Cytological and histopathological bone marrow findings in dogs with natural *Babesia rossi* infection

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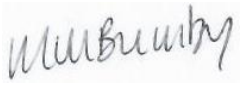
DECLARATION

I, **Maria Magdalena Bumby**, hereby declare that the following work is my own and that no part thereof has been submitted for other degree purposes at any other educational body.



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26 November 2021

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Date

ETHICS STATEMENT

I, Maria Magdalena Bumby, have obtained the necessary research ethics approval (V047-18) for the research described in this dissertation; I declare that I have 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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LIST OF ABBREVIATIONS

ARC	Absolute reticulocyte count
BFU-E	Burst-forming units- erythroid
CACS	Clinical Pathology, Department of Companion Animal Clinical Studies
CFU-BL	Colony-forming unit-B-cells
CFU-E	Colony-forming unit-erythroid
CFU–GEMM	Colony-forming unit–granulocyte, erythrocyte, monocyte/macrophage and megakaryocyte
CFU-lymphoid	Colony-forming unit-lymphoid
CFU-ME	Colony-forming unit - erythroid/megakaryocytic progenitor
CFU-meg	Colony-forming unit-megakaryocyte
CFU-TL	Colony-forming unit-T-cells
CHCMr	Mean haemoglobin concentration of reticulocytes
CHDWr	Distribution width (variability) of CHr
CHr	Reticulocyte haemoglobin content
CNP	Circulating neutrophil pool
CRP	C-reactive protein
CSF	Colony-stimulating factor
DAB	Diaminobenzidine
DALRRD	Department of Agriculture, Land Reform and Rural Development
DIC	Disseminated intravascular coagulation
DPS	Department of Paraclinical Sciences
DVTD	Department of Veterinary Tropical Diseases
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoeitin
FACS	Fluorescence-activated cell sorting
FeLV	Feline leukaemia virus
FVS	Faculty of Veterinary Science
GPI	Glycosylphosphatidylinositol
HB	Haemoglobin concentration
HCT	Haematocrit
HDW	Haemoglobin concentration distribution width
HE	Haematoxylin and eosin
HIER	Heat-induced epitope retrieval
HIF-1	Hypoxia inducing factor 1
HPF	High power field
HSCs	Haematopoietic stem cells
Hz	Haemazoin
IFN-ɣ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
IMHA	Immune-mediated hemolytic anemia
ISA	In-saline agglutination
KC-like	Keratinocyte chemotactic-like
M	Molar

MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Cell Haemoglobin Content
MCP-1	Monocyte-chemotactic protein-1
MCV	Mean cell volume
MCVr	Mean cell volume of reticulocytes
M:E	Myeloid:Erythroid
MHC	Major histocompatibility complex
MIF	Macrophage-inhibitory factor
MNP	Marginal neutrophil pool
MPXI	Myeloperoxidase index
NETs	Neutrophils extracellular traps
NK	Natural killer cells
NO	Nitric oxide
NRBC/100WBC	Nucleated red blood cells per 100 white blood cells
OVAH	Onderstepoort Veterinary Academic Hospital
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PRRs	Pattern recognition receptors
qRT-PCR	Quantitative real-time PCR
RBC	Red blood cell
RDW	Red Cell Distribution Width
RDWr	Distribution width of reticulocyte cell size
RET%	Reticulocyte percentage
RET-He	Reticulocyte haemoglobin equivalent
RLB	Reverse line blot
ROI	Regions of interest
ROS	Reactive oxygen species
RT	Room temperature
SAA	Serum amyloid A
SD	Standard deviation
SMA	Severe malarial anaemia
SOPs	Standard operating procedures
TEG	Thromboelastography
TGF-β	Transforming growth factor beta
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
UP	University of Pretoria
USA	United States of America
WBC	White blood cell
WCC	White cell count
WNL	Within normal limits

SUMMARY

The bone marrow is the primary site for haematopoiesis and is also the largest primary lymphoid organ. Malaria and babesiosis share many similarities regarding their pathogenesis, clinical disease and postmortem findings. Both are vector-borne protozoal diseases and characterized by the presence of anaemia, an inflammatory leukogram and thrombocytopaenia. The bone marrow response during human falciparum malaria has been studied previously, however, the bone marrow response during canine babesiosis has, to the authors knowledge, not been documented as yet. This study's purpose was to document the impact of canine babesiosis on the bone marrow by describing the cytological, histopathological and immunohistopathological changes and comparing the findings to that which has been described in human malaria. Bone marrow smears and sections were prepared and processed for cytological and microscopic examination from each of six *Babesia rossi*-infected dogs and five healthy control dogs. These findings were interpreted in conjunction with the circulating haemogram. A Perls' Prussian blue special stain for iron aided in assessing iron stores within the bone marrow. The application of CD3 (T-lymphocytes), CD20 (B-lymphocytes), Mum-1 (plasma cells), Mac387 (bone marrow derived monocytes and macrophages) and CD204 (resident tissue macrophages) immunohistochemical markers aided in differentiation of the various cell populations, after which analytic software provided the percentage of the above cell populations in the bone marrow of each infected and control case. The haemogram of *Babesia*-infected dogs revealed an inflammatory leukogram and a non-regenerative anaemia as was evident by an inadequate increase in the absolute reticulocyte count. This non-regenerative anaemia is also reported in human falciparum malaria where bone marrow dyserythropoiesis plays a major role in its pathogenesis. The bone marrow of the *Babesia rossi*-infected dogs were hypercellular on the cytological and microscopic examination, mainly because of the proliferation of erythrocyte precursors, notably rubriblasts. Dyserythropoietic changes were evident within the metarubricyte population of *Babesia*-infected dogs, where the number of metarubricytes was also decreased as compared to the control group. This suggests that there may be a suppression of erythropoiesis ultimately resulting in inappropriate erythrocyte regeneration as is evident by the decreased absolute reticulocyte count values. This suppression of erythropoiesis is likely caused by either direct or indirect influence of the disease on the bone marrow. A significant increase in the myeloid lineage was evident within *Babesia*-infected dogs. This is an appropriate response of the bone marrow in view of the systemic inflammation, although half of the infected cases showed a degenerative left shift neutrophilia. A marked decrease in the number of segmented neutrophils within the bone marrow were evident within the infected group as compared to the control group. Increased usage or destruction of neutrophils and prior exhaustion of the neutrophilic bone marrow storage pool may be contributing factors. A significant increase in the number of CD204 positive labelling resident macrophages was evident in the bone marrow of the infected group. This increased number of macrophages observed in tissues during canine babesiosis mirrors the findings of previous studies on canine babesiosis as well as human falciparum malaria. This increase is cytokine driven, forming part of the innate immune system and the first line of defense against the invading parasite. A left shift within the bone marrow megakaryocyte population was observed in the infected group. This is an adequate bone marrow response considering the presence of a peripheral thrombocytopaenia and this study therefore demonstrates that the thrombocytopaenia observed in canine babesiosis is likely of peripheral origin. Perls' Prussian blue stain for iron revealed a significantly increased iron content within the bone marrow of *Babesia*-infected dogs. In this study, reticulocyte indices suggestive of iron-restricted erythropoiesis were not decreased within the infected group and taking this, together with the presence of sufficient iron within the bone marrow into account, it could not be established that iron-restricted erythropoiesis plays a role in the anaemia of canine babesiosis. This study provides the first detailed description on the bone marrow changes during canine babesiosis. Our findings largely mirror what has been recorded in the bone marrow of humans with falciparum malaria and confirms that dyserythropoiesis is partly responsible for the inappropriate erythroid response that is evident in canine babesiosis.

INTRODUCTION

In addition to being one of the body's largest organs, the bone marrow is the primary site for haematopoiesis, and is the largest primary lymphoid organ (pertaining to antigen-independent lymphocyte production) and it also plays a crucial role as a secondary lymphoid organ due to the differentiation of terminal antigen-induced lymphoid cells [1,2]. Furthermore, the regenerative capabilities of the majority of peripheral lymphoid organs are dependent upon the pluripotent progenitor cells within the bone marrow [1]. This review focuses on the bone marrow response of two haemoparasitic diseases, namely canine babesiosis and human malaria, however, the bone marrow response to murine malaria and murine babesiosis are also briefly evaluated. Both malaria and babesiosis are vector-borne protozoal diseases and share many similarities regarding their pathogenesis, clinical disease and postmortem findings [3–5]. Numerous studies exist on malaria, some of which evaluated the bone marrow response, but literature on the bone marrow response during canine babesiosis is scarce or lacking, specifically relating to *B. rossi* infection. Furthermore, there is no comparison between the bone marrow pathology of canine babesiosis, that of human malaria, murine models of malaria or babesiosis in other species. This study investigates the impact of canine babesiosis on the bone marrow by describing the cytological, histopathological and immunohistopathological changes and compares this with malaria in humans and other animal models.

CHAPTER 1. LITERATURE REVIEW

1.1. Bone marrow structure and function

1.1.1. Gross / macroscopic anatomy and structure

In adult animals, bone marrow is located in the medullary cavity of flat and long bones and contributes approximately 5% to the total body weight [6]. Macroscopically bone marrow is a soft gelatinous tissue which can be divided into red and yellow marrow [6]. Red marrow consists of haematopoietic tissue with haeme chromogen responsible for the red colour thereof [7]. Monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets and osteoclasts arise from haematopoietic cells within the red marrow [7]. Yellow marrow consists of adipose tissue and is present in the mid-diaphyseal portions of the medullary cavity of long bones. It also fills the spaces of spongy bone in short bones in adults [7]. The quantity of adipose tissue increases with age and the cellularity decreases to the extent that haematopoietic cells may comprise as little as 25% of the marrow in older animals, compared to 100% at birth [6].

Bony trabeculae divide the bone marrow into haphazard, interconnected spaces. Various cellular components, including stromal cells and accessory cells and their products, form a complex microenvironment which provides a framework and nutrient support for developing haematopoietic cells [6,7]. Stromal cells include fibroblast-like reticular cells, endothelial cells, adipocytes and macrophages. Accessory cells include T-lymphocytes, natural killer cells and monocytes/macrophages. Accessory cell products include extracellular matrix and cytokines. Osteoblasts and osteoclasts of surrounding bone are present within the bone marrow as well [6]. Cells of the microenvironment play a crucial role in stem cell fate and in the regulation of haematopoiesis, either by means of direct cell to cell contact and/or by the release of soluble regulatory molecules. These regulatory molecules have either a positive or a negative influence on the growth of haematopoietic precursor cells [6,8]. This is achieved by the production of extracellular matrix (ECM) molecules, including collagen, fibronectin, laminin and proteoglycans [6]. Additionally, the bone marrow contains mesenchymal stem cells which can differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cells [7].

The bone marrow blood supply is extensive and is acquired from the nutrient artery entering the bony shaft at the nutrient foramen and from the periosteal capillary network (numerous small vessels that perforate the cortical bone) (Figure 1) [2,6]. The nutrient artery divides into branches extending throughout the marrow cavity and outward toward the cortical bone. It eventually branches to give rise to numerous small, thin-walled arterioles and capillaries. These arterioles anastomose with a branching network of venules and sinusoids near the surface of the bone [2,6]. Walls of the sinusoids consist of three layers: a monolayer of endothelial cells which line the vascular space, a middle, supportive layer of the basement membrane and reticular cells on the outside bone marrow space (Figure 2). Adjacent endothelial cells overlap to form loose intercellular junctions which serve as a selective barrier between blood and bone marrow [6]. The basement membrane is irregular and even absent in some areas in order to accommodate stretch/dilatation from hyperplastic bone marrow, whilst the reticular cells that form the outer wall of the sinusoid control access of bone marrow cells into the vascular space. These fibroblast-like reticular cells branch into the marrow space to form a supporting framework for haematopoietic cells and assist in haematopoiesis by binding of growth factors [6]. Migration of mature haematopoietic cells is either through loose intercellular junctions joining endothelial cells or through intracellular pores within endothelial cells. Cytoplasmic pseudopodia of megakaryocytes penetrate through the sinusoid wall to release platelets into the vascular space [6]. Sinusoidal blood drains into the longitudinal central vein which drains into venules in the perforating or nutrient canals. The concentration of sinusoids and venules are highest in the subcortical bone areas and this parallels haematopoietic activity [6,7]. Bone marrow does not possess lymphatic drainage, however, it does have a rich nerve supply via myelinated and non-myelinated nerve fibres that enter through the nutrient canal, accompanying blood vessels [2,6].

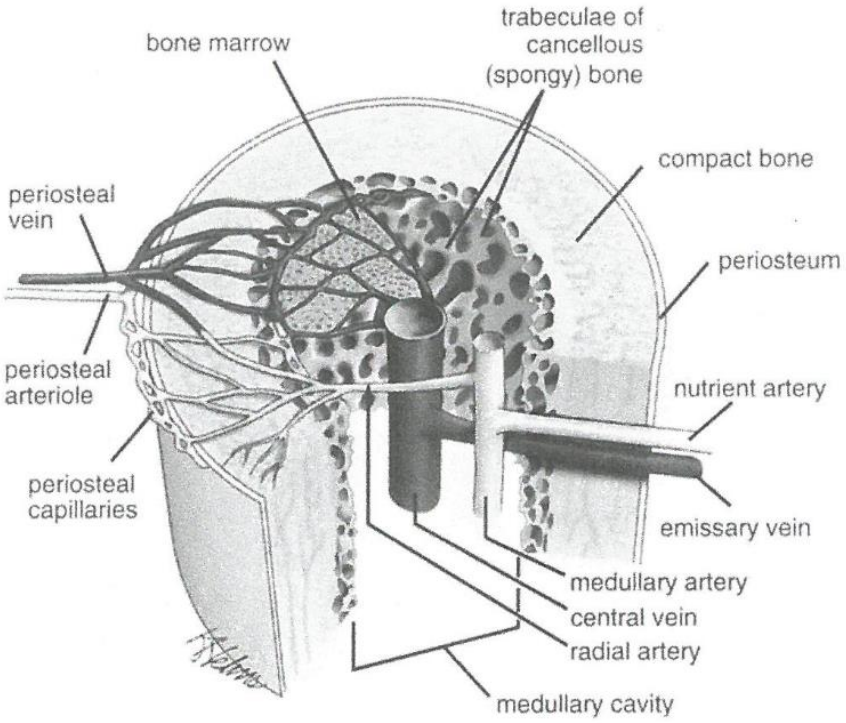

Figure 1: Schematic drawing illustrating the microcirculation of the bone marrow [6].

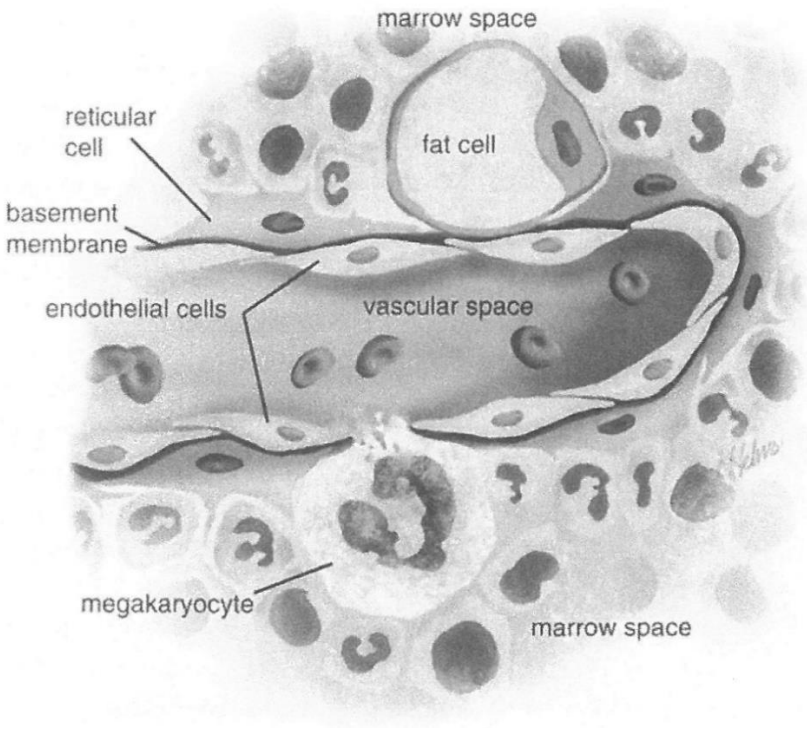

Figure 2: Schematic drawing of the bone marrow illustrating the cellular and vascular compartments. Note the trilaminar structure of the sinusoidal walls, consisting of endothelial cells on the inside, basement membrane forming the middle layer and providing support for haematopoietic cells and the fibroblast-like stromal/adventitial reticular cells comprising the outer layer [6].

1.1.2. Basic function

1.2.1.1. Haematopoiesis

Bone marrow is the primary site for haematopoiesis [7]. Mesenchymal stem cells within adult bone marrow also contribute to the renewal of connective tissues via differentiation into osteoblasts, chondrocytes, myocytes, adipocytes, tenocytes and neuronal cells [7].

In the embryo and foetus, haematopoietic stem cells (HSCs) migrate from the haematopoietic island within the yolk sac to the developing organs, especially the foetal liver which is mainly responsible for foetal haematopoiesis. The spleen, thymus and lymph nodes also contribute to foetal haematopoiesis to a lesser extent [6]. During late gestation, haematopoiesis shifts to the bone marrow, to become the principal site of haematopoiesis at birth. The erythroblastic island is located close to the vasculature and is the functional unit of erythropoiesis. The erythroblastic island comprises a central macrophage (nurse cell) encircled by erythroid precursors. The nurse cell is a minor source of erythropoietin and iron to the growing erythroid cells. HSCs are not morphologically distinct and give rise to erythroid, granulocytic-monocytic, lymphoid and megakaryocytic lineages. HSCs may differentiate into two types of multipotent precursor cells which includes the colony-forming unit-lymphoid (CFU-lymphoid) and the colony-forming unit–granulocyte, erythrocyte, monocyte/macrophage and megakaryocyte (CFU-GEMM). CFU-GEMM can undergo further differentiation to establish committed progenitors that can only multiply and mature along more limited scope of pathways [6]. A bipotent erythroid/megakaryocytic progenitor (CFU-ME) is responsible for the differentiation of erythrocytes and megakaryocytes and further differentiation of progenitors include colony-forming unit-erythroid (CFU-E) and colony-forming unit-megakaryocyte (CFU-meg) which are responsible for the development of erythrocytes and megakaryocytes respectively [6]. Differentiation of CFU-L results in CFU-TL and CFU-BL which are dedicated to becoming T- and B-lymphocytes respectively (Figure 3). When increased blood cells are needed, active haematopoiesis are also present in various extramedullary sites, including the liver and spleen [9].

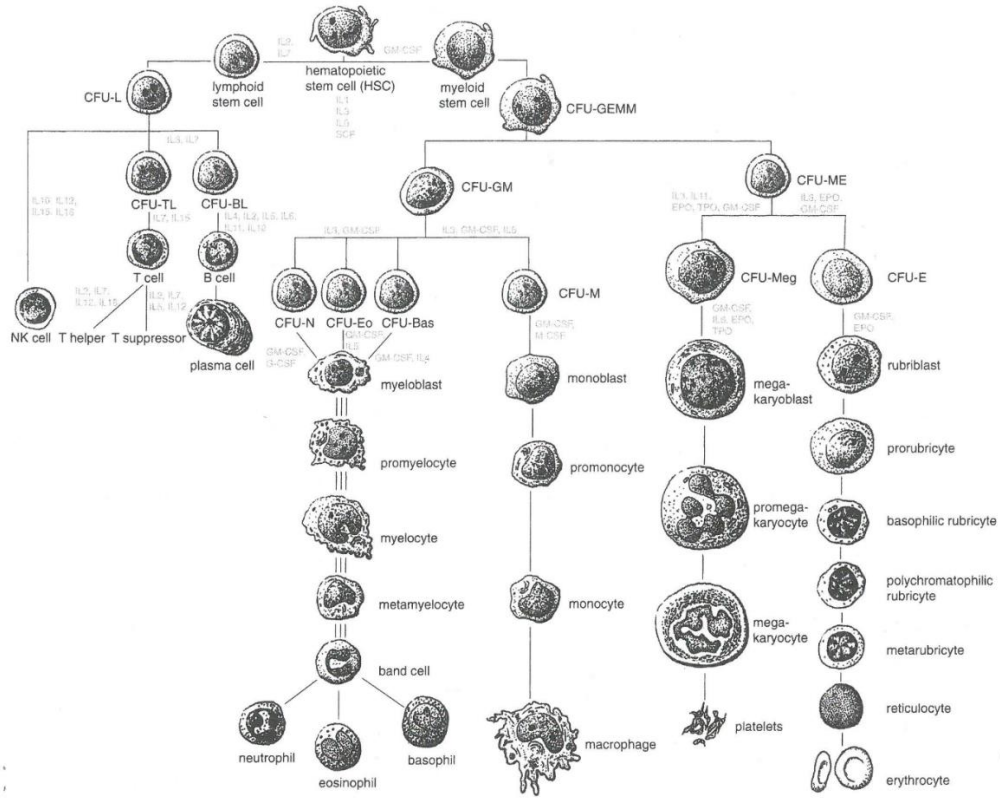

Figure 3: Schematic illustration of haematopoiesis [6].

Cytokines, including colony-stimulating factors, interleukins and growth factors, are crucial for haematopoiesis via regulation at various levels in the developmental pathways of haematopoietic cells. In general, growth stimulatory cytokines include colony-stimulating factor (CSF) and interleukins (ILs). Tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ), transforming growth factor-β (TGF-β) and lactoferrin are inhibitors of haematopoiesis [6].

Erythropoeitin (EPO) is a lineage-specific haematopoietic growth factor and is responsible for regulating the erythroid bone marrow response [9]. Erythropoietin production is induced in the renal cortex in response to renal hypoxia due to various causes, anaemia being most common [9]. The liver can additionally produce 10-15% of plasma EPO [10]. EPO elicits its action predominantly on the bone marrow erythroid precursor cells, i.e. the burst-forming units- erythroid (BFU-E) and the CFU-E. BFU-E are the early erythropoeitic precursors. When stimulated maximally, the bone marrow can produce erythrocytes at 10 to 50 times the normal rate [9]. Additionally, erythropoietin is responsible for the maturation of the early erythroid precursors to fully haemoglobinized erythrocytes, which usually takes one week [9]. It takes approximately 3-4 days for prorubricytes to mature into reticulocytes and reticulocytes persist in the bone marrow for 2-3 days [11]. Erythrocytes will therefore only be evident within the peripheral circulating blood after approximately five days [10]. EPO suppresses apoptosis of recently formed progenitory cells, thereby ensuring their maturation to mature erythrocytes [10]. Partial maturation of reticulocytes occurs in the peripheral circulation in healthy dogs [10].

1.2.1.1.1. Granulopoiesis

Myeloblasts, derived from CFU-GM, are the first stage of granulopoiesis (white blood cell formation). Myeloblasts further differentiate to become promyelocytes characterized by azurophilic (primary) granules, which give rise to myelocytes characterized by specific (secondary) granules [12]. Myeloblasts, promyelocytes and myelocytes can undergo mitotic cell division to replicate into additional cells, after which the myelocyte matures (in the absence of more cell division) into the metamyelocyte, band and mature segmented granulocytes [6]. Upon maturation of the above-mentioned white blood cells, there is a gradual reduction in cell size, condensation of nuclear chromatin and disappearance of nucleoli to ultimately form a nucleus which is segmented and a cytoplasm that progressively loses basophilia with the accumulation of secondary granules [6]. Mature granulocytes enter the bloodstream and these mostly consist of segmented neutrophils (see below).

1.2.1.1.1.a. Neutrophilic granulocytes

Neutrophils comprise the majority of blood leukocytes in healthy dogs and when their number varies, it has a significant impact the total white blood cell (WBC) count as a result [13]. Neutrophils within the bone marrow are grouped into two compartments, namely a proliferation compartment and a maturation and storage compartment. The proliferation compartment comprises myeloblasts, promyelocytes and myelocytes and makes up 10-30% of the bone marrow neutrophil population. The maturation and storage compartment comprises 65-90% of the bone marrow neutrophil population and include metamyelocytes, bands and segmented neutrophils [10]. Their release into the peripheral blood depends on the age of the cell, where more mature cells are released first. In dogs, the whole production and maturation process from myeloblast to segmented forms takes 3.5 – 6 days [13]. Mature neutrophils enter the bloodstream where some form the marginal neutrophil pool (MNP) that line the walls of capillaries and venules, whilst others form the circulating neutrophil pool (CNP) which are more centrally located within the vasculature. Neutrophils within the MNP enter the circulating pool in response to stress and exercise and many mature neutrophils are stored within the bone marrow to be released when the need arises [12]. The MNP and the CNP are approximately similar in size and may vary as they can freely exchange between pools. The total and differential neutrophilic cell count within the complete blood count only reflects the CNP. Neutrophils within the vasculature remain there for a brief period only and may migrate into tissues when the need arises [6]. When there is an increased demand for

neutrophils in tissues, mature neutrophils within the bone marrow storage pool are depleted, whereafter less mature forms are present within the circulation, designating a left shift [10]. A degenerate left shift is when the total leukocyte count is within the normal range or only slightly elevated, but the presence of immature granulocytes is prominent within the circulation.[14]. The degenerative left shift is an area of contention amongst pathologists, but this is how it was defined for the purpose of this study. A degenerative left shift is an inappropriate inflammatory response because the rate of neutrophil movement into inflamed tissue surpasses the ability of the bone marrow to deliver neutrophils to the peripheral circulation [14]. In dogs, neutrophils have a circulating half-life of about 5.5 – 7.5 hours and a tissue survival time of approximately 1-2 days, after which they undergo apoptosis with subsequent phagocytosis by macrophages [13]. Neutrophils' primary function is phagocytosis and destruction of bacteria, but this may also extend to include fungi, yeasts, algae, parasites and viruses [10]. They are also able to induce antibody-dependent cellular cytotoxicity in order to eliminate infected or damaged cells [10]. They reach inflammatory sites by means of chemotaxis.

1.2.1.1.1.b. Eosinophilic and basophilic granulocytes and mast cells

Maturation and differentiation of eosinophilic and basophilic granulocytes are largely similar to that of neutrophilic granulocytes, except for varying size, shape, colour and number of secondary granules between different animal species. Specific granules within eosinophils are lysosomes which contain various enzymes (peroxidase, acid phosphatase and arylsulfatase) and toxic proteins (major basic protein, eosinophil peroxidase and eosinophilic cationic protein). Specific granules within basophils include biogenic amines, heparin, hydrolytic enzymes, proteoglycans and major basic protein. Mast cells also arise from a pluripotent haemopoietic stem cell, but are distinct and differ from monocytic and granulocytic precursors. Mast cells are characterized by large numbers of basophilic granules within their cytoplasm and a spherical nucleus and although normal residents of the bone marrow, they are much fewer in number [6].

1.2.1.1.2. Monocytopoiesis

Monocytes and macrophages are derived from the CFU-GM progenitor cell, which gives rise to the monoblast with the promonocyte being the next stage of differentiation [6]. Macrophages are involved in a variety of host defense mechanisms against infection. This include phagocytosis of microorganisms, secretion of cytokine and chemical mediators of inflammation, antigen presentation to lymphocytes as well as being cytotoxic towards foreign cells (including tumours) [9]. There is no maturation or storage pool for monocytes within the bone marrow and they are released into the peripheral circulation about six days after generation [9,10]. Similar to neutrophils, monocytes also accumulate at inflammatory sites, albeit in lower numbers [10]. The majority of macrophages at sites of inflammation are derived from blood monocytes and although the exact life span of tissue macrophages is not known, it is thought that resident macrophages have an increased life span whereas macrophages or monocytes that respond to inflammatory stimuli have a shorter life span [10]. Monocytosis is a common during a variety of acute and chronic diseases, including infections, neoplasia, immune-mediated diseases, trauma, haemorrhage, haemolysis and corticosteroid treatment [9].

1.2.1.1.3. Erythropoiesis and erythroid precursors

Erythropoiesis is controlled by various stimulatory growth factors, including EPO, IL-6, IL-3 and IL-11, as well as inhibitory factors (TNF-α, IL-2, glucocorticoids) and transcription factors [10]. The first erythroid precursor is the rubriblast, originating from the CFU-E upon EPO stimulation. As the erythroid cell lineage matures, there is a reduction in the size of cells and of nuclei with concomitant aggregation of chromatin [10,11]. The nucleus is eventually released at the metarubricyte or normoblast stage resulting in reticulocytes [11]. Reticulocytes possess abundant ribosomes and are released into the circulation after which the ribosomes are lost and they mature to become circulating erythrocytes. Erythrocytes are ultimately removed by the mononuclear phagocyte system

upon senescence. This erythrophagocytosis occurs predominantly in the spleen although the liver and bone marrow are involved to a lesser extent [11]. The average erythrocyte life span in healthy dogs is 110 days [6,11].

1.2.1.1.4. Thrombopoiesis and platelet precursors

The first megakaryocytic progenitor cell is the megakaryoblast which originates from CFU-Meg upon stimulation with thrombopoietin. Megakaryoblasts undergo endomitosis (nuclear division and cytoplasmic maturation without cellular division) in the vicinity of vascular sinusoids within the bone marrow to form promegakaryocytes and megakaryocytes. The cell size gradually increases, as does the number of nuclei and the cytoplasmic volume (which also loses basophilia and acquires primary granules) upon megakaryocytic maturation. The final product of the megakaryocytic cell lineage are platelets which form via fragments of megakaryocytic cytoplasmic pseudopodia that protrude through the bone marrow vascular sinusoidal endothelium [6]. Platelets' primary function is haemostasis by adhering to the subendothelium with subsequent aggregation resulting in the formation of the primary platelet plug. Further recruitment of platelets to the site and the subsequent genesis of a fibrin thrombus curbs blood loss [10]. The life span of platelets within the peripheral circulation is about ten days [11]. Thrombocytopaenia is due to either a diminished production, increased elimination, sequestration or loss. Thrombocytosis may be reactive / subsequent to, amongst others, acute or chronic inflammation and iron deficiency [14].

1.2.1.1.5. Lymphopoiesis and lymphoid precursors

Lymphoblasts are the first recognizable progenitor cell within the lymphoid lineage. They are present in small numbers within the bone marrow and are almost indistinguishable from rubriblasts and early myeloblasts [6]. Prolymphocytes result from mitotic division and differentiation of lymphoblasts which in turn divide and differentiate into mature lymphocytes, including T-lymphocytes and B-lymphocytes, indistinguishable on their morphology alone. Further differentiation of B-lymphocytes may occur within the bone marrow to become plasma cells. B-lymphocytes are involved in the production of antibodies, i.e. humoral immunity [13]. B-lymphocytes exist for a few days to weeks [9]. A proportion of lymphoid precursors establish themselves within the thymus to mature to T-lymphocytes [6]. Self-tolerance is induced within the thymus by means of positive and negative selection [10]. After selection, lymphocytes develop and operate as either CD4+ T-helper cells or CD8+ cytotoxic T-cells [10]. During antigen presentation, CD4+ T-helper cells recognize antigen only when bound to class II major histocompatibility complex (MHC) antigens, whereas CD8+ cytotoxic T-cells only recognize antigen bound to MHC class I molecules [10]. T-cells form a crucial part of cell-mediated immunity and typically exist for months to years [9]. Lymphocytes leave the bone marrow and thymus (central lymphoid tissues) and are distributed to lymph nodes and other tissues via the peripheral blood [15]. The presence of B-lymphocytes within the peripheral circulation are transient, whereas T-lymphocytes are the predominant circulating lymphocyte [11]. Within the peripheral blood, lymphocytes are distributed into the circulating lymphocyte pool and the marginated lymphocyte pool [14]. Within lymph nodes, lymphocytes migrate to the lymph node cortex by means of specific receptor mediated interactions and chemokine induced transmigration across the endothelium of specialized postcapillary venules. Lymphocytes migrate throughout the node and exit via the efferent lymphatic vessels to reach the peripheral blood via the thoracic duct [14]. Lymphocytes also migrate to tissues (other than lymph nodes) to perform various functions. Here they may undergo blastogenesis, enter lymphatic vessels to return to the peripheral blood, or die [14]. A small proportion of lymphocytes within the bone marrow are large granular lymphocytes which may be either natural killer cells or cytotoxic T-lymphocytes. These cells play a vital role in tumour resistance and host immunity [6]. Second to neutrophils, lymphocytes are the most frequent blood leukocytes in dogs [9]. Less than 5% of the total body lymphocyte population are present within the peripheral circulation [13].

