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Canine Primary Immune Mediated Hemolytic Anemia: A Retrospective Study of 52 Cases from Two Veterinary Teaching Hospitals (2009 - 2015)

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Abstract

Background: Canine primary immune mediated hemolytic anemia (pIMHA) is the most common immune-hematological disease in dogs. It involves the destruction of red blood cells by the immune system resulting in moderate to severe anemia in most patients. Despite its frequent description in the literature, only a few authors have described the vast range of morphological abnormalities of erythrocytes, leukocytes and platelets that may be present in the presentation of the disease.

Objectives: To investigate retrospectively clinical and clinicopathological findings in two groups of dogs with pIMHA, as well as to describe the alterations most commonly observed in patients' peripheral blood smears. Cases were collected at the "Mario Modenato" Veterinary Teaching Hospital of the Department of Veterinary Sciences, Pisa University, Italy (*Pisa*) and the Veterinary Teaching Hospital of the Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Israel (*Beit-Dagan*).

Material and Methods: Fifty-two cases of canine pIMHA between the years 2009 and 2015 were collected. Signalment, clinical findings, and clinicopathological results (complete blood count and biochemical profile) between the two groups and with a control group (only for Pisa cases) were compared statistically.

Results: The entire study group was composed mainly of middle-aged and elderly dogs. Neutered females were overrepresented, as well as the Cocker-Spaniel and the Maltese breeds. No seasonal predisposition was found. The comparison between the two groups (Pisa and Beit Dagan) shows: tachycardia, tachypnea and icterus were significantly more frequent in the Israeli group (P=0.017, 0.001 and 0.048 respectively); MCV, MCHC and RDW varied significantly (P= 0.004, 0.001 and 0.018 respectively) and so did the absolute lymphocyte count (P=0.001) and the total protein measurement (P=0.02).

Conclusions: This study of canine pIMHA from two different sites confirms the findings previously described in the literature in regards to the clinical and clinicopathological alterations occurring in this disorder even if different diagnostic approaches were noticed. The *Beit-Dagan* group was characterized by a more severe and acute onset of IMHA, with more evidence of intravascular hemolysis and a slightly lower Hct median. A significant correlation between NRBCs, band neutrophils and macrothrombocytes was evidenced that could be related to extra-medullary hemopoiesis. The occurrence of schistocytes and macrothrombocytes could help diagnosing a coagulation disorder. The review of the blood smear is valuable in diagnosing and monitoring of the disease.

Keywords: Immune mediated hemolytic anemia; Dog; Retrospective study; Clinical findings; Clinicopathological results; two veterinary teaching hospitals.

Riassunto

Anemia Emolitica Immuno-mediata Primaria nel Cane: Studio Retrospettivo di 52 Casi Osservati Presso Due Ospedali Didattici Veterinari (2009-2015)

Background. L'anemia emolitica immunomediata primaria (pIMHA) è la malattia immunoematologica più comune nei cani. Comporta la distruzione dei globuli rossi da parte del sistema immunitario con conseguente anemia da moderata a grave in molti pazienti. Nonostante sia stata frequentamente descrita in letteratura, solo pochi autori hanno sottolineato la vasta gamma di anomalie morfologiche degli eritrociti, leucociti e piastrine che possono essere presenti nel corso della malattia.

Animali. Questo studio retrospettivo ha indagato le alterazioni cliniche e clinico-patologiche presenti in cinquantadue casi di anemia emolitica immunomediata canina visitati tra gli anni 2009 e 2015. I casi sono stati raccolti in due ospedali didattici veterinari: l'Ospedale Didattico Veterinario "Mario Modenato" del Dipartimento di Scienze Veterinarie, Università di Pisa, Italia e l'Ospedale Didattico Veterinario al " Koret School of Veterinary Medicine", Università Ebraica di Gerusalemme, Israele.

Obiettivi. indagare i risultati clinici e clinico-patologici in due gruppi di cani con pIMHA, nonché per descrivere le alterazioni più comunemente osservate nello striscio di sangue periferico dei pazienti.

Risultati. Il nostro gruppo di studio è composto principalmente da cani di mezza età. Le femmine sterilizzate sono sovrarappresentate, così come la razza Cocker-Spaniel e la razza Maltese. Inoltre si è dimostrato che non esiste una predisposizione stagionale alla patologia. Tachicardia , tachipnea e ittero sono stati osservati in modo significativamente più frequente nel gruppo israeliano rispetto a quello italiano (P=0.017, 0.001 e 0.048 rispettivamente). Inoltre alcuni indici eritrocitari (MCV,MCHC e RDW) risultavano significativamente diversi tra il gruppo israeliano e quello italiano(P=0.004, 0.001 e 0.018 rispettivamente), cosi come la conta linfocitaria (P=0.001) e la misurazione delle proteine totali (P=0.02).

Conclusioni. Lo studio conferma i risultati descritti precedentamente in letteratura per quanto concerne il segnalamento e le alterazioni cliniche e clinico-patologiche di pIMHA anche se differenti approcci diagnostici sono stati osservati. Il gruppo di Beit Degan è caratterizzato da una osservazione in fase più acuta, con maggiore evidenza di casi di emolisi intravascolare e Hct medio più ridotto. Una correlazione significativa tra NRBC, neutrofili banda e macrotrombocitosi è stata osservata e potrebbe essere correlata ad una ematopoiesi extramidollare. La presenza di schistociti con macrotrombocitosi potrebbe facilitare la diagosi di disordini coagulativi. La valutazione dello striscio ematico è utile nella diagnosi e monitoraggio della patologia.

Parole chiave:anemia emolitica immunomediata; cane; casi retrospettivi; riscontri clinici; riscontri clinico-patologici; due ospedali didattici veterinari.

Introduction

The term anemia indicates a pathologic condition characterized by a decreased concentration of hemoglobin (Hb) in blood and/or a decreased number of erythrocytes (RBC) and/or decreased hematocrit (Ht). Anemia can arise in one of two mechanisms; decreased RBC production, which leads to non regenerative anemia or decreased life-span of erythrocytes. The latter provokes a regenerative response with release of reticulocytes from bone marrow in attempt to restore the RBC mass. Reduced RBC life-span due to hemolysis is a common occurrence in small animal veterinary clinical practice. Hemolysis can originate from various etiologies including infective (e.g. *Clostridium* spp., *Leptospira* spp., *Babesia* spp.), toxic (e.g. consumption of onions leading to Heinz Body anemia, zinc intoxication and snake venom), mechanical damage (e.g. microangiopatic hemolytic anemia, vena-caval syndrome) and metabolic disorders (e.g. hypophosphatemia, pyruvate kinase deficiency), to name a few[1]. Nevertheless, the most common hemolytic anemia in dogs is the immune mediated hemolytic anemia (IMHA). In most cases canine IMHA is considered to be auto-immune [2] (idiopathic or primary).

Auto-immune diseases represent some of the most frustrating types of disorders that are diagnosed and treated in veterinary medicine. Given the nature of these diseases, in which an abnormal immune response against the body's own cells arise, treatment is difficult and necessarily includes suppression of immune defenses[3].

Primary IMHA (pIMHA) is a severe disease with high mortality rates, mainly in the first two weeks that follow its presentation[4]. Due to its complex pathophysiology and the numerous components that take part in the pathological mechanisms that cause it, pIMHA has been the subject of numerous studies, in veterinary and human medicine alike.

The first part of this work is aimed to thoroughly review the pathological mechanisms, diagnostic approaches, treatment options and outcome prediction scales described by the scientific veterinary

literature. Some references from human hematology literature were used. These references were used for means of comparison to veterinary medicine with the intra-species variation in mind.

The second part of this work includes a retrospective study of fifty-two cases of canine primary immune mediated hemolytic anemia. Cases were collected from two veterinary teaching hospitals in two different countries; Italy and Israel. The goals of this study were to analyze the various clinical signs, symptoms and clinicopathological alterations of this complex disease and to compare them between the two hospitals and the corresponding findings described in veterinary scientific literature.

Chapter two: Definition and Patient Profiling

2.1 Definition

Immune-mediated hemolytic anemia (IMHA) is the most common severe anemia of dogs and is less frequent in cats[5]. The majority of cases (at least in North America, Northern Europe and Australia) are currently considered to be primary IMHA[6]. It is also reported to be the most common immune-mediated disease of dogs [4].

IMHA involves the destruction of red blood cells via type II hypersensitivity (antibody-dependent cytotoxicity), and may arise the following:

1. binding of autoantibody to a structural component of the erythrocyte membrane in the absence of an underlying disease (Primary idiopathic IMHA)[7]. This type of anemia is also known as Auto Immune Hemolytic Anemia (AIHA) – a term currently used almost exclusively in human medicine since that in dogs, it is virtually impossible to exclude with certainty the presence of a precipitating factor that could have triggered or caused the disease. In Humans, a much lower percentage of cases of AIHA are idiopathic (25 to 30% compared with 60 to 75% in dogs)[8]. This reinforces the importance of thoroughly investigating canine cases for an underlying cause which may be of therapeutic and prognostic value[9].

2. Binding of cross-reactive antibody, i.e., antibody with specificity for a drug or infectious agent, or for membrane antigens exposed or modified by such agents (secondary IMHA) [7].

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2.2 Classification of IMHA

Immune anemias are classified on the basis of mechanism or association. Serologically there are two types: warm-antibody type with maximal activity at 37°C and cold-antibody type with maximal activity at 2-4°C.

Classification by association is based on the identification of an underlying cause and includes primary, idiopathic immune hemolytic anemia (pIMHA) and secondary immune hemolytic anemia[10].

Secondary IMHA occurs when there is an underlying reason for the attachment of immunoglobulin to RBCs. For example, IMHA may occur as a secondary phenomenon in neoplastic disease or when the antibody has affinity for an infectious agent or drug that is associated with the RBC surface. In these latter cases, the RBC destruction is due to - "bystander hemolysis " as the causative antibody is not specific for the RBC itself [6].

In dogs, there are many conditions that can cause secondary IMHA; blood parasites such as *Ehrlichia spp* and *Babesia spp*, lymphoid malignancies [11](such as lymphoma and myeloproliferative disease), erythroid malignancies (such as heamangiosarcoma [6]) and poorly differentiated malignancies [12]. Some drugs such as Cephalosporins [13], Carprofen [14] and others, may also be considered as a cause for secondary IMHA.

The most controversial association is the one proposed between the development of canine IMHA and vaccination in the immediately preceding 4 - week period. Anecdotal evidence for this association has been available for some time;

A pivotal study by Duval and Giger provided evidence for a temporal relationship of vaccine - associated IMHA in the dog[15]. However, a large epidemiologic survey that was subsequently conducted in the United Kingdom failed to support these findings. In this study, there was no clear evidence that dogs with Coombs' - positive IMHA were more likely to have been recently vaccinated. Overall, it appears that vaccine - associated IMHA does occur in dogs but with low incidence. The report of the United Kingdom Veterinary Products Committee provided specific data from a pharmaco-vigilance data base which suggested that vaccine - associated with IMHA was a relatively rare occurrence (incidence of 0.001 per 10,000 doses of vaccine sold). The immunological mechanisms by which these reactions develop are not understood and no particular vaccine has been implicated [6].

By contrast, in primary idiopathic IMHA there is no underlying disease or evidence of recent drug or vaccine administration, and the antibody is considered to be a true autoantibody with affinity for a self - antigen of the RBC membrane. Only this form of disease is true autoimmune hemolytic anemia (AIHA). This may occur as a single clinical entity, or may be recognized concurrently with primary immune - mediated thrombocytopenia (IMT; the combined disease is Evans' syndrome), primary immune - mediated neutropenia (IMN) or be part of the multi systemic autoimmune disease, i.e. systemic lupus erythematosus (SLE)[6,11]. Activation of auto-reactive lymphocytes is generally considered to reflect a failure of natural regulation (suppression) of such cells [6].

The onset of the disease might also be the result of a well documented immunological mechanism known as molecular mimicry. Molecular mimicry has been proposed as a pathogenic mechanism for autoimmune disease in humans as well as in animals. It is defined as similar structures shared by molecules from dissimilar genes or by their protein products. Either the molecules' linear amino acid sequences or their conformational fits may be shared, for example, by a virus and a normal host–self determinant. An immune response against the determinant shared by the host and the virus can evoke a tissue-specific immune response that is presumably capable of eliciting cell and tissue destruction. The probable mechanism is generation of cytotoxic cross-reactive effector lymphocytes or antibodies that recognize specific determinants on target cells [16]. In the latter case the anemia would still be considered primary.

2.3 Factors Predisposing to Canine Primary IMHA

A number of factors predisposing to the development of primary IMHA are defined in the dog. Domestic dogs have been actively line-bred for the past few hundred years to achieve extreme phenotypic variation between different pedigrees, but minimal genetic variation still remains between breeds. Such breeding programs have inadvertently resulted in differences in breed susceptibility to certain immune-mediated diseases, including IMHA, diabetes, hypothyroidism and Addison's disease. With the completion of the Dog Genome Assembly and initiation of ongoing breed genetic diversity projects, comparative genomic studies in

diseases, including IMHA, could provide important information into such complex genetic disorders.

There is a strong genetic influence suggested by the greater prevalence of the disease in particular breeds (e.g. Old English Sheep dog, Cocker Spaniel, Border Collie, Poodle, English Springer Spaniel, Irish Setter, Miniature Schnauzer) and within particular pedigrees. Studies have revealed associations with allotypes and haplotypes of genes of the major histocompatibility complex (MHC), which are strongest when specific breed groups are considered[6,17]. In one study the overall patient group was divided on the basis of individual breeds with more than six animals represented and each of the haplotypes could be shown to be implicated in one of the breeds. Thus, it is apparent that different breeds had different MHC associations with canine IMHA[18]. Some MHC haplotypes increase susceptibility to IMHA, whereas others appear to confer protection [18]. One report has suggested that expression of the blood group antigen DEA 7 in Cocker Spaniel dogs reduces susceptibility to IMHA [19].

