8 | 52,3 | 40,3 | 742,1 | 14,0 |
| 20 | 6 | 10,4 | Female | Infected | Survived | 7,87 | 4,09 | 0,31 | 74,2 | 165,1 | 2313,8 | 128,7 | 124,8 | 112,4 | 293,5 | 17,7 |
| 21 | 2 | | Female | Infected | Died | 5,78 | 3,47 | 0,46 | 53,4 | 86,5 | 791,8 | 1590,3 | 82,7 | 62,3 | 657,1 | 25,7 |
| 22 | 10 | 25,0 | Male | Infected | Survived | 11,22 | 8,53 | 1,01 | 19,4 | 55,1 | 2577,1 | 162,7 | 43,7 | 20,7 | 211,4 | 16,8 |
| 23 | 32 | 10,8 | Male | Infected | Survived | 3,73 | 2,54 | 0,45 | 2389,8 | 6718,3 | 5639,0 | 12786,5 | 2249,8 | 2920,8 | 5950,5 | 28,7 |
| 24 | 84 | 65,0 | Male | Infected | Survived | 16,60 | 10,62 | 1,83 | 53,4 | 64,5 | 10065,4 | 628,8 | 97,9 | 75,9 | 307,0 | 16,1 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 3 | 10,4 | Female | Infected | Survived | 7,44 | 2,98 | 0,00 | 3,4 | 18,6 | 857,8 | 412,8 | 16,0 | 9,1 | 769,0 | 14,9 |
| 26 | 3 | 11,4 | Male | Infected | Survived | 7,38 | 4,72 | 0,07 | 3,4 | 29,7 | 1964,7 | 969,7 | 32,4 | 9,1 | 800,2 | 20,3 |
| 27 | 7 | 14,0 | Male | Infected | Survived | 4,63 | 2,64 | 0,14 | 136,8 | 241,8 | 834,7 | 851,2 | 110,8 | 119,9 | 330,1 | 17,4 |
| 28 | 12 | 13,4 | Male | Infected | Survived | 5,19 | 3,32 | 0,26 | 273,2 | 495,8 | 171,9 | 3019,6 | 306,1 | 204,4 | 459,5 | 21,8 |
| 29 | 84 | 30,0 | Female | Infected | Survived | 5,90 | 4,78 | 0,06 | 37,2 | 99,1 | 21,6 | 555,2 | 31,2 | 43,7 | 563,7 | 17,0 |
| 30 | 48 | 49,6 | Male | Infected | Survived | 4,29 | 1,89 | 0,69 | 39,9 | 77,1 | 1097,2 | 607,5 | 67,5 | 54,6 | 372,3 | 28,2 |
| 31 | 7 | 16,6 | Male | Infected | Survived | 5,78 | 3,76 | 0,23 | 3,4 | 3,6 | 880,8 | 906,2 | 24,8 | 9,1 | 323,7 | 16,5 |
| 32 | 48 | 20,0 | Female | Infected | Survived | 6,07 | 4,49 | 0,18 | 84,2 | 121,1 | 3610,1 | 470,0 | 166,9 | 75,9 | 555,9 | 17,3 |
| 33 | 18 | 32,0 | Male | Infected | Survived | 6,08 | 3,71 | 0,00 | 3,4 | 3,6 | 256,7 | 781,8 | 24,8 | 9,1 | 203,3 | 15,9 |
| 34 | 4 | 18,4 | Male | Infected | Died | 6,73 | 4,04 | 0,54 | 363,9 | 1925,9 | 2737,8 | 3702,9 | 981,2 | 640,5 | 1665,1 | 27,4 |
| 35 | 32 | 33,0 | Male | Infected | Survived | 9,04 | 4,70 | 2,35 | 15,9 | 73,2 | 4610,1 | 961,2 | 36,6 | 9,1 | 527,4 | 20,6 |
| 36 | 28 | 9,0 | Male | Infected | Survived | 5,32 | 2,77 | 1,38 | 164,7 | 218,2 | 447,3 | 228,5 | 587,9 | 263,8 | 544,2 | 23,8 |
| 37 | 35 | 13,0 | Male | Infected | Died | 17,78 | 11,02 | 3,20 | 3,4 | 788,0 | 2627,9 | 303,5 | 5,7 | 9,1 | 500,0 | 11,0 |