1.2. Bone marrow collection and evaluation

Bone marrow is collected from sites with active haematopoietic tissue. In dogs and cats this include the medullary cavities of the proximal femurs or humeri and the iliac crest [14]. Bone marrow collection is achieved by two methods, namely aspiration and core collection/trephine biopsy. It is common practice to obtain both samples routinely at the same time and at the same site [14,16,17]. Bone marrow evaluation via aspiration is a quick, easy and reliable method and it provides insight into the number and cytological features of marrow cells [17]. Additionally, marrow cells obtained via aspiration can also be further examined via cytogenetics, molecular and flow cytometric methods [17]. Better assessment of the M:E ratio and the maturation progression can be achieved via cytologic examination of aspirate samples since individual cells and cell population can be differentiated and evaluated [14]. Other advantages include improved detection of phagocytosis of haematopoietic cells or organisms, improved detection of dysplastic or neoplastic cell features, relatively low processing costs and the use of cytochemical staining and immunocytohemistry where some antibodies do not work on fixed tissues [14]. Pitfalls of bone marrow aspiration include minimal assessment of tissue architecture, difficult detection of necrosis or myelofibrosis, difficulty in the determination whether a sample is representative (i.e. to differentiate hypocellular bone marrow from an inadequate sample) and occasionally aspiration may yield a dry tap where no material was obtained [14,17]. Bone marrow trephine biopsies are useful to assess the spatial relationships between cells, to evaluate the overall bone marrow structure where bone marrow aspirates have failed or are inadequate, where there is a suspected focal lesion, overall haematopoiesis or in cases of bone marrow fibrosis [16,17]. Other advantages of histologic examination of core samples/trephine biopsies include better assessment of necrosis, bone marrow cellularity, megakaryocyte numbers and evaluation of any abnormalities of bone and vessels (which cannot be assessed via cytological examination). Furthermore, additional tissue sections can also be cut for special stains. Disadvantages of bone marrow biopsies include the pain and clinical risk associated with a more invasive procedure, the difficult detection of phagocytosis of haematopoietic cells as well as many dysplastic features, it is more expensive and takes longer to process, and cell differentiation by morphology is more difficult to determine (particularly if sections are not cut thin enough) [14]. Potential difficulties with core sampling include samples consisting mostly of cortical and subcortical bone, damaged sample due to prior aspiration, traumatic collection leading to a crush artefact, improper fixation and damage induced during sectioning [14]. Bone marrow aspirations surpass bone marrow biopsies in conditions such as megaloblastic anaemia, iron deficiency anaemia and idiopathic thrombocytopaenia, whereas myeloproliferative disorders, aplastic/hypoplastic anaemia, granulomatous lesions, non-Hodgkin's lymphoma and acute leukaemia are more readily diagnosed with bone marrow biopsies [17]. Additionally, myelofibrosis relies solely on a bone marrow biopsy for diagnosis since bone marrow aspiration yields a diluted marrow or dry tap [17]. Conducting a simultaneous evaluation of bone marrow aspirations and bone marrow biopsies prove to be complementary to one another and additionally provide more material for evaluation. Correlation of aspirate and trephine appearances with other modalities such as flow/fluorescence-activated cell sorting (FACS) results, molecular tests and cytogenetics is the gold standard for diagnosis of haematopoetic abnormalities in humans and animals [14,16].

1.2.1. Bone marrow cellularity

Bone marrow cellularity evaluates the ratio of haematopoeitic cells to adipose tissue and this assessment supplies important information regarding bone marrow activity [18]. Cellularity is most accurately assessed via histopathological examination on H&E-stained sections. Active haematopoietic tissue fills nearly the entire marrow space at birth but this increased cellularity declines with age due to a decrease in osteoblastic progenitors, loss of haematopoietic tissue and a decrease in the amount of bone [18]. This decreased cellularity within the bone marrow in aged animals is accompanied by a corresponding increase in the amount of adipose tissue. Bone marrow hyperplasia, i.e. increased cellularity, is a typical response to peripheral cytopaenia due to extramedullary cell destruction (e.g. regenerative anaemia, leukopaenia or thrombocytopaenia), or a higher

demand for haematopoietic components (e.g. hypoxia, infection) [18]. Bone marrow hypoplasia or aplasia, i.e. decreased bone marrow cellularity, is seen where one or more of the haematopoietic cell lines are damaged, e.g. drugs, toxins, infectious agents and immune-mediated disease [18].

1.2.2. Differential cell count

A manual or electronic differential cell counter determines the percentage of the various cell types. This differential cell count is then compared with normal values and the total cellularity of the specimen. This differential cell count, in conjunction with the total leukocyte count, establishes whether a specific cell line is increased / hyperplastic or decreased / hypoplastic [18].

1.2.3. Myeloid:Erythroid (M:E) ratio

This is a semi-quantitative estimation of the relative proportion of granulocytic and erythrocytic cells, determined by dividing the proportion of cells in the myeloid series by the proportion of erythroid precursors up to and including the metarubricytes [18]. The normal ratio varies from 0.9 – 1.8 in the dog [19]. Increased values are indicative of decreased erythropoiesis and / or increased granulopoiesis and a decreased value is indicative of erythroid hyperplasia and / or decreased granulopoeisis [18].

1.3. Bone marrow and iron storage

Iron forms an essential part of various biochemical pathways, such as the formation of haemoglobin and myoglobin, production of neurotransmitters and myelin, energy metabolism, the formation of collagen, immune system function, synthesis of DNA and RNA and a vast array of enzyme systems [20]. Although iron is needed in small quantities by all cells, most of the body's iron is used by erythroid precursors in haemoglobin production and, as such, any abnormality in iron homeostasis leading to decreased iron levels would therefore result in anaemia[20]. Iron distribution within the body can be divided into three pools: transport, functional and storage. The storage pool encompasses hepatocytes and macrophages of the mononuclear phagocyte system, of which the bone marrow is a major storage site [18,20]. In the transport pool, i.e. circulation, iron is bound to transferrin, a plasma protein and this pool amounts to 1% of total body iron [20]. The functional pool encompasses iron in haemoglobin, myoglobin and enzymes [20]. Haemoglobin in mature erythrocytes and within erythroid precursors make up more than 67% of the body's iron and 10-15% is present within myoglobin in muscle or enzymes and cytochromes in various tissues [20].

Intracellular iron within the bone marrow is mainly in the form of haemosiderin present in macrophages and to a lesser percentage within erythroblasts/sideroblasts [18]. Haemosiderin content increases as a result of inappropriate utilization or release, and can be evaluated with the application of a Perl's special stain. There is an increase in the amount of iron within the bone marrow in conditions such as 1). anaemia of chronic disease, 2). Iron overload, 3). Myelodysplastic syndromes and 4). Sideroblastic anaemia. Bone marrow iron stores are depleted or decreased in iron deficiency anaemia [18] (refer to section 1.4.2.1.a for discussion on iron-restricted erythropoeisis).

1.4. Canine babesiosis

Canine babesiosis is a tick-borne protozoal disease and commonly encountered in South Africa [21,22]. It affects approximately 10% of clinically ill dogs presented at veterinary practices nationwide [21,22]. Morphologically, canine babesiosis parasites are classified into small (*Babesia gibsoni, B. conradae* and *B. annae)* and large piroplasms (*B. canis, B. vogeli* and *B. rossi*) [23,24]. *Babesia annae* has recently been reclassified as *Babesia vulpes* [25]. The *Babesia* sp. are transmitted by different vectors, are antigenically distinct and vary according to their geographical distribution and pathogenicity [26]. *Babesia rossi,* transmitted by the tick vector *Haemaphysalis elliptica,* is the most pathogenic of all the canine *Babesia* parasites, is endemic to South Africa and causes

peracute and acute disease [22,27,28]. *Babesia* species are intracellular protozoa that parasitize erythrocytes. Clinical signs of canine babesiosis include fever, anaemia, icterus, tachypnoea, tachycardia, anorexia and splenomegaly [28]. Marked haemolytic anaemia [which may be immune-mediated and warm in saline agglutination (ISA) positive], marked acid-base disturbances with secondary multiple organ failure and complications, including acute renal failure, hypoglycaemia, lactacidosis, hepatopathy with marked icterus, pancreatitis, acute respiratory distress syndrome and cerebral pathology may occur [28–31]. *Babesia vogeli* is endemic to the United States of America (USA), South Africa, Japan, Brazil and Australia. It is associated with mild, often inapparent disease in adult dogs, however, some puppies may experience severe disease [28,32]. *Babesia canis* is of moderate pathogenicity and occurs throughout Europe [28]. The smaller *Babesia* species have not been detected in South Africa yet. *B. gibsoni* occurs in the Middle East, Japan, southern Asia, North Africa, South America, the USA, Italy, Hungary, and Australia and *B. vulpes (*formerly *annae)* is endemic in dogs in northwest Spain [22,28]. *Babesia conradae* occurs in southern California and is reported to be more pathogenic than *B. gibsoni*, resulting in increased parasitaemia and more significant anaemia in comparison to *B. gibsoni*-infected dogs [24].

Although disease caused by *B. vogeli* does occur in South Africa, it is relatively rare [22] and as such, this study will focus on the bone marrow changes in disease caused by mono-infections with *B. rossi.*

1.4.1. Haematologic parameters of canine babesiosis

Canine babesiosis is characterized by anaemia, an inflammatory leukogram and thrombocytopaenia, however, the haematological changes vary between the different parasites that infect dogs. This is likely as a result of differing pathogenicity and virulence among the various *Babesia* species [27].

Regenerative anaemia, leukocytosis with neutrophilia, lymphocytosis and thrombocytopaenia were reported in a litter of puppies infected by *B. vogeli* in a kennel in north Florida [33]. Furlanello reported a mildly regenerative normocytic normochromic anaemia and variable leukocyte abnormalities in dogs infected with canine babesiosis in Italy which recently travelled from Eastern Europe (Bosnia, Herzegovina, Croatia and Hungary) [34]. Although polymerase chain reaction (PCR) analysis was not performed in the study to determine the babesial species involved, it is presumed to be either *B. canis* or *B. vogeli* as these are prevalent in Eastern Europe [35,36]. A broad range of haematological abnormalities were reported in dogs infected with large *Babesia* species in northern Australia [37] and although PCR was not performed in this study to determine the *Babesia* species involved, *B. canis and B. vogeli* are predominant in northern Australia [38]. Haematological abnormalities reported for canine babesiosis in dogs from Poland and Spain include thrombocytopaenia, variable anaemia, left shift neutropaenia, lymphocytosis and lymphopaenia [39,40]. The *Babesia* species involved were not determined in the above two studies from Poland and Spain, but *B. canis* and *B. vogeli* occur in both countries [26,41].

Clinical signs associated with *B. rossi* infection are reported to be more severe than disease caused by *B. canis* and *B. vogeli* [22,31,32,42]. Compared with the haematolgical changes observed in canine babesiosis of dogs in Poland and Spain, dogs infected with *B. rossi* had a more severe anaemia, neutrophilia and monocytosis [31,42].

The anaemia of canine babesiosis is the result of both antibody-mediated red blood cell destruction and direct parasitic damage resulting in intravascular haemolysis [43]. *B. canis* infection is associated with a mild to moderate normocytic, normochromic non-regenerative anaemia during the early stages of infection, which progress to macrocytic and regenerative anaemia after a few days [44]. The non-regenerative anaemia that is evident during the acute stages of infection has been related to the time that is needed for the bone marrow to respond to acute erythrocyte breakdown, generally three to five days [28,44]. Scheepers et al (2011) reported a slight to

moderate regenerative normocytic and normochromic anaemia in canine babesiosis, however, they reported an inadequate regenerative response to the anaemia [45].

A study of 662 hospitalized canine babesiosis case was conducted by Reyers et al. (1998) in order to determine whether there were notable differences in the haematological values in dogs grouped according to the degree of anaemia and mortality [42]. In this study, 50% of dogs had severe anaemia (defined as a haematocrit of ≤0.15 litre/litre) and 32% had moderate anaemia (defined as a haematocrit of 0.15±0.29 litre/litre), associated with a mortality rate of 8.8% and 12.3% within the respective groups. A leukocytosis, a left shift neutrophilia, monocytosis and thrombocytopaenia were observed in dogs of both groups. Lymphocyte counts were within the normal reference range. Eighteen percent of dogs had no anaemia (defined as a haematocrit of 0.30 litre/litre) and total white blood counts, segmented neutrophil and monocyte counts were within the normal reference range, but marginal lymphopaenia and thrombocytopaenia were reported [42]. A mortality rate of 29.2% was recorded for this group. This study demonstrated an inverse relationship between the differential leukocyte counts for neutrophils (both band and segmented) and lymphocytes to the haematocrit, where a moderate leukocytosis was observed in severely anaemic dogs and a marked lymphopaenia were observed in the non-anaemic dogs. Younger dogs were mostly categorized in the severely anaemic group, with older dogs present in the non-anaemic group. Poor prognostic indicators included a high haematocrit, low lymphocyte count and increased numbers of metamyelocytes. To conclude, this study demonstrated that non-anaemic cases comprised mostly an older age group and were associated with a lack on inflammatory response, whereas the younger age group were associated with an inflammatory response and anaemia. The authors hypothesized that older dogs exhibit a massive inappropriate inflammatory response and that this lack of inflammatory response was due to a rapid and overwhelming disease process within the non-anaemic group which allowed no time for an inflammatory reaction to become apparent [42]. These findings supported the hypotheses that a fraction of dogs contract a complicated form of babesiosis similar to the complicated form of falciparum malaria in humans, sharing similar age predisposition and the tendency to progress to a severe inflammatory response [42]. Leisewitz et al. (2019) in their study of 320 dogs naturally infected with *Babesia* parasite found that 35.6% were severely anaemic, 25.5% were moderately anaemic and 22.1% were mildly anaemic. Mortalities were recorded as 14.2%, 8% and 11.3%, respectively. Fifty of these *Babesia* infected dogs had a normal haematocrit and a 4% mortality was recorded within this group. [46]. In the above study, the degree of left shift seen in *B. rossi* infections was correlated with poor outcome [46]. The left shift is also indicative of the inflammatory nature of the host's response to infection.

Leukopaenia is a frequent finding in early canine babesiosis and may be due to marked consumption, a sepsis-like state or possibly splenic sequestration and it is largely due to a neutropaenia and lymphopaenia [44]. A leukaemoid response has also been described in two dogs with canine babesiosis in which the *Babesia* species involved were not determined by PCR analysis [47]. Pancytopaenia has also been reported with canine babesiosis, albeit rare [48,49]. Van de Maele *et al.,* reported an unusual case of acute canine babesiosis (*B. canis*) in a 10-year old male Akita Inu in Belgium that was characterized by neurological signs and pancytopaenia. Cytological examination of bone marrow aspirates revealed erythroid hyperplasia (myeloid:erythroid <1), normal numbers of the myeloid series with orderly maturation and adequate numbers of megakaryocytes. This hypercellular bone marrow with a decreased myeloid to erythroid ratio indicated early or ineffective erythropoiesis due to hemolysis [49].

A study conducted by Rautenbach et al., 2017, showed that mean blood lymphocyte counts were within laboratory reference intervals throughout the duration of the study for both complicated and uncomplicated canine babesiosis cases, but were however significantly lower in contrast to the control cases [50]. There was a drastic rise in the lymphocyte counts in both complicated and uncomplicated cases within 24 and 48 – 72 hours

post-presentation, which may be ascribed to redistribution of the lymphocyte pool [50,51]. The CD3+ T-lymphocytes levels within peripheral blood were reduced in *B. rossi* infected dogs, including reduced numbers of both CD4+ T-helper cells and CD8+ cytotoxic T-cells [50]. Dogs with complicated babesiosis showed a noteworthy decrease in the percentage of T-lymphocytes, especially the percentage of CD4+ T-helper cells, versus those with uncomplicated babesiosis [50]. This may be suggestive of functional immunosuppression caused by the parasite [50]. A significant reduction in CD8+ T-lymphocytes was present in both complicated and uncomplicated cases of canine babesiosis compared to healthy control dogs, which may have been due to increased apoptosis or lack of recruitment of CD8+ T-lymphocytes [50,52,53]. At presentation, there were no significant differences within the B-lymphocyte counts of *B. rossi* infected dogs compared to the healthy control dogs, however, the proportion of B-lymphocytes increased after 48 – 72 hours in dogs with complicated babesiosis [50]. This increased B-lymphocyte percentage in canine babesiosis may represent a delayed T-helper 2 mediated B-lymphocyte rebound response [50].

Thrombocytopaenia is invariably present in *B. rossi* infections and it may be severe [44,46,54]. The mechanism of thrombocytopaenia is not understood, however, immune-mediated destruction and increased consumption [i.e. disseminated intravascular coagulation (DIC)] have been implicated [44,55,56]. Evaluation of the bone marrow of *Babesia canis* infected dogs in one study revealed normal to increased numbers of megakaryocytes [44] and based on the presence of large sized platelets within the circulation indicating a strong bone marrow response, diminished production from the bone marrow is unlikely to be the cause for the thrombocytopaenia observed [57]. Despite thrombocytopaenia which would normally interfere with maintenance of primary haemostatic function, clinical haemorrhage is not observed [45,46,54,57,58]. Goddard et al. (2015) found in their study evaluating the platelet indices in dogs infected with *Babesia rossi* that the number of large activated platelets were notably increased and the authors postulated that this may contribute to the absence of haemorrhage in spite of the marked thrombocytopaenia observed [57]. Liebenberg et al (2013) demonstrated by means of thromboelastography (TEG) a normocoagulable state in dogs with uncomplicated babesiosis. This finding also correlates with the clinical presentation of these cases [58]. Sufficient numbers of normally functioning platelets are crucial for primary haemostasis and several studies in both humans and dogs have illustrated that platelet counts below 66 x 10^9/L influence TEG variables [59,60]. However, despite median platelet counts of 20 x 10^9/L, TEG variables were within the reference interval for dogs with uncomplicated babesiosis in the study conducted by Liebenberg et al. (2013). The authors hypothesized that this might be due to increased platelet activation secondary to the systemic inflammation which subsequently provides a procoagulant membrane surface for the assembly of coagulation factors[58]. A relative hypercoagulable state was observed in these uncomplicated *Babesia* cases when compared to what is normally observed in dogs with such markedly decreased platelet counts and that this explains the normal TEG variables [58]. A systemic inflammatory response is seen with both *B. canis* and *B. rossi* infections and is associated with elevated C-reactive protein, hyperfibrinoginaemia, thrombocytopaenia and leukopaenia [29,61,62]. The presence of this hyperfibrinoginaemia could also influence the TEG variables [60] and subsequently mask the changes associated with thrombocytopaenia. DIC can ensue which is associated with severe multi-organ damage [63]. Inflammation triggers coagulation and inflammation can also be caused by thrombin, which is also the most potent platelet activator [64]. This triggering of the coagulation system can result in a consumptive coagulopathy, which has is a negative prognostic indication in both human and canine medicine [55,65,66].

1.4.2. Assessing the degree of red cell regeneration in the canine haemogram

1.4.2.1. Anaemia

Anaemia is defined as a decrease in the blood haemoglobin (HGB) concentration, the red blood cell count and haematocrit (HCT) [11]. It is the consequence of various pathological conditions influencing the erythrocyte population by changing the equilibrium between the production and destruction of erythrocytes [10]. HCT is the

most used measure of red cell mass. The red blood cell indices of mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC) are used to further assess and classify the type of anaemia [67]. In contrast to the absolute reticulocyte count, these indices are insensitive indicators of regeneration but helpful to evaluate the pathophysiological mechanism of the anaemia [10,68]. Anaemia is classified morphologically via MCV and MCHC as macrocytic, normocytic or microcytic and hypochromic or normochromic, respectively [11,67]. The extent of reticulocytosis is used to examine the bone marrow response and for further classification into regenerative (responsive) or nonregenerative (nonresponsive) anaemia [14]. Reticulocytosis is evident 2-4 days after an incident of haemolysis or blood loss, where it reaches a peak at 4-7 days and slowly decreases 2-3 weeks after restoration of the depleted red cell mass [10]. This regenerative response takes 2-5 days to become apparent in the peripheral circulation and anaemia may therefore appear nonregenerative in the initial stages [68]. An increased regenerative response to haemolytic anaemia is significantly stronger when compared to that seen with an anaemia due to external haemorrhage. This is because iron and proteins from haemolysed erythrocytes can be recycled and is more easily available for erythropoiesis than stored iron [11,68]. Haemolytic anaemias are typically regenerative and may be macrocytic and hypochromic when associated with intense regeneration [10].

1.4.2.1.a. Regenerative response during canine babesiosis

The degree of erythropoiesis is best assessed with the absolute reticulocyte count [68,69]. In a study conducted by Furlanello et al (2005), three of 23 *Babesia* sp. infected dogs (13%) were associated with a poor regenerative response and the anaemia was classified as normocytic and normochromic [34]. Similarly, Scheepers et al (2011) reported a normocytic and normochromic anaemia in contrast to the macrocytic and hypochromic anaemia one would expect in cases with a strong regenerative response [45]. They also observed a left shift in most dogs without neutrophilia. These erythrocytic and leukocytic responses were less vigorous than anticipated based on the extent of haemolysis and inflammation with the possibility of diminished haematopoiesis in these cell lines or other factors [45]. In the study conducted by Reyers et al (1998), the reticulocyte response was reported as normal (non-regenerative) for non-anaemic and moderately anaemic dogs, whereas mild regeneration was noted in dogs with severe anaemia [42]. After evaluating the reticulocyte percentages, a moderate to strong reticulocyte response was noted by Maegraith et al (1957) [70]. However, the absolute reticulocyte count is a far better way to evaluate for effective erythropoiesis, as reticulocyte percentages may be falsely increased with anaemia which my lead to wrongly interpreting the anaemia as regenerative [45]. Comparing the reticulocyte counts of *Babesia* infected dogs with normovolaemic dogs with acute phlebotomy-induced anaemia and a comparable haematocrit, it was found the reticulocyte counts were much lower in *Babesia* infected dogs than in the dogs with phlebotomy-induced anaemia [45,71]. Similar to Scheepers et al (2011), a subsequent study showed that the absolute reticulocyte count was <100 x 10^9/L in approximately 70% of *Babesia* infected cases where the reticulocyte counts were available, compatible with a minimal regenerative response to the anaemia [46]. Seejarim et al. (2020) in their study comparing the absolute reticulocyte count (ARC) in dogs naturally infected with *B. rossi* with the ARC in dogs suffering from immune-mediated hemolytic anemia (IMHA) unrelated to babesiosis, as well as healthy control dogs, demonstrated an inappropriate regenerative response during canine babesiosis relative to the observed anaemia [72]. They found that despite no difference in the haematocrit values between the *Babesia* and IMHA group, the ARC in the *Babesia* group was significantly lower than the ARC of the IMHA group. Inappropriately low reticulocytosis relative to the degree of anaemia is also a frequent finding in malaria patients [73,74]. Seejarim et al. (2020) hypothesized that their findings, similar to what has been reported in *falciparum* malaria, may suggest a direct effect of the organism on the bone marrow or that other factors (such as inflammation) still requiring further investigation, may be involved [72]. Scheepers et al (2011) postulated that the ineffective erythropoiesis may be directly caused by the parasite, or that secondary immune mediated mechanisms may be involved. No bone marrow samples were evaluated, but no findings suggestive of

dyserythropoeisis were detected in their study [45]. Leisewitz et al (2019) suggested that dyserythropoeisis may play a role in this poor regenerative response to the anaemia as erythropoietin levels during canine babesiosis, similar to what has been found in human malaria, are deemed sufficient for the degree of anaemia [46,75].

1.4.2.1.b. Iron-restricted erythropoeisis

Iron-restricted erythropoiesis is seen in conditions where iron stores are too low for normal red blood cell production, such as an absolute iron deficiency, sequestration of iron and functional iron deficiency [76]. An absolute iron deficiency is seen when there is a reduced storage of iron, especially within the bone marrow and is seen in conditions where iron is lost due to chronic external haemorrhage (gastrointestinal ulcers, neoplasms, haemophagic parasites) or in cases of nutritional iron deficiency [77]. Iron sequestration within cells (enterocytes, hepatocytes, macrophages) is hepcidin-mediated and occurs in chronic inflammatory and infectious diseases [77]. With functional iron deficiency, there are adequate iron stores but there is an imbalance between the demand for iron and the rate of iron mobilization and can be seen in conditions with marked erythropoietic stimuli (i.e. regenerative response to anaemia) [77]. Iron-deficient anaemia is typically microcytic and hypochromic[76]. Iron-restricted erythropoiesis is best assessed by means of reticulocyte indices (specifically the size and haemoglobin content of reticulocytes). Assessment of the reticulocyte indices ensure that microcytosis and hypochromasia are detected earlier as compared to assessing the MCV and MCHC indices [76]. This is because reticulocytes are evident within the circulation of dogs for 1 to 2 days before maturation and MCV and MCHC indices mostly capture the cell size and haemoglobin content of mature erythrocytes which can circulate in the blood for many months [76]. Immune-mediated haemolytic anaemia (IMHA) in dogs is associated with a strong erythropoietic response, although a proportion of dogs with IMHA have unexpectedly weak regeneration, as indicated by an inappropriately low ARC [78]. This weak regeneration may be due to functional iron deficiency and / or inflammation-mediated iron sequestration, as decreased reticulocyte haemoglobin content (CHr) was reported in dogs with IMHA [76]. Similarly, reticulocyte haemoglobin equivalent (RET-He) is reported to be a sensitive diagnostic indicator of iron-deficient erythropoiesis [79].

1.5. Human malaria

Most human malaria is caused by four *Plasmodium* species, namely *P. falciparum, P. vivax, P. malariae* and *P. ovale,* of which *P. falciparum* is the most pathogenic [80,81]. A fifth malarial parasite, *P. knowlesi* has also been identified and like *P. vivax, P. malariae* and *P. ovale,* may cause acute and severe disease, however, mortalities are low [82,83]. Conversely, *P. falciparum* is associated with one or more life-threatening complications, including severe malarial anaemia (SMA), hypoglycaemia, cerebral malaria and respiratory distress [84].

1.5.1. Haematologic changes during falciparum malaria

1.5.1.1. Erythrocyte response

Normocytic and normochromic anaemia characterizes human malaria but microcytosis and hypochromasia due to the complication of iron deficiency and / or thalassaemia may be present [81]. Patients with cerebral malaria often present with severe anaemia and in most cases, the anaemia intensifies and reaches a nadir 1-17 days after the start of treatment [85]. Reticulocytosis is not a feature for several days after malarial infection and a significant lack of reticulocytes is reported [80,81]. This inadequate erythropoiesis seen in some malarial patients may fail to ease the malarial anaemia and worsen the severity of the disease [73,86].

Anaemia in human malaria is as a result of the destruction of parasitized erythrocytes during merogony, phagocytosis of intact infected and non- infected erythrocytes by macrophages (with a rise in the macrophage number and activity within the bone marrow and spleen), splenic sequestration of erythrocytes, dyserythropoiesis (improper growth / maturation of erythrocytes) and bone marrow suppression [84,85,87,88]. During anaemia, increased EPO is produced within the kidneys in response to the tissue hypoxia. EPO stimulates

erythropoiesis within haematopoietic organs, including the bone marrow and spleen, by binding to the EPO receptor expressed on erythroid progenitors, particularly CFU-E and proerythroblasts [86] . This would normally result in a high number of reticulocytes being released into the bloodstream. It therefore follows that the inadequate number of reticulocytes present during malarial infection may be either the result of 1.) insufficient EPO production and / or 2.) insufficient response of red blood cell progenitors to EPO stimulation. The latter is most likely, as several studies revealed that deficiency in erythropoietin levels are not a feature of malarial infection [75,89–91]. In fact, high levels of erythropoietin during malaria infection is observed due to high levels of TNF-α and hypoxia inducing factor 1 (HIF-1) [92,93]. Therefore, the absence of reticulocytosis despite adequate levels of erythropoietin characterizes malarial anaemia [94]. Chang et al (2004) postulated in their study that red blood cell progenitors may possibly have a suboptimal response to erythropoietin which may explain the underlying mechanism of inadequate erythropoiesis during blood-stage malaria, as opposed to insufficient erythropoietin production [95]. Further studies by Chang et al (2004) showed that development of late erythroid precursors is markedly suppressed during blood stage malaria with subsequent decline in reticulocyte production in malaria infected experimental animals and patients [86]. Therefore, the lack of a sufficient red blood cell response during malarial infection may be due to unresponsiveness of red blood cell progenitors to the erythropoietin produced, a decline in the production of red blood cell progenitors and/or a decline in the production of multipotent progenitor stem cells [75,89,90]. The reticulocyte response to exogenous administration of erythropoietin in *Plasmodium*-infected mice was suppressed and the degree of suppression was correlated with rising parasitaemia [75]. This suppression of the reticulocyte response to administration of exogenous erythropoietin suggests defective development of erythroid precursors [75]. Suppression in erythropoiesis may be mediated by the parasite itself, parasite products or host factors, as increased reticulocytosis follows close on the heels of parasite clearance [75].

1.5.1.1.a. The role of malarial parasite factors in ineffective erythropoiesis

Haemazoin (Hz) is a malarial pigment formed by haemoglobin digestion. It is the main route of haeme detoxification in various species of haematophagous organisms, including *P. falciparum* [96]. Hz has been found to directly induce apoptosis within erythrocytes [97]. It is considered a potential mediator of inflammation which may be partly responsible for the inadequate erythropoietic response in falciparum malaria. It is hypothesized that its toxic effects may inhibit erythropoiesis by direct or indirect means. In addition to inhibition of erythropoiesis, Hz may also contribute to anaemia by other mechanisms, such as decreased red cell deformability [98]. Hz is sequestered in leukocytes, bone marrow and erythroid precursors and the proportion of Hz-containing monocytes has been found to have a stronger suppressive effect on reticulocytes than that of TNF-**α** levels [98]. In addition to erythopoiesis inhibition, Hz also impairs the function of human endothelial cells, dendritic cells and monocytes *in vitro* [99–101].

In addition to Hz, other parasite or host factors may be at least partially responsible for ineffective erythropoiesis. These include glycosylphosphatidylinositol (GPI) and macrophage-inhibitory factor (MIF). GPI modulates cytokine secretion and *in vitro* responses of macrophages and endothelial cells, although it has not been demonstrated to be directly or indirectly inhibitory for erythropoiesis [102]. MIF contributes to bone marrow failure in rodent models of malaria by suppressing the development of early erythroid and myeloid progenitors [103]. MIF is a proinflammatory mediator which impedes erythroid (BFU-E), multipotential (CFU-GEMM) and granulocyte-macrophage (CFU-GM) progenitor derived colony formation [103]. MIF is released by macrophages after phagocytosis of erythrocytes infected with *Plasmodium* spp or containing haemozoin. Martiney, JA et al (2000) demonstrated that MIF is responsible for inhibition of erythropoiesis *in vitro*, despite the presence of erythropoietin. MIF levels also correlate with the severity of the disease in *P. chabaudi* infected BALB/c mice. MIF has been demonstrated to be produced within the bone marrow, liver and spleen in mice with active *P.*

chabaudi infection [103]. Haemozoin is not produced by *Babesia* parasites [104], but GPI molecules have been demonstrated to be present within *Babesia* parasites [105,106].

1.5.1.2. Leukocyte response

In human malaria, leukocyte count changes include a leukocytosis although leukopaenia may also be observed and infrequent leukemoid reactions have been documented [107]. After two days of pyrexia due to falciparum malarial infection, the neutrophil count may rise (left shift neutrophilia) with a subsequent drop [85]. A neutrophilia has been associated with a poor prognosis [80]. Both chronic and acute falciparum malarial cases may show monocytosis [85]. During acute falciparum malaria, a lymphocytosis is present [81]. Lymphopaenia may however also occur which may be due to a redistribution of lymphocytes and splenic pooling [81,85,108]. The absolute CD4+ and CD8+ lymphocyte counts were reduced in children with acute malaria [109]. Acute falciparum malarial cases usually present with eosinopaenia which gradually corrects over a period of 5 to 10 weeks [85].

1.5.1.3. Thrombocyte response

Acute malaria is often accompanied by mild or moderate thrombocytopenia, the severity of which correlates with the level of parasitaemia, occurs without bleeding tendencies and is mostly unrelated to disseminated intravascular coagulation [85,108]. Thrombocytopaenia does not correlate with the disease severity [81]. Thrombocytopaenia is mostly as a result of a shortened life span of platelets due to either attachment of malarial antigens onto platelets with subsequent antibody response and platelet phagocytosis, or due to platelet activation and sequestration within the spleen [85,110]. A normal number of megakaryocytes are present within the bone marrow and defective thrombopoiesis is not evident [80,81].

1.5.2. Bone marrow changes during human malaria

The macroscopic appearance of the bone marrow in patients with repeated infection with the malaria parasite may appear grey or black due to build-up of phagocytosed malaria pigment [85]. On light microscopy, some studies have shown the bone marrow as either hypocellular, normocellular or slightly hypercellular with normal or a decreased percentage of erythroblasts in patients with acute uncomplicated *P. falciparum* malaria [85,111]. Others have described the marrow as hypercellular with notable gross abnormalities in the erythroid precursor development, i.e. dyserythropoiesis [85,111–115]. Dyserythropoiesis is characterized by an irregular nuclear outline in the developing erythroid cell line, erythroblasts with markedly deformed nuclei, bi- and multinucleated erythroid cells, megaloblastic change, nuclear budding, internuclear chromatin bridges and karyorrhexis [85,115,116].