2.4 Signalment and Clinical Presentation

Canine primary IMHA is generally a disease of middle-aged (6 – 8 years) dogs [6,7,17]. Although there is no *clear* gender predisposition, there is a slight overrepresentation of female neutered dogs[11,17]. The disease can also be precipitated in bitches by the stress of whelping or estrus[6,7]. Some studies suggest that intact male dogs might be less prone to the disease. The latter may indicate that androgens are protective in the face of the disease [20].

There are two main clinical presentations of canine IMHA:

Acute onset (1-2 days)

May be presented with severe intravascular hemolysis with jaundice, hemoglobinemia and hemoglobinuria (red or dark brown urine), As well as pyrexia and vomiting [6,7]. This form of the disease is less common.

Chronic onset (days or weeks)

May be presented with weakness, lethargy, exercise intolerance, anorexia, pyrexia, pallor of mucous membranes, tachypnoea, tachycardia, hepatosplenomegaly, lymphadenomegaly [6,7]. This is the more common form of the disease.

Hematological examination may reveal: Moderate to severe anemia (5-20 PCV); Marked reticulocytosis and polychromasia (3- 4+); gross or microscopic auto agglutination of blood and a moderate to severe spherocytosis [5]. Marked leukocytosis is typically present and is characterized by a strong neutrophilia [7], possibly in combination with monocytosis. The presence of band neutrophils has also been reported [8].

The definitive diagnostic test is the demonstration of erythrocyte-bound antibody and/or complement by flow-cytometry or Coombs' test [7]. A proportion of dogs with IMHA will subsequently develop disseminated intravascular coagulation and/or pulmonary thromboemboli.

Chapter three: Immunopathogenesis of Primary IMHA

3.1 Pathophysiology of Hemolysis

Anemia of hemolysis develops when the rate of destruction exceeds that of production. Since the products of hemoglobin catabolism are readily reutilized, the anemia of hemolysis is characteristically and predominantly highly responsive with reticulocytosis and an upward shift in mean corpuscular volume (MCV). It is stated that human marrow can increase output 6-fold in hemolytic anemia and cats and dogs have a similar capacity for regeneration.

Intracellular destruction of red cells is the normal route of removal and occurs in the monocyte-macrophage system in the bone marrow, spleen, liver and lymph nodes. In hemolytic states, the spleen may become quantitatively more important than marrow. The actual process of lysis is rapid and if erythrophagocytosis is observed, the level of destruction is likely also rapid. This catabolism releases iron from heme to transferrin or to storage as ferritin or hemosiderin, while the protoporphyrin ring is opened and released to the plasma as unconjugated bilirubin. Intravascular destruction of red cells occurs in normal circumstances at a very low rate as a result of high velocity trauma in small arteries. In some cases of accelerated hemolysis with complement fixation, red cells are lost by intravascular lysis at very rapid rates resulting in hemoglobinuria.

Adherence of antibody to the red cell membrane, with or without complement fixation, can cause red cells to undergo partial erythrophagocytosis which gives them the spherical shape.

Their lifespan is then reduced either by destruction by the monocyte - macrophage system or by intravascular hemolysis. Since the damage is usually to the most mature cells, most hemolytic anemias are highly responsive [6,10].

3.2 Auto - antibody Etiology

The etiology of most RBC auto-antibodies is not well understood. Given the association between IMHA and other autoimmune disorders, generalized immune system dysfunction likely plays a role. The relationship between pIMHA and lymphoproliferative disorders and other neoplasms likewise suggest generalized dysfunction of immune surveillance. The immune system has meny control points that keep a balance between the need to tolerate self-antigens and the need to appropriately respond to foreign antigens. Distruption of any of those processes may be a potential cause of auto-immune disease.

The variety of mechanisms that are involved in the control of self - reactive lymphocytes include:

(1) central deletion of T cells with such specificity, as part of intrathymic development and of auto- reactive B cells during development in the bone marrow;

(2) peripheral deletion of self - reactive T cells upon exposure to autoantigenic peptide outside of the thymus;

(3) the induction of anergy by exposure to autoantigen in the absence of delivery of costimulatory signals;

(4) immunological ignorance due to failure to present autoantigen to the autoreactive populations, and

(5) active suppression by regulatory lymphocytes.

Particular advances have been made recently with respect to understanding of the last component. A population of "natural suppressor" T cells defined by expression of CD4 and CD25 is thought to be constitutively present within the immune system in order to prevent the activation of pathogenic T cells specific for autoantigen or allergen. These "Treg" cells are further defined by expression of specific genes (e.g. Foxp3, GITR). Although Treg cells secrete the immunoregulatory cytokine IL – 10, they are thought to require direct contact

("cognate interaction") with the target cells they intend to regulate. Treg cells have now been identified in the dog [21] and it is likely that suboptimal function of this population occurs in canine autoimmunity. Other T cell types are also known to have suppressive function. Overall, T regulatory cells are involved in dampening an immune response after they have successfully tackled invading organisms as well as keeping in check immune responses that may potentially attack one's own tissues [6].

Another central player in maintaining peripheral tolerance is the dendritic cell (DC). The DC is a antigen-presenting cell that processes and presents antigen in the context of MHC I and II to naïve T cells. $CD4^+$ T cells that engage the MHC II/Ag complex may differentiate into T-Helper (Th) subtype in the presence of a co-stimulatory signal produced by the DC. Dendritic cells do not express the requisite co-stimulatory signal unless they have been activated by certain triggers, such as signaling through pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) and some cytokines including TNFα. In the absence of a co-stimulatory signal the T cell is rendered anergic or undergoes apoptosis. DCs also appear to direct the differentiation and proliferation of a subset of T regulatory cells known as inducible T regs. Signals produced during interaction of T cells with DC may induce differentiation into various T cell subtypes that mediate different responses in the immune system. Since activation of particular subsets is associated with immune-mediated diseases, the ability of DC to facilitate differentiation of certain T cell subtypes may be important for understanding the mechanisms involved in auto-immunity [9].

Less generalized autoimmune processes also exist. For example auto immune disease can arise from the response to a foreign antigen, if it shows sufficient homology with a self-antigen ("molecular mimicry"). Another theoretical source of auto-antibodies is a malignant B cell clone, but RBC auto-antibodies are generally polyclonal. On the basis of studies made with other human auto-immune disorders, such as ankylosing spondilitis and multiple sclerosis, as well as in veterinary auto-immune disorders, both genetic and environmental factors likely play a role in the production of RBC auto-antibodies [6,22].

In the course of Primary IMHA, both IgM and IgG autoantibodies are found, and particular subclasses (IgG1 and IgG4) dominate the IgG response [23]. Significant quantities of IgA may also be associated with the erythrocyte membrane in AIHA, but the presence of this immunoglobulin is of questionable relevance. The specificity of the IgG autoantibodies has

been characterized by eluting them from the surface of patient RBCs and incubating them with biotin - labeled normal canine RBC in the technique of immunoprecipitation. These autoantibodies are directed against various components including erythrocyte membrane glycophorins, the anion exchange molecule (band 3) and the cytoskeletal molecule spectrin[24,25]. Recent studies have identified reactivity to calpain, complement component 3, and peroxiredoxin 2 in some dogs with AIHA, but not in healthy dogs. These proteins are involved in oxidative stress and apoptosis (calpain), inflammation (complement), and scavenging of reactive oxygen species (peroxiredoxin 2). It remains to be determined if these proteins are important in initiating autoimmunity or if immunoglobulins targeting these proteins develop during IMHA[26].

3.3 Immunopathogenesis of pIMHA

Factors underlying the development of a true autoimmune response are complex, and even now, not entirely understood. As mentioned previously, the expression of any autoimmune disease requires that a combination of predisposing factors permit the development of an immunological alteration resulting in the observed autoimmune pathology. Such immunological alterations have been experimentally investigated using murine models of pIMHA. For example, pIMHA may be induced in particular inbred strains of mice that are immunized with rat RBCs, or that are transgenic for high expression of interleukin (IL) - 4, or expression of an RBC autoantibody. IL - 2 deficient mice, created by targeted disruption of the IL - 2 gene, develop a lymphoproliferative syndrome with multisystemic autoimmunity, including Coombs' positive hemolytic anemia. New Zealand black (NZB) mice spontaneously develop autoimmunity including IMHA or a SLE - like disease, characterized by immune complex glomerulonephritis and serum antinuclear antibody (ANA). The IMHA is mediated by CD4 + Th1 lymphocytes that are first activated in very young mice before the appearance of auto-antibodies and anemia. A strain of non - obese diabetic (NOD) mice that develop autoimmune destruction of pancreatic beta cells mediated by T cells, culminating in insulin - dependent diabetes mellitus, may also develop Coombs' - positive hemolytic anemia late in life. However, the immune mediated anemia in the NOD strain may occur in both diabetic and non - diabetic mice [6].

It is now also believed that microbial infection is of particular importance in the induction of autoimmune diseases. Clinically normal individuals have circulating lymphocytes that are

programmed to recognize self - antigens, but these cells are normally incapable of responding to autoantigens (self - tolerance). The altered immune regulation that follows infection may permit loss of self - tolerance and the subsequent expression of autoimmune disease. For example, mice infected with a particular substrain of the lymphocytic choriomeningitis virus develop transient IMHA. In this instance the anemia is not caused by antibodies that react with a shared epitope on the virus and RBC, but by true erythrocyte specific autoantibodies [27]. The induction of this autoimmune response is thought to be due to inappropriate activation of autoreactive T cells by virally - derived peptides that are " molecular mimics " of erythrocyte - derived peptides; these peptides would normally not be presented by the antigen presenting cells (APCs) of the immune system and thus, autoreactive T cells are maintained in a state of " immunological ignorance ". It is also possible that the presence of infectious agents or their molecular components (LPS from bacteria, RNA from virus etc.) facilitate T-cell activation by acting as costimulatory molecules during APC antigen presentation[9].

A series of investigations has revealed many parallels between canine pIMHA and the disease in humans and experimental rodent models. The autoantibodies that characterize the canine disease are heterogeneous in their class and specificity, suggesting that a range of different underlying mechanisms may be involved in triggering the disease. Both IgM and IgG autoantibodies are found, and particular subclasses (IgG1 and IgG4) dominate the IgG response [25]. Significant quantities of IgA may also be associated with the erythrocyte membrane in pIMHA, but the presence of this immunoglobulin is of questionable relevance.

T cell reactivity in canine pIMHA has also been examined. Like other species, clinically normal dogs harbour RBC - reactive lymphocytes that can be induced to proliferate in vitro when challenged with RBC - derived antigens. Such cells have a greater degree of reactivity when they are obtained from dogs recovered from pIMHA (memory lymphocytes) or from normal dogs that are closely related to pIMHA cases[28]. The latter observation suggests an immunological mechanism for genetic susceptibility to pIMHA in dogs. It is important to further investigate the fine specificity of such autoimmune responses, as this knowledge will form the basis for developing novel immuno therapeutic agents in future years.

3.4 Mechanisms of immune destruction of red blood cells

In patients with pIMHA there is strong evidence that the coating of auto antibodies per se, does not damage the red cells but causes erythrocyte destruction by complement activation and/or by inducing interactions with cells of the mononuclear phagocyte system (opsonization). Erythrocyte destruction may be extra- or intravascular. In most patients, hemolysis is extravascular and involves mononuclear phagocytes reacting with erythrocytes coated with antibodies of lgG, IgM class and with C3b. IgA may also be associated with the erythrocyte membrane, but as mentioned before this antibodies don't seem to be involved in the pathological process [6]. Destruction of IgG coated cells usually occurs in the spleen, but when the coating is heavy it may occur anywhere in the mononuclear phagocyte system. Less frequently, destruction occurs intravascularly, either through the activation of complement, or more controversially, through the erythrocytes interacting with lymphoid and granulocytic cells [29].

3.4.4 Intravascular Destruction by Complement

Intravascular hemolysis is seen in less than 20% of patients with pIMHA; it occurs if complement activation proceeds to completion, when the resultant defect in the erythrocyte membrane causes osmotic lysis of the cell. Deposition of the terminal C5b-9 complex on erythrocytes of patients with AIHA has been demonstrated using a radioisotopic method. Antibodies which trigger this process are called hemolysins; most are of IgM class though some are lgG. Complement-induced lysis is the most efficient method of red cell destruction and causes severe anemia; fortunately it is uncommon since the in vivo activity of hemolysins is restricted by their optimal temperature of activity being well below 37ºC. Hemolysis is never complete since regulatory inactivators operate at several levels of the complement cascade. In patients with pIMHA, inhibition occurs at the level of C3, however decrease in complement regulatory proteins that protect host cells from complement-mediated destruction has been shown in people with IMHA[9]. Erythrocytes coated with C3b can then be destroyed extravascularly by cells of the mononuclear phagocyte system [29].