| 38 | 24 | 25,8 | Male | Infected | Survived | 5,60 | 3,47 | 0,50 | 14,0 | 19,9 | 754,1 | 93,2 | 45,1 | 20,3 | 230,8 | 19,2 |
| 39 | 96 | 37,0 | Female | Infected | Survived | 5,91 | 5,44 | 0,12 | 103,4 | 229,1 | 111,4 | 512,8 | 142,1 | 117,6 | 700,6 | 22,3 |
| 40 | 23 | 8,0 | Male | Infected | Survived | 5,19 | 3,22 | 0,16 | 3,4 | 3,6 | 5693,8 | 197,4 | 22,7 | 9,1 | 322,9 | 13,7 |
| 41 | 24 | 7,2 | Female | Infected | Survived | 3,68 | 2,28 | 0,26 | 43,3 | 57,4 | 101,0 | 169,7 | 80,6 | 44,3 | 360,7 | 25,4 |
| 42 | 144 | 18,2 | Male | Infected | Survived | 5,91 | 4,43 | 0,00 | 3,4 | 3,6 | 686,4 | 938,4 | 33,1 | 9,1 | 485,0 | 17,2 |
| 43 | 8 | 22,0 | Male | Infected | Survived | 11,49 | 4,25 | 3,91 | 15,0 | 540,6 | 9608,0 | 97,3 | 43,6 | 18,5 | 687,8 | 14,1 |
| 44 | 96 | 3,0 | Female | Infected | Survived | 6,22 | 4,48 | 0,12 | 26,9 | 143,4 | 1066,3 | 889,4 | 49,5 | 22,0 | 1484,3 | 25,8 |
| 45 | 120 | 54,0 | Male | Infected | Survived | 6,21 | 4,04 | 0,37 | 3,4 | 3,6 | 50,1 | 140,8 | 31,4 | 9,1 | 327,5 | 26,7 |
| 46 | 9 | 28,0 | Male | Infected | Survived | 6,43 | 4,31 | 0,32 | 261,5 | 520,5 | 838,1 | 276,1 | 396,1 | 296,3 | 459,4 | 15,2 |
| 47 | 78 | 9,2 | Male | Infected | Survived | 3,06 | 1,53 | 0,12 | 3,4 | 29,6 | 60,5 | 1799,5 | 31,4 | 9,1 | 402,1 | 21,4 |
| 48 | 11 | 19,8 | Male | Infected | Survived | 3,80 | 1,06 | 1,44 | 16,0 | 94,1 | 229,1 | 2191,9 | 35,7 | 17,9 | 1470,9 | 21,2 |
| 49 | 20 | 15,6 | Male | Infected | Survived | 12,88 | 11,08 | 0,90 | 3,4 | 46,2 | 1188,0 | 325,5 | 22,7 | 9,1 | 883,9 | 25,5 |
| 50 | 35 | 33,2 | Male | Infected | Survived | 33,12 | 20,53 | 2,65 | 766,1 | 549,5 | 1054,8 | 656,7 | 1097,0 | 410,1 | 535,0 | 15,9 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | 21 | 4,4 | Female | Infected | Survived | 5,18 | 3,06 | 0,41 | 98,0 | 159,7 | 1851,5 | 78,0 | 97,8 | 94,9 | 539,8 | 17,3 |
| 52 | 18 | 36,0 | Female | Infected | Survived | 11,36 | 9,09 | 0,11 | 17,6 | 35,7 | 859,5 | 176,5 | 28,8 | 19,8 | 651,8 | 16,6 |
| 53 | 54 | 14,2 | Male | Infected | Survived | 13,67 | 7,11 | 2,32 | 36,6 | 74,0 | 300,8 | 150,1 | 25,3 | 26,7 | 395,4 | 20,6 |
| 54 | 10 |  | Male | Infected | Survived | 8,25 | 4,95 | 0,58 | 86,6 | 391,4 | 682,4 | 776,3 | 128,7 | 133,1 | 1218,0 | 27,8 |
| 55 | 6 | 3,0 | Female | Infected | Died | 5,06 | 3,14 | 0,71 | 652,4 | 1022,3 | 776,2 | 366,9 | 734,3 | 515,3 | 1017,1 | 27,0 |
| 56 |  | 7,0 | Female | Infected | Survived | 10,01 | 7,01 | 1,00 | 23,7 | 48,8 | 9135,9 | 633,5 | 46,2 | 24,2 | 426,6 | 13,6 |