In a retrospective study of the bone marrow in human malaria performed by Gandapur, Malik et al. (1997), changes included myeloid hyperplasia, erythroid hyperplasia, megaloblastosis and hypoplasia in varying proportions. They found that out of 26 bone marrows evaluated during a 39-month period, 61% were positive for the presence of *P. falciparum* parasites and of these, 50% were normocellular, 31% hypercellular and 12% hypocellular and all of the patients with hypercellular bone marrow showed severe anaemia. The hypercellular bone marrows in this study were as a result of erythroid and myeloid hyperplasia [87]. The bone marrow hypercellularity in 20 children from Gambia with longstanding malaria infection and marked anaemia was found to be as a result of erythroid hyperplasia only [117]. In another study of falciparum malaria in children from Gambia, the bone marrow showed severe erythroid hyperplasia and dyserythropoiesis, with 14% showing megaloblastic and 71% normoblastic erythropoiesis and 18% showed erythroid and myeloid hyperplasia [114]. In a study done by Fleming (1989) on the bone marrow changes of 34 pregnant Zambian women infected with falciparum malaria, 26 (76%) showed erythroid hyperplasia, 25 (74%) had myeloid hyperplasia, 3 (9%) had abundant eosinophil precursors, 3 (9%) had increased lymphocytes and 11 (38%) had normal plasma cell densities. Twenty-three (68%) of the bone marrows had megaloblastic erythropoiesis and myelopoiesis and

dyserythropoiesis was present in only one (3%) of the bone marrows studied [113]. The number of marrow macrophages has been shown to be increased and they often contain phagocytosed malarial pigment and demonstrate erythrophagocytosis (parasitized and non-parasitized erythrocytes) [85,114]. These changes were more evident in acute than in chronic malaria and in cerebral than in uncomplicated acute malaria [85,114]. Other changes within the bone marrow include lymphocytosis, the presence of giant metamyelocytes, macrophage hyperplasia, plasmacytosis, increased eosinophil granulocytopoiesis, an increased number of megakaryocytes and a monocytosis and mild neutrophilia in the peripheral blood [85,111,112]. Some megakaryocytes may show dysplastic features, including hyperlobulated or unlobulated nuclei [114,115]. In one study the bone marrow pathology in humans infected with *Plasmodium vivax* was not associated with noteworthy pathology, apart from evidence of iron deficiency anaemia [87].

Bone marrow has been proposed to be a major reservoir for malarial gametocyte maturation and proliferation [118]. One study investigated the accumulation of *P. vivax* parasites within tissues of infected *Aotus lemurinus* monkeys using stage-specific quantitative real-time PCR (qRT-PCR) markers [118]. Parasites were found to be sequestered within the one marrow and may provide an explanation as to why many malarial infections are undetected and may also raise the risk of recurrent blood-stage parasitaemias arising from the bone marrow [118]. Similar to *P. vivax,* bone marrow sequestration of *P. falciparum* malarial parasite has been demonstrated [118–120]. Erythropoiesis may be directly influenced by sequestering of the malarial parasite within the bone marrow [121]. This parasite sequestration is essential for the completion of the erythrocyte cycle to avoid recognition by surveillance macrophages and splenic clearance should the parasites be released into the bloodstream [119,120]. This sequestration of infected erythrocytes containing the asexual-stage involves adherence via specific endothelial cell receptors [119]. Parasite sequestration is partially responsible for the severe pathology of falciparum malaria [119].

1.6. The role of cytokines in canine babesiosis and human malaria

Babesia rossi infection has been associated with an excessive pro-inflammatory cytokine response [46,122]. Similar findings are evident in malaria, where cytokines [interferon gamma (IFN-ɣ), TNF-α, IL-2, IL-12 and IL-10] inhibit erythroid progenitor cells or blunt their response to erythropoietin, thereby inhibiting / suppressing erythropoiesis [85]. A successful inflammatory response is initiated by pro-inflammatory cytokines and chemokines, including TNF-α, IFN-ɣ, IL-1, IL-2, IL-6, IL-8, IL-12, IL-18 and monocyte-chemotactic protein-1 (MCP-1) [123,124]. Cytokines responsible for controlling and down-regulation of inflammation include IL-4, IL-10 and transforming growth factor beta (TGF-β), which exert their effect by suppressing gene expression of pro-inflammatory cytokines [124,125]. Host regulatory imbalances between the pro-inflammatory systemic response and the modulating inflammatory inhibitory response results in multiple organ dysfunction and death in some cases of human falciparum malaria and various other septic conditions [125,126]. A vigorous cytokine-mediated inflammatory response is elicited by the human host upon infection of erythrocytes with *P. falciparum* shizonts with subsequent rupture [126,127]. Cytokines play an important role in the regulation of disease progression in human malaria [127–129]. This entails a sufficient and early pro-inflammatory cytokine response in order to resolve and control the malarial parasitaemia, followed by a timely suppression of this pro-inflammatory response by inflammatory modulating cytokines [130]. Severe malaria and death have been associated with an overzealous pro-inflammatory response with increased concentrations of TNF-α, IFN-ɣ, IL-6, IL-8, IL-18 and MCP-1 [129,131]. Similar to human malaria, cytokines IL-6, IL-8, IL-10 and MCP-1 were demonstrated to play an important role in *B. rossi* infected dogs [122]. The extent to which some of these cytokines were increased was related to a poor prognosis and studies of *B. canis* and *B. gibsoni* infections reported similar elevated cytokine levels [122,132–134]. Goddard et al. (2016) as well as Leisewitz et al. (2019) found that IL-6 and MCP-1 were negative prognostic markers for *B. rossi* infected dogs [122,135]. IL-6 induces the production of acute phase proteins, including C-reactive protein (CRP) and serum amyloid A (SAA) by hepatocytes, it stimulates adrencortioctropic hormone and cortisol

secretion, it subdues the hypothalamic-pituitary-thyroid axis, it activates B- and T-lymphocytes and modulates haematopoiesis [123,134,136]. MCP-1 is a significant mediator for the recruitment of monocytes from the bone marrow upon an inflammatory stimulus as well as monocyte trafficking to inflammatory sites [137,138]. A rise in the number and activity of macrophages are observed within the bone marrow and spleen during human malarial infection [85]. Macrophages and monocytes are activated directly by the malarial toxin (GPI) which in turn promotes the release of pro-inflammatory cytokines (TNF and IL-1) [85]. The increased cytokine production, in turn, stimulates further activation of macrophages which may be advantageous in the removal of parasitized erythrocytes, however, an increased quantity of TNF may lead to a reduction of erythropoiesis as well as dyserythropoiesis [85]. Furthermore, inadequate IL-12 production leads to dyserythropoiesis in malarial infection [139].

1.7. Bone marrow changes in murine malaria

Murine malaria is caused by various strains of *Plasmodium* species and include *P. chabaudi*, *P. berghei*, *P. yoelii,* and *P. vinckei* [94]. Erythrocytes during all stages of development are infected by *P. chabaudi,* similar to *P. falciparum* whilst *P. yoelii* preferentially infects reticulocytes, similar to *P. vivax* [140,141]. A study conducted by Maggio-Price *et al.,* 1985 evaluated the changes in the haematopoietic stem cells within the bone marrow in mice infected with *Plasmodium berghei* and noted changes within the bone marrow cellularity and erythroid progenitors 24 hours postinfection, prior to the onset of anaemia [89]. Haematocrit, leukocyte, reticulocyte, parasite and differential counts were determined on whole blood samples and bone marrow and spleen samples were examined via light microscopy. Erythroid colony and burst-forming cells (CFU-E and BFU-E) were assessed via the plasma clot culture technique for ex vivo evaluation in cultured samples. Changes included a decrease in bone marrow cellularity, a decline in the number of erythroblasts, BFU-E and CFU-E. These changes became evident before there was any change in the heamatocrit. After anaemia developed, there was expansion of the CFU-E compartment but the BFU-E remained drained and an inadequate reticulocyte response was evident [89]. Other studies similarly reported diminished BFU-Es and diminished or mild proliferation of CFU-Es and erythroblasts within the bone marrow during murine malaria, depending on the mouse strain and parasite species [89,142–144]. Within the study conducted by Maggio-Price et al. (1985), minimal changes within the haematocrit, parasite and reticulocyte count were evident within the first five days after infection, however, after a period of seven days, the haematocrit levels were half the normal values with a concomitant increase in the reticulocyte count and parasite numbers [89]. The authors postulated that the decline in bone marrow red blood stem cells may be due to stem cell migration from the marrow to the spleen, defective stem cell growth in culture, or possible competition among stem cells compartments. The migration of BFU-E from the bone marrow to the spleen was deemed likely since the histologic evaluation of the spleen revealed erythropoiesis within the splenic red pulp at the same time that bone marrow erythropoiesis returned (i.e. five days post-infection). Bone marrow CFU-E increased with rising serum levels of erythropoietin, as well as splenic erythroblasts, therefore suggesting rising levels of CFU-E in the spleen and as such the authors suggested that marrow BFU-E levels are lower due to migration to the spleen and differentiation to CFU-E in the bone marrow. However, this increased CFU-E levels and erythroid proliferation and differentiation is not sufficient to provide adequate numbers of reticulocytes for a sustainable haematocrit. The malarial parasite appears not to infect bone marrow stem cells as electron microscopic studies revealed parasitized reticulocytes within vascular sinuses one day after infection, however, parasites were seldom detected within erythroblasts in haematopoietic cords [89]. Several haematopoietic cell lines increase with malaria infection and competition among the various cell lines may account for the reduction of the erythroid marrow pool [89].

Similar to the findings of the above study conducted by Maggio-Price et al (1985), a study performed by Silverman *et al.,* 1987 also revealed adequate erythropoietin levels during infection of mice with *P. berghei, P. vinckei vinckei* and *P. chabaudi adami*, however, erythroid precursors are unable to respond to this hormone [90].

The bone marrow cellularity of mice infected with the non-fatal *P. chabaudi adami* decreased to 60% of normal after seven to nine days post-infection. No increase in the number of stem cells were observed during early infection with *P. vinckei vinckei,* however, after seven to nine days of infection, the stem cell population was diminished by more than half, shortly before death occurred. There was an increase in the common pluripotent stem cells (CFU-S) during the early stages of infection with *P. berghei,* but the stem cell numbers decreased to 25% of the normal value during the later stages of infection shortly before death. The bone marrow stem cell content of mice infected with *P. chabaudi adami* only showed a decline after approximately seven days, when the peripheral blood parasitaemia levels were about 15%. After peak parasitaemia (14 days), a maximum depression of the CFU-S content and CFU-S per 10^6 nucleated bone marrow cells were observed. Normal levels of CFU-S and normal bone marrow cellularity were observed after 23 days when the infection resolved [90]. It should be noted that the peak parasitaemia of murine malaria models is significantly higher than either human malaria or canine *Babesia* infections at their peak. Murine models peak at around 50%. Human malaria and canine *Babesia* peak at similar levels generally less than 5%. This difference in parasite biomass may well have significant implications for the pathology in the bone marrow [50].

Additionally, mice were leukopaenic and lymphopaenic within the first days of infection, however, this progressed to a leukocytosis characterized by a severe neutrophilia and monocytosis as the infection progressed [89]. The first three days after infection was characterized by myeloid-erythroid ratios (M:E) that showed a reduction in the erythroblasts and an increase in granulocytic precursors. The erythroblasts started to increase by day five after infection and steadily increased to reach an M:E ratio of 1 during advanced infection. Bone marrow was examined histologically one to three days post-infection and revealed essentially granulopoiesis with decreased or lack of erythroblasts. Clusters of large immature cells were evident within some of the bone marrows examined and these appeared to be granulocytic or monocytic precursors. Other bone marrows showed a prevalence of mature granulocytes. After 10 days of infection, the number of erythroblasts in all maturational stages were increased and this was accompanied by active granulopoiesis [89].

1.8. Bone marrow changes in babesiosis of species other than the dog

Babesiosis has been reported in many species, however, reports on the bone marrow changes during babesiosis are sparse. Hussein (1982) reported the bone marrow changes in LACA mice infected with *Babesia hylomysci* (40 mice) and *B. microti* (40 mice)[145]. Quantification of nucleated cells were conducted on bone marrow obtained from the right tibia and the differential count of marrow elements were performed on the bone marrow obtained from the left tibia. The results were compared to the tibial marrow of 10 control mice. The total number of nucleated marrow cells showed a progressive decline with a rising parasitaemia during *B. hylomysci* infection. A count of 2.4 ± 0.8 x 10^6 (normal is 15.96 ± 2.08 x 10^6) was reached at peak parasitaemia (10 days). The nucleated cell count progressively increased thereafter, correlating with a declining parasitaemia. The mean myeloid:erythroid ratio (M:E) decreased from 1.58 on day two of infection, to 0.02 during peak parasitaemia, due to the increase of erythroid elements at the cost of the myeloid elements. The myelocytic series remained unchanged and no degenerative changes were detected [145]. Most nucleated cells within the bone marrow of *B. hylomysci* and *B. microti* infected mice were normocytes in diverse developmental stages. Intermediate normocytes were the principal cell type in normal mice, whereas these were decreased with a rise in the early and late-stage normocytes in the bone marrow of *B. hylomysci* and *B. microti* infected mice. This may be indicative of intense differentiation of stem cells into early normocytes and a speedy maturation of intermediate normocytes to late normocytes [145]. The author stated that depression of bone marrow activity during *B. hylomysci* and *B. microti* infection is unlikely to occur (in contrast to malaria and canine babesiosis) since an exaggerated reticulocyte response within the peripheral circulation was observed during both infections [146]. Therefore, the bone marrow activity appeared to be adequate to handle the erythrocyte destruction caused by the less pathogenic *B. microti* as extramedullary compensatory haematopoiesis was

scarce during this infection [145]. Parasitaemia levels of 40% to 50% have been reported in murine *Babesia* models, however, this can reach as high as 80%, which occurs at approximately day six for *B. hylomysci* infection and day 10 for *B. microti* infection[3,145].

Dawood *et al.,* 2013 reported infection of the eastern grey kangaroo in Australia with a novel *Babesia* species, namely *B. macropus* and subsequently this parasite has also been reported in the wallaby[147,148]. A retrospective study conducted by Donahoe *et al.,* 2015 found a regenerative anaemia characterized by bone marrow hyperplasia in conjunction with anisocytosis and polychromasia within the peripheral blood in eastern grey kangaroos infected with *B. macropus* [148].

In humans, babesiosis is an emerging disease with most cases reported in Europe and the USA. The infection is mostly self-limiting in the immunocompetent host, however, it may be life-threatening with multi-organ failure in immunocompromised and splenectomized patients [104,149,150]. Akel and Mobarakai, 2017 reported infection of a 70-year old woman in New York, USA with *B. microti* [104]. Haematology revealed abnormal cell counts characterized by neutropaenia, lymphopaenia, anaemia and thrombocytopaenia and a bone marrow biopsy showed dyseryhtropoiesis. Similar to canine babesiosis, thrombocytopaenia is a major feature of human babesiosis and is as a result of hypersplenism with resultant increased platelet sequestration and destruction by splenic macrophages [104]. Gupta *et al.,* 1995 reported the presence of *B. microti* infection in a 47-year-old splenectomized renal allograft recipient [151]. Evaluation of the bone marrow revealed marked hypocellularity associated with a large number of plasma cells, rare immunoblasts and morphologically normal neutrophil and red blood cell precursors and megakaryocytes [151]. Bone marrow trephine sections showed near obliteration of the bone marrow parenchyma by many actively phagocytic histiocytes with the presence of haemopoietic precursors within their phagocytic inclusions [151]. Babesiosis is commonly associated with mild to moderate cytopaenias and in this report, pancytopaenia was seen in conjunction with haemophagocytosis by bone marrow macrophages [151]. This finding supports other reports which have suggested that pancytopaenia associated with *B. microti* infection in humans may be as a result of haemophagocytosis [151–154].

1.9. Aims of the study

1. Perform and report the cytological, histopathological and immunohistochemical changes within the bone marrow of *Babesia.rossi*-infected dogs and to compare these findings with normal bone marrow collected from a cohort of healthy dogs without babesiosis.
2. Correlation of the above bone marrow findings with the circulating haemogram in an attempt to explain the poorly regenerative nature of the anaemia caused by *Babesia rossi* infection.
3. To use quantitative immunostaining to investigate subsets of bone marrow haematopoietic cells to establish whether there are any changes that are specific to canine babesiosis.

CHAPTER 2: MATERIALS AND METHODS

2.1. Experimental design

This study was a cohort (prospective observational) matched case-control study of dogs with babesiosis that died naturally or were euthanized on owner's request. Both treated and untreated dogs were included. Five control dogs were obtained from a national welfare organization after humane euthanasia. The University of Pretoria Ethics committee approved this project (V047-18).

2.1.1. Infected animals: Inclusion criteria

· Six dogs naturally infected with *B. rossi,* (as diagnosed on a thin peripheral blood smear) and that died either due to the disease, or were euthanized on owner's request, were included in the study. This group included dogs of any breed, age, sex and body weight and was independent of whether they were treated or not.

· Only dogs for which owners gave permission to conduct routine haematology (complete blood counts) and full post mortem examinations were included.

· Diseased dogs were from the Onderstepoort Veterinary Academic Hospital (OVAH) and from specific, collaborating private veterinary practices in the vicinity of the Onderstepoort Faculty of Veterinary Science (FVS), University of Pretoria (UP).

· All dogs underwent further screening via polymerase chain reaction (PCR) and reverse line blot (RLB), to ensure absence of co-infection with *Ehrlichia*, *Theileria*, *Anaplasma* or *Babesia* species other than *B.rossi* [155].

· Post mortem evaluation and histopathology sample collection were performed within 12 hours of the animal's death.

The two groups were evaluated retrospectively to look for any obvious differences between them (such as bone marrow parasitaemia or parasite phagocytosis).

2.1.2. Infected animals: Exclusion criteria

· Dogs found positive for parasites other than *B. rossi* (by PCR-RLB assay) were automatically excluded from the study.

· Dogs with concurrent (non-*Babesia* related) severe disease determined on post mortem examination (such as neoplasia, organ failure, severe helminthiasis, anaemia, inflammatory disease) were excluded. An interim period (period between death and post mortem) of more than 12 hours was grounds for exclusion from the study.

· Dogs for which routine haematologies [complete blood count (CBC), manual differential count and reticulocyte count] were not performed prior to death were excluded.

2.1.3. Control animals: Inclusion criteria

Control dogs included those that had been euthanized at a welfare organization in accordance with the organizations policies regarding the euthanasia of dogs held in their facility because they had not been rehomed within the specified time period.

2.1.4. Control animals: Exclusion criteria

· Any dog that had significant immunosuppressive or inflammatory disease (e.g. neoplasia, organ failure, severe helminthiasis) as determined during the gross post mortem examination in conjunction with the CBC results was excluded.

· Dogs that had any haemoparasitic or rickettsial infection diagnosed on a peripheral blood smear or on the basis of PCR-RLB assays were excluded.

· Dogs that had significant haematological abnormalities (abnormal CBC and differential count) were excluded.

· Dogs for which routine haematology (CBC, manual differential count and reticulocyte count) had not been collected just prior to death were excluded.

2.1.5. Infected animals: Sources

The majority of dogs were sourced from the OVAH and the remainder were obtained from pre-selected collaborating private and community veterinary clinics close to the FVS, UP. These clinics had previously been involved in another research project managed by Professor Andrew Leisewitz (protocol V034-14). Owner's signed consent in cases of humane euthanasia and permission to use animal tissues was prerequisite (Appendix 1).

2.1.6. Control animals: Sources

Control animals were sourced from a national welfare organization, which provided written consent for the use of tissues from dogs euthanized according to their internal policies.

2.1.7. Post mortem examination and sample collection

Blood samples for full a haematology assessment were collected from all study and control dogs within 3 hours before death. Samples consisted of 5ml of peripheral venous blood collected via jugular venipuncture with a 21-gauge needle and placed into an EDTA vacutainer tube (BD Vacutainer K2E (EDTA) 7,2 mg). A 1ml aliquot of this EDTA anticoagulated blood was archived at -80 degrees Celsius for later PCR-RLB assays to confirm *B. rossi* mono-infection as previously published.[155] This was done in the Department of Veterinary Tropical Diseases (DVTD), FVS, UP.

2.1.8. Haematology samples

An automated CBC was performed on the blood samples (ADVIA 2120, Siemens) and the parameters that were evaluated included:

- Red blood cell count (RBC) measured in 10^{12}/L.
- Haemoglobin concentration [HB] measured in g/dL.
- Packed cell volume (PCV) / haematocrit (HCT) expressed as a %.
- Mean Cell Volume (MCV) measured in femtolitre (fL).
- Mean Corpuscular Haemoglobin Concentration (MCHC) measured in g/dL.
- Mean Corpuscular Haemoglobin (MCH) measured in picogram (pg).
- Red Cell Distribution Width (RDW) expressed as a %.
- Thrombocyte count measured in 10^{9}/L.
- Mean Platelet Volume measured in fL.
- White blood cell count (WBC) measured in 10^{9}/L.

- A differential white blood cell count was conducted manually for each dog by a trained veterinary haematology technician and the differential count (expressed as 10^9 cells/L) was recorded.

- Reticulocyte count

A separate automated reticulocyte count was perfomed (ADVIA 2120, Siemens) for each dog and the absolute reticulocyte count was established (10^9 reticuloctes/L). Various reticulocyte indices as generated by the ADVIA 2120 were also captured and used in the assessment of the anaemia.

2.1.9. Post mortem histopathology and cytology samples

Post mortem examinations were performed at the Section of Pathology, Department of Paraclinical Sciences (DPS), FVS, UP by the principal investigator. To minimize pre-analytic error, the collection and preparation of bone marrow samples were standardized for both study and control cases. The interim was recorded for each case. Bone marrow cytology and histopathology samples were obtained as soon as possible, within 12 hours after death to minimize autolytic change which hampers accurate cytologic and histologic interpretation. Bone marrow samples were collected from the metaphyses of both (left and right) humeri and femori to ensure adequate sample size (approximately $1cm^3$) for both cytology and histopathology. The proximal metaphyses of the humeri and femori were opened using secateurs in order to avoid heat damage to the cells associated with sawing bones.

2.1.9.1 Cytology samples

Samples for bone marrow for cytology were collected by gently breaking off pieces of bony trabeculae using an 18-gauge needle and lightly rolling the bony spicules over a clean microscope glass slide, taking care not to roll the material more than two thirds of the way down the slide. For cytological evaluation, 10 slides per patient were prepared and stained with Wright-Giemsa stain.

2.1.9.2 Histopathology samples

At least four 5mm x 5mm samples of metaphyseal bone marrow (one per humerus and one per femur) were immersed in 10% neutral buffered formalin for evaluation of histomorphology and included the cortical bone of the longitudinally sectioned proximal humeri and femori to avoid the dispersion of the bone marrow sample.

2.1.10 Sample processing

2.1.10.1 Cytology sample processing

Routine cytologic staining of glass slides using a Wright Giemsa special stain via the Siemens Advia 2120i stainer were conducted according to standardized methods employed at the section of Clinical Pathology, Department of Companion Animal Clinical Studies (CACS), FVS, UP. Where slides were of increased thickness (which did not allow for automated staining), slides were manually stained with a Diff-Quik stain according to standardized methods employed at the section of Clinical Pathology, Department of Companion Animal Clinical Studies (CACS), FVS, UP.

2.1.10.2 Histopathology sample processing

Samples for histopathology were fixed for 48 hours after which the samples were wax-embedded. The wax blocks from the study animals were labelled with specific histopathology case identification numbers and slides generated from these blocks were processed and stained with haematoxylin and eosin (HE) according to standardized methods employed at the histotechnology laboratory of the Section of Pathology, DPS, FVS, UP, following Department of Agriculture, Land Reform and Rural Development (DALRRD) -accredited standard operating procedures (SOPs). A total of 11 HE-stained sections were generated. In summary:

- Four 5mm^3 samples of bone marrow were obtained per dog to ensure sufficient formalin-fixed material.

- 6 *Babesia*-infected cases and 5 control cases were selected.

- 5mm x 5mm x 3mm thick portions of bone marrow were placed in tissue cassettes. To ensure the optimum number of bone marrow samples, all collected bone marrow samples were processed, resulting between three and eight wax blocks per case. From the variable number of wax blocks per case, the best / largest specimen were selected for preparation of HE sections.

- A total of 11 HE sections were generated.

2.1.10.3 Tissue sections

From the selected wax blocks, the following sections were obtained for examination:

- 1 x HE-stained tissue section per block = total of 11 HE-stained sections.

- 1x Perls' Prussian blue stained tissue section per block = total of 11 Perls' Prussian blue-stained sections.

- 5 x Immunohistochemistry (IHC)-labelled tissue sections per block = total of 55 IHC-labelled tissue sections.

- Total number of sections: 11 + 11 + 55 = 77.

2.1.10.4 Special stains

Special stains were performed in accordance with accredited methods in the histopathology laboratory, section of Pathology, DPS, FVS, UP.

2.1.10.5 Immunohistochemistry

Indirect chromogen-based immunoperoxidase staining was performed according to standard procedures employed at the Section of Pathology, DPS, FVS, UP, as summarized in Table 1. The wax-embedded tissue sections were adhered to positively charged superfrost glass slides and incubated in an oven overnight at 40°C. Tissues were deparaffinized by treatment in xylene for a period of 10 minutes after which the tissues were rehydrated in decreasing concentrations of ethanol (100%, 96% and 70% ethanol for 3 minutes each time). Distilled water was used to rinse slides three times after which they were incubated for 15 minutes with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. For the purpose of antigen retrieval, slides were microwaved at 96°C in citrate (pH 6) (CD3, Mac387) or EDTA (pH 9) (CD20, MUM-1, CD204) buffer solutions for 14 and 21 minutes, respectively. After cooling to room temperature, the slides were rinsed in distilled water 3 times and then for 10 minutes in 0.1 molar (M) phosphate buffered saline (PBS) buffer solution (pH 7.6), containing 0.1% bovine serum albumin (BSA). This was followed by incubation with the selected primary antibodies (CD3, CD20, MUM-1, Mac387 and CD204; Table 1). Slides were subsequently rinsed 3 times in distilled water, followed by rinsing in PBS-BSA buffer for 10 minutes. Slides were further treated according to BioGenex kit instructions (Reference QD420-YIKE; BioGenex, Fremont, CA 94538, USA). Antibody reactions were visualized using diaminobenzidine (DAB) supplied with the BioGenex immunodetection kit as the chromogen substrate, followed by counterstaining sections with Mayer's haematoxylin for 20 seconds. Finally, slides were rinsed under running tap water for 10 minutes, followed by dehydration of sections by putting them in increasing concentrations of ethanol. Slides were cleared in xylene, mounted in Entellan and coverslipped. Positive labelling was brown in colour in all the cases. Normal bone marrow and validated dog tissue controls (normal reactive lymph node) were used for positive tissue control purposes. These included negative internal tissue controls, e.g. fibrovascular stroma, megakaryocytes, adipocytes etc. not labelling with the selected

antibodies. Positive labelling was interpreted based on the specific pattern of labelling considered specific for each of the antibodies used. The pattern of labelling was as expected for the selected cell markers in dog tissues, namely CD3 and CD204 with cytoplasmic membrane and occasional cytoplasmic reactivity, CD20 with cytoplasmic membrane reactivity, Mac387 with nuclear and cytoplasmic reactivity and nuclear labelling for MUM1.

Table 1: Antibodies and methods used for immunohistochemistry

Antigen	**Antibody (clone)**	**Antibody dilution and incubation time**	**Pretreatment**	**Source**	**Target/s**
Rabbit polyclonal	CD3	1:600 120 minutes	Citrate, pH6, 96°C, 14 min	Dako Catalogue number: A0452	T lymphocytes
Mouse monoclonal	CD20	1:700 120 minutes	EDTA, pH9, 96°C, 21 min	Dako Catalogue number: Thermo scientific - PA5-16701	B lymphocytes
Mouse monoclonal	Mum1	1:50 120 minutes	EDTA, pH9, 96°C, 21 min	Dako Catalogue number: M7259	Mature B lymphocytes and plasma cells
Mouse monoclonal	Mac387	1:800 60 minutes	EDTA, pH9, 96°C, 14 min	Dako Catalogue number: M0747	Monocyte-macrophages within the bone marrow
Mouse monoclonal	CD204	1:400 120 minutes	Citrate, pH6, 96°C, 21 min	Abnova Catalogue number: MAB1710	Resident/tissue macrophages

Abbreviation: EDTA, Ethylenediaminetetraacetic acid

2.1.10.6 Light microscopic examination of tissue sections and cytological preparations

All HE-stained sections of bone marrow were examined using a light microscope in conjunction with a thorough histopathology checklist which had been compiled with the aid of the supervisors to minimize reader error due to inexperience (Appendix 2). All tissue sections were examined by the primary investigator and a subset of sections were double-checked by the supervisors. Light microscopic evaluation included evaluation of bone marrow cellularity. Cellularity was evaluated per 3 high power fields (x400 magnification) and scored on a scale of 1-4. A score of 1 represented 0-25 % cellularity, score 2 represented 26-50 % cellularity, score 3 represented 51-75 % cellularity and score 4 represented 76-100 % cellularity. Megakaryocytes were evaluated per 3 high power fields and the range as well as the mean were recorded. Megakaryocytes numbers were scored as 1, 2 or 3, with 1 representing low numbers, 2 within the normal range and 3 high / increased numbers. Low megakaryocyte numbers were defined as only 1-2 per particle / sample, normal megakaryocyte number was defined as a mean of 2-5 per average sized particle and increased megakaryocytes numbers were defined as a mean of ≥ 3/200x field and ≤ 3/500x field [156]. Endothelial cell hypertrophy was evaluated by comparing the endothelial cell diameter to the diameter to a red blood cell. The score was designated as 0 when the endothelial cell diameter was less than or equal to that of a red blood cell, and 1, when the endothelial cell diameter was greater that the diameter of a red blood cell. The degree of parasitized red blood cells was evaluated per 3 capillary segments. The parasitized red blood cells were evaluated in relation to the unparasitized red blood cells and a score of 0 designated less than 50% parasitized red blood cells and a score of 1 designated more than 50% of red blood cells were parasitized. The presence or absence of congestion, haemorrhages and thrombosis were also recorded (Appendix 3). Virtual slides were generated for the immunohistochemical sections on an Olympus dotSlide scanner at the Medical School, University of the Witwatersrand. The virtual slides were examined by the primary investigator and co-supervisor. The cytological evaluation was performed in a blinded manner by a European board-certified clinical pathologist, Prof. Emma Hooijberg, using check lists (Appendix 4).

2.2. Data analysis for immunohistochemistry

High resolution virtual slide images were created with the Olympus scanner at the University of Witwatersrand Medical School using the VS120-S6-W slide loader system (Wirsam Scientific) for the generation of virtual slide images, scanned in at 200x magnification. Slides were designated A-F for study cases and P,R,M,N and Q for each of the control cases. Thereafter slides were numbered 1-8 to designate the stain applied, e.g. A1 for CD3 stain on study case 1, A2 for CD20 stain on study case 2 etc. Olympus CellSens Dimension software (Olympus CellSens V, Olympus, Japan) was applied to the virtual slide images to count and measure regions of interest (ROI), in particular for the IHC component of the study. Regions of interest were highlighted using the phase separation function according to investigator's needs. The channel separation function was applied to each slide / image to allow for maximum contrast to facilitate enhanced detection and analysis of IHC staining. To increase accuracy, four ROI were determined per section of tissue and the ROI included the largest possible tissue area without the presence of artefacts such as slits, spaces, uneven staining and tissue folds. The results for each of the four ROI were tabulated in an Excel spreadsheet and the mean percentage of positive labelling cells were determined. The analytic software did not allow for the determination of the percentage of positive labelling within a section per cell marker / antibody per slide directly. As such, the percentage of adipose tissue and unlabelled cells, as well as the percentage of unlabelled cells were determined separately by using the Manual Threshold and Count and Measure function. The percentage value of adipose tissue and unlabelled cells were deducted from a 100 to determine the percentage of labelled cells per ROI per section. This obtained percentage value was subsequently divided by the number of unlabelled cells and multiplied by a 100 to get the percentage of positive labelling cells in relation to the percentage of unlabelled cells. The percentage value of positive

labelled cells is provided in Appendix 5. Study cases were designated as *Babesia* case #1, #2 etc. and controls as Control case #1, #2 etc.

2.3. Statistical analysis

The study was descriptive at the haematological, cytological, microscopic and immunohistochemical level and as such did not rely heavily on statistical testing, however, non-parametric tests were conducted to compare medians (Mann Whitney U) and Spearman's rho was used to determine correlations. Statistical analysis was performed by using a statistical software package (SPSS, version 24, IBM). The significance between the medians of the sample groups (control and infected) for the CBC parameters, cytology differential cells counts, immunohistochemical and special stains percentages, were compared by performing a non-parametric statistical test (Mann-Whitney U) and Spearman's rho was used to determine correlations. The significance was set at $P<0.05$. The Shapiro-Wilk test criterion for normality was not satisfied due to the small sample sizes. Figure 11 was generated by means of R software for statistical computing [157].