3.4.5 Erythrocyte Mononuclear Phagocyte Interactions

Erythrocyte-mononuclear phagocyte interactions in patients with AIHA are of particular interest. Their efficiency depends on several factors including the immunoglobulin class and subclass of the autoantibody, the number of antibody molecules bound per erythrocyte, the thermal range of the antibody, the ability of the antibody to activate complement, the amount of free IgG present in the surrounding medium and probably on the activity of the individual's macrophages. In most patients, the erythrocytes are coated with non - complement fixing IgG auto antibodies which induce red cell destruction by the attachment of the Fc portion of the IgG molecule to the macrophage surface at specific receptor sites. This adherence leads to erythrocyte membrane damage by ADCC (antibody dependent cell-mediated cytotoxicity) and to erythrophagocytosis. Whether ADCC or phagocytosis is the prime mechanism of erythrocyte destruction remains to be determined, though both may cause significant hemolysis in vivo. The amount of antibody bound to the cell seems to be important: using human blood monocytes and erythrocytes coated with human anti-Rhesus (Rh) D, it has been shown that the amount of phagocytosis is inversely proportional to the degree of sensitization. At low levels of sensitization, phagocytosis becomes more important than ADCC; the level at which this occurs is much lower with lgG4 than with lgG1 antibodies. Adherence to macrophages may also result in partial engulfment of the erythrocyte, leaving a non-phagocytosed portion to become a rigid spherocyte, which is prematurely destroyed in the spleen. In vivo, splenic macrophages and Kupffer cells are the main effectors of red cell destruction, the blood monocytes appearing to play only a minor role. While IgG-coated cells are usually destroyed in the spleen, in situations where erythrocytes become coated with C3b alone, destruction (by phagocytosis and through the production of spherocytes) occurs mostly in the liver [6,29].

Chapter four: Clinical Aspects of Primary IMHA

4.1 History and Common Owner Complaints

Primary IMHA can be presented either as an acute hemolytic crisis or, more commonly, as a chronic mild onset, with a protracted (days to weeks) history of symptoms [6,7]. Most authors describe the three most common symptoms as: weakness, anorexia and lethargy [2,6,11,20]. These appear in 80-95% of the cases but have very little diagnostic value being highly non-specific. Other symptoms frequently observed by owners are: pigmenturia – red to dark brown colored urine in 44% of cases, vomiting (30%), diarrhea (15%), hemorragic diathesis (15%) and syncopes (3%), based on Piek et al. extensive 2008 study [2]. A thorough and accurate history and anamnesis is recommended in order to identify as much as possible a generating cause for the hemolysis such as tick borne disease or toxin exposure [9].

4.2 Seasonal Incidence of Primary IMHA

Studies have reported conflicting results regarding the seasonal incidence of IMHA, with some reporting a higher incidence in warmer months[11,15,30] and others showing no association[20]. Suggested reasons for the apparent association include the effect of environmental temperature on immune responses, greater risk of dehydration or respiratory distress in warmer months, and the potential effect of an undetected infectious agent, such as Ehrlichia or Babesia, which occur more frequently during warm season[4].

4.3 Physical Examination Findings

Physical examination discloses pale mucosae membrane in 98% of the patients. Pettechiae may be occasionally present as well, on the mucosae or epithelial membranes. This findings may indicate an additional, coexistent condition of thrombocytopenia (Evans' Syndrome). Other regularly observed clinical signs in IMHA patients are: increased body temperature, icterus and abdominal cranial organomegaly in seen in 46, 38 and 34 percent of patients respectively [2]. Moreover, some dogs are presented with tachypnoea, tachycardia and

generalized lymphadenopathy. The latter being more common in the typical chronic onset of the disease [6,7,11,20]. Some dogs with primary IMHA develop disseminated intravascular coagulation (DIC) or pulmonary thromboembolism, the effects and clinical signs of these conditions will be discussed in chapter 5.

4.4 Heamatological and Clinicaopathological Alterations

The hematological profile for primary IMHA is characterized by a moderate to severe macrocytic, hypochromic anemia, accompanied by a presence of spherocytes and/or auto agglutination. The hematocrit (Hct) percentage may range between 4-35% [2], with a most frequently reported median Hct of 15-13% [2,20,31]. The authors have also reported that 80-90% of the dogs had an Hct percentage of less than 20. Mild to marked erythroid regeneration at initial presentation has been demonstrated in two thirds of the patients [2,11,20,31]. As stated before the most common poikilocytosis in pIMHA dogs is spherocytosis, which is reported in 70-90% of cases. The unique form of the spherocyte is due to partial loss of cell membrane in the process of extravascular erythrophagocytosis. Spherocytosis can also be diagnosed using the osmotic fragility test. The increased osmotic fragility reflects the fact that the surface membrane of the erythrocyte is reduced in size relatively to its volume, with the result that its capacity to swell before reaching the rupture point is reduced [32]. Furthermore, reticulocytosis, polychromasia, anisocytosis, and nucleated RBCs are consistent features of pIMHA in the morphologic erythrocyte alterations [6].

Auto agglutination in pIMHA patients is described by many authors [2,7,15,33]. In one retrospective study 78% of pIMHA had evident auto agglutination on blood smear [20]. True auto agglutination may be grossly distinguished from rouleaux formation by adding an equal volume of saline to blood (two drops each). Rouleaux will be dispersed by this procedure but agglutination will persist. It is often said that a positive "in - saline slide autoagglutination test" provides definitive evidence for IMHA and precludes the need for a Coombs' test [6].

The majority of pIMHA dogs exhibit marked neutrophilyc leukocytosis with left shift at time of diagnosis [2,6,20], The mechanism by which this arises is thought to involve tissue necrosis and the effect of pro-inflammatory cytokine (e.g. IL - 1, IL - 6 and TNF - α) production by activated macrophages on granulopoiesis. A minor percentage are presented with normal

leukocyte count, and seldom cases even with leukopenia [34]. Monocytosis has been reported as well[1].

Thrombocytopenia can develop in dogs with IMHA because of consumptive coagulopathy resulting from disseminated intravascular coagulation, concurrent immune-mediated platelet destruction, splenic sequestration of thrombocytes or failure of platelet production (immune-mediated or chemotherapeutic toxicities)[6,20]. 67-85% of patients show some degree of thrombocytopenia[2,11] (<150x10^3/L), 45% of which may be severe thrombocytopenia (<50x10^3 /L), according to one study[2].

Other alterations commonly observed regard the coagulation profile of the patients. Dogs with IMHA may have a range of hemostatic abnormalities including prolonged prothrombin time and activated partial thromboplastin time, elevation of fibrin degradation products, elevation in D-dimer, decrease in anti-thrombin activity[17,35], as well as activation of circulating platelets as assessed by increased expression of membrane P - selectin[36].

Alterations in the biochemical profile often include:

C- reactive protein increase. Recent study conducted using 28 dogs with pIMHA had found that serum CRP concentration is markedly increased in dogs with primary IMHA in comparison to healthy dogs. CRP concentration did not differ based on patient survival, but might be a marker for long-term monitoring of these patients [37].

Pre-hepatic, hemolysis induced hyperbilirubinemia is very commonly encountered. It is due to the fact that the lifespan of red blood cells is greatly reduced, and the rate of hemoglobin degradation is therefore increased. Furthermore, studies show that the bilirubin clearance by the liver is moderately defective in hemolytic disease, as a result of increased metabolic demand in combination of hypoxic centrizonal necrosis [38]. The hyperbilirubinemia is consistent with the clinical findings of icterus in up to 50% of patients [11]. In addition, hemoglobinemia, hemoglobinuria and bilirubinuria are recurrently reported [2,6,11,20]. Mild to moderately high levels of serum alkaline phosphatase (ALP) and alanine aminotransferase (ALT) have also been described[6,20] and may be a result of hypoxic damage to the liver as well as enzymatic induction by endogenous or exogenous corticosteroids.

Creatine kinase (CK) increase is not rare in pIMHA dogs, probably as a consequence of the hypoxic damage to the tissues containing the enzyme (skeletal muscles, myocardium, brain and intestine)[39], muscle injury secondary to impaired perfusion, thromboembolic complications (including embolic myopathy), repeated IV catheter placement (thrombosis in catheters), and SC or IM administered medications. Nonetheless it should be considered as a partial pre-analytic artifact, since the hemolysis causes a release of adenylate kinase from the red blood cells that could interfere with the CK analytical quantification[40].

Hypoalbuminemia is yet another common alteration in the course of the disease[6,20]. It is probably due to reduced hepatic protein synthesis during a generalized inflammatory state; a negative acute-phase response with down-regulation of albumin synthesis in coordination with up-regulation of inflammatory cytokines. Other causes may be proteinuria, enteric protein loss, vascular leakage, or hemorrhagic loss. Hypokalemia in pIMHA dogs has been described by some authors [6,20] and might be caused by the prolonged anorexia and/or diarrhea (if present). Nevertheless, it is the most common electrolyte abnormality in clinical practice seen in 20% of hospitalized human patients [41], therefore having minor specificity to pIMHA hematological alterations.

4.5 Diagnosis of Primary IMHA

Anemia is a common finding in small animal practice; however, the multitude of potential causes can make determining the underlying diagnosis challenging. The diagnosis of IMHA proceeds through a series of laboratory tests after identification of compatible clinical history and presenting signs. An EDTA - anticoagulated blood sample should be collected for hematological examination. This should be examined for the phenomenon of auto agglutination that may be observed by rotating the collection tube, or by placing a drop of blood on a microscope slide. Auto agglutination may only occur at 4° C, so blood should be also refrigerated before making this assessment [6].

Blood smear examination provides instantaneous information about the nature of the anemia through observation of RBC morphology. Additionally, many erythrocyte abnormalities that cannot be detected by other tests, may be revealed on a blood smear. While examining a blood sample of a patient with symptoms compatible with pIMHA, one should examine morphological abnormalities as well as erythrocyte regeneration indicators. Spherocytes appear smaller than normal RBCs and lack central pallor. In dogs, the presence of spherocytes and auto agglutination is highly suggestive of IMHA[42]. Regenerative anemia indicates that the

bone marrow is able to respond to the anemia appropriately and that the likely cause of anemia is blood loss or hemolysis. The most accurate measurement would be the degree of reticulocytosis detected in the patients' blood. Reticulocytes are red blood cells that have been released from the bone marrow before complete extrusion of all RNA and cellular organelles. They can be demonstrated in a blood smear using supravital stains such as new methylene blue or brilliant cresyl blue. These stains precipitate the RNA in the reticulocyte, staining it blue. Reticulocytes stained with new methylene blue appear as erythrocytes with blue granules. Reticulocytes are generally not observed for 2 to 4 days after an acute episode of blood loss or hemolysis. In dogs, the reticulocyte response generally peaks between 4 and 7 days after the insult and starts to decline at 2 to 3 weeks - assuming the underlying cause of the anemia has been treated. A reticulocyte count can be performed by incubating a small amount of EDTA-anticoagulated blood with an equal amount of a supravital stain such as new methylene blue for 10-15 minutes before making a blood smear slide. The number of reticulocytes seen per 1000 RBCs is then counted. A high-power field (40× objective) in which the RBCs are touching or just overlapping contains approximately 200 cells. The number of reticulocytes counted in five such fields is divided by 10 to yield the reticulocyte percentage (number of reticulocytes per 100 RBCs)[42]. A corrected reticulocyte count for the degree of anemia is calculated by multiplying the reticulocyte percentage expressed as a percentage by the ratio of the patient's PCV to the average normal PCV for the species. Additional changes in the RBC indices with regenerative anemia may include macrocytosis (high MCV), heterogeneous cell volume (high RDW), and hypochromasia (low MCHC). The decreased MCHC reflects normal hemoglobin content in a larger-than-normal cell. On blood smear evaluation, RBC morphology shows varying amounts of anisocytosis (variation in volume) and macrocytosis (large cells). Nucleated RBC (meta - rubricytes or late rubricytes) may also be noted with accelerated erythropoiesis. Polychromatophils are immature RBCs that stain blue-purple on Wright-stained blood smears. Polychromasia and reticulocytosis are the hallmarks of regenerative anemia that is a consistent features in pIMHA blood examinations[6,42]. The absence of a regenerative response after 5 days suggests the possibility of bone marrow disease and warrants collection of a bone marrow aspirate and/or core biopsy. Platelet numbers will be adequate in primary IMHA, but significant reduction in platelet count may indicate concurrent IMT, thromboembolism or DIC[42] that are a common complication of the disease.

4.5.1 Coombs' Test

The Coombs' test, also known as the antiglobulin test, is used to detect antibody and complement on the surface of red blood cells (RBCs). The test was previously considered as the gold standard for diagnosis of IMHA, and was recently replaced by flow cytometry. Antibodies directed against the RBC surface are often consisted of incomplete fragments that sensitize or coat RBC but fail to agglutinate them without addition of potentiators to alter the RBC zeta potential or without the addition of antiglobulin reagent. Most incomplete antibodies are IgG and are thus detected by anti - IgG serum (as contained in anti- globulin reagent). A few IgM antibodies also can be incomplete. These incomplete antibodies produce antibody - dependent complement activation; thus antiglobulin reagents are also designed to react with complement components on the red cell surface, with the main components detected being C4 and C3. Polyspecific reagents for veterinary use typically contain anti - IgG, anti - IgM, and anti - C3. Monospecific reagents are also available.

Two forms of the antiglobulin test are used in veterinary medicine: the direct antiglobulin test (DAT) and the indirect antiglobulin test (IAT). The DAT, that detects immunoglobulin and/or complement bound directly to RBC, is used to evaluate IMHA patients. The IAT detects the presence of unbound antibody in the serum and is not typically used in IMHA cases. In this procedure, the addition of antiglobulin reagent to the RBC with bound immunoglobulin or complement causes agglutination to occur.