| 57 | 4 | 13,8 | Male | Infected | Survived | 6,16 | 4,74 | 0,06 | 1342,2 | 2300,3 | 1361,0 | 1931,6 | 616,1 | 568,0 | 1424,2 | 19,5 |
| 58 | 12 | 21,6 | Male | Infected | Died | 14,75 | 11,21 | 1,77 | 21,4 | 971,5 | 1295,9 | 830,9 | 26,2 | 16,0 | 1284,0 | 22,8 |
| 59 | 4 | 8,0 | Male | Infected | Died | 4,62 | 3,42 | 0,18 | 55,6 | 341,3 | 1662,4 | 2183,4 | 236,9 | 133,7 | 1194,7 | 21,4 |
| 60 | 40 | 7,5 | Male | Infected | Survived | 2,30 | 1,59 | 0,07 | 38,9 | 82,8 | 880,2 | 1934,4 | 101,4 | 93,8 | 619,1 | 26,8 |
| 61 | 37 | 26,2 | Male | Infected | Died | 23,93 | 16,99 | 1,91 | 992,9 | 2110,0 | 1907,6 | 330,5 | 1338,8 | 733,9 | 1380,2 | 29,9 |
| 62 | 5 | 17,8 | Female | Infected | Survived | 24,67 | 17,27 | 2,47 | 25,2 | 92,9 | 685,8 | 130,6 | 35,7 | 19,2 | 690,7 | 21,6 |
| 63 | 38 | 21,6 | Female | Infected | Survived | 4,75 | 3,14 | 0,00 | 1606,2 | 3022,4 | 2323,8 | 1378,0 | 2800,2 | 736,4 | 1523,7 | 16,7 |
| 64 | 56 | 28,2 | Female | Infected | Survived | 20,49 | 10,86 | 2,25 | 13,7 | 35,7 | 4005,7 | 105,3 | 31,4 | 19,8 | 283,1 | 21,1 |
| 65 | 72 | 27,0 | Female | Infected | Died | 25,70 | 16,96 | 1,80 | 3,4 | 74,0 | 1047,4 | 309,0 | 20,1 | 9,1 | 826,3 | 26,1 |
| 66 | 132 | 5,8 | Female | Infected | Survived | 3,86 | 2,70 | 0,23 | 375,3 | 663,3 | 362,2 | 48,8 | 865,6 | 410,1 | 1318,8 | 20,5 |
| 67 | 3 | 4,6 | Female | Infected | Survived | 1,80 | 0,14 | 0,22 | 29,0 | 516,7 | 11817,4 | 1117,3 | 51,5 | 33,7 | 1819,9 | 19,2 |
| 68 | 9 | 24,2 | Female | Infected | Survived | 9,78 | 5,77 | 0,78 | 3,4 | 26,6 | 2598,3 | 754,5 | 44,4 | 24,2 | 568,8 | 19,1 |
| 69 | 9 | 5,2 | Male | Infected | Survived | 6,21 | 3,35 | 0,56 | 3,4 | 3,6 | 3300,5 | 219,8 | 22,7 | 9,1 | 418,5 | 19,3 |
| 70 | 11 | 12,0 | Female | Infected | Survived | 7,35 | 4,63 | 0,51 | 3,4 | 3,6 | 239,4 | 141,4 | 20,9 | 9,1 | 396,8 | 23,3 |
| 71 | 4 | 12,6 | Male | Infected | Survived | 7,85 | 5,57 | 0,31 | 3,4 | 3,6 | 4811,9 | 653,4 | 15,7 | 9,1 | 740,3 | 8,7 |
| 72 | 42 | 23,2 | Male | Infected | Survived | 16,80 | 12,26 | 0,34 | 3,4 | 53,8 | 2391,7 | 491,0 | 14,8 | 9,1 | 434,7 | 18,7 |
| 73 | 96 | 40,0 | Male | Infected | Survived | 5,87 | 3,52 | 0,18 | 3,4 | 38,4 | 1377,2 | 875,1 | 33,1 | 19,8 | 362,6 | 16,0 |
| 74 | 36 | 20,0 | Male | Infected | Survived | 4,37 | 2,84 | 0,00 | 35,9 | 84,1 | 30,5 | 510,9 | 66,0 | 63,4 | 455,3 | 20,4 |
| 75 | 18 | 50,0 | Male | Infected | Survived | 12,73 | 6,87 | 0,64 | 61,7 | 110,5 | 4437,8 | 304,0 | 115,9 | 82,0 | 424,4 | 12,1 |