CHAPTER 3: RESULTS

3.1. Study population

Data of seven dogs with babesiosis (based on examination of a peripheral blood smear and the results of PCR/RLB) were initially obtained for this study. One dog was excluded based on the absence of an antemortem complete blood count.

3.2. Signalment

All five control dogs were female. Four of these control dogs were intact and one was neutered. Of the six infected dogs included in this study, four were male and two were female. Of the infected female dogs, one was intact, and one was neutered. Of the infected male dogs, one was intact and three were neutered. The median age of the control dogs was 12 months (range 12 months – 48 months). The median age of the infected dogs was 24 months (range one month - 84 months). Breeds included crossbreed (four control dogs and three infected dogs), Jack Russell terrier (two infected dogs), Beagle (one infected dog) and Africanis (one control dog). The duration of illness was unknown for one of the infected dogs, with a three day median duration of sickness in the remaining infected dogs (range 3 days to 14 days).

3.3. Treatment received

Of the six infected cases, three (cases 1, 3 and 4) received no treatment and were euthanased due to owner financial constraints. One dog (case 6) was treated at home (Doxycycline) by the owner before hospitalization, after which the animal was euthanased due to owner financial constraints. The remaining two cases (cases 2 and 5) received antibabesial treatment with diminazene aceturate (3.5mg / kg) according to the standard treatment protocol for babesiosis employed by the OVAH. Both dogs received a blood transfusion and additional supportive therapy was instituted for one of the dogs, including fluid therapy, oxygen and enteral feeding. One of the dogs died naturally and the other was euthanased due to a hopeless prognosis.

3.4. Haemogram / complete blood count

Erythron, reticulocyte, leukon and platelet variables are summarized in Tables 2-5. All infected cases were anaemic (mean haematocrit 0.11, SD 0.05). This anaemia was deemed as severe in five of the six infected cases (defined as a haematocrit of ≤0.15 litre/litre) and moderate in one of the infected cases (defined as a haematocrit of 0.15±0.29 litre/litre). In half of the infected cases, this anaemia was classified as non-regenerative based on the absence of reticulocytosis (as was evident by decreased ARC, i.e. ARC < $80x10^9$ / L). Significantly increased values in the infected *Babesia* cases compared to the control group were observed for the following parameters: red cell distribution width [(RDW), mean RDW 13.7, SD 0.5, p=0.004], nucleated red blood cells (i.e. erythrocyte precursors) per 100 white blood cells [(NRBC/100WBC), mean NRBC/100WBC 0.00, SD 0.00], reticulocyte percentage [(RET%), mean RET% 0.68, SD 0.52, p=0.000] and reticulocyte cell haemoglobin concentration mean (CHCMr), CHCMr mean 288.74, SD 14.85, p=0.012]. The distribution width (variability) of CHr (CHDWr) was associated with significantly decreased values within the infected cases compared to the control cases (CHDWr mean 4.24, SD 0.81, p=0.018). The absolute reticulocyte count was below the reference range [14] in three of the infected cases (*Babesia* cases 4-6) (mean ARC 47.73, SD 31.25, p=0.290). The reticulocyte haemoglobin content (CHr) was decreased below the reference interval [76] in one of the infected cases (*Babesia* case 5) whereas the remaining CHr values for the *Babesia* cases were within the reference range (CHr mean 25.4, SD 3.57). Compared to the reference interval [76], the CH delta value was decreased in one *Babesia* case (case 5) whereas it was increased in the remainder of the *Babesia* cases (CH delta mean 2.1, SD 2.27). Five of the six infected cases had a leukocytosis (mean white cell count 17.27, SD 11.93) with a left shifted neutrophilia (immature neutrophil count mean 2.06, SD 2.13) whilst one infected cases (*Babesia* case 5) had a leukopaenia

(white cell count 4.01 x10^9/L) with neutropaenia (neutrophil count 1.72 x 10^9/L). A thrombocytopaenia varying from mild to severe was noted in five of the six infected cases (mean 154.67, SD 182.43)) and interestingly platelet levels were within normal limits in *Babesia* case 5, which was also associated with a leukopaenia. Tabulated values for each infected and control case are available in Appendix 6.

Table 2: Median red cell variables for control dogs (n=5) and naturally infected *B. rossi* dogs (n=6), including standard deviation (SD) and P-value

Variable	**Unit**	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P-value***
Haemoglobin concentration (HGB)	g/L	156.0 (17.37)	34.5 (22.127)	**0.004**
Red cell count	x10^{12}/L	7.35 (0.72)	1.17 (0.66)	**0.004**
Haematocrit (HCT)	L/L	0.48 (0.05)	0.11 (0.05)	**0.004**
Mean corpuscular volume (MCV)	fL	69.2 (4.02)	74.8 (15.22)	0.177
Mean corpuscular haemoglobin	pg	23.3 (1.92)	29.5 (12.03)	0.247
Mean corpuscular haemoglobin concentration (MCHC)	g/dL	33.3 (0.65)	30.6 (13.16)	0.082
Red cell distribution width	%	13.1 (1.11)	16.7 (2.17)	**0.004**
NRBC/100WBC		0.00 (0.00)	9.00 (10.38)	**0.004**

* Significant P-values (i.e. P-value < 0.05) are in bold

Table 3: Median reticulocyte indices for control dogs (n=5) and naturally infected *B. rossi* dogs (n=6), including standard deviation (SD) and P-values

Variable	**Unit**	**Reference interval**	**Control group Median (SD)**	***Babesia* group Median (SD)**	**P-value***
Reticulocyte percentage (RET%)	%	0 - 1.0 [a]	0.53 (0.52)	11.4 (5.57)	**0.000**
Absolute reticulocyte count (ARC)	x 10^9/L	0 - 80.0 [a]	41.00 (31.25)	66.6 (51.45)	0.290
Reticulocyte mean cell volume (MCVr)	fL	80.5 - 97.0 [b]	86.7 (5.06)	98.2 (14)	0.242
Reticulocyte cell haemoglobin concentration mean (CHCMr)	g/L	27.8 – 32.0 [b]	288 (14.85)	269 (1.53)	**0.012**
Reticulocyte haemoglobin content (CHr)	pg/cell	24.5 – 28.6 [b]	24.2 (1.81)	26.2 (3.57)	0.489
Distribution width (variability) of CHr (CHDWr)	pg	27.8 – 32.9 [b]	3.06 (0.47)	4.55 (0.81)	**0.018**
Distribution width (variability) of reticulocyte cell size (RDWr)	%	10.7 – 17.5 [b]	13.10 (4.84)	15.0 (3.55)	0.312
CH delta	pg	0.7 – 3.0 [b]	1.80 (0.93)	3.1 (2.27)	0.490

* Significant P-values (i.e. P-value < 0.05) are in bold

[a] RET and ARC reference interval according to Stockham & Scott (2008) [14]

[b] Reference interval as indicated in Schaefer et al (2016) [76].

Table 4: Median leukon variables for control dogs (n=5) and naturally infected *B. rossi* dogs (n=6), including standard deviation (SD) and P-value

Variable	**Unit**	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P- value***
White cell count	$x10^9/L$	11.6 (2.95)	15.25 (11.93)	0.662
Segmented neutrophil	$x10^9/L$	6.26 (3.62)	10.37 (7.41)	0.537
Band neutrophil	$x10^9/L$	0.00 (0.00)	0.81 (2.13)	**0.004**
Lymphocyte	$x10^9/L$	3.86 (1.08)	2.8 (1.12)	**0.030**
Monocyte	$x10^9/L$	0.50 (0.42)	1.15 (1.82)	0.329
Eosinophil	$x10^9/L$	0.77 (0.44)	0.00 (0.00)	**0.004**
Basophil	$x10^9/L$	0.00 (0.06)	0.00 (0.00)	-

* Significant P-values (i.e. P-value < 0.05) are in bold

Table 5: Median platelet variables for control dogs (n=5) and naturally infected *B. rossi* dogs (n=6), including standard deviation (SD) and P-value

Variable	**Unit**	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P- value**
Platelet count	$x10^9/L$	231.00 (85.63)	98.50 (182.43)	0.177

3.5. Bone marrow cytology

3.5.1. Erythroid parameters

Erythroid cellularity was consistently increased amongst the infected cases as compared to the controls (Table 6). Orderly maturation and unremarkable morphology were noted within the control group, whereas a variable erythroid left shift was observed amongst five of the six infected cases. One of the infected cases was associated with orderly maturation. Dysplastic changes, including nuclear pleomorphism and binucleation, were observed within metarubricytes of infected animals, varying from mild to moderate (Figure 4).

Table 6: Bone marrow cytology, median values for the erythron variable as a percentage of all erythroid cells which was derived from a 500 differential cell count (standard deviation). Control dogs (n=5) and naturally infected *B. rossi* dogs (n=6).

Variable	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P- value***
Rubriblasts	2.10 (1.91)	9.80 (4.33)	**0.017**
Prorubricytes / rubricytes	79.60 (11.26)	76.30 (5.72)	1.000
Metarubricytes	17.60 (10.25)	12.75 (6.85)	0.329

* Significant P-values (i.e. P-value < 0.05) are in bold

3.5.2. Myeloid parameters

As opposed to the orderly maturation and unremarkable morphology within the control groups, an increased myeloid cellularity was observed in all infected cases (Table 7). This increased myeloid cellularity varied from a mild to marked left shift, with the maturation pool comprising mostly metamyelocytes and bands with a marked decrease in segmented forms (Figure 5). Apart from normal morphology in one of the infected cases, metamyelocytes / bands / segmenters were often giant with some toxic change visible, including cytoplasmic basophilia and granulation (Figure 4). In five of the six infected cases there was an increase in the number of eosinophils, varying from mild to marked.

Table 7: Bone marrow cytology, median values for the myeloid variable as a percentage of all myeloid cells which was derived from a 500 differential cell count (standard deviation). Control dogs (n=5) and naturally infected *B. rossi* dogs (n=6).

Variable	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P- value***
Myeloblasts	1.8 (1.31)	8.1 (5.12)	**0.017**
Promyelocytes	14.3 (4.25)	38.5 (5.24)	**0.004**
Metamyelocytes / Bands / Segmenters	84.4 (3.34)	52.4 (3.99)	**0.004**

* Significant P-values (i.e. P-value < 0.05) are in bold

3.5.3. Plasma cells, lymphocytes and histiocytes / macrophages

Plasma cells, lymphocytes and histiocytes / macrophage variables are summarized in Table 8. Mature plasma cells were observed in all control cases, whereas four out of the six infected cases had immatures with few plasmablasts noted in one of the cases. Lymphocytes were mostly small with an increase in the number of medium-sized lymphocytes noted in one of the cases. The presence of large granular lymphocytes was noted in one *Babesia* case. The number of histiocytes / macrophages were increased and comprised more than 10% of the total nucleated cell percentage in 4 *Babesia* cases, (Figure 6). Two *Babesia* cases were associated with low histiocyte numbers (<5%), mirroring what was observed in the control dogs.

Table 8: Bone marrow cytology, median values for plasma cells, lymphocytes and histiocytes / macrophages as a percentage of a differential 500-cell count (standard deviation). Control dogs (n=5) and naturally infected *B. rossi* dogs (n=6).

Variable	**Control group Median (SD)**	***Babesia* group Median (SD)**	**P- value**
Plasma cells	1.0 (0.52)	4.0 (7.22)	0.177
Lymphocytes	5.0 (2.34)	2.7 (1.29)	0.537
Histiocytes / macrophages	1.4 (1.28)	11.8 (7.62)	0.082

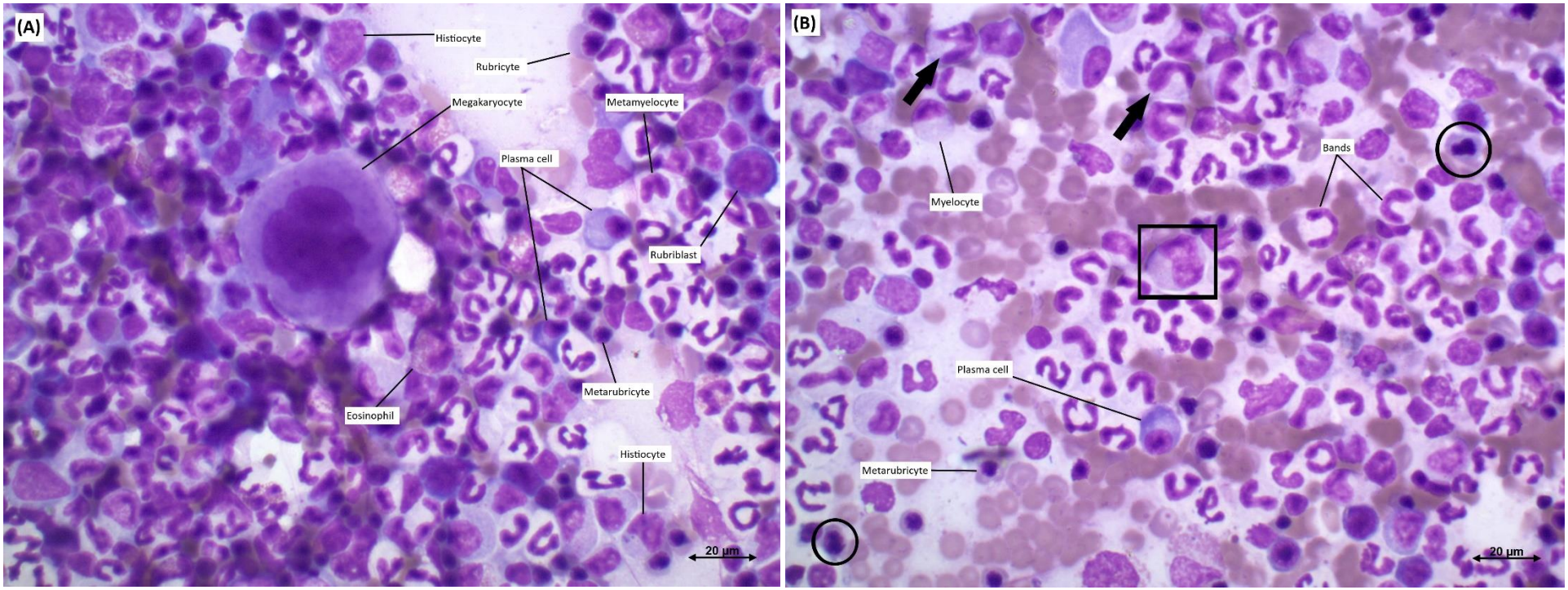

Figure 4: A. Cytology bone marrow smear of control dog no 1, Wright Giemsa stain. The metarubricyte morphology differs compared to what is seen in *Babesia* infection (B) where nuclear pleomorphism and binucleation is evident. Metamyelocytes and bands are smaller than those in the infected cases (B). **B**. Cytology bone marrow smear of *Babesia* case no 5, Wright Giemsa stain. Dysplastic changes (encircled) within the metarubricytes include nuclear pleomorphism and binucleated cells. Metamyelocytes (square) and bands (arrows) are often giant with cytoplasmic basophilia, features not evident in the control dog smear.

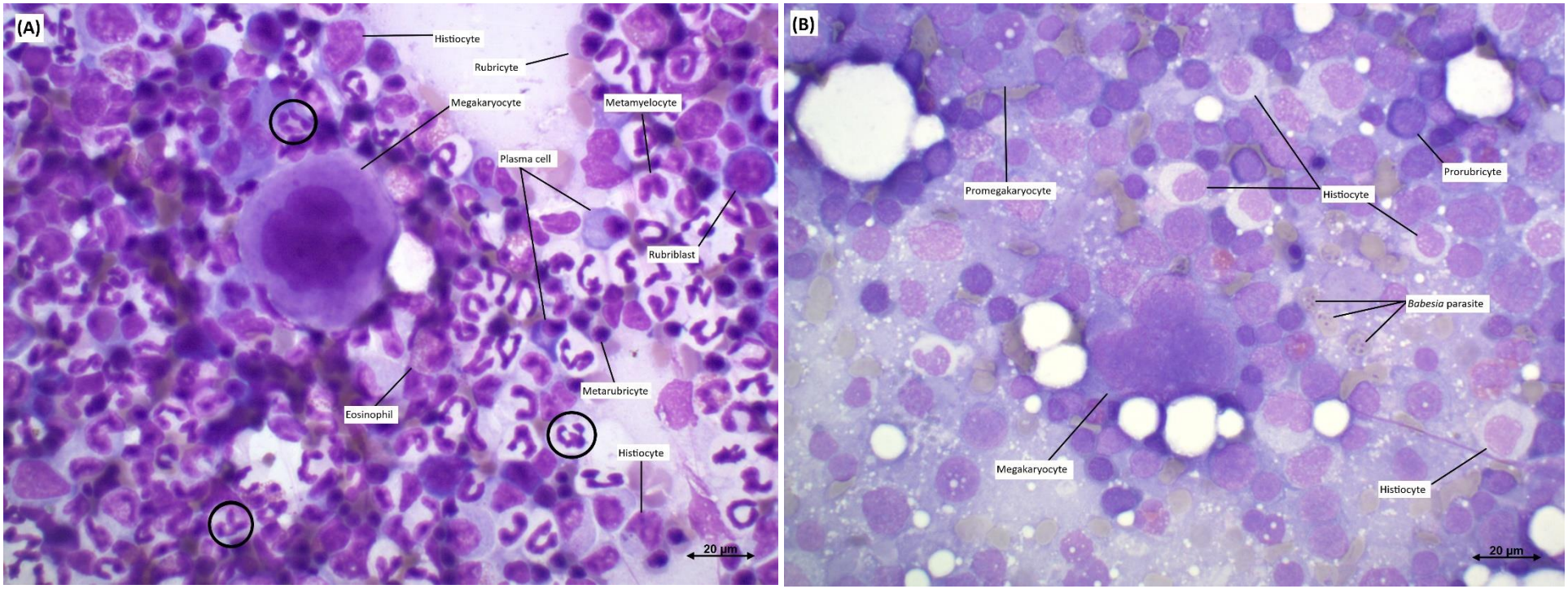

Figure 5: **A.** Cytology bone marrow smear of control dog no 1, Wright Giemsa stain. Mature neutrophils are encircled. **B**. Cytology bone marrow smear of *Babesia* case no 5. Diff-Quik stain. There is a marked decrease in the number of segmented neutrophils. Erythrocytes multifocally contain *Babesia* parasites (labelled).

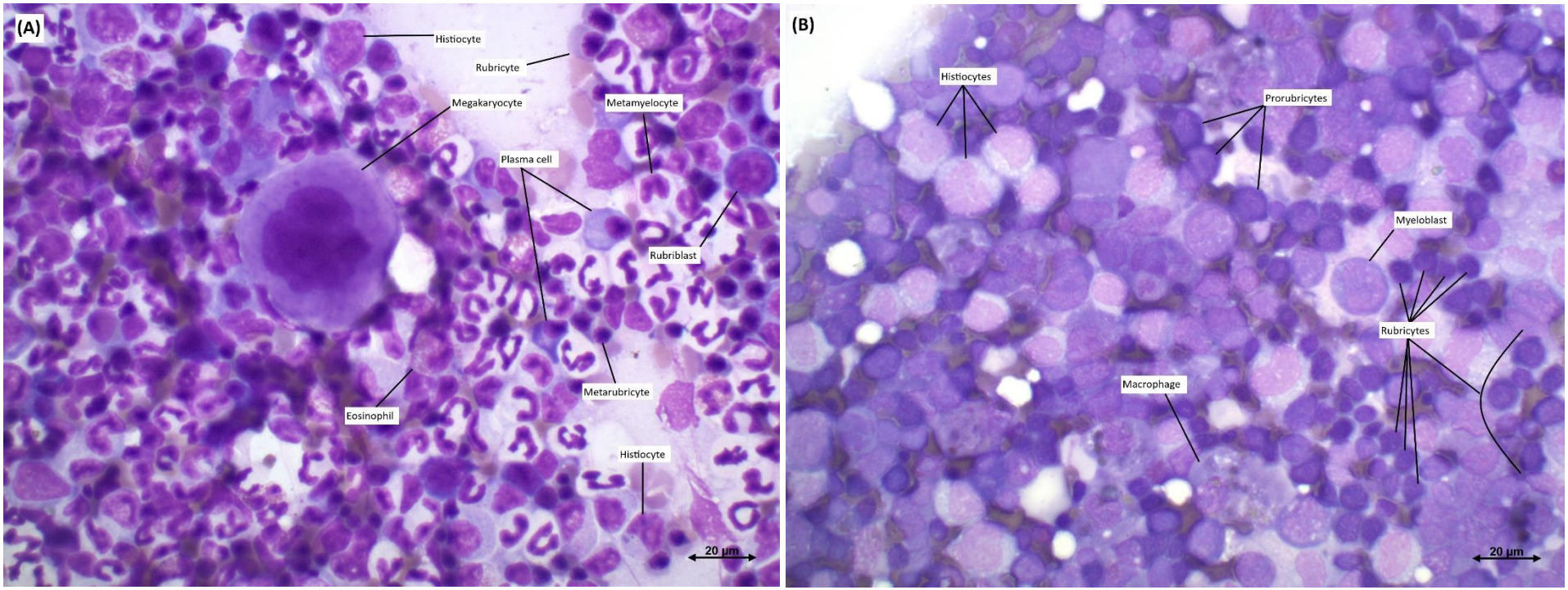

Figure 6: **A.** Cytology bone marrow smear of control case no 1, Wright Giemsa stain. **B.** Cytology bone marrow smear of *Babesia* case no 5, Diff-Quik stain. The macrophages / histiocytes and erythroid precursors are hyperplastic compared to the control (A).

3.5.4. Megakaryocytes

Megakaryocyte numbers were variably increased in 4 of the infected cases. This was associated with a variable left shift. In two of the *Babesia* cases as well as all the control cases, the number of megakaryocytes were within normal limits. A comprehensive table including all cytology results for each individual case is available in Appendix 4.

3.6. Histology

The bone marrow was hypercellular / hyperplastic in all *Babesia* cases (Figure 7). The number of megakaryocytes appeared increased in all *Babesia* cases and were mostly immature (Figure 8). Endothelial cell reactivity score of 0 was designated to four *Babesia* cases whilst two *Babesia* cases had a score of 1, which was associated with diffuse congestion and multifocal haemorrhages (Figure 9). Thrombosis was not observed in any of the cases. One of the infected cases had a parasite score of 1 (Figure 10), whereas five of the six infected cases had a parasite score of 0. Appendix 3 provides a detailed description of the histopathological findings of the bone marrow for each individual case.

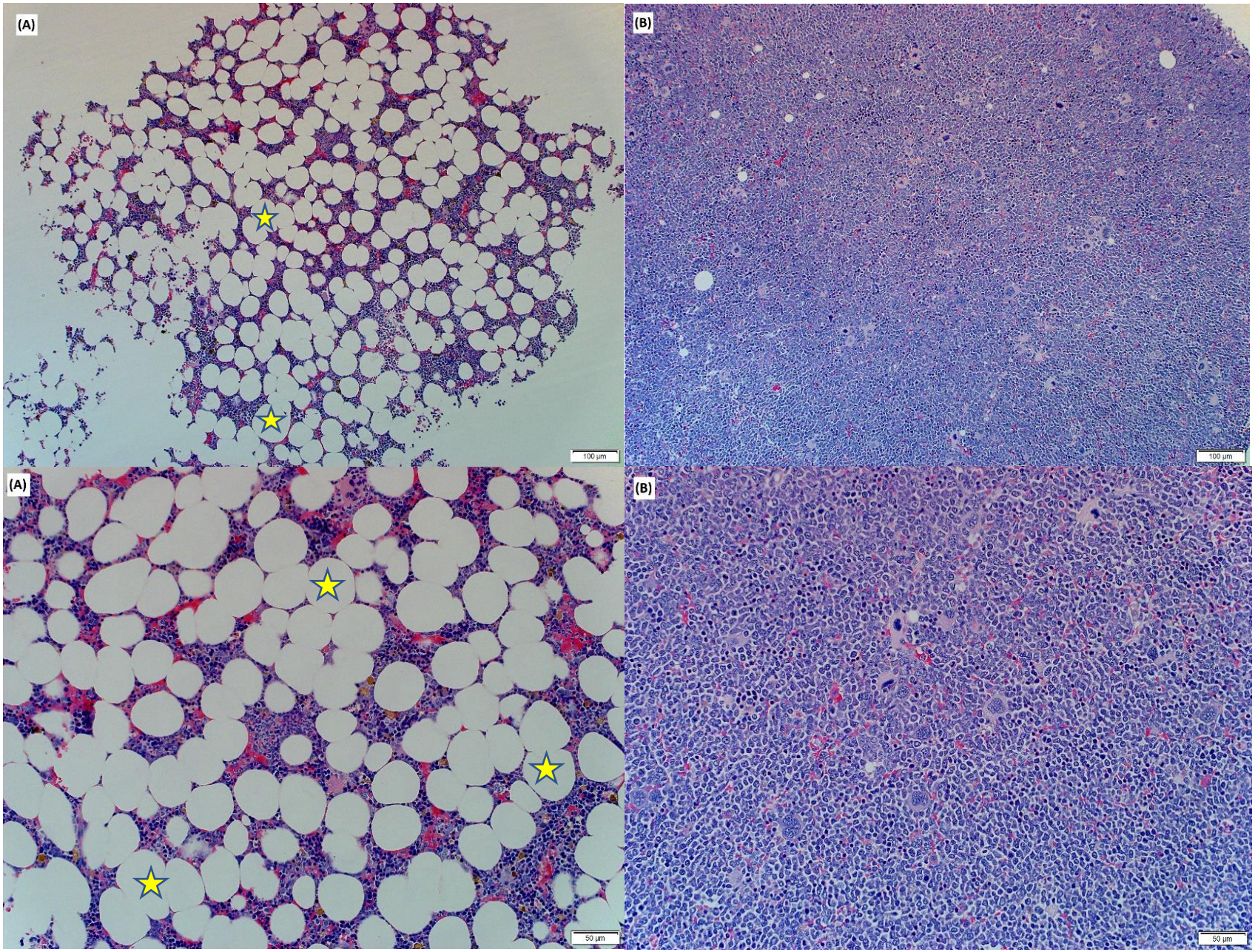

Figure 7 A. Control dog no. 5, haematoxylin and eosin (HE) stain. There are large numbers of adipocytes (stars). **B.** *Babesia* case no 1, Illustrating a hypercellular bone marrow with a paucity of adipocytes.

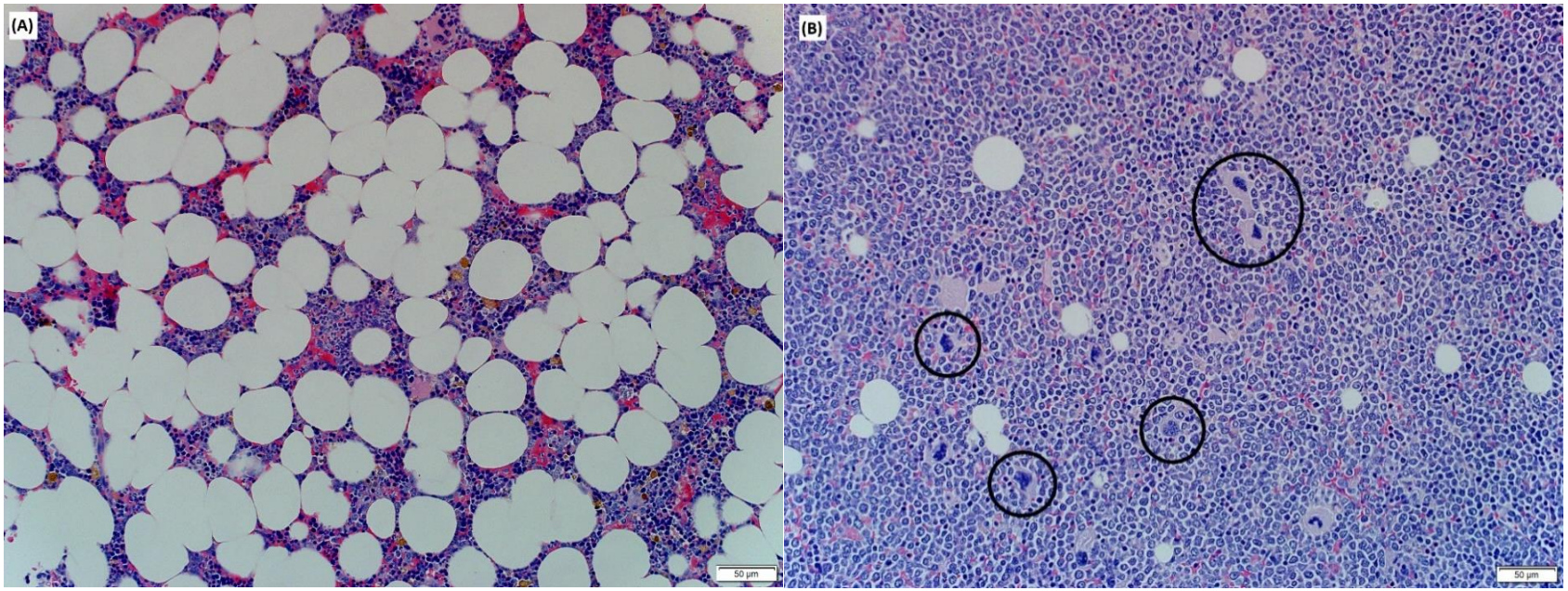

Figure 8 A. Control dog no. 5, HE stain, compared in **B** with *Babesia* case no 1. Immature megakaryocytes (encircled) are prominent and increased in number.

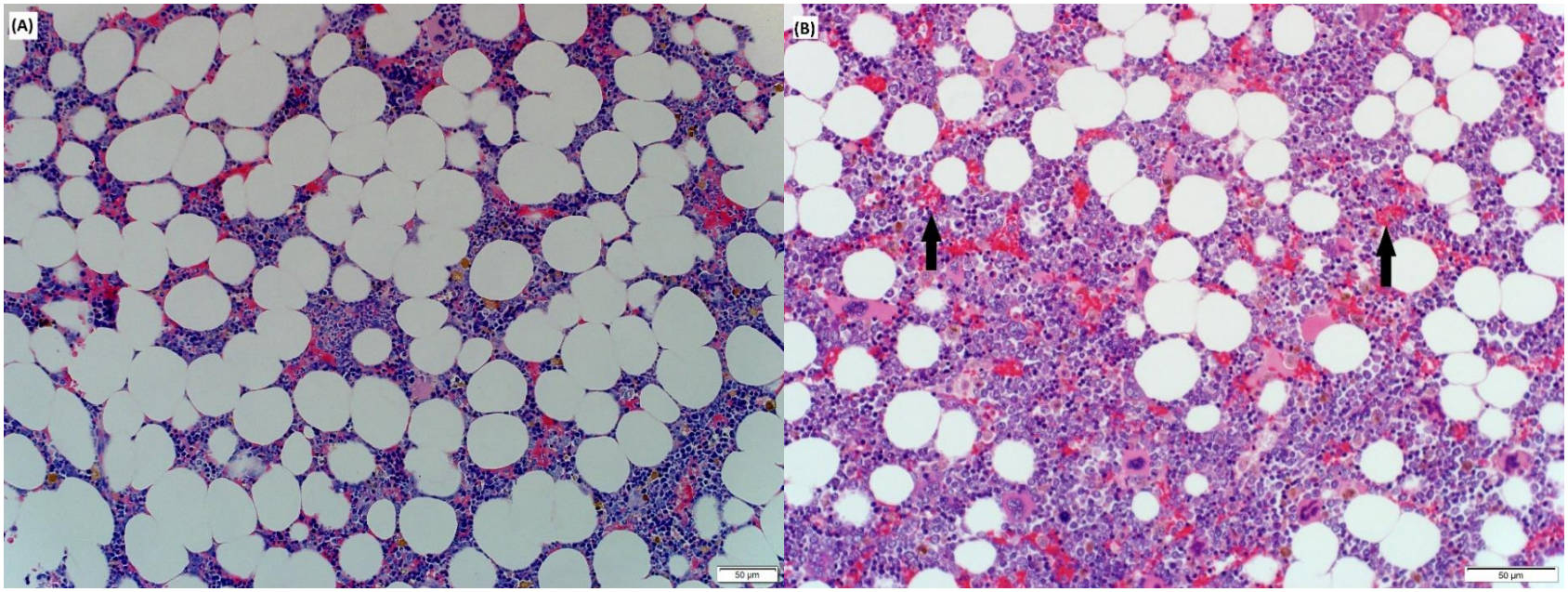

Figure 9. A. Control dog no. 5, HE stain, compared with *Babesia* case no 5 in **B** illustrating scattered haemorrhages within the bone marrow (arrows).