Nonetheless, the DAT test is a direct assay which is relatively insensitive and at best provides only semi-quantitative information on the degree of RBC antibody binding. A negative Coombs' test does not rule out IMHA. Factors that may reduce sensitivity of the assay and cause false - negative results include insufficient quantity of antibody or complement on erythrocytes, improper antiglobulin to antibody ratio, not incorporating the drug into the test that causes a cross-reacting immune response to the drug-red blood cell complex, previous corticosteroid treatment greater than 1 week in duration, and improper or warm temperatures that disperse cold agglutinins [43]. Additionally, there will be variations in the methodology used by different laboratories. A full Coombs' test will be performed using a polyvalent Coombs' reagent (that recognizes IgG, IgM and complement C3), but also with antisera specific for each of these immunoreactants alone. The read - out for the test is erythrocyte agglutination, and the titer of each positive reaction should be determined. The incidence of

false negative reactions is greatly reduced when the full test is performed in this manner [6,44]. Interestingly, positive results in the absence of IMHA can also occur. One study described a high incidence of C3b bound to RBC in dogs with infections, inflammatory disorders, myeloproliferative and lymphoproliferative diseases. Hence, the positive DAT can be useful in dogs with signs of hemolytic disease, but needs to be interpreted with caution in other conditions[6].

In general terms, two broad patterns of Coombs' reactivity are identified and these have some correlation with clinical presentation. The most commonly recorded pattern involves an IgG antibody that may be present with IgM and/or C3, and that reacts equally at 4° C and 37° C. This pattern frequently correlates with disease of chronic onset and is compatible with extravascular RBC removal. The second pattern which manifests C3b and IgM reactivity has been reported with higher frequency in secondary IMHA. IgM reactivity at 4°C was also more often seen in the dogs with secondary IMHA, yet clinical significance of cold agglutinating antibodies is widely debated. There is a belief that they are less significant as they are rarely active at body temperature, but few studies have investigated the complete temperature gradient of the reactivity of these immunoglobulins [45]. The latter pattern of Coombs' reactivity is more often associated with sample auto agglutination, intravascular hemolysis and acute onset, severe clinical disease. Although low titers or the presence of complement alone on the RBC surface are often thought to be more consistent with secondary IMHA than pIMHA, a recent study has not confirmed this [46].

4.5.2 Direct Immunoluorescence Flow Cytometry

Direct immunofluorescence flow cytometry (DIFC) can be used to detect anti-RBC antibodies and it is considered by some authors to be more sensitive and quantitative assay for the detection of anti-RBC antibodies than the Coombs' test [47]. The use of flow cytometry to detect anti-RBC antibodies was reported previously in 2 studies conducted on a small number of dogs with IMHA. The majority of dogs in those studies had IgG antibodies, whereas a few animals had only IgM antibodies[43,48]. In another study, flow cytometry was also compared with the Coombs' test and found it to be more sensitive in dogs with IMHA (100% versus 58%). In contrast, the Coombs' test had better specificity than the DIFC assay (100% versus 87.5%, respectively)[43]. Therefore, DIFC is currently the most frequently used test for the diagnosis of IMHA.

Flow cytometry measures specific characteristics of cells as they flow through a flow chamber, and through the focused beam of a laser. The cells can be fluorescently labeled, typically with a fluorescently conjugated antibody, or can be analyzed unlabeled. When labeled cells pass through the laser, the laser light activates the fluorophore at the excitation wavelength, and the emitted fluorescence as well as the light scattering properties of each cell are detected. The intensity of the emitted light is directly proportional to the antigen density of the cell. More specifically, using this technique for the detection of IgG, IgM fragments and components of complement (C3) on the cell's surface, RBCs are washed and incubated with fluorescein isothiocyanate (FITC) - labeled sheep - antidog IgG, FITC - labeled goat - antidog IgM, or FITC - labeled goat - antidog C3. Cells are then re-washed, re-suspended and fluorescence is analyzed in a flow cytometer [6,44]. The main disadvantage of this test is to necessitate the availability of a flow cytometer in the laboratory[6,48,49].

4.5.3 Gel Microcolumn

A recent innovation is the development of a tube - based test designed for in - practice use. The test is presented in the form of a small plastic card within which there are six columnar tubes with an over- lying reservoir of wider diameter. The tube contains a gel matrix impregnated with rabbit polyvalent DAT- reagent specific for either dog or cat. Separate cards comprised tubes containing only the gel matrix without antibody are used as a negative control. Using the diluent supplied by the manufacturer a 0.8% RBC suspension is prepared and 50 µl of this suspension is pipetted into the reservoir above the gel. The card is then centrifuged in a purpose-designed centrifuge. Following centrifugation, the tubes are examined to determine the distribution of the RBCs. Red blood cell agglutinates become trapped and remain stable in the gel, while free RBCs pass through and form a button at the bottom of the tube [45]. In the negative test the cells are pelleted at the bottom of the tube. Where cells were distributed within the cell matrix the test result is considered as weak positive. Where cells are retained at the top of gel the test it is regarded as positive. A study conducted in 2012 on 247 canine patients had found that the gel test identifies fewer positive samples than the DAT. The positive DAT samples that were missed by the gel test (n = 27) were mainly from dogs with secondary IMHA (n = 20). Only three cases of idiopathic IMHA were missed. Since the gel test is faster and more easily performed than the conventional DAT it might be used as a screening test for IMHA, but if negative, IMHA cannot be excluded and an extended DAT should be performed [45]. Similar results were reported by other

authors [50], but unfortunately for the time being the Gel is not commercially available for dogs [45,50].

4.5.4 Immunochromatographic Strip

The Strip is an innovative and entirely new approach to immunohematology, which has already proven invaluable for canine and feline blood typing [51] and is being developed for in-clinic or laboratory DAT by the same manufacturer (QUICK TEST DAT©**,** Alvedia)**.** It utilizes an immunochromatographic strip with impregnated reagent M to bind antiglobulin-coated RBCs as they diffuse to the top of the strip. It is therefore, a non-agglutination-based test. A recent study had compared the Strip result to other DAT methods [50], in the author's experience, the Strip was easy to perform, but the resulting band strengths were frequently weak, which could make interpretation a little difficult. The Strip results, which included the weak bands, correlated well with those of other DAT methods and thus can readily be used as an in-clinic screening test.

4.6 Coagulopathy - A Common Complication of IMHA

Thromboembolic disease is a major concern affecting survival in dogs with IMHA. It is likely that hemolysis contributes to the prothrombotic state. Thrombosis occurs in both veins and arteries, with pulmonary thromboembolism (a venous thrombus) occurring very commonly [52]. The majority of dogs with idiopathic IMHA are in a hypercoagulable state at the time of diagnosis. A recent study used recalcified unactivated thromboelastography (TEG) and other coagulation assays over a 5 day period to assess the coagulation status of 30 canine patient diagnosed with IMHA [24]. Based on TEG, dogs with IMHA were significantly hypercoagulable vs. controls and over the 5 day period, 3/4 of the TEG parameters reflected progressively increased clotting kinetics. The 30 day survival of these patients was 80% and, at hospital admission, the TEG maximum amplitude (MA) was significantly higher in survivors than non-survivors. Relative hypocoagulability identified by TEG at initial assessment was found to be a negative prognostic indicator [24].

To adequately understand the prothrombotic mechanisms involved in IMHA hypercoagulability state, one should first reflect the complex physiological process of hemostasis.

Physiologic hemostasis

Primary hemostasis refers to the formation of the platelet plug, while secondary hemostasis refers to activation of the coagulation cascade and the formation of a fibrin network. During normal hemostasis, activation of coagulation and platelets occurs simultaneously. To consent proper hemostasis, a platelet-fibrin clot is formed via 3 overlapping phases, initiation, amplification, and propagation. Endothelial damage initiates the formation of a platelet plug through binding of platelets to subendothelial collagen, which is facilitated by vonWillebrand factor (vWf). Tissue factor (TF) within the blood vessel wall simultaneously activates the coagulation protease cascade. The extrinsic cascade is comprised of TF and factor VIIa. The extrinsic cascade initiates coagulation during normal hemostasis and in many prothrombotic

states. Tissue factor is normally absent from the vascular space, being expressed by cells surrounding blood vessels such as pericytes and subendothelial fibroblasts. The initiation phase of coagulation is localized to TF-bearing surfaces. During this phase, coagulation is initiated by exposure of TF to plasma due to endothelial damage, or expression of TF on the surface of activated endothelial cells, monocytes, or microparticles. Circulating microparticles are derived from cell membranes of RBCs, platelets, megakaryocytes, endothelial cells, neutrophils, and monocytes. They express cell surface molecules that are derived from their cell of origin, and are able to interact with, and induce cell signaling in other cell types, including the endothelium. Evidence suggests that activated platelets release phosphatidylserine (PS) exposing microparticles. Microparticles derived from monocytes and endothelial cells and possibly platelets also express TF. Regardless of its source, exposure of TF to plasma factor VII/VIIa initiates coagulation and results in the production of a small amount of thrombin (also referred to as factor IIa). The amplification phase of coagulation occurs mainly on platelets. Thrombin activates platelets, and platelet-associated factor V. Factor Va acts as a cofactor for factor Xa. Together they form the prothrombinase complex that converts prothrombin to thrombin with the result of the production of more thrombin.

The intrinsic pathway consists of high-molecular-weight kininogen, prekallikrien, and the serine proteases factor XII, factor XI, factor IX, and factor VIII. Thrombin activates factor VIII and factor XI. The propagation phase is driven by thrombin activation of the intrinsic pathway downstream of factor XII, and is thought to occur primarily through thrombin-induced activation and formation of the tenase complex (factors VIIIa- IXa) and factor XIa. The TF-factor VIIa complex also activates the intrinsic cascade through activation of factor IX. Formation of the tenase complex, and subsequent further activation of factor X and V, results in further generation of thrombin. Large amounts of thrombin are produced in the propagation phase. Thrombin catalyzes the conversion of fibrinogen to fibrin, and activates the transglutaminase factor XIII which then cross-links fibrin and stabilizes the clot. In addition to its role in the propagation of the coagulation cascade and formation of fibrin, thrombin is a potent activator of platelets and endothelial cells via cleavage of protease-activated receptors. Platelets and other cells release microparticles that enhance clotting by providing a membrane surface for the assembly of the prothrombinase and tenase complexes. Thus, platelets and the clotting cascade work together in the generation of a blood clot.

The major inhibitor of the extrinsic pathway is tissue factor pathway inhibitor (TFPI). TFPI is expressed by endothelium and binds to its surface. There are also small amounts of TFPI in

the circulation. Another anticoagulant expressed by activated endothelium is thrombomodulin. When thrombomodulin binds thrombin, its substrate specificity changes and it becomes an anticoagulant protein by activating protein C. Activated protein C with its cofactor, protein S, cleaves and inactivates factors Va and VIIIa. Antithrombin (AT, formerly antithrombin III) inhibits factors Xa, IIa, VIIa, IXa, XIa, and XIIa. The activity of AT is dramatically increased after binding heparan sulfate, which is expressed on the surface of endothelial cells. Fibrinolysis occurs gradually after clot formation. Activated endothelium and monocytes produce tissue plasminogen activator (tPA), which converts plasminogen to plasmin. Annexin A2 on the surface of endothelial cells facilitates the localization of tPA and plasminogen in close proximity to each other. Tissue plasminogen activator activity is dramatically increased for plasminogen bound to fibrin, thus localizing plasmin production to the region of a blood clot. Plasmin is an endopeptidase that cleaves fibrin which destabilizes the clot and results in the production of fibrin degradation products. Plasmin also inactivates factors V, VIII, IX, and XI, cleaves complement component C3, enhances conversion of factor XII to XIIa and conversion of prekallikrein to kallikrein. Inhibitors of plasmin generation and activity include plasminogen activator inhibitors, α - 2 antiplasmin, α - 2 macroglobulin and other protease inhibitors. Increased procoagulant, decreased anticoagulant, and impaired fibrinolytc activity may shift the hemostatic balance toward thrombosis [52].

4.6.2 The Pathophysiology of Thrombosis in IMHA

Thrombosis is defined as the pathologic formation of a blood clot inside a blood vessel. Thrombosis can occur in either arteries or veins. Importantly, the pathogenesis of the generation of an arterial or venous thrombus differs. Arterial thrombi form primarily as a consequence of platelet activation under high blood flow conditions in arteries and arterioles, and are described as “platelet rich.” Venous thrombi form under low blood flow in veins and venules and are fibrin rich due to the activation of coagulation. Pulmonary thromboembolism (PTE), thought to occur due to the release of venous emboli, is very common in dogs with IMHA[52]. In addition, IMHA is a common underlying disease in dogs with cranial vena cava thrombosis. In recent studies, 80 – 100% of necropsies performed on dogs who died from IMHA revealed evidence of thromboembolism [17,20] . Thromboemboli were located in the lungs, heart, liver, spleen, kidney, lymph nodes and pituitary gland. In many dogs, emboli were found in multiple organs[17]. Thus it appears that IMHA may cause generalized

thromboembolic disease of both veins and arteries, with PTE (a venous thrombus) being a very common clinical manifestation of thrombosis.

The presence of both venous and arterial thrombosis suggests that disregulation of both coagulation and platelets occurs in dogs with IMHA. Indeed, dogs with IMHA exhibit excessive platelet activation [36,53] and activation of coagulation is evidenced by the common findings of decreased AT, thrombocytopenia, prolongation of activated partial thromboplastin time (APTT) and prothrombin time (PT), elevated D-dimers [17,20,24,54] and the finding of widespread fibrin deposition at necropsy. Many dogs with IMHA meet the clinical criteria for disseminated intravascular coagulation (DIC). Liberation of RBC stroma may be one trigger for DIC during hemolysis[17]. Interestingly, the presence of DIC was not a risk factor for thrombosis in one study [17]. However, prolongation of PT, suggesting consumption of factors in the extrinsic pathway, is associated with mortality [55].