| 76 | 24 | 12,0 | Male | Infected | Survived | 6,34 | 3,49 | 0,32 | 27,5 | 41,0 | 410,0 | 365,2 | 72,4 | 42,5 | 368,1 | 22,9 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 77 | 13 | 29,4 | Male | Infected | Survived | 36,00 | 24,48 | 5,76 | 16,0 | 401,0 | 1118,4 | 340,4 | 32,3 | 16,0 | 1253,8 | |
| 78 | 7 | 31,6 | Female | Infected | Survived | 7,14 | 5,14 | 0,00 | 3,4 | 13,5 | 3029,1 | 251,1 | 20,9 | 9,1 | 587,2 | 21,2 |
| 79 | 108 | 35,6 | Male | Infected | Survived | 4,69 | 2,81 | 0,47 | 22,9 | 61,2 | 1377,2 | 620,3 | 41,8 | 21,7 | 709,0 | 13,2 |
| 80 | 8 | 28,2 | Female | Infected | Survived | 6,29 | 3,96 | 0,00 | 50001,0 | 50001,0 | 1677,8 | 937,2 | 50001,0 | 50001,0 | 7974,3 | 20,5 |
| 81 | 29 | 12,8 | Female | Infected | Died | 4,30 | 1,94 | 0,73 | 3,4 | 13,8 | 8861,0 | 576,8 | 22,5 | 9,1 | 472,9 | 8,9 |
| 82 | 24 | 38,0 | Male | Infected | Survived | 4,31 | 3,15 | 0,30 | 546,3 | 860,0 | 840,0 | 837,5 | 1625,4 | 720,2 | 927,9 | 11,2 |
| 83 | 96 | 19,0 | Male | Infected | Survived | 19,09 | 13,55 | 3,25 | 16,0 | 91,2 | 36889,7 | 747,9 | 53,0 | 69,8 | 1021,6 | 25,6 |
| 84 | 9 | | Female | Infected | Died | 4,12 | 0,91 | 0,99 | 28,2 | 1122,4 | 1008,4 | 2680,1 | 47,4 | 29,3 | 1690,2 | 25,6 |
| 85 | 36 | 18,6 | Male | Infected | Died | 4,99 | 3,79 | 0,40 | 165,5 | 499,7 | 242,6 | 867,6 | 182,3 | 113,1 | 563,1 | 18,6 |
| 86 | 96 | 38,0 | Male | Infected | Survived | 5,69 | 4,04 | 0,11 | 174,1 | 835,5 | 385,3 | 1240,4 | 471,2 | 257,2 | 1815,7 | 19,9 |
| 87 | 12 | 20,0 | Male | Infected | Survived | 13,08 | 8,24 | 1,05 | 3,4 | 910,9 | 7450,7 | 4571,4 | 19,1 | 9,1 | 664,7 | 20,5 |
| 88 | 36 | 9,0 | Male | Infected | Survived | 3,34 | 2,47 | 0,20 | 1256,8 | 2690,7 | 68,8 | 357,1 | 1792,4 | 1181,5 | 1283,0 | 28,2 |
| 89 | 24 | 25,6 | Female | Infected | Survived | 6,38 | 4,72 | 0,06 | 3,4 | 3,6 | 321,9 | 768,5 | 20,2 | 9,1 | 365,7 | 19,5 |
| 90 | 12 | 14,2 | Male | Infected | Survived | 5,56 | 3,06 | 0,78 | 2716,1 | 3599,7 | 2287,5 | 2840,3 | 4936,7 | 2446,3 | 707,6 | 29,2 |
| 91 | 36 | 6,6 | Female | Infected | Survived | 4,72 | 2,55 | 0,47 | 112,4 | 135,0 | 551,6 | 1669,9 | 175,9 | 127,1 | 537,9 | 26,9 |
| 92 | 3 | 8,6 | Male | Infected | Survived | 3,06 | 0,92 | 0,24 | 3,4 | 47,2 | 314,0 | 1673,6 | 23,7 | 13,0 | 732,0 | 21,1 |
| 93 | 6 | 20,2 | Male | Infected | Survived | 10,90 | 6,00 | 0,98 | 34,7 | 60,5 | 5686,1 | 313,4 | 66,8 | 38,2 | 325,8 | 19,0 |