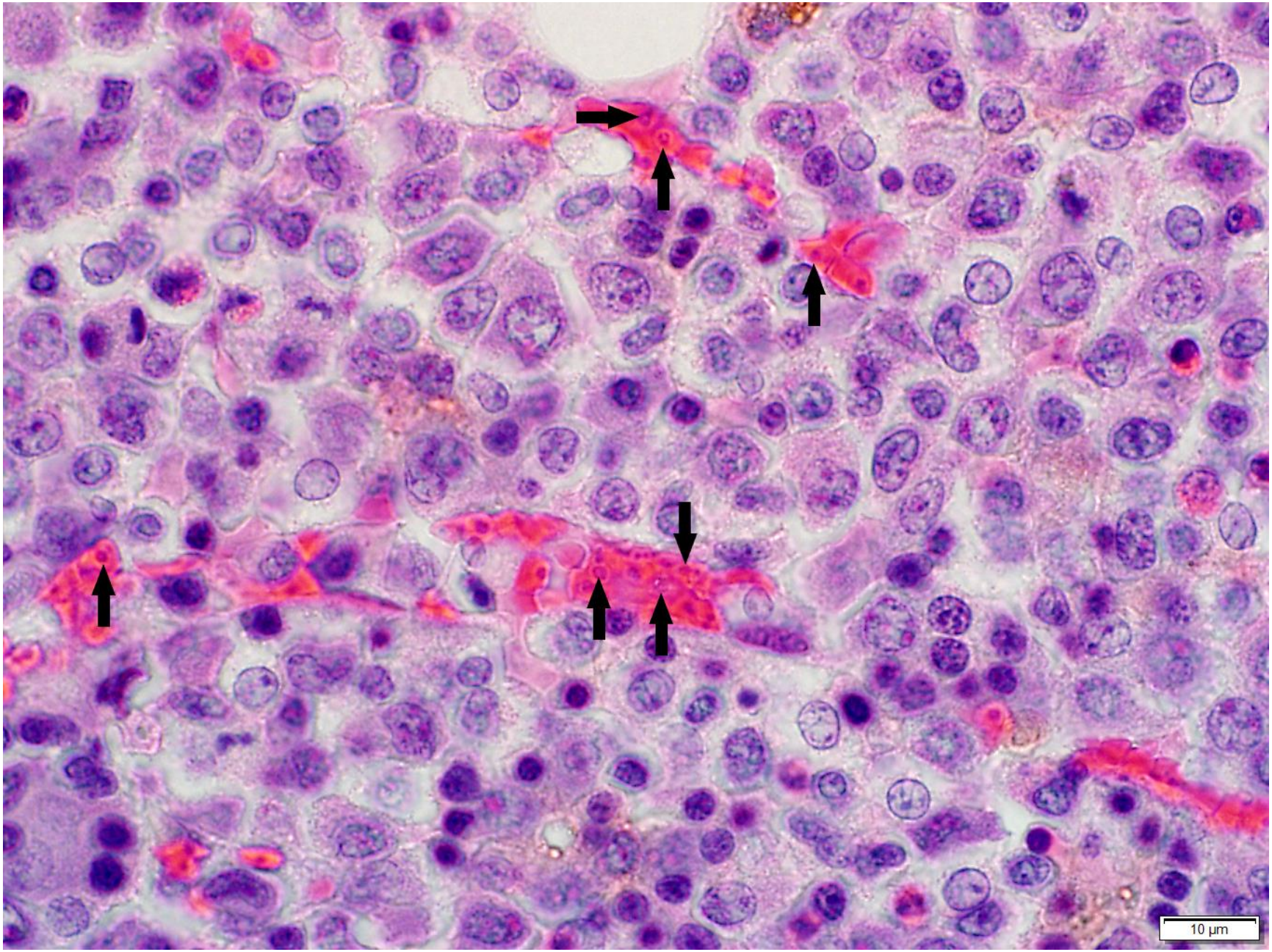

Figure 10: Babesia case no 6, HE stain, illustrating intraerythrocytic Babesia parasites within the vasculature (arrows)

3.7. Immunohistochemical and special stains results

A significant increase in CD204 positive cells (macrophages / histiocytes) and Perls' Prussian blue positive staining was observed in the infected group as compared to the control group (Figures 11-13, Table 9). No significant differences were observed between the control group and infected group with regards to the CD3 (T-lymphocytes, CD20 (B-lymphocytes), Mum-1 (plasma cells) and Mac387 (granulocytes). The percentage of positive labelling per section per cell marker/antibody per slide is provided in Appendix 5.

Table 9: Bone marrow immunohistochemistry, median values as a percentage derived after separate enumeration and subtraction of unstained cells (standard deviation). Control dogs (n=5) and naturally infected *B. rossi* dogs (n=6).

Immunohistochemical stain	**Control group** **Median (SD)**	***Babesia* group** **Median (SD)**	**P- value***
CD3	9.15 (2.44)	9.13 (1.39)	1.000
CD20	11.51 (0.70)	14.04 (4.56)	0.662
MUM1	4.57 (1.54)	7.2 (4.13)	0.329
CD204	15.39 (3.00)	22.64 (2.23)	**0.004**
Mac387	38.74 (3.71)	45.23 (10.11)	0.126
Perls' Prussian Blue	10.58 (7.79)	28.69 (24.75)	**0.030**

* Significant P-values (i.e. P-value < 0.05) are in bold

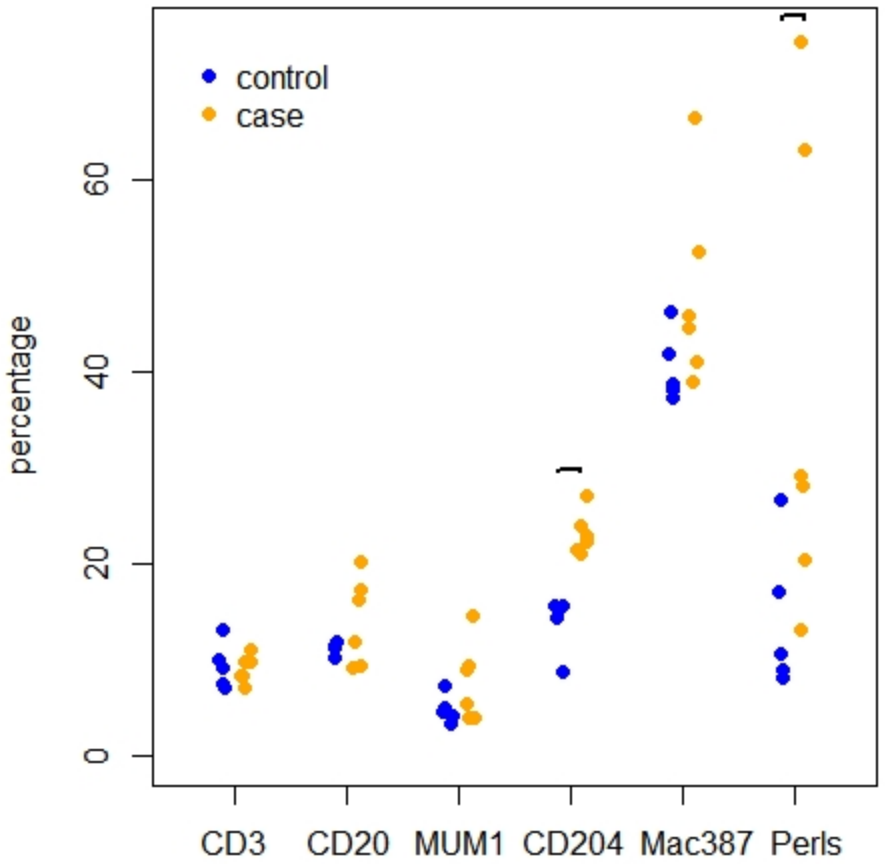

Figure 11: Scatterplot illustrating the median percentage values (derived after separate enumeration and subtraction of unstained cells) for the control cases (blue) and infected (yellow) cases with each immunohistochemical marker. Significant P-values are indicated by black bars above the relevant plots.

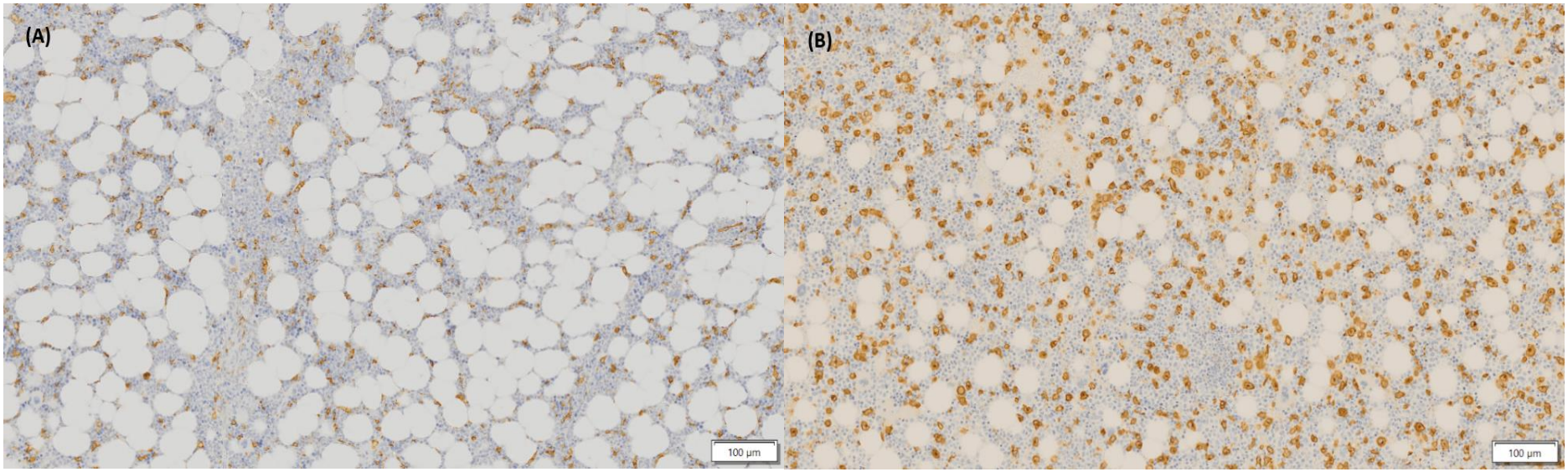

Figure 12: Bone marrow, dog. Immunohistochemistry (IHC) for CD204. A. Control case no 1. B. *Babesia* case no 5 illustrating the significantly increased numbers of histiocytes / macrophages within the infected group as compared to the control group.

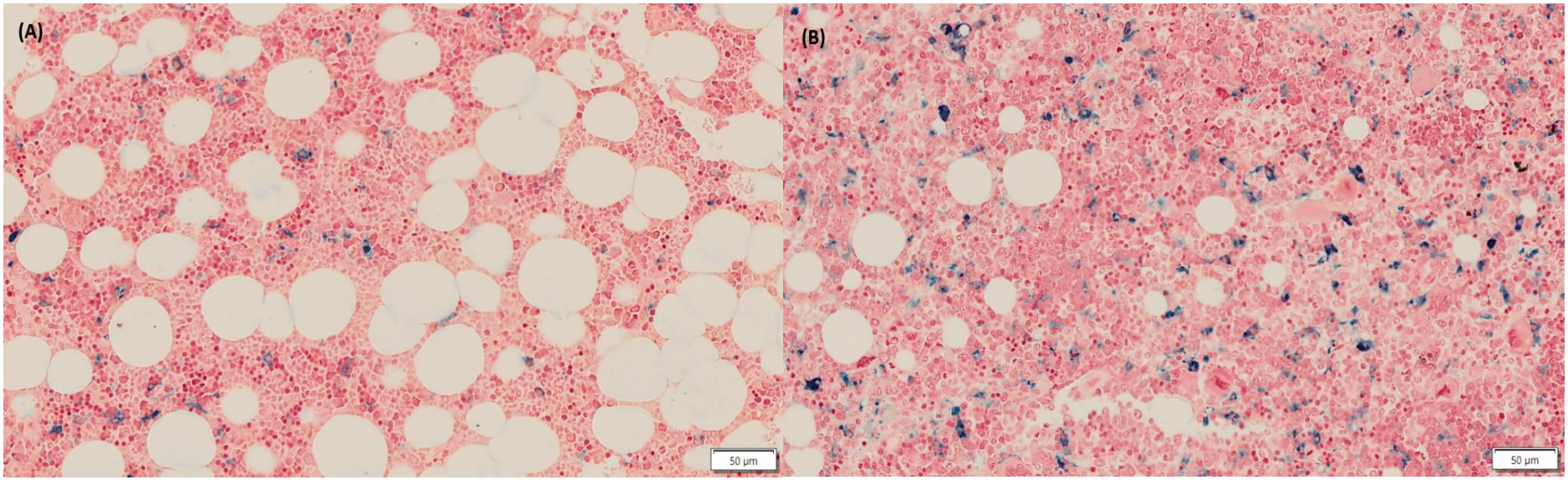

Figure 13: Bone marrow prepared with Perls' Prussian Blue stain. A. Control case no 1. B. *Babesia* case no 6 showing a significant increase in iron stores within the infected group as compared to the control dog.

CHAPTER 4: DISCUSSION

This is the first study reporting the bone marrow findings in dogs with canine babesiosis. The present study describes the cytological, histological and immunohistochemical (IHC) changes in the bone marrow of dogs infected with *B. rossi.* These changes were evaluated in parallel with the complete blood count obtained before the dogs died or were euthanized. The combination of haematology, cytology, histopathology, special stains and IHC ensured a thorough description of the pathology of the bone marrow in dogs with *Babesia rossi* compared to healthy control dogs. The main findings included bone marrow hypercellularity with significant increases in the number of rubriblasts, myeloblasts, promyelocytes and CD204 positive labeling histiocytes. Metamyelocytes / bands and segmented / mature neutrophils were evaluated as a unit and this number was significantly decreased within the infected group, owing mostly to the marked decrease in the segmented neutrophils in this group. However, a left shift was observed within the infected group as was evident by the increased numbers of metamyelocytes and bands. The megakaryocyte maturity was decreased, and a left shift was observed, i.e. shift to young / immature megakaryocytes. The amount of iron present within the bone marrow (evaluated by a Perls' Prussian blue stain) showed a significant increase in the infected dog group. In half of the infected dogs, the bone marrow response was considered an appropriate response to the haemolytic anaemia and systemic inflammation caused by the *Babesia* parasite. However, an inappropriate bone marrow response was observed in the other half of the infected dog group. These findings are discussed below.

4.1. Erythrocyte response

In essence, this study has shown that the anaemia in canine babesiosis is inappropriately regenerative, as observed by an insufficient increase in the absolute reticulocyte count (ARC). This inappropriate regeneration is likely due to a variety of causes, amongst which, host factors [i.e. release of pro-inflammatory cytokines with subsequent reduced erythrocyte survival, impaired iron mobilization or utilization, and impaired red blood cell production due to an unresponsiveness to erythropoietin (EPO)] and the presence of dyserythropoiesis may play a major role.

To compensate for the presence of anaemia in the infected group, the bone marrow responded by means of proliferation of erythroid precursors. The bone marrow of the infected dogs was hypercellular on cytologic and histopathologic examination, largely due to the proliferation of the red cell precursors. The bone marrow erythroid hyperplasia in this study was associated with a significant increase in the number of rubriblasts in infected dogs compared to the control dogs, i.e. left shift. This moderate to marked left shift in the erythroid line in the bone marrow was evident within five of the six *Babesia* cases. This left shift within the bone marrow erythroid lineage is regarded as an appropriate response considering the presence of haemolytic anaemia caused by the *Babesia* parasite. Left-shifted maturation within the bone marrow shows that the normal pyramidal maturation of bone marrow cells is absent and instead there is a proliferation of early progenitors with a decrease in the later stage progenitors [158]. This finding is associated with various aetiologies, including destruction of cells further down the lineage (as is the case with *Babesia*) or an early response of the bone marrow to injury or cytopaenia (i.e. a recovering / regenerating bone marrow) [158]. The significantly increased number of rubriblasts within the infected group was expected given the presence of anaemia. These progenitor cells are generally associated with a higher mitotic rate as there is an increase in mitotic activity within the earlier maturational stages within the bone marrow progenitor cells. However, the median value of metarubricytes was decreased within the infected group compared to the control group, albeit not significantly. This may suggest that there is suppression of erythropoiesis ultimately resulting in an inappropriate red cell regeneration as is evidenced by the low ARC values (discussed below). This is also supported by the presence of dyserythropoiesis within metarubricytes (discussed below). This suppression of erythropoiesis is likely caused by either direct or indirect (e.g. parasite derived soluble factors or as a consequence of the host inflammatory

response) influence of the disease on the bone marrow. Comparing bone marrow histology and cytology between a sterile haemolytic disease (such as with primary immune mediated haemolytic anaemia) with *Babesia* infected dogs would be important to investigate in future. It has already been shown that the ARC in dogs with IMHA is significantly higher than that seen in *Babesia* infected dogs [72]. A study conducted by Chatterjee et al (2016), aiming to identify which erythroid maturational stage within the bone marrow are preferentially targeted for destruction in immune mediated haemolytic anaemia in mice, found that autoantibodies are bound to all erythroid maturational stages within the bone marrow as well as to mature erythrocytes within the peripheral circulation [159]. However, decreased numbers of the late stage erythroid bone marrow precursors as well as the younger red blood cells in the peripheral blood was also found, thereby implying that there is a preferential destruction of the later stage erythroid populations during IMHA [159]. Increased levels of membrane bound autoantibodies were also present in the last stage of erythroid differentiation within the bone marrow (Erythroblast C), as well as in reticulocytes and the most immature erythrocytes within the peripheral circulation [159]. Apart from activation of complement and antibody mediated phagocytosis as a result of increased antibody binding to erythroid cells, the authors also suggested that other factors may cause cell destruction such as the production of reactive oxygen species (ROS), either as a reaction to autoantibody binding or the fact that erythroid cells are generally associated with a higher production of ROS [159]. Bone marrow fibrosis, hypercellularity not only restricted to the erythroid line, and dyserythropoiesis were present in a study of 47 human patients with primary autoimmune haemolytic anaemia [160]. Bone marrow fibrosis was not observed histologically in this study. Weiss (2008) reported on the bone marrow pathology observed in non-regenerative IMHA in dogs and found that various mechanisms were likely involved in the lack of red blood cell regeneration [161]. These included dyserythropoiesis, necrosis, myelofibrosis, haemophagocytosis, altered vascular permeability (as is evident by haemorrhage and oedema) and acute inflammation. The resultant cytopaenia was likely due to a combination of antibody mediated suppression / destruction of precursor cells, macrophage derived cytokines leading to suppression of haematopoiesis as well as a variety of other bone marrow pathologic changes (i.e. dyserythropoiesis, necrosis, fibrosis, inflammation) leading to ineffective erythropoiesis. Apart from a non-regenerative anaemia, neutropaenia and / or thrombocytopaenia were also observed within the peripheral circulation [161]. Bone marrow evaluation of cats with primary IMHA revealed erythroid, myeloid, megakaryocytic and lymphoid hyperplasia, erythrophagocytosis (including phagocytosis of erythroid precursors), dyserythropoiesis and iron deposits [162]. This is similar to what was observed in our study, although we did not observe phagocytosis of erythroid precursors or foci of lymphoid hyperplasia.

One of the *Babesia* cases (case number 4) had a peripheral anaemia with a lack of erythroid left shift within the bone marrow. In this case, orderly maturation within the erythroid line was present with some mild dysplastic changes in metarubricytes, including nuclear pleomorphism and binucleation. Tommasi et al (2014) evaluated the blood and bone marrow findings in a few canine vector-borne diseases and found that in a few cases of dogs infected with *Anaplasma platys* and *Hepatozoon canis,* a non-regenerative anaemia was present associated with a decreased or lack of proliferation of erythroid precursors (rubriblasts and prorubricytes), i.e. lack of left shift [163]. It was speculated that this finding may be due to the induction of a humoral and / or cell-mediated immune response towards the early erythroid precursors [163,164].

In the current study, despite the presence of bone marrow erythroid hyperplasia, this finding was not reflected within the peripheral circulation, i.e. there was a poorly regenerative / non regenerative anaemia (discussed below). Bone marrow erythroid hyperplasia can broadly be divided into disorders / conditions associated with effective erythropoiesis and disorders / condition associated with ineffective erythropoiesis [14]. In conditions where effective erythropoiesis is present, EPO stimulation of precursor cells leads to the proliferation of erythroid cells with subsequent increased release of reticulocytes, nucleated erythrocytes or erythrocytes into

the peripheral circulation. This is usually seen secondary to haemolytic conditions [e.g. autoimmune haemolytic anaemia, haemolysis induced by protozoal, bacterial and viral infections such as *Babesia* spp., *Theileria* spp., *Trypanosoma* spp., *Mycoplasma* spp., *Anaplasma* spp, feline leukaemia virus (FeLV)] or blood loss disorders [14]. It may also occur secondary to persistent hypoxia (e.g. cardiac disease, pulmonary disorders, hyperthyroidism, living at high altitudes) [14]. Various neoplasias (e.g. hepatoma, schwannoma, leiomyosarcoma), renal neoplasms or renal cysts may also lead to inappropriate EPO production (i.e. inappropriate due to production of EPO that is autonomous and not induced by systemic hypoxia), resulting in bone marrow erythroid hyperplasia [14]. In conditions where ineffective erythropoiesis is present, erythroid hyperplasia occurs together with a nonregenerative or poorly regenerative anaemia [14], as was evident in this study. This may be seen in conditions where there is an immune mediated destruction of nucleated red blood cells (ISA positive IMHA was however not demonstrated in this study) and in various nutritional deficiencies, including iron-deficiency anaemia (discussed below) [14]. Iron-restricted erythropoiesis could not be demonstrated in this study, but may require future investigation.

To assess whether the bone marrow is responding adequately to the presence of anaemia, i.e. regenerative versus non-regenerative anaemia, the ARC should be considered. The ARC is the best indicator of regeneration and the bone marrow response to anaemia [68]. In this study, the degree of red cell regeneration in the reticulocyte population (as indicated by the low ARC values) was largely consistent with poor or inadequate regeneration. The extent of reticulocytosis (defined as an increased number of reticulocytes within the peripheral circulation[165]) is used to classify the anaemia as regenerative (responsive) or nonregenerative (nonresponsive) [14]. Anaemia can also be classified according to its pathophysiological mechanisms into blood loss anaemia (internal versus external blood loss), haemolytic anaemia (intravascular versus extravascular haemolysis) and anaemia caused by reduced red blood cell production (e.g. inflammatory diseases, renal disease, bone marrow hypoplasia / aplasia and erythroid hypoplasia / ineffective erythropoiesis) [14]. *B. rossi* infection is characterized by the presence of severe haemolytic anaemia [28,31]. The regenerative response to haemolytic anaemia is significantly stronger when compared to that seen with an anaemia due to external haemorrhage. This is because iron and proteins from haemolysed erythrocytes can be recycled and are more readily available for erythropoiesis than stored iron [11,68]. Haemolytic anaemias are typically regenerative and may be macrocytic and hypochromic when associated with intense regeneration [10]. The mean reticulocyte percentage (RET%) indicates the percentage of reticulocytes within a given blood sample [14] and the significantly increased RET% in the infected group is consistent with *Babesia*-induced reticulocytosis. This reflects a regenerative attempt by the bone marrow, but it does not reflect the adequacy of that response. As mentioned, to assess whether regeneration is adequate or not, the ARC must be considered [166,167]. There is increased stimulation for erythrocyte production during severe anaemia [168], which results in increased ARC values. Regenerative anaemia is characterized by an ARC that is above the upper reference limit of 95 x 10^9 / L [76]. In this study, although the median ARC values were increased compared to the control group, this value was not adequately increased for the degree of anaemia and, in fact, the ARC values in half of the infected cases were less than 70 x 10^9 / L. The anaemia was therefore classified as non-regenerative. This non-regenerative response is similar to what Leisewitz et al. (2019) and Seejarim et al (2020) found in their studies. Possible explanations for this finding include alterations in erythropoiesis before reticulocytes are released into circulation, mediated by increased cytokine concentrations (particularly IL-1, IL3, IL-9, TNF and IL-11), reduced colony stimulating factors (GM-CSF or G-CSF), reduced EPO, reduced maturation of progenitor cells (BFU-E and CFU-E) and the reduced development of erythroid precursors (rubriblasts, prorubricytes, rubricytes and metarubricytes) [72].

The significant decrease in haemoglobin concentration, red cell count and haematocrit in the infected group are expected given the presence of the *Babesia* parasite. The significantly increased number of nucleated red blood cells (i.e. erythrocyte precursors) per 100 white blood cells (NRBC/100WBC) in the infected group indicates an

increased degree of rubricytosis, which refers to the presence of nucleated red blood cells within the peripheral circulation [169]. Rubricytosis is an indication of regenerative anaemia and is characterized by the appearance of at least 0.5 nRBC/100 white blood cells [169,170]. The significant rubricytosis in the infected group is an appropriate response of the bone marrow in view of the haemolytic anaemia [169], however, as discussed above, the anaemia was not considered appropriately regenerative despite the bone marrow showing an erythroid left shift and erythroid hyperplasia. The major cause for the ongoing nonregenerative anaemia is reduced erythrocyte production, although defective erythropoiesis (dyserythropoiesis) may also be a contributing factor [14]. If only reduced erythropoiesis is the cause for the inappropriately regenerative anaemia, this anaemia would take weeks to months to manifest as a dog's erythrocyte life span is 100 days [14]. There are various causes for nonregenerative anaemia, including inflammatory disease, chronic renal disease, diseases causing bone marrow hypoplasia or aplasia of multiple cell lineages and diseases causing selective erythroid hypoplasia / ineffective erythropoiesis without generalized marrow hypoplasia [14]. A select few are discussed below, namely inflammatory disease, diseases causing bone marrow hypoplasia or aplasia of multiple cell lineages and diseases causing selective erythroid hypoplasia or ineffective erythropoiesis.

Inflammation, notably chronic inflammation, causes anaemia of inflammatory disease, also known as anaemia of chronic disease. This is the most common cause of nonregenerative anaemia, typified by a normocytic (rarely microcytic) normochromic anaemia [14]. Any chronic disorder associated with inflammation will result in anaemia, including chronic infections (bacterial, fungal, viral and protozoal) and noninfectious conditions (immune, toxic and neoplastic) [14]. The pathogenesis of anaemia of chronic disease involves a reduced erythrocyte lifespan, impaired iron mobilization or utilization and impaired production of erythrocytes [14]. A reduced erythrocyte lifespan is associated with increased IL-1 concentrations and oxidant damage to red blood cell membranes with subsequent binding of immunoglobulin molecules and erythrophagocytosis. Cytokines IL-1, IL-6, interferon and TNF play a role in altered iron kinetics with subsequent impaired iron mobilization or utilization. The production of erythrocytes is impaired due to the unresponsiveness of erythroid cells to increased EPO levels. This is due to the effects of inflammatory cytokines IL-1, interferon and TNF on erythroid precursors as well as the effects of IL-1, TNF and TGFβ where EPO is being produced at an increased rate but not as much as expected [14]. *Babesia rossi* infection has been associated with an excessive pro-inflammatory cytokine response [46,122]. Similar findings are evident in malaria, where cytokines (IFN-ɣ, TNF-α, IL-2, IL-12 and IL-10) inhibit erythroid progenitor cells or blunt their response to EPO, thereby inhibiting / suppressing erythropoiesis [85]. Severe malaria and death have been associated with an overzealous pro-inflammatory response with increased concentrations of TNF-α, IFN-γ, IL-6, IL-8, IL-18 and MCP-1 [129,131]. Similar to human malaria, cytokines IL-6, IL-8, IL-10 and MCP-1 were demonstrated to play an important role in *B. rossi* infected dogs [122]. As discussed previously, IL-6 and MCP-1 were negative prognostic markers for *B. rossi* infected dogs [122,135]. MCP-1 is a significant mediator for the recruitment of monocytes from the bone marrow upon an inflammatory stimulus as well as monocyte trafficking to inflammatory sites [137,138] and activated macrophages promotes the release of pro-inflammatory cytokines TNF and IL-1 [85]. The increased cytokine production, in turn, stimulates further activation of macrophages which may be advantageous in the removal of parasitized erythrocytes, however, an increased quantity of TNF may lead to a reduction of erythropoiesis as well as dyserythropoiesis (see below) [85]. Furthermore, inadequate IL-12 production leads to dyserythropoiesis in malarial infection [139]. Several studies have showed that a deficiency in EPO levels are not a feature of malarial infection [75,89–91]. In fact, high levels of EPO during malaria infection is observed due to high levels of TNF-α and HIF-1 [92,93]. A blunted response of red blood cell progenitors to EPO may play a role in the inappropriate regenerative anaemia that is observed in canine babesiosis, as postulated in malaria infections [95]. The reticulocyte response to exogenous administration of EPO in *Plasmodium*-infected mice was suppressed and the degree of suppression was correlated with rising parasitaemia [75]. This suppression of the reticulocyte response to administration of exogenous EPO suggests defective development of erythroid precursors [75]. Therefore, the lack of a sufficient red blood cell response during babesial and malarial infection

may be due to unresponsiveness of red blood cell progenitors to EPO [75,89,90]. Suppression in erythropoiesis may be mediated by the parasite itself, parasite products (e.g. parasite associated factors / toxin) or host factors (i.e. cytokine release) [75].

Diseases causing marrow hypoplasia or aplasia of multiple cell lineages can be idiopathic or caused by infectious agents, toxicoses, radiation damage or caused by marrow replacement (e.g. myeloproliferative diseases, lymphoproliferative neoplasia, metastatic neoplasia, myelofibrosis) [14]. Infectious agents may suppress haematopoiesis via direct haemic cell infection, myelitis or via secondary effects such as immune reactions [14]. Suppression of haematopoiesis may occur with bacterial sepsis, disseminated mycosis, viral infections (e.g. feline leukaemia virus) or protozoal infections (e.g. leishmaniasis) [14].

Diseases causing selective erythroid hypoplasia or ineffective erythropoiesis include pure red cell aplasia, immune-mediated nonregenerative anaemia, nutrient deficiencies, endocrine disorders, hepatic disease and dyserythropoiesis [14]. Mild to moderate dyserythropoiesis was indeed observed in all *Babesia* infected cases. These changes were observed in metarubricytes and included nuclear pleomorphism and binucleation. Dyserythropoiesis refers to a variety of disorders where erythrocytes mature abnormally and / or where erythrocyte morphology is associated with inadequate erythropoiesis [171,172]. Dyserythropoiesis may be seen in conjunction with immune-mediated disorders, haematopoietic neoplasms, myelofibrosis, drug toxicity and also forms a prominent component of some congenital disorders in Hereford calves and English springer spaniel dogs [171]. It has also been reported in conjunction with various parasite-induced anaemias, including falciparum malaria and leishmaniasis, where it is related to TNF-α and IL-12 production [85,111–113,115,139,173,174]. Interestingly, in our study the infected cases associated with an inappropriate bone marrow response (*Babesia* cases four to six) were not associated with a drastic increased number of CD204 and MAC387 positive labelling cells (to suggest an increased cytokine production) as compared to the other infected cases where the bone marrow reaction is deemed appropriate. Parmar et al (2015) reported the morphological changes observed during dyserythropoiesis. These changes were evident within the nucleus as well as within the cytoplasm of erythroblasts [172]. The quintessential feature of dyserythropoiesis include asynchrony between the growth and maturation of the nucleus and the cytoplasm of erythroblasts [172]. Nuclear changes were reported to include aberrant mitotic figures, nuclear lobulation and fragmentation with karyorrhexis and pyknosis, bi- and multinucleated cells, internuclear bridging and occasionally chromatin changes resulting in megaloblastosis [172]. Cytoplasmic changes included persistent cytoplasmic basophilia with or without vacuolation or stippling, cytoplasmic bridging and increased deposition of iron within the lysosome or mitochondria [172]. Leisewitz et al (2019) postulated that dyserythropoiesis may be responsible for the inappropriate erythroid regenerative response in canine babesiosis [46]. In human malaria, the host immune response to infection, as well as the presence of parasite derived products are speculated to be the cause of dyserythropoiesis and the inappropriate bone marrow red cell response [94].

Nutrient deficiencies responsible for selective erythroid hypoplasia / ineffective erythropoiesis include iron deficiency, copper deficiency, folate or vitamin B12 deficiency and vitamin B6 deficiency. During copper deficiency there is defective iron transport resulting in abnormal erythrocytes (i.e. functional iron deficiency) [14]. Iron deficiency may also occur due to chronic external haemorrhage or insufficient dietary iron intake. Iron deficiency anaemia is typically microcytic hypochromic but may also present as microcytic and normochromic [14]. During iron-deficiency, anaemia results in spite of erythroid hyperplasia and is due to defective erythropoiesis, improper maturation of late-stage erythroid precursors and inadequate release of erythrocytes into the peripheral circulation [14]. Hypoferrinaemia is an important finding in anaemia of chronic disease and is also present in malarial infections [81,175]. Hypoferrinaemia in malaria infection arises due to iron sequestration within macrophage stores, the production of proteins by inflammatory cells that can bind iron more readily than

transferrin and by a reduction in the production of transferrin in the liver [175]. The malarial parasite can extract iron from the host by the insertion of a transferrin-like receptor on the host erythrocyte membrane and as such, the malarial parasite can cause further iron deficiency within the host. This mechanism is more significant in situations where nutritional iron deficiency is frequent [93,175].