The convoluted mechanism that leads to hypercoagulability in IMHA dogs was recently described by Kidd and Mackman [52]. According to the authors, the extrinsic pathway plays a role in the formation of thromboemboli. Increased tissue factor (TF) expression may initiate intravascular coagulation in pathologic states associated with thrombus formation. Pathologically increased TF originates mainly from endothelial cells. Studies of humans and laboratory animals suggest that free hemoglobin may cause TF expression in these cells. Free hemoglobin results in the scavenging of nitric oxide (NO)[56], reduced levels of NO may contribute to increased TF expression in the pulmonary endothelium of mice with hereditary sickle cell anemia and human endothelial cells in vitro[57,58] .

Moreover, inflammatory cytokines can also induce expression of TF on the surface of endothelial cells. Monocytes are activated by erythrophagocytosis and release inflammatory cytokines that induce TF expression. In fact, a recent study showed that cytokines associated with macrophage and monocyte activation were significantly elevated in dogs with IMHA who died in comparison to survivors [59].

Microparticles (MPs) which carry superficial phosphatidylserine (PS) and express TF might also play a role in thrombosis in human patiants[54]. MPs are derived from blebbing (or protrusion) of the cytoplasmatic membrane of many cell types, and are released upon cell activation or injury. They are then cleared primarily by the reticuloendothelial system[54,60,61] .

Activation and injury of cells also cause plasma membranes to lose phospholipid asymmetry and expose anionic phospholipids, such as PS, on their surface. PS exposure provides a negatively charged docking site for tenase and prothrombinase complexes of the coagulation cascade[62]. Damaged and senescent RBCs also express PS on their surface and form procoagulant MPs. Triggers for increased RBC micro vesciculation include complement attack, oxidative injury, and even stress - conditions that are present in dogs with IMHA[54]. Reticulocytes also express increased levels of cell surface PS, and it has been postulated that reticulocytosis contributes to the risk of thrombosis in hemolytic disease [63]. Auto agglutinating RBCs may also contribute to the direct occlusion of vasculature and worsening thrombosis. Auto agglutination is common in dogs with IMHA and is associated with decreased survival in some studies [2,20].

Platelets also play an important role in the formation of thrombus since sequestration of NO by plasma hemoglobin results in platelet aggregation[52]. Furthermore, loss of asymmetry and exposure of PS on the outer surface occurs after platelet activation. Platelet MPs exposing PS are 50- to 100-fold more procoagulant than activated platelets. MPs from platelets have been demonstrated to be 100% higher in dogs with IMHA than in healthy dogs [53].

Recent study preformed on 32 dogs demonstrated that some dogs with IMHA have increased procoagulant activity associated with phosphatidylserine-positive (PS+) and tissue factor-positive (TF+) MPs in peripheral blood. However, in that study the cellular resource of MPs was not identified, there was no standardization of therapeutic protocols and no exclusion criteria ware established on the base of concomitant disease. Therefore, larger studies with comprehensive underlying disease screening and standardized medical treatment to determine whether MPs can be used as markers of thrombotic risk, and studies to determine the cellular origin of MPs in dogs with IMHA are warranted [54].

The excessive activation of coagulation in dogs with IMHA is accompanied by decreased levels of anticoagulants. Decreased AT activity is common in these patients. Decreased AT likely contributes to the prothrombotic state, although no association with AT activity and mortality was found in one study[35]. Impaired fibrinolysis, characterized by increased levels of plasminogen activator inhibitors, antibodies to annexin A2 and other mechanisms, contribute to the prothrombotic state in some thrombotic and autoimmune diseases in people [64]. Antiphospholipid-antibody-syndrome (APLA) is a common complication of SLE in humans [65]. It has been suggested that a similar mechanism may in part be associated with the

enhanced platelet aggregation and depresses regulation of the coagulation pathways. However, in a recent case series only two of 20 dogs had evidence of anti - phospholipid activity[35]. Whether decreases in inhibitors of coagulation other than AT, or an imbalance of the fibrinolytic pathways may occur in human or canine patients with IMHA has not been investigated.

Therapy and supportive measures used in the treatment of dogs with IMHA may also contribute to thrombosis. Microparticles are cleared by phagocytic cells in the spleen that contain receptors for PS or opsonins on the microparticle surface, such as complement [66]. It is possible that immunosuppressive and immunomodulatory therapy directed at inhibition of phagocytosis in patients with IMHA might increase levels of circulating microparticles and contribute indirectly to the prothrombotic state. Indeed, splenectomy and human intravenous immunoglobulin G (hIVIG) are associated with an increased risk of thrombosis in people, and hIVIG administration is prothrombotic in normal dogs [67,68]. However, in one retrospective study describing splenectomy as an adjunctive therapy for 10 dogs with idiopathic IMHA, no clinical signs of thromboembolic events were observed after splenectomy was performed and 9/10 dogs were alive at 30 days [69].

Drugs used in the treatment of IMHA may exacerbate thrombosis by other mechanisms. Glucocorticoids increase circulating levels of some coagulation factors and decrease fibrinolysis, although this is yet to be proven in human or canine IMHA patients [52]. Thrombotic events are a well-known complication of cyclosporine use in people[70]. Whether the use of glucocorticoids or cyclosporine in dogs with IMHA contributes to the risk of thrombosis is not known. Other supportive measures commonly used in the treatment of dogs with IMHA, such as the use of IV catheters, have been associated with thrombosis in people and in dogs [71,72]. Cage confinement and prolonged recumbency may also exacerbate hypercoagulability [20].

Chapter five: Prognostic Factors and Outcome

5.1 Mortality Rates and Prognosis for Primary IMHA Patients

Measurement and evaluation of prognostic factors has the potential to improve the clinical management of cases of IMHA in dogs and to allow resources to be targeted appropriately .The current literature well-describes prognostic factors for dogs with IMHA, but these differ widely among studies. The studies consider mainly hematologic, biochemical, and clinical variables as potential prognostic factors and several studies evaluate the effect of different treatment protocols on survival[73].

It is clear that canine IMHA must be regarded as a severe disease. Reported mortality rates for idiopathic IMHA range from 22 to 80%, with significantly increased mortality early in the disease course[74]. The most recent study evaluating prognostic indicators and mortality rates reported a 43,9% mortality with a survival range of 2–96 days; mean 19 days; median 7.5 days [31]. However the group of animals used for the latter study was quite small and consisted of only 41 dogs. Considering a larger scale study performed by Piek et al. at 2008 using 149 dogs, the estimated half-year survival for the whole group was 72.6%. The estimated half-year survival rate for the 96 dogs that survived the 1st 14 days was 92.5%, thus confirming the hypothesis of a higher mortality rate with chronological proximity to the onset of the disease.

By contrast, some dogs with IMHA make excellent recovery with appropriate supportive and immunosuppressive therapy, but remain at risk for a disease relapse. There is often very rapid clinical and hematological response to therapy in such patients; however, serial monitoring of the Coombs' test has revealed striking persistence (for many months) of RBC - bound autoantibody in many cases. Re-occurrence of the disease is defined as a decrease in hematocrit after an initial improvement of full recovery[2]. Relapses may occur months or years after the initial episode (one report indicated a median of 112 days ranging between 32 and 1750 days [2]) and are often more severe, resulting in death. Relapse in clinical disease is reported in 15% of dogs that survived beyond 60 days. During this time interval, treatments

had either been discontinued or were on a tapering course. Relapse was as likely to occur while on a tapering course of medication as after treatment was discontinued[20].

Other manifestations of autoimmunity may appear or develop in dogs recovered from IMHA, sometimes several years later. For example, dogs may present with IMT (and no anemia) subsequent to IMHA (without thrombocytopenia). SLE and immune - mediated skin disease are also reported sequels.

Therefore, recovery from IMHA necessitates regular monitoring for the life of the patient. Hematological monitoring is the most cost - effective means; it is recommended that a PCV be performed every 2 weeks throughout the course of treatment, extending for 6 – 12 months after withdrawal of therapy. In dogs recovered from IMHA associated with vaccination, care should be taken with administration of subsequent booster vaccines. A risk - benefit analysis should be conducted in consultation with the client. Measurement of serum antibody titre to core virus vaccine may be performed to support any decision not to re-vaccinate, but where re-vaccination is required, it is recommendable to use of a product from a different manufacturer and a product with the longest possible duration of immunity is advised. There is, however, little clear evidence that booster vaccination induces relapse of disease or disease of greater severity [6].

5.2 Prognostic Factors

Factors associated with reduced survival in the large case series of Weinkle et al., Carr et al., Goggs et al. and Piek et al. included:

(1) Auto agglutination; was more common in dogs that died or were euthanatized during initial hospitalization, compared with dogs that survived to discharge (36/37 [97%] vs 82/114 [72%])[20].

(2) Thrombocytopenia; lower platelet count was associated with worst outcome in three of the studies.

(3) Hyperbilirubinemia; all four studies indicate increased serum TB as a poor prognostic factor. Two of which refer to a lower rate of serum TB concentrations (> 1.5 mg/dL ; reference range, < 0.3 mg/dL). This serum concentration is the concentration above which hyperbilirubinemia is manifested clinically as jaundice. Most dogs that died had jaundice,

whereas 50% of surviving dogs did not[2,20]. Another study refers to a higher TB serum concentration of >5mg/dL[17].

(4) Hypoalbuminemia; associated with poor prognosis in two studies, but in a lower degree of significance [17,20].

(5) Band neutrophilia; a presence of ≥ 3,000 cells/μL was indicated as a poor prognosis indicator by Weinkle et al. and Piek et al. This finding may reflect ischemic or hypoxic tissue necrosis accompanying severe anemia [2,20].

(6) Hypokalemia; Although an association between hypokalemia (serum potassium concentration < 3.5 mEq/L) and an increased rate of mortality has not been reported, the association between critical illness and hypokalemia is not novel. Hypokalemia promotes vascular constriction and may increase the risk for vascular and thromboembolic complications[20].

(7) Increased serum urea concentration; Reported by by Piek et al. and Goggs et al. defined as one of the four main predictors of mortality in dogs with idiopathic IMHA [2].

5.3 Scoring Systems

Many attempts have been made to link clinical pathologic changes with the prognosis in patients with IMHA, and although individual studies have detected associations between specific laboratory criteria and outcomes, few prognostic indicators are consistently reproducible in multiple studies[75].

Scoring schemes are routinely used in human medicine to predict patient outcome and compare treatment protocols. So far two different grading scales that predict the outcome of the disease in dogs with IMHA. The *canine hemolytic anemia objective score* (CHAOS) and the *Tokyo* [76] score were developed to predict the survival of dogs with hemolytic anemia.

Table 5.1 Instruction for calcolation of illnes severity scores by two scoring systems; in both scales the prognosis improves with the increase of the score.

Canine hemolytic anemic objective score (CHAOS).	
Age (year)	If ≥7 score 2, otherwise score 0
Temperature (°F)	If ≥102.0 score 1, otherwise score 0
Agglutination	If present score 1, otherwise score 0
Albumin (g/dL)	If <3.0 score 1, otherwise score 0
Bilirubin (mg/dL)	If ≥5.0 score 2, otherwise score 0
Total	Maximum score 7

Published by Whelan et al. 2006

Tokyo score	
Sex	Male score 1, Female score 0
Season	Apr-Sept score 1, Oct-Mar score 0
Packed cell volume (%)	If <20 score 1, otherwise score 0
Platelet count ($\times 10^3/\mu L$)	If <200 score 1, otherwise score 0
Total protein (g/dL)	If <6.0 score 1, otherwise score 0
Total	Maximum score 5

Published by Ishihara et al. 2009

Alternatives to these disease-specific illness severity scores that might be easier to estimate are the American Society of Anesthesiologists (ASA) health classification and the presence or absence of markers of a systemic inflammatory response syndrome (SIRS). The ASA classification is typically used to evaluate patient risk for anesthesia, but the classification is easy to apply and has been used as a marker of disease severity in other canine populations. A recent study preformed in 10 referral veterinary centers in the UK has evaluated the previously mentioned illness-severity scores [77]. The Study found no association between the *Tokyo* score and the mortality either at time of discharge or within 30 days. The CHAOS univariate analyses, when dichotomized as <3 or ≥3, was associated with death in hospital and death within 30 days of admission. The authors also found that ASA classification ≥3 was also associated with death, suggesting the subjective assessment of experienced clinicians can be a reasonable gauge of illness severity in IMHA. Markers of kidney function, bilirubin concentration were likewise identified as independently associated with outcome. Therefore, a multivariate model combining illness severity scores and the above mentioned

clinicopathological data was established. The new model correctly predicted outcome at discharge in 82% cases [77].

It is hoped that the identification of "at high risk" patients will allow early intervention and resource management to improve the clinical decisions making and the outcome of this frustrating and common disease.

Chapter six: Immunosuppressive and Supportive Care

Immune-mediated diseases represent some of the most frustrating types of disorders that are diagnosed and treated in veterinary medicine. Drug-induced immunosuppression is an attempt to control the aberrant immune response against self antigens, but the immunosuppression can result in sepsis or other unacceptable adverse effects. If the pathophysiology of immune-mediated and autoimmune disease is considered, the immune response can be divided into

several components and attempts can be made to selectively deal with each component separately. The components of the immune response that can be manipulated by therapy include antibodies, effector cells, mononuclear phagocytic system and clinical manifestations of disease [3]. In true autoimmune responses such as in Auto Immune Hemolytic Anemia, an autoantibody (antibody with activity against self antigens) is the inciting cause of the immunologic damage. As formerly mentioned in chapter 3, these autoantibodies can either cause intravascular hemolysis by Complement - induced lysis, or, more frequently membrane damage by ADCC (antibody dependent cell-mediated cytotoxicity) and complete phagocytosis of the cell primarily in the liver and spleen. It is therefore apparent that immune-modulatory treatment should be directed at suppressing these specific mechanisms of the immune response.