| 94 | 11 | 55,0 | Male | Infected | Survived | 13,94 | 7,81 | 0,14 | 27,2 | 53,2 | 1252,4 | 325,9 | 56,1 | 33,4 | 149,6 | 21,8 |
| 95 | 10 | 20,0 | Female | Infected | Survived | 3,62 | 2,24 | 0,04 | 445,6 | 469,4 | 27723,4 | 846,9 | 680,7 | 302,9 | 391,9 | 12,8 |
| 96 | 36 | 10,0 | Male | Infected | Survived | 3,58 | 1,00 | 0,14 | 13768,3 | 19018,6 | 567,5 | 1519,2 | 4052,2 | 6412,9 | 2450,5 | 19,6 |
| 97 | 12 | 28,8 | Male | Infected | Survived | 6,67 | 4,54 | 0,13 | 14,1 | 102,9 | 804,6 | 140,0 | 39,7 | 18,7 | 501,9 | 18,5 |
| 101 | 23 | 19,3 | Male | Infected | Survived | 6,27 | 4,45 | 0,50 | | | | | | | | 10,9 |
| 102 | 11 | 28,6 | Male | Infected | Survived | 5,03 | 3,07 | 0,15 | | | | | | | | 7,6 |
| 103 | 9 | 11,8 | Female | Infected | Survived | 6,38 | 4,47 | 0,57 | | | | | | | | 18,3 |
| 104 | 9 | 4,7 | Male | Infected | Survived | 5,30 | 1,86 | 1,38 | | | | | | | | 13,3 |
| 105 | 48 | 18,0 | Female | Infected | Survived | 7,14 | 5,43 | 0,43 | | | | | | | | 14,0 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 106 | 43 | 9,8 | Male | Infected | Survived | 5,27 | 3,27 | 0,00 | | | | | | | | 15,8 |
| 107 | 60 | 7,4 | Male | Infected | Survived | 2,55 | 1,89 | 0,15 | | | | | | | | 21,0 |
| 108 | 24 | 11,6 | Male | Infected | Survived | 11,63 | 6,28 | 2,44 | | | | | | | | 15,8 |
| 109 | 36 | 32,0 | Male | Infected | Survived | 6,01 | 4,39 | 0,24 | | | | | | | | 16,5 |
| 110 | 6 | 23,0 | Female | Infected | Survived | 6,53 | 3,85 | 0,33 | | | | | | | | 21,0 |
| 111 | 9 | 18,6 | Male | Infected | Survived | 5,58 | 3,29 | 1,28 | | | | | | | | 11,4 |
| 112 | 3 | 8,8 | Female | Infected | Survived | 23,60 | 20,53 | 0,24 | | | | | | | | 17,3 |
| 113 | 18 | 6,8 | Male | Infected | Survived | 1,99 | 0,44 | 0,52 | | | | | | | | 17,3 |
| 114 | 19 | 23,8 | Male | Infected | Survived | 4,75 | 2,76 | 0,81 | | | | | | | | 19,7 |
| 115 | 5 | 5,8 | Female | Infected | Survived | 3,20 | 2,37 | 0,13 | | | | | | | | 22,8 |
| 116 | 6 | 12,7 | Male | Infected | Survived | 6,36 | 4,07 | 0,13 | | | | | | | | 18,8 |
| 117 | 36 | 14,6 | Male | Infected | Survived | 10,21 | 7,66 | 0,31 | | | | | | | | 7,6 |
| 118 | 24 | 14,7 | Male | Infected | Survived | 11,50 | 5,87 | 0,69 | | | | | | | | 11,1 |
| 119 | 48 | 31,6 | Male | Infected | Survived | 5,02 | 2,81 | 0,25 | | | | | | | | 15,0 |
| 120 | 72 | 10,6 | Male | Infected | Survived | 7,56 | 5,14 | 0,30 | | | | | | | | 13,9 |