Reticulocyte indices [including mean cell volume of reticulocytes (MCVr), mean haemoglobin concentration of reticulocytes (CHCMr) and haemoglobin content of reticulocytes (CHr)] are used to evaluate the aetiopathogenesis of anaemia and are invaluable in assessing the functional state of erythropoiesis [72]. These indices provide information regarding the size of reticulocytes, as well as the haemoglobin content of reticulocytes [76]. In this study, data on the reticulocyte indices were sparse. Reticulocyte indices are not routinely processed in non-anaemic cases and as such reticulocyte data in healthy dogs is not easily available. A set of control data originating from healthy dogs was however available for our use [72]. Iron-restricted erythropoiesis is best assessed by means of reticulocyte indices such as reticulocyte microcytosis and reticulocyte hypochromasia which are detected earlier in comparison to other mature erythrocyte indices [76]. These include reduced values of MCVr, CHCMr and CHr [76,176–178]. That iron-restricted erythropoiesis plays a role in the anaemia of canine babesiosis could not be established in this study based on the lack of significantly decreased values within reticulocyte indices MCVr and CHr that are most likely to reflect iron deficiency. In this study, significantly decreased values were only noted for CHCMr in the infected dogs (discussed below). A Perls' Prussian blue stain for iron was significantly increased within the bone marrow of the infected group. Thus, iron stores appear to be sufficient during canine babesiosis although whether this iron is available for red cell production is uncertain. Functional iron deficiency may play a role in the non-regenerative anaemia observed, although in this study, CHr values were mostly within the normal range for the *Babesia* infected cases.

In addition to the significantly increased RET% and NRBC/100WBC values, significant increases were also evident in the RDW and CHCMr parameters of the infected dogs. The significantly increased RDW in the infected group reflects the extent of variation within erythrocyte volumes and therefore indicates increased anisocytosis [14]. This is due to the release of immature erythrocytes into the peripheral circulation [179]. This significantly increased RDW value in the infected group is expected given the presence of haemolytic anaemia [180,181]. Although the distribution width of reticulocyte cell size (RDWr) was increased within the infected group as compared to the control group, this increase was not as significant as one would expect during cases of accelerated erythropoiesis. Although RDW is mainly used to classify anaemia (the higher the RDW the more regenerative the anaemia), it has also been studied as an inflammatory marker and increased levels were found to be associated with a poorer outcome and higher risk for morbidity and mortality in various conditions, including autoimmune disease, cardiovascular disease, malignancy, chronic renal disease and chronic lower respiratory disease [180,182]. However, Lippi et al (2009) speculated that the link between RDW and inflammatory markers is a consequence of underlying anaemia. The distribution width (variability) of CHr (CHDWr) indicates the distribution width in haemoglobin content of all reticulocytes measured [14]. It equates to the haemoglobin concentration distribution width (HDW) within erythrocytes [14]. The significantly increased value within the infected group indicates that there is a greater variation in the haemoglobin content of reticulocytes within this group. Significantly decreased values were observed for CHCMr in the infected dogs. CHCMr is a measure of the average haemoglobin concentration in reticulocytes (reticulocyte hypochromasia) [14]. Although the reticulocyte MCV and RDW values were not significantly increased within the infected group, the median values were higher within the infected group for both parameters. Taking these findings together with the significantly decreased CHCMr and significantly increased CHDWr, it suggests that a proportion of these reticulocytes are so called "stress" reticulocytes, released upon enhanced erythropoietic stimulation [183]. These large reticulocytes have increased residual RNA compared to normal reticulocytes. Their size is due to skipped steps in cell division within the bone marrow because of accelerated erythropoiesis and a reduced time period between stem cell

differentiation and reticulocyte emergence [183,184]. During intense erythropoiesis / severe anaemia, the maturation time for reticulocytes within the bone marrow is reduced, followed by an increased maturation time within the peripheral circulation [183,185].

4.2. Leukocyte response

This study demonstrated an overall increased number of leukocytes within the bone marrow and the peripheral circulation of the infected group. This increase was mainly due to significantly increased numbers of band neutrophils within the peripheral circulation, a significantly increased number of myeloblasts and promyelocytes within the bone marrow smears and a significantly increased number of CD204-reactive mononuclear leukocytes within the bone marrow. The number of lymphocytes and plasma cells within the peripheral circulation and bone marrow of infected dogs was not altered significantly. The myeloid left shift and histiocytic inflammatory response are deemed an appropriate response of the bone marrow in view of the systemic inflammation caused by *Babesia* [186].

4.2.1. Monocyte-macrophages

Although there were increased numbers of CD204 and Mac387 reactive mononuclear leukocytes within the infected animal group, only the CD204 reactive macrophages were significantly increased. This finding is similar to previous IHC studies investigating the effects of canine babesiosis on the lung and spleen [187,188]. An increase in macrophages within the bone marrow has also been demonstrated in human falciparum malaria [85,114]. Macrophages play a pivotal role in innate immunity where they are crucial for parasite phagocytosis as well as the release of cytokines, chemokines and toxic mediators and their increased numbers in the infected group in this study was therefore not surprising [189]. In human falciparum malaria, macrophages and monocytes are directly activated by glycosylphophatidylinositol produced by the parasite [thought to be a typical protozoal pathogen associated molecular pattern (PAMP) molecule, discussed below], thereby promoting release of pro-inflammatory cytokines TNF and IL-1 [85]. The release of these cytokines in turn stimulates further activation of macrophages, leading to quantities of TNF being produced which may lead to dyserythropoiesis and reduced erythropoiesis, as was observed in our study [85]. Increased levels of MCP-1 were found in *B. rossi* infected dogs, where prognosis was directly related to MCP concentrations [122,135,190]. Various inflammatory cytokines, oxidative stress and some growth factors are responsible for the release of MCP-1 [138]. MCP-1 is one of the principal chemokines regulating migration and infiltration of macrophages / monocytes to inflammatory sites [138]. MCP-1 is also crucial for macrophage / monocyte activation with release of supplementary inflammatory mediators as well as increasing their phagocytic activity [138]. This increased number of macrophages / monocytes observed in tissues during canine babesiosis is therefore cytokine driven, forming part of the innate immune system and the first line of defense against the invading parasite. Apart from their crucial role in innate immunity, macrophages also play an integral part in adaptive immunity and their increased numbers in canine babesiosis forms part of the body's attempt to eliminate infection. The role of macrophages in the innate and adaptive immune response is discussed below.

The body's first line of defense against infection is via the innate immune system and includes the recognition of infectious agents by pattern recognition receptors (PRRs) that are expressed on both haematopoietic and non-haematopoietic cells, including dendritic cells, monocytes, macrophages and endothelial cells [191,192]. Consequently, a variety of inflammatory cytokines, chemokines and chemical mediators, including IL-6, IL-1β, CXCL-8, IL-12, TNF-α, leukotriene B4, prostaglandin E2 and histamine, are released by innate immune cells following binding of pathogen derived molecules to PRRs [192]. The secretion of these inflammatory cytokines, chemokines and chemical mediators in turn results in the recruitment of immune effector cells from the peripheral circulation to the site of infection [192]. Activation of macrophages via IFN-γ and parasite derived products results in phagocytosis of the parasite with the production of toxic metabolites such as nitric oxide

(NO) [193]. Toll-like receptors (TLRs) also play an important role in innate immunity against protozoan parasites. These receptors belong to a family of host receptors capable of recognizing PAMP, molecules which are maintained among distinct classes of microbes but not available in the host [194,195]. Generally, antigen presentation on MHC molecules and PAMP receptors alert the immune system to the presence of intracellular pathogens, however, in order to optimize oxygen delivery, mammalian erythrocytes do not possess nuclei and do not express MHC molecules and most PAMP receptors [196]. The lack of MHC association contributes to the success of *P. falciparum* in avoiding the host's adaptive immune response [197]. Malarial merozoites are briefly susceptible to the adaptive and innate immune responses of the host when they are released into the plasma after replicating in erythrocytes, however, they swiftly infect other erythrocytes, whereafter they are invisible again to the host's adaptive immune response [198]. Most innate immune responses induce inflammation rather than targeted destruction of infected erythrocytes. The spleen is responsible for the removal of damaged infected red blood cells where infected red blood cells encounter the macrophage-rich red pulp sinusoids and have to traverse their way through slender gaps lined by specialized endothelial cells [199]. Although exactly how infected erythrocytes are distinguished from healthy erythrocytes is still uncertain, but increased rigidity of the infected red blood cell is speculated to be an important factor resulting in phagocytosis by the splenic red pulp macrophages [199]. The high haeme content within red blood cells make them susceptible to oxidative stress. Intraerythrocytic malarial and *Babesia* parasites exploit the fact that red blood cells do not contain MHC bound molecules, but encounter toxicity as a result [196]. Oxidative stress within erythrocytes lead to the aggregation of plasma membrane high molecular weight aggregates, carrying N-linked high-mannose glycans [200]. These N-linked high-mannose glycans are subsequently recognized by macrophages containing mannose receptor CD206 and therefore parasitized red blood cells are targeted for phagocytosis [199–201]. Mannose receptors are also expressed by bone marrow macrophages [202].

In addition to the innate immune system, an adaptive immune response is also elicited by the production of neutralizing antibodies aimed at extracellular malarial merozoites and the surface antigens on parasitized red blood cells, a process that is dependent on the action of CD4+ T-cells [193] and sequestration of parasitized erythrocytes in various organs [203]. Three subsets of monocytes are recognized, differentiated by the expression of CD14 and CD16 on their surface [204]. These include classical / inflammatory (CD14++ CD16−), non-classical / patrolling (CD14+ CD16++) and intermediate (CD14++ CD16+) [203]. The largest subtype is the classical monocyte which expresses chemokine receptor CCR2 responsible for leukocyte recruitment to inflammatory sites and differentiation of monocytes to macrophages or dendritic cells within tissues [203]. Patrolling monocytes are the non-classical monocytes, responsible for the clearance of damaged cells and debris and the resolution of inflammation within damaged tissues [203]. Macrophages form part of the innate immune system based on their phagocytic role and the release of cytokines during the acute stages of inflammation, however, they are one of the principal triggers of the adaptive immune response based on their processing and presentation of antigen and regulation of T cell activity [205]. Macrophages act as antigen presenting cells, presenting processed peptide fragment of antigen to T lymphocytes thereby inducing a cell-mediated immune response [205]. Apart from their crucial role in the innate immune response by means of phagocytosis, cytokine and chemokine release and ultimately parasite clearance, macrophages are also involved in the removal of erythrocytes during haematoprotozoal infections [187,206]. The important role of macrophages during *Babesia* infection is demonstrated by a study conducted by Terkawi et al (2015) [206]. In this study, BALB/c mice infected with *Babesia microti* demonstrated a significant rise in parasitaemia correlating with notable mortality upon depletion of macrophages after treatment with clodronate liposome [206]. The production of Th1 cell cytokines, namely IFN-γ and TNF-α, was decreased in macrophage depleted mice. The authors speculated that severe infections in an immunocompromised host may be as a result of diminished macrophage function [206]. Similarly, experimental macrophage depletion has also demonstrated an adverse outcome for mice infected with *Babesia duncani* and *Plasmodium chabaudi* resulting in high parasitaemia and increased mortalities [144,207]. The secretion of IFN-γ by

natural killer cells and T cells stimulates the production of proinflammatory cytokines (TNF-α, IL-6) by macrophages resulting in a primarily Th1 immune response [206]. A Th1 immune response therefore characterizes the body's immune reaction against intracellular protozoal infections, where Th1 lymphocytes produce and secrete IL-1 and IFN-y for stimulation of macrophages and ultimately parasite destruction via phagocytosis [205]. Natural killer (NK) cells similarly play an important role in the immunity against protozoan parasites. Kitaguchi et al (1996) demonstrated in their study that disease is more severe in mice infected with *Plasmodium chabaudi* and depleted of NK cells and associated with higher parasitaemias and mortalities [208]. Chen et al (2014) similarly demonstrated that insufficient NK cells (but not macrophages) in mice infected with *P. falciparum* resulted in increased parasitaemia [209]. Red blood cells infected with *P. falciparum* parasites are recognized by human NK cells with ultimate IFN-y secretion [210,211]. NK cells are also responsible for perforin and granzyme production leading to erythrolysis of *P. falciparum* infected erythrocytes [212]. NK cells are bone marrow derived nonspecific cytotoxic cells of lymphocytic lineage [205]. They contain granules within their cytoplasm and are commonly known as large granular lymphocytes [205]. These intracytoplasmic cytotoxic granules contain perforin and granzymes responsible for lysis of target cells [205]. These cells are not MHC restricted, they don't develop memory cells and they form part of the early innate immune response to intracellular pathogens [205]. They are the innate immune system's analogue to the cytotoxic T-cells of the adaptive immune response [205].

4.2.2. Myeloid cells

Within the bone marrow of the infected animal group, myeloid cellularity was significantly increased and a left shift of mainly the maturation pool was present (myeloblasts, promyelocytes, metamyelocytes and bands). The hyperplasia and left shift were an appropriate response of the bone marrow considering the presence of systemic inflammation and an inflammatory leukogram. The degree of left shift did however demonstrate the characteristic of a degenerative left shift (see below). Neutrophilic metamyelocytes, bands and segmented neutrophils within the infected group were often enlarged / giant with some toxic changes evident (basophilia, granulation), indicative of severe inflammation [186].

Babesiosis is characterized by an inflammatory leukogram with especially a monocytosis being prominent, as discussed above [42,45,46,213]. This monocytosis is particularly severe in dogs presenting with severe anaemia [42]. However, the inflammatory leukogram characterizing canine babesiosis is dominated by a predominantly left shift neutrophilia with increased band neutrophils in the absence of a coinciding mature neutrophilia, i.e. degenerative left shift [213,214]. A degenerative left shift was also reported in one study, where a high number of immature neutrophils was found with concomitant low or normal numbers of mature neutrophils [213]. The degree of left shift has also been directly correlated with disease severity and poor prognosis [46]. This is an inappropriate inflammatory response [14] and may therefore suggests increased usage or destruction of neutrophils, an inadequate bone marrow response, early stages of the bone marrow response (bone marrow response generally requires three to four days [186]) or prior exhaustion of the neutrophilic bone marrow storage pool [213]. In this study, there was a marked decrease in the segmented forms within the bone marrow of the infected group as compared to the control group. Three of the infected cases (numbers 1, 5 and 6) demonstrated a degenerative left shift neutrophilia with increased bands within the peripheral circulation accompanied by normal to low numbers of mature neutrophils. The bone marrow myeloid changes in these cases demonstrated a left shift with increased numbers of mostly the metamyelocytes and bands, associated with a marked decrease in the segmented forms. From the various possible scenarios described above, it appears as if an increased usage or destruction of neutrophils and previous exhaustion of the neutrophilic bone marrow storage pool may play a role here. The marked left shift is expected given the systemic inflammation and the bone marrow therefore responds adequately. Also, the fact that there is this marked left shift would suggest that adequate time has elapsed for the bone marrow to respond.

Neutrophils function in parasite clearance via phagocytosis, production of reactive oxygen species (ROS), production of antimicrobial products or by the generation of neutrophils extracellular traps (NETs) [215]. They also produce cytokines and chemokines and may be associated with antigen presentation, thereby playing a crucial role in activating and regulating the immune response [216,217]. In human malaria, various changes are described within the neutrophil population in the peripheral circulation [218]. Stable neutrophil numbers have been reported during the asymptomatic liver stage of non-immune persons with controlled human malaria infection [219]. Olliaro et al (2011) demonstrated a 43% rise in circulatory neutrophil numbers during the acute stage in persons with uncomplicated malarial infections. This neutrophil rise was directly related to the level of parasitaemia [220]. Neutrophils have different subsets with different functional properties [221]. Differing behaviour amongst the neutrophil sub-populations are detected amongst persons infected with falciparum malaria compared to uninfected persons [218], where a subgroup of neutrophils in persons with falciparum malaria is associated with decreased respiratory oxidative burst function suggesting changes within neutrophil subgroups as the disease progresses [222]. During falciparum malaria, increased levels of neutrophil chemoattractants are present, but malarial pathology is rarely associated with notable neutrophil infiltration at parasitized red blood cell sequestration sites [218]. In brain microvasculature, as well as in placentas and pulmonary tissue during falciparum malaria, the number of neutrophils were not significant [223–225]. Similarly, in canine babesiosis neutrophils were found to be scant in the splenic and pulmonary tissues from infected animals [187,188]. In these studies, it was concluded that neutrophils are not a major contributor to the injury observed during canine babesiosis [187,188]. Data suggests the recruitment of neutrophils to infection sites during falciparum malaria and canine babesiosis is not always present and Aitken et al (2018) postulated that neutrophil chemotaxis may be inhibited by infection [218]. Decreased neutrophil chemotaxis is observed in human falciparum malaria, with parasite antigens likely involved as is evident by the restoration of chemotaxis after treatment [226]. Neutrophil chemotaxis is suppressed by *P. falciparum* protein MSP1-19 blocking the neutrophil's response to S100P, a proinflammatory protein [227]. A similar mechanism may be involved with canine babesiosis. It is possible that neutrophil nets play a role in the acute inflammation, coagulopathy and platelet activation that are present in babesiosis.

A study conducted by Atkinson et al (2021), demonstrated an inverse relationship between the number of segmented neutrophils and the cytokine keratinocyte chemotactic-like (KC-like) [190]. This cytokine plays an important role in the migration and activation of neutrophils [228]. Similarly, an inverse relationship was also demonstrated with IL-8 and the number of segmented neutrophils [190]. This mirrors what Goddard et al (2016) found in their study where the concentration of IL-8 was notably decreased in *Babesia* infected dogs as compared to control dogs and this low level of IL-8 serum concentration correlated with a notable decreased circulating neutrophil count[122]. IL-8 plays and important role in neutrophil recruitment and activation [229]. A decrease in the segmented neutrophil number has also been reported in acute human falciparum malaria and various mechanisms for this have been proposed, namely a shift from the circulating pool to the marginated pool and early release of premature granulocytes from the bone marrow [230]. Increased levels of myeloperoxidase index (MPXI) in neutrophils were noted in dogs infected with *B. rossi*, ascribed to possible hastened maturation and release of immature neutrophils from the bone marrow [231].

4.2.3. Lymphocytes

There was no appreciable difference in the lymphocyte counts between the infected and control groups. The number of plasma cells were increased (as is evident by the number of MUM-1 positive labelling cells), but the increase was not statistically significant.

4.2.3.1. T-lymphocytes

This negligible difference in the T-cell population between the infected and control animal group mirrors what has been found in the canine spleen during babesiosis [187]. Rautenbach et al (2017) in their study assessing the

peripheral immunophenotypes in dogs naturally infected with *Babesia rossi*, demonstrated a decline in CD3+ lymphocytes in dogs with complicated babesiosis, compared to uncomplicated babesiosis cases. The authors hypothesized about the possibility of a functional immunosuppression due to apoptosis or redistribution of the effector T-cells, or a combination of the above and other mechanisms [50]. Martin (2019) in his study on the pathology associated with complicated *Babesia rossi* acute lung injury, demonstrated a significant increase in T-lymphocytes within alveolar walls of the lung, thereby suggesting that redistribution of these circulating T-cells may partially explain the relative decrease of T-cells observed within the Rautenbach et al (2017) study [188]. Henning et al (2019) demonstrated in their study on the splenic pathology during canine babesiosis, that there is a negligible difference in the T-cell population between the infected and control animal group. This lack of significant T-cell increase may, apart from previously mentioned possibilities of rapid redistribution and accelerated apoptosis, be due to the influence of alternatively activated macrophages [187]. Whilst classically activated macrophages are instrumental in protection of the host against a variety of microbial pathogens, alternatively activated macrophages play a major role in immune regulation and tissue repair [232]. These macrophages are characterized by a distinct phenotype due to differing gene expression profiles [233]. Alternatively activated macrophages have been demonstrated to suppress T-cell function in a variety of infectious disease, including *Theileria* spp., *Trypanosoma* spp., and *Toxoplasma* spp. [234–237].

4.2.3.2. B-lymphocytes and plasma cells

There was a slight increase in B-lymphocytes within the infected animal group as compared to the control group in this study, but this increase was not statistically significant. The number of plasma cells were increased within the infected group, albeit not statistically significant. This plasma cell hyperplasia is consistent with inflammation and is deemed an appropriate bone marrow response. In human falciparum malaria, it has been demonstrated that the malarial parasite can negatively influence the generation and long-term maintenance of memory B-cells, ultimately with a negative effect on the humoral immune response[238]. Severe disruption of B-cell lymphopoiesis has also been demonstrated in mice infected with acute *Plasmodium chabaudi* [239]. Rautenbach et al (2017) demonstrated an increase in the B-lymphocyte population two to three days after presentation in dogs with complicated babesiosis [50]. This increase in B-lymphocytes and plasma cells is time dependent with an increase noted in increasing chronicity. However, during the early stages of infection, several studies have demonstrated that resistance to babesiosis and malaria is antibody independent and an absence of B lymphocytes is not associated with impaired resistance [3,207,240,241]. That said however, antibody responses are essential for parasite clearance after the acute infection is resolved [242]. Although the time frame of disease within the infected animal group in this study is known, there is insufficient data to explore the possibility of parasite suppression of B-cell lymphopoiesis and memory B-cell production.

4.3. Megakaryocyte response

There was a left shift within the bone marrow megakaryocyte population of the infected group in this study. This is an appropriate response of the bone marrow considering the presence of a peripheral thrombocytopaenia, which is common in canine babesiosis [54]. The pathogenesis of thrombocytopaenia during canine babesiosis is unknown, although from this study, bone marrow suppression with decreased platelet production appears unlikely. The finding of an adequate number of megakaryocytes in this study is similar as to what has been found in Kirtz, Leschnik et al.(2012) study. As concluded in their study, the thrombocytopaenia during canine babesiosis is likely of peripheral origin via either immune-mediated destruction and increased consumption [44]. An immune-mediated thrombocytopaenia was also proposed by Scheepers et al (2011), where they found resolution of the thrombocytopaenia in most *Babesia*-infected dogs after six days of infection, thereby suggesting a sufficient bone marrow response [45], which has now been confirmed with this study.

4.4. Bone marrow sequestration of parasites

Babesia parasites were present within vasculature, in contrast to what has been reported for *P. vivax* parasites, where the majority was located within the extravascular parenchyma of the bone marrow [118]. Sexual forms (i.e. gametocytes) of the malarial parasite are sequestered within the extravascular environment of the bone marrow [118]. Preliminary studies have also shown that this is the case in mouse models and has also suggested the occurrence of an asexual reservoir in the bone marrow [120]. In order to complete its life cycle, it is essential for the malarial parasite to sequester within the bone marrow as the release of parasites into the peripheral blood circulation allows for parasite recognition by surveillance macrophages and subsequent splenic clearance [120]. Asexually replicating parasites are sequestered via the attachment of the parasitized red blood cell to the vascular endothelium of various tissues, including the bone marrow [120]. Sexual conjugation and sporogony of the *Babesia* parasite are completed within the transmitter tick and the asexual stage is present within the vertebrate host [243]. Whether the bone marrow serves as a reservoir of the asexual stages of the *Babesia* parasite still needs to be investigated in future studies, including the changes within the bone marrow endothelium upon infection.

In our study vascular endothelial cell hypertrophy was observed in the bone marrow of *Babesia* infected dogs. Endothelial cells play a crucial role in the regulation of haematopoiesis during inflammation. PPRs are expressed on endothelial cells and upon activation by TLR4, G-CSF is produced, thereby ensuring neutrophil production within the bone marrow with simultaneous neutrophil recruitment to the site of infection [192]. Upon stimulation by TNF-α and IL-1β, bone marrow endothelial cells also produce GM-CSF leading to neutrophil recruitment and proliferation of haematopoietic progenitor cells in the bone marrow [192]. Future studies should evaluate the role of bone marrow vascular endothelium during canine babesiosis, including the interaction of the *Babesia* parasite with the host vasculature, the role of endothelium in the regulation of haematopoiesis and the role of endothelium as to potential parasite sequestration within the bone marrow.

No obvious differences were noted between the bone marrow smears and HE sections from the treated and non-treated infected cases. The degree of parasitaemia varied from low to high in the various infected cases, including the treated and non-treated cases. The highest parasitaemia was noted in the infected case that received Doxycycline treatment at home (case 6). This study is limited in the number of infected cases. As such, no conclusions can be drawn on the effect of treatment on the parasitic load within the bone marrow. This would have to be investigated in future studies.

4.5. Role of parasite factors on the bone marrow response

As discussed previously, an inappropriate erythroid bone marrow response was detected in half of the *Babesia* cases in this study. Included in the various hypotheses for this phenomenon (as discussed previously), is the influence of various parasite products, e.g. haemozoin produced by malarial parasites. Haemozoin is not produced by *Babesia* parasites [104], however, GPI molecules have been demonstrated to be present within *Babesia* parasites [105,106]. Therefore, apart from haemozoin, other factors, e.g. GPI need to be considered as a cause of the inappropriate bone marrow response and reticulocyte suppression in cases of *Babesia*-induced anaemia.

4.6. Comparison between bone marrow cytology and bone marrow histopathology

The bone marrow cytological findings largely mirrored the histopathological findings. However, a discrepancy was noted in the number of macrophages within the infected group, where significantly raised values were not evident on bone marrow cytology as compared to the significantly increased CD204-reactive macrophages within histological specimens in the infected group. In *Babesia* case 4, the number of macrophages was decreased to such an extent that its value was comparable to the control dogs and this decreased value caused

for a statistically insignificant raise within the histiocytes. This discrepancy may be explained by the fact that the bone marrow smears were relatively thick in areas and evaluation of cellular niches were not always possible. Hence, evaluation focused on areas where the smears were thinner and as a result, the number of histiocytes may have been underestimated with cytological examination.

CONCLUSION

The bone marrow changes during canine babesiosis were characterized by a significant erythroid (rubriblast) and histiocytic response with sufficient iron stores. This macrophage response is characteristic of *Babesia rossi* infection, correlating with what other studies have found in the spleen and lungs of *Babesia* infected dogs and in natural and experimental malarial infections [85,187,188]. The thrombocytopaenia consistently observed during canine babesiosis is deemed to be of peripheral origin via either immune mediated destruction or increased consumption as megakaryocytes were adequate / increased in number in this study. The bone marrow response in infected dogs was inappropriate within the erythroid lineage in this study, mirroring what has been found in previous studies [45,46,72]. This inappropriate response is likely due to a variety of causes, amongst which host factors (i.e. release of pro-inflammatory cytokines with subsequent reduced erythrocyte survival, impaired iron mobilization or utilization and impaired red blood cell production due to a nonresponsiveness to EPO) and dyserythropoiesis play a major role. Dyserythropoiesis was demonstrated in all *Babesia*-infected cases. Iron availability may play a role in this inappropriate response to the anaemia during canine babesiosis, although in this study there were sufficient iron stores within the bone marrow and reticulocyte indices did not support iron-restricted erythropoiesis. As demonstrated in previous studies [122], it is likely that a cytokine dysregulation may be responsible for the inappropriate bone marrow response, but further investigation is necessary to explain this phenomenon. In contrast to what has been found in human and murine malaria in which parasitized erythrocytes are sequestered within the bone marrow, a similar finding is not supported by the findings of this study. The small sample size of *Babesia*-infected dogs is a limitation in this study and therefore future research should incorporate a larger infected animal group in an attempt to answer various research questions, including: 1). Is there sequestration of the *Babesia* parasite within the bone marrow? 2). What are the mechanisms behind the inappropriate bone marrow erythroid responses observed in the infected cases in this study? This should include an investigation of the role of various host factors (e.g. TNF, IL-1) and parasite factors (e.g. MIF, GPI) and 3.) What role does the vascular endothelium play in potential parasite sequestration within the bone marrow? From the results obtained in the present study, it is clear that evaluation of the bone marrow should be conducted in concordance with evaluation of the complete blood count / haemogram and bone marrow cytology to ensure accurate interpretation of bone marrow findings.

REFERENCES

1. Elmore, S. A. Enhanced histopathology of the bone marrow. *Toxicol Pathol* **34**, 666–686 (2006).

2. Travlos, G. S. Normal structure, function, and histology of the bone marrow. *Toxicol Pathol* **34**, 548–565 (2006).

3. Homer, M. J., I. Aguilar-Delfin, S. R. Telford, P. J. K. and D. H. P. Babesiosis. *Clin. Microbiol. Rev.* **13**, 451–469 (2000).

4. Engwerda, C. R., L. B. and F. H. A. The importance of the spleen in malaria. *Trends Parasitol.* **21**, 75–80 (2005).

5. Krause, P. J., J. Daily, S. R. Telford, E. Vannier, P. L. and A. S. Shared features in the pathobiology of babesiosis and malaria. *Trends Parasitol.* **23**, 605–610 (2007).

6. Eurell, J. and B. F. *Dellmann's Textbook of Veterinary Histology*. (Oxford UK, Blackwell Publishing Ltd, 2006).

7. Gurkan, U. A. and O. A. The mechanical environment of bone marrow: a review. *Ann Biomed Eng* **36**, 1978–1991 (2008).

8. Smith, J. N. and L. M. C. Concise review: Current concepts in bone marrow microenvironmental regulation of hematopoietic stem and progenitor cells. *Stem Cells* **31**, 1044–1050 (2013).

9. Ettinger, S. and E. F. *Textbook of Veterinary Internal Medicine*. (Elsevier Saunders, 2005).

10. Feldman BF, Zinkl JG, J. N. Schalm's Veterinary Hematology. in 281–296 (Lippincott Williams & Wilkins, 2000).

11. Duncan JR, Prasse KW, M. E. *Veterinary Laboratory Medicine: Clinical Pathology*. (Iowa State Press, 2003).

12. Young, B., J. S. Lowe, A. S. and J. W. H. *Wheater's Functional Histology: A Text and Colour Atlas*. (Philadelphia, Elsevier, 2006).

13. Latimer KS, R. P. Clinical interpretation of leukocyte responses. *Vet. Clin. North Am. Small Anim. Pract.* **19**, 637–668 (1989).

14. Stockham, S. L. and M. A. S. *Fundamentals of Veterinary Clinical Pathology*. (Blackwell publishing, 2008).

15. Pereira, M. *et al.* Development of Dog Immune System: From in Uterus to Elderly. *Vet. Sci.* **6**, 83 (2019).

16. Toi, P., R. G. V. and R. R. Comparative evaluation of simultaneous bone marrow aspiration and bone marrow biopsy: an institutional experience. *Indian J Hematol Blood Transfus* **26**, 41–44 (2010).

17. Gilotra, M., M. Gupta, S. S. and R. Sen. Comparison of bone marrow aspiration cytology with bone marrow trephine biopsy histopathology: An observational study. *J Lab Physicians* **9**, 182–189 (2017).

18. Roger S. Riley, David Williams, Micaela Ross, Shawn Zhao, Alden Chesney, Bradly D. Clark, and J. M. B.-E. Bone Marrow Aspirate and Biopsy: A Pathologist's Perspective. II. Interpretation of the Bone Marrow Aspirate and Biopsy. *J. Clin. Lab. Anal.* **23**, 259–307 (2009).

19. Raskin, R. E. and J. B. M. Bone marrow cytologic and histologic biopsies: indications, technique, and evaluation. *Vet Clin North Am Small Anim Pr.* **42**, 23–42 (2012).

20. Jennifer L McCown, A. J. S. Iron homeostasis and disorders in dogs and cats: a review. *J Am Anim Hosp Assoc* **47**, 151–160 (2011).

21. Collett, M. G. Survey of canine babesiosis in South Africa. *J S Afr Vet Assoc* **71**, 180–186 (2000).

22. Penzhorn, B. L. Why is Southern African canine babesiosis so virulent? An evolutionary perspective. *Parasit Vectors* **4**, 51 (2011).

23. Birkenheuer, A. J., J. Neel, D. Ruslander, M. G. L. and E. B. B. Detection and molecular characterization of a novel large Babesia species in a dog. *Vet Parasitol* **124**, 151–160 (2004).

24. Kjemtrup, A. M. & Conrad, P. A. A review of the small canine piroplasms from California: Babesia conradae in the literature. *Vet. Parasitol.* **138**, 112–117 (2006).

25. Baneth, G., Cardoso, L., Brilhante-Simões, P. & Schnittger, L. Establishment of Babesia vulpes n. sp. (Apicomplexa: Babesiidae), a piroplasmid species pathogenic for domestic dogs. *Parasit. Vectors* **12**, 129 (2019).

26. Uilenberg, G., F. F. Franssen, N. M. P. and A. A. S. Three groups of Babesia canis distinguished and a proposal for nomenclature. *Vet Q* **11**, 33–40 (1989).