For the time being, the majority of clinicians use a non-selective immune-suppressive approach. This nonselective immune-suppressive approach has several negative aspects. Because conventional therapy suppresses protective as well as destructive immune responses, the individual is predisposed to infectious diseases. Death due to overwhelming infection is a risk of immunosuppression. In addition, adverse effects of currently used immunosuppressive agents are considerable. Glucocorticoids commonly cause iatrogenic hyperadrenocorticism and may possibly be associated with pulmonary thromboembolism. Cytotoxic agents can induce significant bone marrow suppression as well as gastrointestinal upset. Therefore, nonselective immunosuppression is a crude approach, and the current therapeutic strategies should be improved[3]. In the following chapter conventional immuno-supressive agents will be presented along side with some of the new non conventional agents not yet applicable for veterinary use.

6.1 Immunosuppressive treatment

6.1.1 Glucocorticoids

Glucocorticoids (GC) act primarily by binding to a cytosolic glucocorticoid receptor (GR), which then translocates to the nucleus, binding specific DNA sequences (glucocorticoid responsive elements) where they act to enhance or inhibit transcription of corresponding

genes [78]. Recent evidence suggests that more rapid effects are mediated through GRs influencing intracellular signalling, non-specific interactions with cell membranes and specific interactions with membrane-bound receptors. Anti-inflammatory effects of glucocorticoids relate to the stabilization of cell membranes of granulocytes, mast cells and monocytes-macrophages and inhibition of phospholipase A2 (thereby preventing release of the arachidonic acid metabolites of the cyclooxygenase and lipoxygenase pathways). In addition, glucocorticoids prevent release of the pro-inflammatory cytokines IL-1 and IL-6. Effects on complement and rapid down-regulation of Fc receptor expression on macrophages reduce phagocytosis of opsonised red blood cells. The latter could explain much of the reported early efficacy of glucocorticoids in treating IMHA.

Immunosuppressive effects in dogs are less clear from published studies but may include reduction in antigen processing and presentation by effects on macrophages and dendritic cells, direct suppression of T cell function (including T cell help for B cells) and reduced affinity of antibody to cell membrane epitopes[33].

The widespread distribution of GRs and the vast number of genes affected by GR ligation goes some way to explain the severity and wide range of side effects caused by systemic administration of glucocorticoids [79]. Side effects from long-term high dose GC treatment include serious endocrine alterations. Decreased beta-cell insulin production and insulin resistance may lead to diabetes mellitus. Prolonged suppression of adrenocorticotropin levels leads to atrophy of the adrenal cortex and secondary adrenal insufficiency[80]. Iatrogenic Cushing syndrome will eventually develop in all patients undergoing prolonged GC therapy[80] and includes: polydipsia, polyuria with reduced urine specific gravity[81], atrophy of epidermis and dermis, decreased fibroblast proteo-synthesis that results in wound healing and scaring disorders, catabolic effect on skeletal muscle protein, muscle atrophy[80] and lethargy. The most predominant findings on biochemical are elevated alkaline phosphatase and alanine transferase ; hypercholesterolemia ; hyperglicemia; elevated aspartate transaminase; and elevated triglycerides[81] .In one study the mean time to complete remission of GC adverse effects was 12 weeks [81].

Currently, prednisolone (prednisone), methylprednisolone and dexamethasone are the most widely used glucocorticoids for systemic effect. Prednisolone has an anti-inflammatory potency roughly equal to hydrocortisone, whereas methylprednisolone and dexamethasone are

approximately 5 and 30 times more potent, respectively. Prednisolone and methylprednisolone have an intermediate duration of action and dexamethasone has a long duration of action relative to hydrocortisone. Doses of prednisolone up to 0,5 mg/kg twice daily are considered anti-inflammatory and higher doses immunosuppressive. In reality, there is likely to be a continuum of effect due to the large number of cellular processes influenced by glucocorticoids and such a division is somewhat arbitrary. Immunosuppressive protocols invariably involve tapering the glucocorticoid dose down to zero (according to clinical effect) over several weeks or months. Glucocorticoids should not be abruptly discontinued due to time required for resolution of the iatrogenic suppression of the hypothalamic- pituitary-adrenal axis[33].

It is common to combine a cytotoxic drug with glucocorticoids in the therapy of IMHA. The rational behind this is obvious when one looks at the various aspects of the immune response suppressed by each drug. The addition of a cytotoxic drug usually allows reduction in dosage or more rapid tapering of the dosage of glucocorticoids [3,2]. Nevertheless this is yet to be demonstrated scientifically since some authors offer disputable results [73,82]. Further work should be directed at examining the effects of treatment regimen and combination of drugs on outcome, using larger study groups, more standardized protocols of treatment and uniform patient inclusion and exclusion criteria.

6.1.2 Azathioprine

Azathioprine is a cytotoxic synthetic imidazole derivative of 6-mercaptopurine (6MP). Both are thiopurines, which interfere with purine synthesis and result in production of fraudulent nucleotides. Hence, DNA and RNA syntheses are inhibited, and mitosis and cellular metabolism is disrupted. Two studies found that azathioprine does not reduce serum immunoglobulin concentrations[83,84]. Rinkardt et. al also reported no reduction in circulating blood lymphocytes or specific subsets of these cells. However, these observations were only made at 14 days after commencing of therapy, and it is generally accepted that azathioprine has a "lead-in time" of 11 days before having clinical effect. The main effects of this drug are thought to be on Cell Mediated Immunity (CMI) with a reduction in lymphocyte number and T cell-dependent antibody synthesis. Azathioprine has long been used in combination with glucocorticoids, with which it is thought to have a synergistic effect, to allow more rapid dose

reduction or tapering of glucocorticoids (the "glucocorticoid sparing" effect). Speed of onset of immunosuppression induced by azathioprine alone is reported to be variable. One study has documented a reduction in T cell blastogenic response to mitogens at 7 days [84], whereas another suggested a lag period of 3 to 5 weeks [85]. Azathioprine has been extensively used in management of canine immune-mediated disease, with relatively low cost and good tolerability making it an attractive choice. A few large retrospective studies lend limited support to its use but there are no prospective controlled studies.

The principle adverse effects of azathioprine are myelosuppression resulting in thrombocytopenia, acute pancreatitis, hepatopathy and gastrointestinal distress[85,86,87] . Safety and efficacy of azathioprine therapy in dogs might be refined by considering drug metabolism in more depth: azathioprine is initially converted in the liver and other tissues to 6MP. Thiopurine methyltransferase (TPMT) is an important enzyme in the metabolism of 6MP and can be measured in canine erythrocytes. Variation in TPMT activity correlates with clinical outcomes in human patients receiving azathioprine, with high TPMT activity associated with reduced efficacy and low TMTP activity associated with increased risk of bone marrow toxicity. Notably, significant breed-related variation in TMTP activity has been documented in dogs, with TMTP activity being much lower in giant Schnauzers and much higher in Alaskan Malamutes than in other breeds [88]. Six single-nucleotide polymorphisms have been found to account for much of the variation in TMTP activity in the canine population [89]. Standard treatment protocol of Azathioprine is 2 mg/kg/day PO for dogs weighing under 20 kg. The daily azathioprine dose in dogs of 25, 30, 40, and 50 kg can be maximized at 45, 50, 60, and 70 mg, respectively [2], commencing at 2mg/kg SID [6].

6.1.3 Cyclophosphamide

Cyclophosphamide is a cytotoxic alkylating agent that cross-links DNA, preventing its separation. It is toxic to resting and dividing cells, particularly proliferating lymphocytes. It suppresses CMI and humoral immunity and was shown to suppress the function of B lymphocytes preferentially as compared with T lymphocytes in guinea pigs and presumably in humans, but non in canines[90]. Paradoxically, Cyclophosphamide increases the severity of inflammatory disease and reduces regulatory T cell numbers in some experimental rodent models. The latter study found that the drug aggravated features of allergic inflammation by reducing expression of suppressive cytokines due to toxicity of regulatory T cells [91].

Cyclophosphamide has been used extensively in the dog for management of primary IMHA and sporadically for other immune-mediated diseases. It has been proposed that the primary indication for cyclophosphamide therapy in the dog should now be for cancer therapy. As for most other immunosuppressive agents, there is a lack of published efficacy data. Studies suggesting increased morbidity and the increased incidence of serious adverse effects of cyclophosphamide do not justify its use as an immunosuppressive agent. The toxic effects of the drug include: leukopenia, anemia, hemorrhagic gastro-enteritis and cystitis, adrenal insufficiency and liver function alterations[86].

Despite the fact that cyclophosphamide is still in common use as an immune-suppressive drug for the treatment of pIMHA, recent studies have demonstrated that it has little if no beneficial value. According to a randomized controlled prospective clinical trial completed in 2003, the addition of cyclophosphamide to glucocorticoid therapy did not accelerate the resolution of spherocytosis or the resolution of a positive Coombs' test result, suggesting that cyclophosphamide had no additional effect on reducing the production of anti-erythrocyte antibody compared to prednisone alone[92]. Furthermore, two retrospective studies suggested that the use of this drug resulted in an increased mortality compared with other interventions [82,93]. Based on this evidence, some authors do not consider the use of cyclophosphamide to be warranted when alternative products are available[73].

The standard treatment protocol with cyclophosphamide is the following: either 50 mg/m2 body surface area PO q24h for 4 days a week or 200 mg/m2 given IV as a bolus in combination with glucocorticoids for additional immunosuppression in the initial acute management of IMHA or in an attempt to lower the necessary glucocorticoid dosage earlier [92].

6.1.4 Cyclosporine A

The use of cyclosporine A for immune mediated disease has been reported in human as well as in veterinary medicine[3]. The drug has a selective T-cell immunosuppressive activity that inhibits phosphatase activity of calcineurin, thereby preventing the transcription of many cytokines and particularly IL-2, which is necessary for proliferation and maturation of T-cells[94]. Activated T cells are the most susceptible to its effects. Antibody formation against T-cell-dependent antigens is thus reduced[3]. In humans it is used primarily to prevent transplant rejection and is very useful for the treatment of chronic inflammatory skin conditions, and its

efficacy for these pathologies has been demonstrated in dogs too[94].The original vegetable oil formulation was found to have poor and unpredictable oral bioavailability, but the current micro-emulsified formulations (cyclosporine modified or micro-emulsified) have markedly improved bioavaliability and attain more consistent and predictable therapeutic blood levels[75]. Interestingly, the concurrent use of ketoconazole to inhibit cyclosporine metabolism has been shown to reduce the required dose and hence the cost of cyclosporine therapy[95]. Several studies have found that this drug combination resulted in financial savings of 40% to 70% compared to the use of cyclosporine alone [75]. Another recent study found cyclosporine in combination with prednisone to be less effective then azathioprine in combination with prednisone [96]. The authors estimated that since cyclosporine has a low availability (35%) after oral dosing in healthy animals, it is to be expected that this absorption would be reduced in hypovolemic animals with constricted splanchnic vessels. It is thus possible that although cyclosporine may be an effective immunosuppressive agent, it may not be absorbed in sufficient concentrations to control the immune response in collapsed animals, including those with IMHA[96]. Further studies regarding the pharmacokinetics of the drug in hypovolemic dogs are warranted.

Unlike cyclophosphamide and azathioprine, cyclosporine is not myelosuppressive and is suitable for use in patients with non-regenerative forms of IMHA [75]. Adverse effects of cyclosporine in dogs include vomiting, diarrhea, anorexia, gingival hyperplasia, weight loss, alopecia, hypertension, and papillomatosis. Some more severe neoplastic side-effects such as lymphoma and squamous cell carcinoma are sporadically reported in dogs[3,97], and are well known in cats [98] and in humans[99]. In dogs, given the commonly used oral dose of 5 mg/kg per day there have been few adverse reactions- mostly limited to vomit and diarrhea[96], but some clinicians report that the immunosuppressive starting dose of oral microemulsified cyclosporine is 10 mg/kg q12–24h[75].

6.1.5 Mycophenolate mofetil

Mycophenolate mofetil (MMF) was developed as an alternative to azathioprine with reduced myelotoxicity and hepatotoxicity and is now widely used in multi-drug regimes for prevention of human renal allograft rejection and for treating several human immune-mediated diseases such as inflammatory myopathy [100], lupus nephritis [101] and even AIHA and IMT [102]. MMF

has also exhibited antimicrobial activity against pathogens including hepatitis C and human immunodeficiency virus [103].

The drug is metabolized in the plasma and the liver into mycophenolic acid (MPA), a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in de-novo purine biosynthesis. T- and B-lymphocytes are more dependent on this pathway than other cell types. Moreover, MPA is a fivefold more potent inhibitor of the type II isoform of IMPDH, which is expressed in activated lymphocytes, than of the type I isoform of IMPDH, which is expressed in most cell types. MPA has therefore a more potent cytostatic effect on lymphocytes than on other cell types. This is the principal mechanism by which MPA exerts immunosuppressive effects.

Three other mechanisms may also contribute to the efficacy of MPA in immune response modulation. First, MPA can induce apoptosis of activated T-lymphocytes, which may eliminate clones of cells responding to antigenic stimulation. Second, by depleting guanosine nucleotides, MPA suppresses glycosylation and the expression of some adhesion molecules, thereby decreasing the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection. Third, by depleting guanosine nucleotides MPA also depletes tetrahydrobiopterin, a co-factor for the inducible form of nitric oxide synthase (iNOS). MPA therefore suppresses the production by iNOS of NO, and consequent tissue damage mediated by peroxynitrite. Additionally, the drug also suppresses primary, but not secondary, antibody responses by inhibiting differentiation, maturation and allostimulatory function of human monocyte-derived dendritic cells[103–105] .