| 121 | 10 | 3,4 | Female | Infected | Survived | 4,20 | 1,76 | 0,59 | | | | | | | | 12,7 |
| 122 | 10 | 7,8 | Female | Infected | Survived | 3,90 | 2,65 | 0,39 | | | | | | | | 10,8 |
| 123 | 4 | 3,6 | Female | Infected | Survived | 3,76 | 3,24 | 0,25 | | | | | | | | 14,1 |
| 124 | 18 | 6,4 | Male | Infected | Survived | 3,63 | 2,87 | 0,15 | | | | | | | | 13,2 |
| 125 | 72 | 6,2 | Male | Infected | Survived | 5,80 | 2,32 | 0,99 | | | | | | | | 5,6 |
| 126 | 30 | 30,6 | Male | Infected | Died | 103,55 | 67,31 | 21,75 | | | | | | | | 6,4 |
| 127 | 9 | 45,6 | Male | Infected | Died | 21,60 | 15,12 | 0,86 | | | | | | | | 23,7 |
| 128 | 23 | 20,0 | Female | Infected | Survived | 9,94 | 8,35 | 0,40 | | | | | | | | 20,3 |
| 129 | 29 | 21,0 | Male | Infected | Survived | 8,81 | 5,81 | 0,44 | | | | | | | | 16,2 |
| 130 | 36 | 6,5 | Female | Infected | Survived | 1,12 | 0,66 | 0,09 | | | | | | | | 13,0 |
| 131 | 60 | 34,0 | Male | Infected | Died | 14,36 | 9,19 | 2,87 | | | | | | | | 17,7 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 132 | 10 | 26,7 | Male | Infected | Survived | 5,20 | 3,95 | 0,16 | | | | | | | | 9,6 |
| 133 | 30 | 29,0 | Female | Infected | Survived | 7,11 | 4,55 | 0,36 | | | | | | | | 19,9 |
| 134 | 18 | 6,0 | Female | Infected | Survived | 9,63 | 8,28 | 0,10 | | | | | | | | 19,8 |
| 135 | 12 | 10,6 | Female | Infected | Survived | 5,14 | 2,26 | 0,26 | | | | | | | | 26,4 |
| 136 | 3 | 11,5 | Male | Infected | Survived | 9,27 | 7,05 | 0,65 | | | | | | | | 20,6 |
| 137 | 42 | 5,6 | Male | Infected | Survived | 5,40 | 2,16 | 0,54 | | | | | | | | 13,9 |
| 138 | 91 | 9,8 | Male | Infected | Survived | 5,19 | 2,39 | 0,42 | | | | | | | | 15,1 |
| 139 | 10 | 2,6 | Male | Infected | Survived | 4,90 | 2,65 | 0,39 | | | | | | | | 8,9 |
| 140 | 84 | 8,6 | Male | Infected | Survived | 4,04 | 2,10 | 0,00 | | | | | | | | 14,5 |
| 141 | 11 | 11,6 | Male | Infected | Survived | 9,59 | 7,10 | 0,48 | | | | | | | | 2,5 |
| 142 | 8 | 12,6 | Male | Infected | Survived | 12,81 | 7,69 | 2,18 | | | | | | | | 6,9 |
| 143 | 24 | 6,8 | Female | Infected | Survived | 4,47 | 2,99 | 0,18 | | | | | | | | 13,3 |
| 144 | 20 | 8,8 | Male | Infected | Survived | 4,84 | 1,94 | 0,19 | | | | | | | | 13,6 |
| 201 | 24 | 40,6 | Male | Control | Control | 14,07 | 9,29 | 0,00 | 12,2 | 42,4 | 1655,3 | 8,4 | 34,9 | 18,2 | 115,0 | 19,9 |
| 202 | 84 | 34,0 | Female | Control | Control | 8,58 | 5,83 | 0,00 | 227,3 | 291,7 | 7898,4 | 401,3 | 851,9 | 387,7 | 278,9 | 19,2 |