27. Schetters, T. P., K. Moubri, E. Precigout, J. Kleuskens, N. C. S. and A. G. Different Babesia canis isolates, different diseases. *Parasitology* **115**, 485–493 (1997).

28. Schoeman, J. P. Canine babesiosis. *Onderstepoort J Vet Res* **76**, 59–66 (2009).

29. Welzl, C., A. L. Leisewitz, L. S. Jacobson, T. V.-S. and E. M. Systemic inflammatory response syndrome and multiple-organ damage/dysfunction in complicated canine babesiosis. *J S Afr Vet Assoc* **72**, 158–162 (2001).

30. Keller, N., L. S. Jacobson, M. Nel, M. de Clerq, P. N. T. and J. P. S. Prevalence and risk factors of hypoglycemia in virulent canine babesiosis. *J Vet Intern Med* **18**, 265–270 (2004).

31. Jacobson, L. S. The South African form of severe and complicated canine babesiosis: clinical advances 1994-2004. *Vet Parasitol* **138**, 126–139 (2006).

32. Matjila, P. T., B. L. Penzhorn, C. P. Bekker, A. M. N. and F. J. Confirmation of occurrence of Babesia canis vogeli in domestic dogs in South Africa. *Vet Parasitol* **122**, 119–125 (2004).

33. Harvey JW, Taboada J, L. J. Babesiosis in a litter of pups. *J. Am. Vet. Med. Assoc.* **192**, 1751–1752 (1988).

34. Furlanello, T., Fiorio, F., Caldin, M., Lubas, G. & Solano-Gallego, L. Clinicopathological findings in naturally occurring cases of babesiosis caused by large form Babesia from dogs of northeastern Italy.

Vet. Parasitol. **134**, 77–85 (2005).

35. Duh, D., Tozon, N., Petrovec, M., StraŠek, K. & AvŠic-Županc, T. Canine babesiosis in Slovenia: Molecular evidence of Babesia canis canis and Babesia canis vogeli. *Vet. Res.* **35**, 363–368 (2004).

36. Földvári, G., Hell, É. & Farkas, R. Babesia canis canis in dogs from Hungary: detection by PCR and sequencing. *Vet. Parasitol.* **127**, 221–226 (2005).

37. IRWIN, P. & HUTCHINSON, G. Clinical and pathological findings of Babesia infection in dogs. *Aust. Vet. J.* **68**, 204–209 (1991).

38. Jefferies R, Ryan UM, Muchlnickel CJ, I. P. Two species of canine Babesia in Australia: detection and characterization by PCR. *J. Parasitol.* **89**, 409–412 (2003).

39. Ruiz de Gopegui, R. *et al.* Clinico-pathological findings and coagulation disorders in 45 cases of canine babesiosis in Spain. *Vet. J.* **174**, 129–132 (2007).

40. Zygner, W., Gójska, O., Rapacka, G., Jaros, D. & Wędrychowicz, H. Hematological changes during the course of canine babesiosis caused by large Babesia in domestic dogs in Warsaw (Poland). *Vet. Parasitol.* **145**, 146–151 (2007).

41. Criado-Fornelio, A. *et al.* New advances in molecular epizootiology of canine hematic protozoa from Venezuela, Thailand and Spain. *Vet. Parasitol.* **144**, 261–269 (2007).

42. Reyers, F., A. L. Leisewitz, R. G. Lobetti, R. J. Milner, L. S. J. and M. van Z. Canine babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria? *Ann Trop Med Parasitol* **92**, 503–511 (1998).

43. Taboada, J. and S. R. M. Babesiosis of companion animals and man. *Vet Clin North Am Small Anim Pr.* **21**, 103–123 (1991).

44. Kirtz, G., M. Leschnik, E. Hooijberg, A. T. and E. L. In-clinic laboratory diagnosis of canine babesiosis (Babesia canis canis) for veterinary practitioners in Central Europe. *Tierarztl Prax Ausg K Kleintiere Heimtiere* **40**, 87–94 (2012).

45. Scheepers, E., A. L. Leisewitz, P. N. T. and M. M. C. Serial haematology results in transfused and non-transfused dogs naturally infected with Babesia rossi. *J S Afr Vet Assoc* **82**, 136–143 (2011).

46. Leisewitz, A. L. *et al.* A clinical and pathological description of 320 cases of naturally acquired Babesia rossi infection in dogs. *Vet. Parasitol.* **271**, 22–30 (2019).

47. Lobetti, R. G. Leukaemoid response in two dogs with Babesia canis infection. *J S Afr Vet Assoc* **66**, 182–184 (1995).

48. Boozer, A. L. and D. K. M. Canine babesiosis. *Vet Clin North Am Small Anim Pr.* **33**, 885–904, viii (2003).

49. Van de Maele, I., K. Savary-Bataille, I. G. and S. D. An unusual form of canine babesiosis. *Can Vet J* **49**, 283–286 (2008).

50. Rautenbach, Y., A. Goddard, P. N. Thompson, R. J. M. and A. L. L. A flow cytometric assessment of the

lymphocyte immunophenotypes in dogs naturally infected with Babesia rossi. *Vet Parasitol* **241**, 26–34 (2017).

51. Kumararatne, D. S., R. S. Phillips, D. Sinclair, M. V. P. and J. B. F. Lymphocyte migration in murine malaria during the primary patent parasitaemia of Plasmodium chabaudi infections. *Clin Exp Immunol* **68**, 65–77 (1987).

52. Brown, W. C. Molecular approaches to elucidating innate and acquired immune responses to Babesia bovis, a protozoan parasite that causes persistent infection. *Vet Parasitol* **101**, 233–248 (2001).

53. White, C. E., N. F. Villarino, S. S. Sloan, V. V. G. and N. W. S. Plasmodium suppresses expansion of T cell responses to heterologous infections. *J Immunol* **194**, 697–708 (2015).

54. Kettner, F., F. R. and D. M. Thrombocytopaenia in canine babesiosis and its clinical usefulness. *J S Afr Vet Assoc* **74**, 63–68 (2003).

55. Goddard, A., Wiinberg, B., Schoeman, J. P., Kristensen, A. T. & Kjelgaard-Hansen, M. Mortality in virulent canine babesiosis is associated with a consumptive coagulopathy. *Vet. J.* **196**, 213–217 (2013).

56. Paim, C. B. *et al.* Thrombocytopenia and platelet activity in dogs experimentally infected with Rangelia vitalii. *Vet. Parasitol.* **185**, 131–7 (2012).

57. Goddard, A., Leisewitz, A. L., Kristensen, A. T. & Schoeman, J. P. Platelet indices in dogs with Babesia rossi infection. *Vet. Clin. Pathol.* **44**, 493–497 (2015).

58. Liebenberg, C. *et al.* Hemostatic Abnormalities in Uncomplicated Babesiosis (Babesia rossi) in Dogs. *J. Vet. Intern. Med.* **27**, 150–156 (2013).

59. Bowbrick, V. A., Mikhailidis, D. P. & Stansby, G. Influence of platelet count and activity on thromboelastography parameters. *Platelets* **14**, 219–224 (2003).

60. Wiinberg, B. *et al.* Thromboelastographic Evaluation of Hemostatic Function in Dogs with Disseminated Intravascular Coagulation. *J. Vet. Intern. Med.* **22**, 357–365 (2008).

61. Koster LS, Van Schoor M, Goddard A, Thompson PN, Matjila PT, K.-H. M. C-reactive protein in canine babesiosis caused by Babesia rossi and its association with outcome. *J S Afr Vet Assoc* **80**, 87–91 (2009).

62. Schetters, T. P. M. *et al.* Systemic inflammatory responses in dogs experimentally infected with Babesia canis; a haematological study. *Vet. Parasitol.* **162**, 7–15 (2009).

63. DJ, M. Disseminated intravascular coagulation: a review of its pathogenesis, manifestations and treatment. *J. S. Afr. Vet. Assoc.* **50**, 259–264 (1979).

64. Esmon, C. T. *et al.* Inflammation, sepsis, and coagulation. *Haematologica* **84**, 254–9 (1999).

65. Weiss, D. J. & Rashid, J. The Sepsis-Coagulant Axis: A Review. *J. Vet. Intern. Med.* **12**, 317–324 (1998).

66. Laforcade, A. M. *et al.* Hemostatic Changes in Dogs with Naturally Occurring Sepsis. *J. Vet. Intern. Med.* **17**, 674–679 (2003).

67. AM, B. The complete blood cell count: a powerful diagnostic tool. *Vet. Clin. North Am. Small Anim. Pract.* **33**, 1207–1222 (2003).

68. Cowgill ES, Neel JA, G. C. Clinical application of reticulocyte counts in dogs and cats. *Vet. Clin. North Am. Small Anim. Pract.* **33**, 1223–1244 (2003).

69. Tvedten, H. W. Hematology of the normal dog and cat. *Vet. Clin. North Am. Small Anim. Pract.* **11**, 209–17 (1981).

70. Maegraith, B., Gilles, H. M. & Devakul, K. Pathological processes in Babesia canis infections. *Z. Tropenmed. Parasitol.* **8**, 485–514 (1957).

71. Spotswood, T. C., Kirberger, R. M., Kirb, L. M. P. K., Reyers, F. & Van der Merwe, L. A canine model of normovolaemic acute anaemia. *Onderstepoort J Vet Res* **72**, (2005).

72. Seejarim, C. Reticulocyte count and indices in dogs naturally and experimentally infected with Babesia rossi. (2020).

73. Weatherall DJ, Abdalla S, P. M. The anaemia of Plasmodium falciparum malaria. *Ciba Found Symp* **94**, 74–97 (1983).

74. Kurtzhals JA, Rodrigues O, Addae M, Commey JO, Nkrumah FK, H. L. Reversible suppression of bone marrow response to erythropoietin in Plasmodium falciparum malaria. *Br J Haematol* **97**, 169–174 (1997).

75. Chang, K. H. and M. M. S. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int J Parasitol* **34**, 1501–1516 (2004).

76. Schaefer, D. M. W. & Stokol, T. Retrospective study of reticulocyte indices as indicators of iron-restricted erythropoiesis in dogs with immune-mediated hemolytic anemia. *J. Vet. Diagnostic Investig.* **28**, 304–308 (2016).

77. Goodnough, L. T. Iron deficiency syndromes and iron-restricted erythropoiesis (CME). *Transfusion* **52**, 1584–1592 (2012).

78. Klag, A. R., Giger, U. & Shofer, F. S. Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990). *J. Am. Vet. Med. Assoc.* **202**, 783–8 (1993).

79. Fuchs, J. *et al.* Canine reticulocyte hemoglobin content (RET-H e) in different types of iron-deficient erythropoiesis. *Vet. Clin. Pathol.* **46**, 422–429 (2017).

80. White, N. J. and M. H. The pathophysiology of malaria. *Adv Parasitol* **31**, 83–173 (1992).

81. Casals-Pascual, C. and D. J. R. Severe malarial anaemia. *Curr Mol Med* **6**, 155–168 (2006).

82. Tangpukdee, N., C. Duangdee, P. W. and S. K. Malaria diagnosis: a brief review. *Korean J Parasitol* **47**, 93–102 (2009).

83. Kantele, A. and T. S. J. Review of cases with the emerging fifth human malaria parasite, Plasmodium knowlesi. *Clin Infect Dis* **52**, 1356–1362 (2011).

84. Perkins, D. J., T. Were, G. C. Davenport, P. Kempaiah, J. B. H. and J. M. O. Severe malarial anemia: innate immunity and pathogenesis. *Int J Biol Sci* **7**, 1427–1442 (2011).

85. Wickramasinghe, S. N. and S. H. A. Blood and bone marrow changes in malaria. *Baillieres Best Pr. Res Clin Haematol* **13**, 277–299 (2000).

86. Chang KH, Tam M, S. M. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. *Blood* **103**, 3727–3735 (2004).

87. Gandapur, A. S., S. A. M. and F. R. Bone marrow changes in human malaria: a retrospective study. *J Pak Med Assoc* **47**, 137–139 (1997).

88. McDevitt, M. A., J. Xie, V. G. and R. B. The anemia of malaria infection: role of inflammatory cytokines. *Curr Hematol Rep* **3**, 97–106 (2004).

89. Maggio-Price, L., D. B. and L. W. Changes in hematopoietic stem cells in bone marrow of mice with Plasmodium berghei malaria. *Blood* **66**, 1080–1085 (1985).

90. Silverman, P. H., J. C. S. and L. J. M. Murine malaria decreases hemopoietic stem cells. *Blood* **69**, 408–413 (1987).

91. Harvey, J. W. *Atlas of Veterinary Hematology*. (Saunders, 2000).

92. Sandau, K. B., J. Zhou, T. K. and B. B. Regulation of the hypoxia-inducible factor 1alpha by the inflammatory mediators nitric oxide and tumor necrosis factor-alpha in contrast to desferroxamine and phenylarsine oxide. *J Biol Chem* **276**, 39805–39811 (2001).

93. Ghosh, K. and K. G. Pathogenesis of anemia in malaria: a concise review. *Parasitol Res* **101**, 1463–1469 (2007).

94. Pathak, V. A. and K. G. Erythropoiesis in Malaria Infections and Factors Modifying the Erythropoietic Response. *Anemia 2016* **9310905**, (2016).

95. Chang K-H, Tam M, S. M. Modulation of the course and outcome of blood-stage malaria by erythropoietin-induced reticulocytosis. *J Infect Dis* **189**, 735–743 (2004).

96. J.Egan, T. Haemozoin formation. *Mol. Biochem. Parasitol.* **157**, 127–136 (2008).

97. Abigail A. Lamikanra, Michel Theron , Taco W. A. Kooij, D. J. R. Hemozoin (Malarial Pigment) Directly Promotes Apoptosis of Erythroid Precursors. *PLoS One* **4**, e8446 (2009).

98. Climent Casals-Pascual, Oscar Kai, Joyce O. P. Cheung, Senani Williams, Brett Lowe, Mike Nyanoti, Thomas N. Williams, Kathryn Maitland, Malcolm Molyneux, Charles R. J. C. Newton, Norbert Peshu, Suzanne M. Watt, D. J. R. Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood* **108**, 2569–2577 (2006).

99. Schwarzer E, Turrini F, Ulliers D, Giribaldi G, Ginsburg H, A. P. Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment. *J Exp Med.* **176**, 1033–1041 (1992).

100. D Taramelli, N Basilico, A M De Palma, M Saresella, P Ferrante, L Mussoni, P. O. The effect of synthetic malaria pigment (beta-haematin) on adhesion molecule expression and interleukin-6 production by human endothelial cells. *Trans R Soc Trop Med Hyg* **92**, 57–62 (1998).

101. Skorokhod OA, Alessio M, Mordmuller B, Arese P, S. E. Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-gamma-mediated effect. *J Immunol* **173**, 4066–4074 (2004).

102. Schofield L, Hewitt MC, Evans K, Siomos MA, S. P. Synthetic GPI as a candidate antitoxic vaccine in a model of malaria. *Nature* **418**, 785–789 (2002).

103. J A Martiney, B Sherry, C N Metz, M Espinoza, A S Ferrer, T Calandra, H E Broxmeyer, R. B. Macrophage migration inhibitory factor release by macrophages after ingestion of Plasmodium chabaudi-infected erythrocytes: possible role in the pathogenesis of malarial anemia. *Infect Immun* **68**, 2259–2267 (2000).

104. Akel, T. and N. M. Hematologic manifestations of babesiosis. *Ann Clin Microbiol Antimicrob* **16**, 6 (2017).

105. Rodriguez AE, Couto A, Echaide I, Schnittger L, F.-C. M. Babesia bovis contains an abundant parasite-specific protein-free glycerophosphatidylinositol and the genes predicted for its assembly. *Vet Parasitol* **167**, 227–235 (2010).

106. Carcy B, Precigout E, Schetters T, G. A. Genetic basis for GPI-anchor merozoite surface antigen polymorphism of Babesia and resulting anitgenic diversity. *Vet Parasitol* **138**, 33–49 (2006).

107. Roberts DJ, Casals-Pascual C, W. D. The clinical and pathophysiological features of malarial anaemia. *Curr Top Microbiol Immunol* **295**, 137–167 (2005).

108. Kueh, Y. K. and K. L. Y. Haematological alterations in acute malaria. *Scand J Haematol* **29**, 147–152 (1982).

109. Lisse, I. M., P. Aaby, H. W. and K. K. A community study of T lymphocyte subsets and malaria parasitaemia. *Trans R Soc Trop Med Hyg* **88**, 709–710 (1994).

110. Skudowitz, R. B., J. Katz, A. Lurie, J. L. and J. M. Mechanisms of thrombocytopenia in malignant tertian malaria. *Br Med J* **2**, 515–518 (1973).

111. Abdalla, S., D. J. Weatherall, S. N. W. and M. H. The anaemia of P. falciparum malaria. *Br J Haematol* **46**, 171–183 (1980).

112. Wickramasinghe, S. N., S. Looareesuwan, B. N. and N. J. W. Dyserythropoiesis and ineffective erythropoiesis in Plasmodium vivax malaria. *Br J Haematol* **72**, 91–99 (1989).

113. Fleming, A. F. The aetiology of severe anaemia in pregnancy in Ndola, Zambia. *Ann Trop Med Parasitol* **83**, 37–49 (1989).

114. Abdalla, S. H. Hematopoiesis in human malaria. *Blood Cells* **16**, 401–416; discussion 417-409 (1990).

115. Knuttgen, H. J. The bone marrow of non-immune Europeans in acute malaria infection: a topical review. *Ann Trop Med Parasitol* **81**, 567–576 (1987).

116. Abdalla, S. H. Peripheral blood and bone marrow leucocytes in Gambian children with malaria: numerical changes and evaluation of phagocytosis. *Ann Trop Paediatr* **8**, 250–258 (1988).

117. Abdalla, S. H., S. N. W. and D. J. W. The deoxyuridine suppression test in severe anaemia following Plasmodium falciparum malaria. *Trans R Soc Trop Med Hyg* **78**, 60–63 (1984).

118. Obaldia, N. *et al.* Bone Marrow Is a Major Parasite Reservoir in Plasmodium vivax Infection. *MBio* **9**, e00625-18 (2018).

119. Joice, R., S. K. Nilsson, J. Montgomery, S. Dankwa, E. Egan, B. Morahan, K. B. Seydel, L. Bertuccini, P. Alano, K. C. Williamson, M. T. Duraisingh, T. E. Taylor, D. A. M. and M. M. Plasmodium falciparum transmission stages accumulate in the human bone marrow. *Sci Transl Med* **6**, 244–245 (2014).

120. CORDIS & Results, E. research. Mechanisms of bone marrow sequestration during malaria infection. https://cordis.europa.eu/project/id/682360.

121. Ruth Aguilar, Cinta Moraleda, Ariel H. Achtman, Alfredo Mayor Llorenc Quinto´, Pau Cistero, Augusto Nhabomba, Eusebio Macete, Louis Schofield, P. L. A. and C. M. Severity of anaemia is associated with bone marrow haemozoin in children exposed to Plasmodium falciparum. *Br. J. Haematol.* **164**, 877 (2014).

122. Goddard, A., Leisewitz, A. L., Kjelgaard-Hansen, M., Kristensen, A. T. & Schoeman, J. P. Excessive Pro-Inflammatory Serum Cytokine Concentrations in Virulent Canine Babesiosis. *PLoS One* **11**, e0150113 (2016).

123. Blackwell, T. S. & Christman, J. W. Sepsis and cytokines: current status. *Br. J. Anaesth.* **77**, 110–7 (1996).

124. Gogos, C. A., Drosou, E., Bassaris, H. P. & Skoutelis, A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J. Infect. Dis.* **181**, 176–80 (2000).

125. Dinarello, C. A. Proinflammatory cytokines. *Chest* **118**, 503–8 (2000).

126. Day, N. P. *et al.* The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J. Infect. Dis.* **180**, 1288–97 (1999).

127. Ioannidis, L. J., Nie, C. Q. & Hansen, D. S. The role of chemokines in severe malaria: more than meets the eye. *Parasitology* **141**, 602–13 (2014).

128. Hunt, N. H. & Grau, G. E. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol.* **24**, 491–9 (2003).

129. Artavanis-Tsakonas, K., Tongren, J. E. & Riley, E. M. The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clin. Exp. Immunol.* **133**, 145–52 (2003).

130. Riley, E. M., Wahl, S., Perkins, D. J. & Schofield, L. Regulating immunity to malaria. *Parasite Immunol.* **28**, 35–49.

131. Awandare, G. A. *et al.* Increased levels of inflammatory mediators in children with severe Plasmodium

falciparum malaria with respiratory distress. *J. Infect. Dis.* **194**, 1438–46 (2006).

132. Brown, A. L., Shiel, R. E. & Irwin, P. J. Clinical, haematological, cytokine and acute phase protein changes during experimental Babesia gibsoni infection of beagle puppies. *Exp. Parasitol.* **157**, 185–96 (2015).

133. Zygner, W., Gójska-Zygner, O., Bąska, P. & Długosz, E. Increased concentration of serum TNF alpha and its correlations with arterial blood pressure and indices of renal damage in dogs infected with Babesia canis. *Parasitol. Res.* **113**, 1499–503 (2014).

134. Zygner, W., Gójska-Zygner, O., Bąska, P. & Długosz, E. Low T3 syndrome in canine babesiosis associated with increased serum IL-6 concentration and azotaemia. *Vet. Parasitol.* **211**, 23–7 (2015).

135. Leisewitz, A. *et al.* Disease severity and blood cytokine concentrations in dogs with natural Babesia rossi infection. *Parasite Immunol.* **41**, (2019).

136. Tanaka, T. & Kishimoto, T. Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases. *Int. J. Biol. Sci.* **8**, 1227–36 (2012).

137. Duffy, A. L., Olea-Popelka, F. J., Eucher, J., Rice, D. M. & Dow, S. W. Serum concentrations of monocyte chemoattractant protein-1 in healthy and critically ill dogs. *Vet. Clin. Pathol.* **39**, 302–5 (2010).

138. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* **29**, 313–26 (2009).

139. Mohan, K. and M. M. S. Dyserythropoiesis and severe anaemia associated with malaria correlate with deficient interleukin-12 production. *Br J Haematol* **103**, 942–949 (1998).

140. Garnham, P. C., R. G. Bird, J. R. Baker, S. S. D. and H. M. el-N. Electron microscope studies on motile stages of malaria parasites. VI. The ookinete of Plasmodium berghei yoelii and its transformation into the early oocyst. *Trans R Soc Trop Med Hyg* **63**, 187–194 (1969).

141. Carter, R. and D. W. New observations on the malaria parasites of rodents of the Central African Republic - Plasmodium vinckei petteri subsp. nov. and Plasmodium chabaudi Landau, 1965. *Ann Trop Med Parasitol* **69**, 187–196 (1975).

142. Villeval JL, Lew A, M. D. Changes in hemopoietic and regulator levels in mice during fatal or nonfatal malarial infections, I: erythropoietic populations. *Exp Parasitol* **71**, 364–374 (1990).

143. Weiss L, Johnson J, W. W. Mechanisms of splenic control of murine malaria: tissue culture studies of the erythropoietic interplay of spleen, bone marrow, and blood in lethal (strain 17XL) Plasmodium yoelii malaria in BALB/c mice. *Am J Trop Med Hyg* **41**, 135–143 (1989).

144. Yap GS, S. M. Plasmodium chabaudi AS: erythropoietic responses during infection in resistant and susceptible mice. *Exp Parasitol* **75**, 340–352 (1992).

145. Hussein, H. S. The bone marrow response during Babesia hylomysci and Babesia microti infections in mice. *J Comp Pathol* **92**, 613–620 (1982).

146. Hussein, H. S. The effect of a milk diet on Babesia microti and B. hylomysci infections in mice. *Trans R Soc Trop Med Hyg* **67**, 5 (1973).

147. Dawood, K. E., J. A. Morgan, F. Busfield, M. Srivastava, T. I. Fletcher, J. Sambono, L. A. Jackson, B. Venus, A. W. P. and A. E. L.-T. Observation of a novel Babesia spp. in Eastern Grey Kangaroos (Macropus giganteus) in Australia. *Int J Parasitol Parasites Wildl* **2**, 54–61 (2013).

148. Donahoe, S. L., C. S. Peacock, A. Y. Choo, R. W. Cook, P. O'Donoghue, S. Crameri, L. Vogelnest, A. N. Gordon, J. L. S. and K. R. A retrospective study of Babesia macropus associated with morbidity and mortality in eastern grey kangaroos (Macropus giganteus) and agile wallabies (Macropus agilis). *Int J Parasitol Parasites Wildl* **4**, 268–276 (2015).

149. Krause, P. J., B. E. Gewurz, D. Hill, F. M. Marty, E. Vannier, I. M. Foppa, R. R. Furman, E. Neuhaus, G. Skowron, S. Gupta, C. McCalla, E. L. Pesanti, M. Young, D. Heiman, G. Hsue, J. A. Gelfand, G. P. Wormser, J. Dickason, F. J. Bia, B. Hartman, S. R. Te, J. E. G. and A. S. Persistent and relapsing babesiosis in immunocompromised patients. *Clin Infect Dis* **46**, 370–376 (2008).

150. Vannier, E. and P. J. K. Human babesiosis. *N Engl J Med* **366**, 2397–2407 (2012).

151. Gupta, P., R. W. Hurley, P. H. Helseth, J. L. G. and D. E. H. Pancytopenia due to hemophagocytic syndrome as the presenting manifestation of babesiosis. *Am J Hematol* **50**, 60–62 (1995).

152. Auerbach, M., A. H. and G. S. Systemic babesiosis. Another cause of the hemophagocytic syndrome. *Am J Med* **80**, 301–303 (1986).

153. Slovut, D. P., E. B. and A. J. M. Babesiosis and hemophagocytic syndrome in an asplenic renal transplant recipient. *Transplantation* **62**, 537–539 (1996).

154. Go, S. A., Phuoc, V. H., Eichenberg, S. E., Temesgen, Z. & Beckman, T. J. Babesia microti infection and hemophagocytic lymphohistiocytosis in an immunocompetent patient. *Int. J. Infect. Dis.* **65**, 72–74 (2017).

155. Matjila, P. T., A. L. Leisewitz, F. J. and B. L. P. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol* **155**, 152–157 (2008).

156. Lucidi, C. A., C. L. E. de Rezende, L. A. J. and M. A. S. Histologic and cytologic bone marrow findings in dogs with suspected precursor-targeted immune-mediated anemia and associated phagocytosis of erythroid precursors. *Vet Clin Pathol* **46**, 401–415 (2017).

157. R Core Team. R: A language and environment for statistical computing. https://www.r-project.org/. (2021).

158. EClinPath.com, C. U. Bone marrow - General interpretation. *eClinPath.com, Cornell University* https://eclinpath.com/cytology/bone-marrow/general-interpretation/.

159. Chatterjee, S., Bhardwaj, N. & Saxena, R. K. Identification of Stages of Erythroid Differentiation in Bone Marrow and Erythrocyte Subpopulations in Blood Circulation that Are Preferentially Lost in Autoimmune Hemolytic Anemia in Mouse. *PLoS One* **11**, e0166878 (2016).

160. Fattizzo, B. *et al.* Prognostic impact of bone marrow fibrosis and dyserythropoiesis in autoimmune hemolytic anemia. *Am. J. Hematol.* **93**, E88–E91 (2018).

161. Weiss, D. J. Bone Marrow Pathology in Dogs and Cats with Non-Regenerative Immune-Mediated Haemolytic Anaemia and Pure Red Cell Aplasia. *J. Comp. Pathol.* **138**, 46–53 (2008).

162. Swann, J. W., Szladovits, B. & Glanemann, B. Demographic Characteristics, Survival and Prognostic Factors for Mortality in Cats with Primary Immune-Mediated Hemolytic Anemia. *J. Vet. Intern. Med.* **30**, 147–156 (2016).

163. Anna S De Tommasi, Domenico Otranto, Tommaso Furlanello, Silvia Tasca, Cinzia Cantacessi, Edward B Breitschwerdt, Dorothee Stanneck, Filipe Dantas-Torres, Gad Baneth, Gioia Capelli, D. de C. Evaluation of blood and bone marrow in selected canine vector-borne diseases. *Parasit. Vectors* **7**, 534 (2014).

164. Weiss, D. J. & Aird, B. Cytologic Evaluation of Primary and Secondary Myelodysplastic Syndromes in the Dog. *Vet. Clin. Pathol.* **30**, 67–75 (2001).

165. EClinPath.com. Regeneration. *eClinPath.com, Cornell University* https://eclinpath.com/hematology/anemia/assessment-regeneration/.

166. EClinPath.com, C. U. Absolute retic count. *eClinPath.com, Cornell University* https://eclinpath.com/hematology/tests/absolute-reticulocyte-count/.

167. EClinPath.com, C. U. Reticulocyte %. *eClinPath.com, Cornell University* https://eclinpath.com/hematology/tests/reticulocyte-percentage/.

168. OISHI, A., SAKAMOTO, H., SHIMIZU, R., OHASHI, F. & TAKEUCHI, A. Evaluation of Phlebotomy-Induced Erythropoietin Production in the Dog. *J. Vet. Med. Sci.* **55**, 51–58 (1993).

169. Dank, G., Segev, K., Elazari, M., Tovi-Mazaki, M. and Aroch, I. Diagnostic and Prognostic Significance of Rubricytosis in Dogs: A Retrospective Case-Control Study of 380 cases. *Isr. J. Vet. Med.* **75**, 193–203 (2020).

170. Mandell, C.P.; Jain, N.C.; Farver, T. B. The significance of normoblastemia and leukoerythroblastic reaction in the dog. *J. Am. Anim. Hosp. Assoc.* **26**, 665–672 (1989).

171. Harvey, J. W. Evaluation of Erythrocytes. in *Veterinary Hematology A Diagnostic Guide and Color Atlas* 49–121 (Elsevier Saunders, 2012).

172. Parmar, J. & Sheikh, S. Study of bone marrow: dyserythropoiesis for etiological evaluation of anemia. *Int. J. Res. Med. Sci.* 3734–3738 (2015) doi:10.18203/2320-6012.ijrms20151431.

173. Abdalla, S. H. Iron and folate status in Gambian children with malaria. *Ann Trop Paediatr* **10**, 265–272 (1990).

174. A, S. Dyserythropoiesis in 105 Patients with Visceral Leishmaniasis. *Lab. Hematol. Off. Publ. Int. Soc. Lab. Hematol.* **10**, 206–211 (2004).

175. Oppenheimer, S. J. Iron and malaria. *Parasitol Today* **5**, 77–79 (1989).

176. Brugnara, C. Reticulocyte Cellular Indices: A New Approach in the Diagnosis of Anemias and Monitoring of Erythropoietic Function. *Crit. Rev. Clin. Lab. Sci.* **37**, 93–130 (2000).

177. Brugnara, C. Iron Deficiency and Erythropoiesis: New Diagnostic Approaches. *Clin. Chem.* **49**, 1573–1578 (2003).

178. Steinberg, J. D. & Olver, C. S. Hematologic and biochemical abnormalities indicating iron deficiency are associated with decreased reticulocyte hemoglobin content (CHr) and reticulocyte volume (rMCV) in dogs. *Vet. Clin. Pathol.* **34**, 23–27 (2005).

179. Feng, G.-H., Li, H.-P., Li, Q.-L., Fu, Y. & Huang, R.-B. Red blood cell distribution width and ischaemic stroke. *Stroke Vasc. Neurol.* **2**, 172–175 (2017).

180. Lippi, G. *et al.* Relation Between Red Blood Cell Distribution Width and Inflammatory Biomarkers in a Large Cohort of Unselected Outpatients. *Arch. Pathol. Lab. Med.* **133**, 628–632 (2009).

181. Evans, T. C. & Jehle, D. The red blood cell distribution width. *J. Emerg. Med.* **9**, 71–74 (1991).

182. Katsaros, M., Paschos, P. & Giouleme, O. Red cell distribution width as a marker of activity in inflammatory bowel disease: a narrative review. *Ann. Gastroenterol.* 1–7 (2020).

183. Brugnara, C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. *Int. J. Clin. Lab. Res.* **28**, 1–11 (1998).

184. D'onofrio, G., Zini, G. & Brugnara, C. Clinical Applications of Automated Reticulocyte Indices. *Hematology* **3**, 165–176 (1998).

185. Hillman, R. S. Characteristics of marrow production and reticulocyte maturation in normal man in response to anemia. *J. Clin. Invest.* **48**, 443–453 (1969).

186. EClinPath.com, C. U. White blood cells - Left shift. https://eclinpath.com/hematology/morphologic-features/white-blood-cells/left-shift/.

187. Leisewitz, A. H. S. J. C. A. L. The pathology of the spleen in lethal canine babesiosis caused by Babesia rossi. *Parasite Immunol.* **42**, (2020).

188. C, M. Pathology of complicated Babesia rossi-associated acute lung injury and respiratory distress syndrome in dogs. (2020).

189. Corbett, Y. *et al.* Phagocytosis and activation of bone marrow-derived macrophages by Plasmodium falciparum gametocytes. *Malar. J.* **20**, 81 (2021).