Clinical use in the dog has emerged from extensive use in canine renal and bone marrow transplantation models. Significant potential advantages of MMF as an immunosuppressive agent in the dog include availability of a parenteral preparation, rapid onset of IMPDH inhibition (and hence immunosuppression) occurring 2 to 4 hours after dosing and low toxicity with signs primarily limited to gastrointestinal effects, although mild suspected allergic reactions have been reported with the parenteral preparation [33]. Veterinary clinical usage of MMF for treatment of refractory immune-mediated disease is now quite widespread, especially for acquired myasthenia gravis [106] , IMHA and pemphigus vulgaris. One study performed on 30 cases of canine IMHA found no difference in short-term outcome for dogs with idiopathic IMHA between those treated with glucocorticoids and MMF or glucocorticoids and another adjunctive immunosuppressive agent. This was despite MMF

dogs presenting with clinicopathological data shown to be associated with a poorer prognosis. More importantly MMF administration was only associated with transient diarrhea in a small number of dogs[74]. This is an encouraging fact considering the wide spectrum of adverse affects other cytotoxic drugs cause. Nonetheless, the latter study had a few limitations regarding the uniformity of treatment protocols and further research is needed to establish the efficacy of MMF in comparison to other cytotoxic drugs. The standard dosage for MMF is 1,3 to 4 mg/kg/day[74].

6.1.6 Human intravenous immunoglobulin

Human intravenous immunoglobulin (IVIG) has broad but incompletely understood immune-modulatory effects in people and has multiple clinical indications supported by controlled studies. In dogs, in vitro evidence that IVIG competitively inhibits the binding of canine IgG to monocytes by saturation of Fc receptors provides rationale for use in IMHA and IMT by prevention of phagocytosis of antibody-coated erythrocytes and platelets [107,108]. The authors of the latter study have also documented IVIG binding to canine T (CD4 and CD8) and B cells in vitro. Human data suggest that IVIG interferes with Fas (CD95) – Fas ligand (CD95R) induced apoptosis in lymphocyte-mediated cytotoxicity of target cells, providing a possible explanation for suggested efficacy in canine immune-mediated skin diseases such as erythema multiforme. Additional mechanisms of immunosuppression in people recently described by Ballow[109] include:

Saturation of the FcRn receptors to enhance the clearance of autoantibodies; inhibition of complement uptake on target tissues; inhibition of the differentiation and maturation of dendritic cells; restoration of the idiotypic–anti-idiotypic network; suppression or neutralization of cytokines by specific antibodies in the IVIG; blockage of binding of adhesion molecules on leukocytes to vascular endothelium; blockage of Fas ligand–mediated apoptosis by anti-Fas antibodies in the IVIG; induction of apoptosis with anti-Fas antibodies at high concentrations of IVIG; neutrophil apoptosis by anti–Siglec-9 antibodies in IVIG; Induction of inhibitory FcgRIIb receptors on effector macrophages; neutralization of growth factors for B cells such as B-cell activating factor and inhibition of T cell–proliferative responses; expansion and activation of a population of Treg cells[107,108,110–112].

Although IVIG has been used for various conditions in dogs, efficacy data are limited to case reports and two small controlled studies. In one study, a group receiving hIVIG tended to have stabilization of PCV approximately 1 day sooner than the placebo group, yet this

difference was not significant. Survival to discharge was comparable between the dogs receiving hIVIG and placebo. The authors therefore concluded that the therapeutic benefit of hIVIG is not enough to warrant the additional costs of the treatment [55].While this situation persists, use of IVIG may be difficult to justify on ethical (and financial) grounds at times when demand is high for human patient[33].

IVIG has recently been shown to promote hypercoagulability and an inflammatory state in healthy dogs in a small controlled trial [113], but not in IMHA[55] or IMT dogs [114] (although the latter report only included 5 dogs). This would question the application of this treatment to dogs with IMHA in which a hypercoagulable state is already recognised[33].

The protocol frequently used by some clinicians is the following: 0.5 g/kg daily reconstituted with sterile water, for 3 consecutive days. Infused hIVIG must be refrigerated and administered within 24 hours of reconstitution to minimize risk of bacterial contamination[55].

6.1.7 Danazol

Danazol is a synthetic androgen which is reported to down-regulate macrophage Fc receptor expression, reduce antibody binding to erythrocytes, stabilize erythrocyte membranes and alter T cell homeostasis. It has been used as an adjunctive agent with glucocorticoids in canine IMHA and IMTP, but its efficacy is only supported by a few published case-studies [115,116] and its usage is now uncommon. Danazol can be hepatotoxic in dogs[33].

6.1.8 Leflunomide

Leflunomide is an isoxazole immune-modulatory drug, licensed in some countries for treatment of human rheumatoid arthritis. The primary metabolite reversibly inhibits dihydro-orotate dehydrogenase, the rate-limiting enzyme in de novo pyrimidine synthesis. At high concentrations, cytokine and growth factor receptor-associated tyrosine kinase activity are inhibited [117]. T and B cell proliferation is inhibited and the drug has significant anti-inflammatory effects. Work in rodent models of contact allergy and inflammatory brain disease also suggests that leflunomide may induce regulatory T cells and that induction of CD86 expression and IL-10 production by microglial cells could play a role in the CNS effects. Leflunomide was first used in dogs as an adjunctive agent in renal transplantation but has been applied to treatment of a range of immune-mediated and inflammatory diseases. It is

used primarily in cases refractory to conventional medications or where glucocorticoid use is contraindicated – like in one case of diabetic miniature schnauzer that was diagnosed with Evans' syndrome and treated successfully with leflunomide and hIVIG[118]. Despite having a long elimination half-life and potential hepatotoxicity and myelotoxiticty, these effects seem rare. Some controlled studies have been performed in the dog, mainly revolving the use in renal transplantation[119] and polyarthritis [120], some presenting promising results[33,75].

6.1.9 Splenectomy

The spleen has a central role in the pathogenesis of IMHA. The spleen is typically the major site of mononuclear phagocytic system removal of IgG- coated RBCs in patients with IMHA and has an integral role in antigen presentation and autoantibody production. Removal of the spleen can eliminate a major contributor to the pathogenesis of RBC destruction in patients with IMHA but is reserved for patients that do not respond to standard immunosuppressive therapy or that experience adverse effects associated with drug therapy[69]. A recent preliminary study in a relatively small group of dogs described the use of early splenectomy to treat patients with acute IMHA and found that dogs treated with glucocorticoids and azathioprine as well as splenectomy within 48 hours of presentation had shorter recovery times and higher survival rates than dogs treated with glucocorticoids and azathioprine alone. Based on these preliminary results, further investigation into early splenectomy is warranted. Risks associated with splenectomy include complications associated with anesthesia and surgery and an increased predisposition to infectious diseases (bacterial sepsis, blood-borne parasitemia) that would normally be controlled by the spleen[75].

6.1.10 Plasmapheresis

Plasmapheresis (PP), or the removal of plasma and its constituents from whole blood, can be acutely effective in decreasing the antibody and immune complex concentrations within the blood. A wide variety of immune mediated diseases have been treated using plasmapheresis with mixed results. In humans, immune-mediated thrombocytopenia (IMT), immune-mediated hemolytic anemia (IMHA), SLE, myasthenia gravis, rheumatoid arthritis, glomerulonephritis have all been treated successfully with plasmapheresis. Dogs with SLE, monoclonal gammopathy and myasthenia gravis have also been treated successfully with this modality[121–124]. The clinical effectiveness of PP in the treatment of autoimmune disease may

be due to partial removal of autoantibodies, immune complexes, complement components, pro-inflammatory agents, and soluble adhesion molecules[94]. Plasmapheresis may be used as an emergency procedure before cytotoxic therapy has had time to take effect. Temporary stabilization of acute hemolysis occurred in response to plasmapheresis in two IMHA dogs but was suspected to have contributed to the death of one of them[121]. Another case study reported a complete resolution of anemia with minimal complications following a plasma exchange with membrane separation PP technique and fresh-frozen plasma[125]. The author therefore concluded that since transfusion requirements appeared to be reduced, and the procedure was well tolerated, there may be a place for this modality in severe cases to act as a bridge until medical therapy takes full effect. Because of the cost of performing this therapy, and the potential requirement for multiple treatments, it should be reserved for selected patients[3,125]. PP as a therapeutic approach is yet controversial since the main location of hemolysis in IMHA is extra-vascular, and the vast majority of auto-antibodies are adhesive to the cellular component and therefore further study is required.

6.2 Supportive therapy

Supportive care for IMHA dogs is directed at two primary goals: to reduce organ damage caused by hypoxia and to prevent the formation of life threatening thromboemboli. Complimentary treatment such as antibiotic coverage, gastro-protectors, antioxidants, electrolyte imbalance correction and fluid-therapy are highly suggested as well.

In severely anemic dogs (PCV < 15%), supportive therapy in the form of cross - matched whole blood or packed RBC transfusion may be required. The benefits of transfusion in providing short - term oxygen carrying capacity outweigh any possibility of enhancing hemolysis by providing greater antigenic load [6]. The consequences of severe anemia include tissue hypoxia, acid-base disturbances and hypotension. Blood transfusion addresses many of these problems by improving oxygen carrying capacity and intravascular volume; supporting blood colloidal pressure; and improving tissue perfusion. Importantly, blood replacement normalizes the body's buffering capacity, which is crucial in states of lactic acidosis and mixed acid-base disturbances and is frequently depleted in states of severe anemia [126].The decision to administer a blood transfusion to a patient with IMHA should be based on the presence of anemia-related clinical signs, such as tachypnea, tachycardia, and weakness[75], as

well as the velocity of Hct decline. Several studies demonstrated a relationship between reduced short-term survival and transfusion, but this is partially due to the fact that dogs that receive blood transfusion are more anemic[2,20]. The possible correlation between hypercoagulability or PTE and PRBC transfusion is yet to be determined.

6.2.1 Thromboprophylaxis in IMHA

Unfractionated heparin, ultra low doses of aspirin (ULDA), and clopidogrel have been used as thromboprophylactic agents in dogs with IMHA. Given that venous thrombi and PTE are common death cause, fibrin rich thrombi have been documented, and TF-induced activation of coagulation likely initiates thrombus formation in hemolysis, the use of drugs that target coagulation is a logical choice for dogs with IMHA. Preferences for the use of one or another combination of these therapies in treatment of IMHA vary among clinicians and are based on personal experience, method of administration, cost and monitoring requirements.

Heparin

Heparin is the standard of care for prevention of deep vein thrombosis and PTE. Unfractionated heparin (UFH) facilitates the AT-mediated inactivation of thrombin and factor Xa. Factors IXa, XIa, VIIa, and XIIa are also inactivated by this complex. Heparin also facilitates release of tissue factor plasminogen inhibitor from endothelial cells and inhibits the binding of phosphatydilserin to thrombospondin which reduces the protrombinase and tenase complex formation. Clinically relevant doses of heparin inhibit this binding in a concentration- dependent manner. Additionally, it inhibits complement. Complement inhibition is thought to contribute to the anti-thrombotic effects of heparin in women with APLA syndrome. Heparin inhibits hemolysis-induced thrombin generation in vitro. Thus, heparin appears to target many of the pathways that might contribute to the prothrombotic state in IMHA[52,54]. Importantly, UFH therapy requires diligent and appropriate monitoring for each individual patient since standardized dosing results in highly variable levels of anticoagulation in different patients. In fact, some previous studies suggest UFH is not effective in preventing thromboembolism in IMHA; however, it has been hypothesized that sub-therapeutic dosing could explain the seeming lack of efficacy [127]. The same study suggests that individually adjusted UFH therapy using anti-Xa monitoring reduced case fatality rate in dogs with IMHA when compared with dogs receiving fixed low dose UFH therapy[127]. Measuring anti-factor Xa activity in individual patients is the gold standard for determining whether therapeutic UFH heparin levels are reached in human medicine[52]. Even

so, the range of anti-factor Xa activity needed to prevent thrombosis in dogs with IMHA has not been established. A prospective controlled clinical trial by Helmond et al. showed that doses ranging from 150U/kg to 566U/kg QID were necessary to achieve target anti-factor Xa activity of 0.35–0.7 U/mL in individual dogs [127]. APTT was suggested as a correlated indicator to anti-factor Xa activity, and is more available for clinicians to perform. However, predicting whether plasma anti-factor Xa activity is within a target range based on prolongation of APTT in individual normal dogs is difficult. Furthermore, the relationship between APTT and heparin activity in normal dogs depends on the reagent used in the APTT assay. In diseased patients, APTT can be affected by many additional factors: hyperfibrinogenemia, hyperbilirubinemia and corticosteroid treatment are only some of them. Monitoring heparin therapy is also important due to the risk of bleeding associated with its use. However, it appears that the risk of bleeding associated with UFH therapy in dogs with IMHA might be less than in normal dogs[52], this may be due to reduced bioavailability caused by the presence of inflammatory mediators found in the blood of IMHA dogs[127].

Low-molecular-weight heparin (LMWH) has been suggested as an alternative to UFH in dogs because of more predictable pharmacokinetics and similar efficacy and safety as compared to individually adjusted UFH therapy in people with deep vein thrombosis and other thrombotic disease. This translates to a reduced need for monitoring anti-factor Xa in most human patients, although monitoring is still recommended for critically ill patients. APTT is not used to monitor LMWH because the smaller heparin molecules are not large enough to inhibit thrombin while complexed to AT. In healthy dogs, enoxaparin administered at a dosage of 0.8 mg/kg subcutaneously (SC) every 6 hours maintained target anti-Xa activity without hemorrhagic complications[128] but data describing the use of LMHW in IMHA is limited[129]. Unfortunately, a recent study had failed to determine whether enoxaparin therapy can reduce mortality and thrombotic complications in dogs with primary IMHA compared with other anticoagulation protocols [130].