| 203 | 72 | 31,0 | Female | Control | Control | 7,80 | 4,91 | 0,00 | 4162,6 | 4393,4 | 1528,1 | 19,5 | 5241,0 | 2501,7 | 334,0 | 20,7 |
| 204 | 84 | 23,6 | Male | Control | Control | 12,22 | 8,55 | 0,24 | 3,4 | 16,1 | 837,3 | 1015,8 | 15,7 | 16,6 | 20,9 | 20,0 |
| 205 | 24 | 30,0 | Female | Control | Control | 11,60 | 5,80 | 0,00 | 16,0 | 43,6 | 2930,3 | 56,6 | 32,3 | 27,4 | 234,5 | 12,5 |
| 206 | 84 | | Female | Control | Control | 8,05 | 5,31 | 0,08 | 34,3 | 68,9 | 3769,1 | 280,9 | 43,6 | 21,1 | 208,6 | 21,9 |
| 207 | 48 | 15,0 | Female | Control | Control | 8,99 | 4,14 | 0,00 | 13,1 | 23,4 | 4063,1 | 6612,4 | 24,8 | 9,1 | 174,2 | 16,0 |
| 208 | 64 | 8,8 | Female | Control | Control | 10,57 | 7,50 | 0,11 | 405,5 | 663,1 | 4886,1 | 8,4 | 367,1 | 9,1 | 275,3 | 20,4 |
| 209 | 78 | 35,0 | Male | Control | Control | 4,72 | 3,12 | 0,00 | 3,4 | 3,6 | 1291,1 | 8,4 | 5,7 | 9,1 | 94,5 | 19,1 |
| 210 | 84 | 27,0 | Female | Control | Control | 10,84 | 7,59 | 0,11 | 12,2 | 26,5 | 6033,5 | 30,5 | 33,7 | 34,5 | 103,8 | 17,7 |
| 211 | 18 | 65,0 | Male | Control | Control | 7,86 | 3,54 | 0,24 | 937,0 | 1355,5 | 1261,0 | 8,4 | 1393,5 | 783,3 | 270,7 | 13,5 |
| 212 | 3 | 9,0 | Male | Control | Control | 17,23 | 8,96 | 0,17 | 19,0 | 29,7 | 5810,5 | 135,9 | 53,1 | 21,8 | 211,7 | 10,0 |
| 213 | 3 | 8,0 | Female | Control | Control | 13,28 | 6,11 | 0,00 | 168,2 | 189,3 | 3998,5 | 95,5 | 264,1 | 166,3 | 321,2 | 10,4 |

| Case | Age (mos) | Weight (kg) | Gender | Status | Outcome | WBC (x10^9/L) | Neut Seg (x10^9/L) | Neut Band (x10^9/L) | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) | IL-18 (pg/mL) | GM-CSF (pg/mL) | MCP-1 (pg/mL) | MPXI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 214 | 27 | 29,0 | Female | Control | Control | 8,39 | 4,20 | 0,08 | 54,0 | 348,3 | 3316,7 | 8,4 | 420,1 | 125,8 | 222,6 | 15,2 |
| 215 | 24 | 23,0 | Female | Control | Control | 11,46 | 5,50 | 0,00 | 3,4 | 3,6 | 1767,2 | 44,6 | 5,7 | 9,1 | 160,3 | 17,5 |
| 216 | 76 | 30,9 | Male | Control | Control | 7,68 | 5,22 | 0,00 | | | | | | | | 8,5 |
| 217 | 72 | 35,0 | Female | Control | Control | 10,84 | 6,83 | 0,11 | | | | | | | | 18,7 |
| 218 | 44 | 30,0 | Female | Control | Control | 14,43 | 7,50 | 0,00 | | | | | | | | 14,0 |
| 219 | 24 | 20,0 | Male | Control | Control | 9,21 | 5,16 | 0,00 | | | | | | | | 11,1 |
| 220 | 42 | 25,0 | Female | Control | Control | 8,25 | 5,78 | 0,00 | | | | | | | | 15,8 |