190. Atkinson, B.K,; Thompson, P; Van Zyl, E; Goddard, A; Rautenbach, Y; Schoeman, J.P.; Mukorera, V and Leisewitz, A. Kinetics of the inflammatory response during experimental Babesia rossi infection of beagle dogs. *BioRxiv* (2021) doi:https://doi.org/10.1101/2021.09.16.460686.

191. Leimkühler, N. B. & Schneider, R. K. Inflammatory bone marrow microenvironment. *Hematology* **2019**, 294–302 (2019).

192. Kovtonyuk, L. V., Fritsch, K., Feng, X., Manz, M. G. & Takizawa, H. Inflamm-Aging of Hematopoiesis, Hematopoietic Stem Cells, and the Bone Marrow Microenvironment. *Front. Immunol.* **7**, (2016).

193. Brown, W. C., Norimine, J., Knowles, D. P. & Goff, W. L. Immune control of Babesia bovis infection. *Vet.*

Parasitol. **138**, 75–87 (2006).

194. Janeway, C. A. & Medzhitov, R. Innate Immune Recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).

195. Baccarella, A., Fontana, M. F., Chen, E. C. & Kim, C. C. Toll-Like Receptor 7 Mediates Early Innate Immune Responses to Malaria. *Infect. Immun.* **81**, 4431–4442 (2013).

196. Cao, H. & Vickers, M. A. Oxidative stress, malaria, sickle cell disease, and innate immunity. *Trends Immunol.* **42**, 849–851 (2021).

197. Insights into malaria susceptibility using genome-wide data on 17,000 individuals from Africa, Asia and Oceania. *Nat. Commun.* **10**, 5732 (2019).

198. Weiss, G. E. *et al.* Revealing the Sequence and Resulting Cellular Morphology of Receptor-Ligand Interactions during Plasmodium falciparum Invasion of Erythrocytes. *PLOS Pathog.* **11**, e1004670 (2015).

199. Henry, B. *et al.* The Human Spleen in Malaria: Filter or Shelter? *Trends Parasitol.* **36**, 435–446 (2020).

200. Cao, H. *et al.* Red blood cell mannoses as phagocytic ligands mediating both sickle cell anaemia and malaria resistance. *Nat. Commun.* **12**, 1792 (2021).

201. Martinez-Pomares, L. The mannose receptor. *J. Leukoc. Biol.* **92**, 1177–1186 (2012).

202. Schreiber, S. *et al.* Regulation of mouse bone marrow macrophage mannose receptor expression and activation by prostaglandin E and IFN-gamma. *J. Immunol.* **151**, 4973–81 (1993).

203. Ortega-Pajares, A. & Rogerson, S. J. The Rough Guide to Monocytes in Malaria Infection. *Front. Immunol.* **9**, (2018).

204. Mitchell, A. J., Roediger, B. & Weninger, W. Monocyte homeostasis and the plasticity of inflammatory monocytes. *Cell. Immunol.* **291**, 22–31 (2014).

205. McGavin, M. Z. F. *Pathologic Basis of Veterinary Disease*. (Elsevier Saunders, 2007).

206. Terkawi, M. A. *et al.* Macrophages Are the Determinant of Resistance to and Outcome of Nonlethal Babesia microti Infection in Mice. *Infect. Immun.* **83**, 8–16 (2015).

207. Aguilar-Delfin, I., Wettstein, P. J. & Persing, D. H. Resistance to Acute Babesiosis Is Associated with Interleukin-12- and Gamma Interferon-Mediated Responses and Requires Macrophages and Natural Killer Cells. *Infect. Immun.* **71**, 2002–2008 (2003).

208. Kitaguchi, T., Nagoya, M., Amano, T., Suzuki, M. & Minami, M. Analysis of roles of natural killer cells in defense against Plasmodium chabaudi in mice. *Parasitol. Res.* **82**, 352–357 (1996).

209. Chen, Q. *et al.* Human natural killer cells control Plasmodium falciparum infection by eliminating infected red blood cells. *Proc. Natl. Acad. Sci.* **111**, 1479–1484 (2014).

210. Artavanis-Tsakonas, K. *et al.* Activation of a Subset of Human NK Cells upon Contact with Plasmodium falciparum -Infected Erythrocytes. *J. Immunol.* **171**, 5396–5405 (2003).

211. Artavanis-Tsakonas, K. & Riley, E. M. Innate Immune Response to Malaria: Rapid Induction of IFN-γ from Human NK Cells by Live Plasmodium falciparum -Infected Erythrocytes. *J. Immunol.* **169**, 2956–2963 (2002).

212. Korbel, D. S., Newman, K. C., Almeida, C. R., Davis, D. M. & Riley, E. M. Heterogeneous Human NK Cell Responses to Plasmodium falciparum -Infected Erythrocytes. *J. Immunol.* **175**, 7466–7473 (2005).

213. Scheepers, E. The haematological kinetics of canine babesiosis in South Afric. (2008).

214. Weltan, S. M., Leisewitz, A. L. & Goddard, A. A case-controlled retrospective study of the causes and implications of moderate to severe leukocytosis in dogs in South Africa. *Vet. Clin. Pathol.* **37**, 164–172 (2008).

215. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).

216. Tecchio, C. & Cassatella, M. A. Neutrophil-derived chemokines on the road to immunity. *Semin. Immunol.* **28**, 119–128 (2016).

217. Lin, A. & Loré, K. Granulocytes: New Members of the Antigen-Presenting Cell Family. *Front. Immunol.* **8**, (2017).

218. Aitken, E. H., Alemu, A. & Rogerson, S. J. Neutrophils and Malaria. *Front. Immunol.* **9**, (2018).

219. van Wolfswinkel, M. E. *et al.* Changes in total and differential leukocyte counts during the clinically silent liver phase in a controlled human malaria infection in malaria-naïve Dutch volunteers. *Malar. J.* **16**, 457 (2017).

220. Sirima, S. B. *et al.* Hematologic Parameters in Pediatric Uncomplicated Plasmodium falciparum Malaria in Sub-Saharan Africa. *Am. J. Trop. Med. Hyg.* **85**, 619–625 (2011).

221. Silvestre-Roig, C., Hidalgo, A. & Soehnlein, O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood* **127**, 2173–2181 (2016).

222. Cunnington, A. J. *et al.* Prolonged Neutrophil Dysfunction after Plasmodium falciparum Malaria Is Related to Hemolysis and Heme Oxygenase-1 Induction. *J. Immunol.* **189**, 5336–5346 (2012).

223. Feintuch, C. M. *et al.* Activated Neutrophils Are Associated with Pediatric Cerebral Malaria Vasculopathy in Malawian Children. *MBio* **7**, (2016).

224. Carmona-Fonseca, J., Arango, E. & Maestre, A. Placental Malaria in Colombia: Histopathologic Findings in Plasmodium vivax and P. falciparum Infections. *Am. J. Trop. Med. Hyg.* **88**, 1093–1101 (2013).

225. Ampawong, S. *et al.* A potential role for interleukin-33 and γ-epithelium sodium channel in the pathogenesis of human malaria associated lung injury. *Malar. J.* **14**, 389 (2015).

226. NIELSEN, H., KHARAZMI, A. & THEANDER, T. G. Suppression of blood monocyte and neutrophil chemotaxis in acute human malaria. *Parasite Immunol.* **8**, 541–550 (1986).

227. Waisberg, M. *et al.* Plasmodium falciparum merozoite surface protein 1 blocks the proinflammatory

protein S100P. *Proc. Natl. Acad. Sci.* **109**, 5429–5434 (2012).

228. Ritzman, A. M. *et al.* The Chemokine Receptor CXCR2 Ligand KC (CXCL1) Mediates Neutrophil Recruitment and Is Critical for Development of Experimental Lyme Arthritis and Carditis. *Infect. Immun.* **78**, 4593–4600 (2010).

229. Harada, A. *et al.* Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukoc. Biol.* **56**, 559–64 (1994).

230. Dale, D. C. & Wolff, S. M. Studies of the neutropenia of acute malaria. *Blood* **41**, 197–206 (1973).

231. Celliers, A., Rautenbach, Y., Hooijberg, E., Christopher, M. & Goddard, A. Neutrophil Myeloperoxidase Index in Dogs With Babesiosis Caused by Babesia rossi. *Front. Vet. Sci.* **7**, (2020).

232. Leopold Wager, C. M. & Wormley, F. L. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. *Mucosal Immunol.* **7**, 1023–1035 (2014).

233. Tomioka, H. *et al.* Characteristics of Suppressor Macrophages Induced by Mycobacterial and Protozoal Infections in relation to Alternatively Activated M2 Macrophages. *Clin. Dev. Immunol.* **2012**, 1–19 (2012).

234. Schleifer KW, M. J. Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. *J. Immunol.* **151**, 5492–5503 (1993).

235. Flynn J, S. M. The role of the macrophage in induction of immunosuppression in Trypanosoma congolense-infected cattle. *Immunology* **74**, 310 (1991).

236. Fry LM, Schneider DA, Frevert CW, Nelson DD, Morrison WI, K. D. East coast fever caused by Theileria parva is characterized by macrophage activation associated with vasculitis and respiratory failure. *PLoS One* **11**, e0156004 (2016).

237. Khan IA, Matsuura T, K. L. IL-10 mediates immunosuppression following primary infection with Toxoplasma gondii in mice. *Parasite Immunol.* **17**, 185–195 (1995).

238. Weiss, G. E. *et al.* Atypical Memory B Cells Are Greatly Expanded in Individuals Living in a Malaria-Endemic Area. *J. Immunol.* **183**, 2176–2182 (2009).

239. Bockstal, V., Geurts, N. & Magez, S. Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage Plasmodium chabaudi Infection in Mice. *J. Parasitol. Res.* **2011**, 1–11 (2011).

240. Brown, W. . & Palmer, G. . Designing Blood-stage Vaccines against Babesia bovis and B. bigemina. *Parasitol. Today* **15**, 275–281 (1999).

241. Grun, J. L. & Weidanz, W. P. Immunity to Plasmodium chabaudi adami in the B-cell-deficient mouse. *Nature* **290**, 143–145 (1981).

242. Fell, A. . & Smith, N. . Immunity to Asexual Blood Stages of Plasmodium: Is Resistance to Acute Malaria Adaptive or Innate? *Parasitol. Today* **14**, 364–369 (1998).

243. Krämer, F. Canine babesiosis – a never-ending story. *CVBD® Dig.* **4**, (2009).

APPENDICES

Appendix 1: Informed consent form

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

Encircle Yes or No where necessary

1.	Have you read the information sheet on canine babesiosis?	Yes	No
2.	Have you had the opportunity to ask questions about the research project?	Yes	No
3.	Have you received satisfactory answers to your questions?	Yes	No
4.	Have you received enough information about this study	Yes	No
5.	Supply the name of the person to whom you have spoken to:		
	.....		
6.	Do you grant consent that blood and urine samples can be drawn from your dog?	Yes	No
7.	Do you grant consent that a post mortem examination can be performed in the case of death?		
		Yes	No

I, ……, hereby give permission that my dog ……., a …….. may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore I understand that the costs of the additional tests will be borne by the trial fund, and that I will only be liable for costs pertaining the treatment that would in any event be required by my dog, including any complications that may arise as a result of canine babesiosis.

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

Signature Owner/Agent ……..

Home Tel: ……..

Work Tel: ……

Cell No: ……

Appendix 2: Histopathology check-list

Observational matrix for histopathology of the bone marrow [modified from Raskin & Messick (2012)]

OVERALL CELLULARITY (%) (x10 objective)	Hypocellular	Mild		
		Moderate		
		Severe		
	Normocellular (25% - 75%)			
	Hypercellular	Mild		
		Moderate		
		Severe		
MEGAKARYOCYTES (x10 objective)	Number		Decreased	
			Adequate	
			Increased	
	Distribution			
	General maturity		Mature	
			Immature	
IRON CONTENT (PERLS' PRUSSIAN BLUE STAIN)	Absent / decreased			
	Within normal limits			
	Decreased			
Presence of erythrophagocytosis	Yes			
	No			
Parasite score				
Presence of vascular necrosis / vasculitis	Yes			
	No			
Thrombosis	Yes			
	No			
Haemorrhage	Yes			
	No			
Congestion / hyperaemia	Yes			
	No			
Endothelial cell hypertrophy	Yes			
	No			

Appendix 3: Histopathological findings of the bone marrow for each individual case

***Babesia* case #1** (Hospitalization number: N/A; Post mortem number: S847-19)

On low power magnification the sections show sparse fragments of bony trabeculae and few adipocytes. The bone marrow is hypercellular, approximately 90%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 4 to 11 per high power field, with a mean of 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident, approximately 6-7 per high power field (x40) and 62 per 10 high power fields (x400). Erythrophagocytosis is observed. Sparse haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 4-11; mean 7.33
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

***Babesia* case #2** (Hospitalization number: 2237315; Post mortem number: S2678-17)

On low power magnification the sections show few fragments of bony trabeculae and a low number of adipocytes. The bone marrow is hypercellular, approximately 85%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-7 per high power field, with a mean of 5.67. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines appear to predominate but few differentiated cells are seen. Multifocal mitoses are evident, approximately 59 per 10 high power fields (x400). Erythrophagocytosis is observed. A moderate number of haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 5-7; mean 5.67
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

***Babesia* case #3** (Hospitalization number: 5191717; Post mortem number: S1951-17)

On low power magnification the sections show scattered fragments of bony trabeculae and sparse adipocytes. The bone marrow is hypercellular, approximately 90 - 95%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 6-9 per high power field with the mean 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power

magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident - 54 per 10 high power fields (x400). Erythrophagocytosis is observed. Sparse haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 6-9, mean 7.33
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

***Babesia* case #4** (Hospitalization number: 4944217; Post mortem number: S879-17)

On low power magnification the sections show sparse fragments of bony trabeculae and moderate numbers of adipocytes. The bone marrow is hypercellular, approximately 90%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-6 per high power field, with the mean 5.33. Megakaryocytes of all maturational stages are evident and mature cells appear to be slightly predominating. On high power magnification (x40) immature cell lines appear to predominate and fewer differentiated cells are seen. Multifocal mitoses are evident - 43 per 10 high power fields (x400). Erythrophagocytosis is observed. A moderate to large amount of haemosiderin pigment is evident.

Cellularity score: 3
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 5-6, mean 5.33
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

***Babesia* case #5** (Hospitalization number: Ozzy; Post mortem number: S776-19)

On low power magnification the sections show rare fragments of bony trabeculae and moderate numbers of adipocytes. The bone marrow is hypercellular, approximately 80%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 6-9 per high power field with a mean of 7.33. Megakaryocytes of all maturational stages are evident, but immatures appear to be predominating (left shift). On high power magnification (x40) immature cell lines predominate fewer differentiated cells are seen. Multifocal mitoses are evident - 42 per 10 high power fields (x400). Erythrophagocytosis is observed. Moderate number of haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 0
Endothelial cell reactivity score: 1
Megakaryocyte: Range 6-9, mean 7.33
Megakaryocyte score: 3

Congestion: Yes
Haemorrhage: Yes
Thrombosis: No

***Babesia* case #6** (Hospitalization number: 5376417; Post mortem number: S2710-17)

On low power magnification bony trabeculae are not clear. Few adipocytes are observed. The bone marrow is hypercellular, approximately 80-85%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-10 per high power field and a mean of 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident, approximately 59 per 10 high power fields (x400). Erythrophagocytosis is observed. Small amounts of haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 1
Endothelial cell reactivity score: 1
Megakaryocyte: Range 5-10, mean 7.33
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Control case #1 (Hospitalization number: N/A; Post mortem number: S4191-18)

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 4-9 per high power field and a mean of 6.67. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 21 per 10 high power fields (x400). Small to moderate amounts of haemosiderin pigment is evident.

Cellularity score: 3
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 4-9, mean 6.67
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Control case #2 [Hospitalization number: N/A; Post mortem number: S4191-18(2)]

On low power magnification few fragments of bony trabeculae are evident. Moderate to occasionally large numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –2-9 per high power field with a mean of 5. Megakaryocytes of various maturational stages are evident but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 13 per 10 high power fields (x400). Small amounts of haemosiderin pigment is evident.

Cellularity score: 2
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 2-9, mean 5
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

Control case #3 [Hospitalization number: N/A; Post mortem number: S4191-18(3)]

On low power magnification few fragments of bony trabeculae are evident. Moderate to occasionally large numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –3-5 per high power field and a mean of 4. They are mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 23 per 10 high power fields (x400). Moderate amounts of haemosiderin pigment is evident.

Cellularity score: 3
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 3-5, mean 4
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Control case #4 [Hospitalization number: N/A; Post mortem number: S4191-18(4)]

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –4-9 per high power field and a mean of 6.67. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 18 per 10 high power fields (x400). Sparse haemosiderin pigment is evident.

Cellularity score: 3
Parasite score: 0

Endothelial cell reactivity score: 0
Megakaryocyte: Range 4-9, mean 6.67
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

Control case #5 [Hospitalization number: N/A; Post mortem number: S4191-18(5)]

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 1-4 per high power field with the mean of 2.33. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 18 per 10 high power fields (x400). Moderate amounts of haemosiderin pigment is evident.

Cellularity score: 2
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 1-4, mean 2.33
Megakaryocyte score: 2
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Appendix 4: Cytological evaluation of the bone marrow for each infected and control case

Specimen	*Babesia* case #1	*Babesia* case #2	*Babesia* case #3	*Babesia* case #4	*Babesia* case #5	*Babesia* case #6	Control case #1	Control case #2	Control case #3	Control case #4	Control case #5
CBC: Erythron	Severe normocytic, normochromic anaemia	Moderate normocytic normochromic regenerative anaemia, moderate increase in nucleated RBCs	Severe macrocytic, hypochromic appropriately regenerative anaemia	Severe macrocytic hypochromic non-regenerative anaemia, RBC agglutination	Severe normocytic hypochromic non-regenerative anaemia	Severe normocytic hypochromic non-regenerative anaemia; moderate increase in nucleated RBCs	WNL. No retic count	WNL. No retic count	WNL. No retic count	WNL. No retic count	WNL. No retic count
CBC: Leukon	Mild left shift neutrophilia and eosinopaenia	Inflammatory leukogram: Moderate leukocytosis with left shift neutrophilia and monocytosis, moderate neutrophil toxicity	Leukocytosis with left shift neutrophilia and an eosinopaenia	Inflammatory leukogram: Moderate leukocytosis with left shift neutrophilia and monocytosis, moderate neutrophil toxicity	Leukopenia with neutropenia and a left shift	Inflammatory leukogram: Normal WBC with mildly left shifted neutrophils and many moderately toxic neutrophils	WNL.	WNL	WNL	Mild neutropenia and mild lymphocytosis	Mild mature neutrophilia and monocytosis - stress leukogram
CBC: Thrombon	Severe thrombocytopenia	Severe thrombocytopenia, although also severe platelet aggregation on smear so platelet count not accurate	Mild thrombocytopaenia	Mild thrombocytopenia	WNL	Severe thrombocytopenia	WNL	WNL	WNL	Platelet count low with platelet aggregates - so probably WNL	WNL.
CBC: Other		*Babesia* noted		*Babesia* noted	*Babesia* noted.	*Babesia* noted. Plasma haemolytic					
Bone marrow evaluation:											
Adequate specimen	Very thick	Yes	Yes	Yes	Yes	Yes	Yes	Many degenerate cells	Yes	Yes	Yes
Cellularity %	>75%	>75%	>75%	50-75%	>75%	>75%	50-75%	50-75%	50%	50%	50-75%
Cellularity interpretation	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Normal - hypercellular depending on age	Normal - hypercellular depending on age	Normocellular	Normocellular	Normal - hypercellular depending on age
Iron stores	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present

Megakaryocytes cellularity	4/10 high power field (hpf)	6/ 10x hpf	10/ 10x low power field (lpf)	3/ 10x hpf	8/ 10x lpf	10/ 10x lpf	11/ 10x lpf	9/ 10 x lpf	6/10 hpf	9/10 hpf	4/10 hpf
Megakaryocytes maturity	Approximately 50% mature	Approximately 20% mature	Approximately 50% mature	Approximately 90% mature	Approximately 60% mature	Approximately 50% mature	Approximately 60% mature	Approximately 60% mature	Approximately 90% mature	Approximately 80% mature	Approximately 80% mature
Myeloid:Erythroid (M:E)	1: 1.2	1: 1.9	1: 1.9	1: 1.4	1:4	1: 1.9	1.92:1	1: 0.44	1:1.09	1: 1.3	1: 0.9
Myeloid cellularity	Increased	Increased	Increased	Hypercellular	Normal - increased	Increased	Normal	Normal	Normal	Normal	Normal
Myeloid morphology	Left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Marked left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/ segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Moderate left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/ segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Left shifted, normal morphology	Marked left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/ segmented neutrophils are often giant with some toxic change (basophilia, granulation). Marked increase in eosinophils.	Moderate left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/ segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology
Erythroid cellularity	Increased	Increased	Increased	Hypercellular	Increased	Increased	Normal	Normal	Normal	Normal	Normal
Erythroid morphology	Moderate left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Orderly maturation, mild dysplastic changes in metarubricytes: pleomorphic nuclear shapes, binucleation	Moderately left shifted, mild dysplastic changes in metarubricytes: pleomorphic nuclear shapes	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology
Differential 500-cell count Of All Nucleated Cells (%):											

Rubriblasts	3.6	5.2	6.6	1.4	4.2	6.6	2.0	0.8	1.0	1.0	0.8
Prorubricytes/ Rubricytes	46.0	31.4	38.2	39.0	49.4	38.2	21.0	22.6	31.4	47.8	39.8
Metarubricytes	4.4	2.8	7.6	14.2	6.0	7.6	8.6	5.0	15.4	4.0	5.6
Total Erythroid %	54.0	39.4	52.4	54.6	59.6	52.4	31.6	28.4	47.8	52.8	46.2
Of All Erythroid Cells:											
Rubriblasts	6.7	13.2	12.6	2.6	7.0	12.6	6.3	2.8	2.1	1.9	1.7
Prorubricytes/ Rubricytes	85.2	79.7	72.9	71.4	82.0	72.9	66.5	79.6	65.7	90.5	86.1
Metarubricytes	8.1	7.1	14.5	26.0	11.0	14.5	27.2	17.6	32.2	7.6	12.1
Of All Nucleated Cells (%):											
Myeloblasts	0.8	2.8	2.2	2.4	2.4	2.2	0.8	1.5	0.8	0.4	2.2
Promyelocytes/ myelocytes	19.0	7.2	10.4	19.8	5.8	10.4	8.6	6.0	6.8	7.8	5.0
Metamyelocytes/Bands/Segmented neutrophils	23.0	10.6	14.4	18.0	7.0	14.4	50.8	57.4	36.2	32.0	44.4
Total Myeloid %	42.8	20.6	27.0	40.2	15.2	27.0	60.2	64.9	43.8	40.2	51.6
Of All Myeloid Cells:											
Myeloblasts	1.9	13.6	8.1	6.0	16.0	8.1	1.3	2.3	1.8	1.0	4.3
Promyelocytes/ myelocytes	44.4	35.0	38.5	49.3	38.0	38.5	14.3	9.2	15.5	19.4	9.7
Metamyelocytes/Bands/Segmented neutrophils	53.7	51.5	53.3	44.8	46.0	53.3	84.4	88.4	82.6	79.6	86.0
Of All Nucleated Cells (%):											
Plasma cells	0.0	19.4	6.6	1.4	1.2	6.6	1.0	1.2	1.6	0.2	0.8
Plasma cell comment		Many immatures back to plasmablasts	Some immatures	Normal morphology	Some immatures	Some immatures	mature	mature	mature	mature	mature
Lymphocytes	1.2	4.8	2.2	3.2	3.8	2.2	5.8	1.6	5.4	5.0	0.8
Lymphocytes comment	small	Small, medium, large granular lymphocytes	Small	Mostly small, normal morphology	Increase in medium-sized	Small	small	small	Small and medium	small	small
Histiocytes/macrophages	2.2	15.6	11.8	0.6	20.2	11.8	1.4	4.0	1.4	1.8	0.6
Histiocytes / macrophages comment	Moderate activation, about 30% show vacuolisation, phaogcytosis of mature RBC, contain haemosiderin	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Not active	Very active, some erythrophagocytosis and haemosiderin-laden	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Mild activation	Moderate activation, about 30% show vacuolisation, phaogcytosis of mature RBC, contain haemosiderin	Mild activation	Mild activation	Mild activation
Other cells comment										Occasional osteoclasts	
Stromal reaction	None	None	None	None	None	None	None	None	None	None	None
Infectious agents	*Babesia* +++	*Babesia* +++	*Babesia* +++	None seen	*Babesia* +++	*Babesia* +++	None	None	None	None	None

Megakaryocytes interpretation	Left shift. This would be an appropriate response if there was a peripheral thrombocytopenia	Marked left shift - this would be an appropriate response in the face of a peripheral thrombocytopenia	Left shift - this would be an appropriate response in the face of a peripheral thrombocytopenia	WNL - would expect some left shifting and hyperplasia given the CBC findings	WNL	Left shift - this is an appropriate response in the face of a peripheral thrombocytopenia	WNL	WNL	WNL	WNL	WNL
Erythroid interpretation	Hyperplasia and left shift. This would be an appropriate response if there was a peripheral anaemia	Marked hyperplasia with a left shift, appropriate in the face of haemolytic anaemia; mild dysplasia	Marked hyperplasia with a left shift, appropriate in the face of haemolytic anaemia; mild dysplasia	Some dysplastic changes. Lack of left shift unusual given the peripheral anaemia.	Marked hyperplasia with a left shift, appropriate given the haemolytic anaemia; mild dysplasia	Marked hyperplasia with a left shift, this is appropriate in the face of haemolytic anaemia; mild dysplasia	WNL	WNL	WNL	WNL	WNL
Myeloid interpretation	Hyperplasia and left shift. This would be an appropriate response if there was an inflammatory leukogram	Hyperplasia and left shift consistent with systemic inflammation	Hyperplasia and left shift consistent with systemic inflammation	Mild left shift - consistent with inflammatory leukogram	Marked left shift, increased eosinophilic granulocytes	Hyperplasia and left shift consistent with systemic inflammation	WNL	WNL	WNL	WNL	WNL
Plasma cells and lymphoid interpretation	WNL Unexpected given the *Babesia*	Marked plasma cell hyperplasia consistent with inflammation, reactive lymphoid hyperplasia	Marked plasma cell hyperplasia consistent with inflammation	Normal	WNL	Marked plasma cell hyperplasia consistent with inflammation	WNL	WNL	Mild lymphocytosis	WNL	WNL
Histiocyte/ macrophage interpretation	Moderate activation. Expected given the *Babesia*	Marked macrophagic/ histiocytic inflammation	Marked macrophagic/ histiocytic inflammation	Normal	Marked macrophagic/ histiocytic inflammation	Marked macrophagic/ histiocytic inflammation	WNL	Mild macrophagic activation	WNL	WNL	WNL
Other interpretation											
Summary	Apart from the lack of plasma cell hyperplasia this would be an appropriate bone marrow response to	Appropriate bone marrow response to haemolytic anaemia, thrombocytopenia and systemic	Appropriate bone marrow response to haemolytic anaemia and systemic inflammation	Myeloid left shift appropriate in the face of inflammatory leukogram. Lack of erythroid	Appropriate bone marrow response to severe haemolytic anaemia. The myeloid reaction may	Appropriate **bone marrow** response to severe thrombocytopenia and haemolytic anaemia. The	Normal bone marrow apart from a mild lymphocytosis	Apart from some macrophage activity, this represents normal	Normal bone marrow apart from a mild lymphocytosis	Normal bone marrow, no evidence of a response or reason	Normal bone marrow

	haemolytic anaemia and systemic inflammation caused by *Babesia*	inflammation caused by *Babesia*	caused by *Babesia*	hyperplasia in the face of peripheral anaemia not appropriate; interesting that no parasites noted in BM smears.	not be sufficient, given the peripheral leukopenia. Histiocytic inflammation due to presence of parasite.	presence of increased nucleated RBCs in peripheral blood in the face of no increase in reticulocytes and a clear erythroid hyperplasia in bone marrow is an unusual finding. Either these samples have been taken just at the point where metarubricytes are about to mature into retics (i.e. 2-3 days after start of anaemia), or perhaps reticulocytes are being released but are being destroyed at high rate in the haemolytic process? The myeloid reaction may not be sufficient, given the left shift without neutrophilia and the toxic changes. Histiocytic inflammation due to presence of parasite.		bone marrow		for the mild neutropenia	

Abbreviations: CBC: Complete blood count; RBC: Red blood cell; WNL: Within normal limits; HPF: High power field; LPF: Low power field

Appendix 5: The percentage of positive labelling per immunohistochemical marker/antibody

	B1	B2	B3	B4	B5	B6	C1	C2	C3	C4	C5
CD3 ***	9.78	8.3	11	7.12	9.92	8.47	7.06	13.23	9.15	10	7.61
CD20 ***	16.26	11.81	9.51	17.33	20.24	9.13	11.51	10.3	11.95	11.21	12.01
MUM1 ***	4	9.47	5.44	14.64	8.96	4.01	3.27	7.39	4.17	5.01	4.57
CD204 ***	21.41	24.06	22.94	22.34	27.19	21.17	8.82	14.32	15.39	15.76	15.61
MAC387 ***	66.6	45.82	44.63	52.62	39.1	41.01	38.17	42	38.74	37.4	46.38
Perls' Prussian blue stain***	74.43	20.44	63.19	28.22	29.15	13.12	9.01	17.17	8.23	10.58	26.78

* *Babesia* cases are denoted by the letter B and include case numbers 1 to 6
** Control cases are denoted by the letter C and include control case numbers 1 to 5
*** This represents the percentage of positive labelling per cell marker for each case

Appendix 6: Complete blood count values for each infected and control case (significantly decreased values are in bold and significantly increased values are underlined)

Case number	Case identifier	Haemoglobin (g/L)	Red cell count (x10^{12}/L)	Haematocrit (L/L)	Mean corpuscular volume (f/L)	Mean corpuscular haemoglobin (pg)	Mean corpuscular haemoglobin concentration (g/dL)	Red cell distribution width (%)	White cell count (x10^{9}/L)	Segmented neutrophil (x10^{9}/L)	Band neutrophil (x10^{9}/L)	Lymphocyte (x10^{9}/L)	Monocyte (x10^{9}/L)	Eosinophil (x10^{9}/L)	Basophil (x10^{9}/L)	Platelet count (x10^{9}/L)	NRBC/100 WBC	Reticulocyte percentage	Absolute reticulocyte count (x10^{9}/L)
1	B1	**22**	**1.03**	**0.06**	63.1	20.1	31.8	15.4	14.18	8.65	0.71	3.83	0.99	**0**	0	**20**	3	-	-
2	B2	**60**	**2.48**	**0.19**	75.7	24.1	31.9	21.3	24.83	14.9	3.97	3.48	2.48	**0**	0	**43**	16	5.9	147.1
3	B3	**30**	**1.16**	**0.12**	101.3	25.8	**25.5**	17	16.31	12.08	0.82	2.12	1.31	**0**	0	**154**	28	11.4	132.9
4	B4	**13**	**0.57**	**0.06**	96.7	51.5	**23.3**	16.4	36.64	22.35	5.5	3.66	5.13	**0**	0	**182**	2	11.7	66.6
5	B5	**70**	**1.67**	**0.12**	70.8	42.2	59.5	18.2	**4.01**	**1.72**	0.8	1.12	0.36	**0**	0	500	3	1.9	31.6
6	B6	**39**	**1.18**	**0.09**	73.9	33.2	**29.4**	15.8	7.65	4.82	0.54	1.91	0.38	**0**	0	**29**	15	1.8	20.6
7	C1	153	6.29	0.47	75.4	24.3	32.2	15.1	11.6	6.26	0	3.83	0.35	1.16	0	231	0	-	-
8	C2	137	6.18	0.41	65.8	26.4	33.6	13.1	12.45	6.47	0	4.36	0.5	1	0.12	**196**	0	-	-
9	C3	177	7.57	0.53	70.1	23.3	33.3	12.8	11.03	5.63	0	3.86	0.66	0.77	0.11	321	0	-	-
10	C4	156	7.35	0.48	65.5	21.1	32.4	14.7	9.29	**2.79**	0	5.85	0.37	0.28	0	**96**	0	-	-
11	C5	178	7.66	0.53	69.2	23.2	33.5	12.8	17.15	12.69	0	2.92	1.37	0.17	0	276	0	-	-
Reference range		120-180	5.5-8.5	0.37-0.55	60-77	No reference range	32-36	No reference range	6-15	3-11.5	0-0.5	1-4.8	0.15-1.35	0.1-1.25	0-0.1	200-500	0-9	0-1 *	0-80*

* Reticulocyte percentage reference interval according to Stockham & Scott (2008) [14]
** *Babesia* cases are denoted by the letter B and include case numbers 1 to 5.
*** Control cases are denoted by the letter C and include case numbers 1 to 5.