Platlet inhibitors: Aspirin and Clopidogrel

Ease of use and lack of need for intensive monitoring make platelet inhibitors, such as aspirin and clopidogrel, attractive thromboprophylactic agents for dogs with IMHA. Aspirin is a cycloxygenase inhibitor. Thromboxane A2 is produced by platelets in a COX-1 dependent manner. Thromboxane A2 is a potent platelet agonist. Ultra low doses of aspirin (ULDA)

inhibit platelet thromboxane A2 production while preserving COX-1 dependent prostacyclin (which is antithrombotic) production from endothelial cells. The dose of aspirin needed to achieve this effect in normal dogs or dogs with IMHA has not been established. The dose of 0.5 milligrams of aspirin per kilogram per day has been shown to inhibit normal canine platelet aggregation in vitro when Adenosine diphosphate (ADP) and collagen are used as agonists. Nevertheless, up to 30% of dogs did not exhibit an anti-platelet effect depending on the parameter measured, suggesting that the dose may be inadequate to inhibit platelet function in some individuals. Another study showed that dosing every 12 hours was more effective at inhibiting platelet aggregation than administering aspirin every 24 hours in normal dogs using this dose. This dose does not increase the risk of gastrointestinal ulceration in normal dogs treated with immunosuppressive doses of prednisone. Whether concurrent use of prednisone and aspirin in dogs with IMHA increases the risk of gastrointestinal ulceration has not been determined, however anecdotally it does not appear to be common [20,52,131].

Clopidogrel has been shown to inhibit platelet aggregation in dogs. The drug irreversibly inhibits the platelet ADP receptor. ADP is a potent activator of platelets. A recent prospective study showed that dogs with IMHA treated with clopidogrel had comparable short-term survival to dogs treated with ULDA (0.5mg/kg/day) or clopidogrel combined with ULDA. The overall mortality rate at 90 days for this study was 21%, and thrombotic events were suspected or confirmed in 3/5 fatalities. Therefore, clopidogrel may represent a safe and equally effective alternative means of anticoagulation in dogs unable to tolerate aspirin therapy[132].

Whether UFH is a superior thromboprophylactic agent to ultralow-dose aspirin or clopidogrel for canine IMHA is not clear, and authors have presented contradicting results [20,127,133]. It seems like future research should focus on understanding the pathophysiology of thrombosis in IMHA and provided solutions that will confront specific mechanisms of hypercoagulation. Microparticles expressing Phosphatydilserin and Tissue-factor might be used as markers for thrombotic risk as well as the target of future treatment[54]. For instance, a platelet GPIIb-IIIa receptor antagonist (abciximab), an anti-platelet drug used after percutaneous coronary intervention in humans has been associated with the reduction in microparticle release by platelets at high shear stress. In addition to integrin antagonists, statins are another class of

drugs already reported to reduce circulating microparticles levels. Statins also reduced endothelial microparticle release in TNF-alpha stimulated cells in culture[134].

Other future prospective currently research for human application are Biomimetic Nanoparticles. The selective depletion of disease-causing antibodies using nanoparticles offers a new model in the management of type II immune hypersensitivity reactions. The demonstration of pathophysiologically inspired nano engineering serves as a valuable prototype for additional therapeutic improvements with the goal of minimizing therapy-related adverse effects. Through the use of cell membrane-cloaked nanoparticles, nanoscale decoys with strong affinity to pathological antibodies can be administered to disrupt disease processes in a minimally toxic manner. These biomimetic nanoparticles enable indiscriminate absorption of pathological antibodies regardless of their epitope specificities. Through both in vitro and in vivo studies on rodent models, Copp et al. demonstrated efficacy of RBC membrane-cloaked nanoparticles to bind and neutralize anti-RBC polyclonal IgG effectively, and thus preserve circulating RBCs [135].

In conclusion the quality of evidence available to guide clinical decisions in the treatment of IMHA is generally poor and many times ambiguous. Further research of a higher quality will be required to investigate existing and novel therapeutic regimens for the disease. The low incidence of the disease also emphasizes the importance of multi-center cooperation to maximize the enrollment of suitable cases, the need to apply robust and consistent enrollment criteria and report consistent treatment protocols, which take account of long-term treatment, tapering, and responses to relapses [73].

Chapter seven: Experimental part

7.1 Introduction

Immune-mediated hemolytic anemia (IMHA) is the most common severe anemia of dogs and is less frequent in cats[5]. The majority of cases are currently considered to be primary IMHA[6]. It is also reported to be the most common immune-mediated disease of dogs [4]. Auto-immune diseases represent some of the most frustrating types of disorders that are diagnosed and treated in veterinary medicine. Given the nature of these diseases, in which an abnormal immune response against the body's own cells occurs, treatment is difficult and necessarily includes suppression of immune defenses[3].

Primary IMHA (pIMHA) is a severe disease with high mortality rates, mainly in the first two weeks that follow its presentation[4]. Due to its complex pathophysiology and the numerous components that take part in the pathological mechanisms that cause the disease, pIMHA has been the subject of numerous studies, in veterinary and human medicine alike.

The following chapter is a retrospective study of fifty-two cases of canine primary immune mediated hemolytic anemia from two veterinary teaching hospitals in two different countries; Italy and Israel. The goals of this study were to analyze the clinical and clinicopathological alterations of this complex disease and to compare them between the two hospitals and the corresponding findings described in veterinary scientific literature.

7.2 Materials and Methods

7.2.1 Materials

Cases of primary IMHA were collected from two veterinary teaching hospitals; "Mario Modenato Veterinary Teaching Hospital" of the Department of Veterinary Sciences, Pisa University, Italy and the Veterinary Teaching Hospital of the Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Israel. For simplification they will be further referred as *Pisa* and *Beit-Dagan* respectively. The search for cases was considerably easier at the *Pisa* facility since an electronic database is used by the clinicians to store all patient data. To retrieve cases from the database it was enough to search for a certain hematocrit cut-off, typical erythrocyte deformation and the test for anti-RBC antibody presence on erythrocyte surface in the computerized data-base. At *Beit-Dagan* data collection was more complicated since there is currently no electronic database system used to store patient data, therefore the data collection was based on searching for discharge letters that contained the words

hemolytic anemia or the abbreviation IMHA. This system of data collection presents a major bias risk since the owners of the patients who died during hospitalization did not receive a discharge letter. For this reason, another search was performed using the laboratory paper archive for low hematocrit, presence of spherocytes or suspect of IMHA as a motive for the exam request. The chart numbers of possible cases were collected and the corresponding clinical charts were then retrieved from the archive present in the hospital. The search in both hospitals yielded 106 potential cases that responded to the inclusion criteria, 54 of which were then excluded from the study for meeting the exclusion criteria as well (see the next section).

7.2.2 Selection of cases

Study inclusion criteria included Hct ≤ 30% associated with either positive or weak positive result of IgG and IgM antibodies on erythrocytes detected by flow-cytometry, or positive osmotic fragility test, or evidence of spherocytosis or auto-agglutination in blood smear. Clinical records were excluded (n = 54) if an underlying disease process was identified or if results were positive for polymerase chain reaction (PCR) or serologic tests for *Ehrlichia* spp., *Borrelia* spp., *Leptospira* spp., *Leishmania* spp. or *Rickettsia* spp. or if a neoplastic process was identified.

The main causes for exclusion varied greatly between the two hospitals. The main cause for exclusion in *Beit-Dagan* was the identification of *Ehrlichia* spp. as a primary cause for anemia, using a PCR technique. *Babesia* spp. was likewise present as a primary cause of IMHA, but with a minor incidence. At the *Pisa* hospital the frequency of exclusion for PCR or serologic positivity to tick borne disease was minor. The most common excluded cases were infected with either *Leishmania* spp. *or Leptospira* spp.. *Ehrlichia* spp. infection was reported as well, but less commonly. Sporadic cases of *Babesia* spp. and *Rickettsia* spp. seropositivity were reported. Other excluded cases from both hospitals were diagnosed with Heinz body anemia, leukemia and autoimmune conditions like Pure Red Cell Aplasia and immune mediated thrombocytopenia without RBC destruction.

7.2.3 Methods

Data collected for each case included: Body weight at initial evaluation; age; gender; history taken by a clinician or a student; physical examination on arrival which included patient's body condition score, rectal temperature, heart rate, color of mucous membranes, evaluation of hydration level, sensorial state and all other clinical observations made by the clinician.

Diagnostic workup included:

(1) Complete blood count (CBC) including a blood smear for microscopic evaluation

Blood sample was collected in a potassium-ethylenediamine tetraacetic acid (EDTA) test tube and analyzed within 2 hours of collection. Complete blood count was performed using hematologic analyzers; IDEXX ProCyte Dx ® (laser flow cytometry, optical fluorescence, and Laminar Flow Impedance) at *Pisa* and Diatron Abacus ® or Arcus ® (volumetric impedance) at *Beit-Dagan*. The Analysis provided the following information: Red blood cells (RBC) 10^6/µL, hematocrit (Hct)%, hemoglobin (HGB) g/dL, mean corpuscular volume (MCV) fL, mean corpuscular hemoglobin (MCH) pG, mean corpuscular hemoglobin concentration (MCHC) g/dL, red blood cell distribution width (RDW) %, platelet (PLT) 10^3/µL, white blood cells (WBC) × 10^3/ µL, absolute numbers (10^3/ µL) and percentage of neutrophils, lymphocytes, eosinophils, monocytes and basophils. When available, a manual differential count of leukocytes was considered to be more accurate than the automated count reported by the automated analyzer.

At *Pisa* the blood smear was stained in an automated slide stainer using a May-Grünwald-Giemsa method. Evaluation included a differential leukocyte count, a leukocyte and erythrocyte morphological evaluation and a platelet morphological and quantitative assessment. The examination was performed by the experienced personnel of the clinical pathology laboratory. Morphologic examination of blood smear indicated alterations according to a scale of 1+ to 4 + or +/- based on frequency of alteration observed per 100 WBC counted.

At *Beit-Dagan* the blood smear was stained in an automated slide stainer using a modified Wright's stain. Evaluation included morphological evaluation of erythrocytes and leukocytes and a morphologic and quantitative assessment of platelets. The blood smear examination was performed in the majority of cases by the clinician responsible for the case. Morphologic examination of blood smear indicated alterations using a brief description of the slide's RBC,

WBC and PLT populations, and were then translated to the same scoring system used in *Pisa* in the following manner ; slight (-/+), mild (1+), moderate(2+), severe (3+), very severe (4+).

(2) A broad biochemistry profile in serum or heparinized plasma;

Samples were analyzed in an automated biochemistry analyzer (Liasys® and Cobas integra 400® at *Pisa* and *Beit-Dagan* respectively). The profile included the following parameters: total plasmatic protein (TP) g/dL, albumin (ALB) g/dL, globuline g/dL, Fructosamine mcmol/L (Pisa only), C-reactive protein mg/dL (Pisa only), total calcium mg/dL, phosphate mg/dL, iron mcg/dL (Pisa only), urea mg/dL, creatinine mg/dL, total bilirubine mg/dL, cholesterol mg/dL, triglyceride mg/dL, glycemia mg/dL, alkaline phosphatase (ALKP) U/L, gamma-glutamyl transpeptidase (GGT) U/L, aspartate transaminase (AST) U/L, alanine aminotransferase (ALT) U/L, creatine kinase (CK) U/L, lactate dehydrogenase (LDH) U/L, Amylase U/L, sodium (Na) mEq/L, potassium (K) mEq/L, chloride (Cl) mEq/L, Total CO_2 (TCO_2) mEq/L (Pisa only). In this study only the following parameters were examined:

- Total plasmatic protein (TP) g/dL
- Albumin (ALB) g/dL
- Total bilirubine mg/dL
- C-reactive protein mg/dL (Pisa only)

(3) Serology or PCR in order to diagnose infective agents as cause of IMHA:

- *Ehrlichia* spp; using indirect immunofluorescence assay (IFA) in *Pisa*, seronegativity was established if the titer was lower than 1/40. PCR technique was used in *Beit-Dagan*.

- *Babesia* spp; using indirect immunofluorescence assay (IFA) in *Pisa*, seronegativity was established if the titer was lower than 1/64. PCR technique was used in *Beit-Dagan*.

- *Leishmania* spp; using a blood marrow/lymph node aspirate examination or an indirect immunofluorescence antibody test (IFA) with a positivity cut-off of 1/160 or RT-PCR technique in *Pisa* as well as in *Beit-Dagan*.

- *Leptospira* spp.; Determination of antibody titer by microscopic agglutination test (MAT).
- *Borrelia* spp, *Rickettsia* spp; using indirect immunofluorescence assay (IFA) in *Pisa* with seronegativity established if the titer was lower than 1/80.

(4) Flow cytometry for detection of IgG and IgM anti-RBC on the surface of RBCs;

This assay was performed in twenty-two cases out of thirty cases in the *Pisa* group. Osmotic fragility test for detection of spherocytes in blood sample was performed in thirteen cases out of twenty-two cases in the *Beit-Dagan* group. In-slide saline auto agglutination test was performed in four cases.

Out of fifty two cases of primary IMHA identified for this study, forty eight patients were presented during a first hemolytic crisis and four patients were in relapse after being formerly diagnosed and treated for the disease. Eight patients were in treatment with corticosteroids.

Anemia was classified based on MCV and MCHC values for each patient. Anemia was defined as microcytic, macrocytic or normocytic if the value of MCV was lower, higher or within reference ranges, respectively. Anemia was defined as hypochromic, hyperchromic or normochromic if the value of MCHC was lower, higher or within reference ranges, respectively. The definition "Hyper-chromic" is inaccurate since the erythrocyte cannot "over-saturate" with hemoglobin. This error usually indicates biological or mechanical hemolysis or that the sample is lipemic. For reference ranges see APPENDIX.

Statistics

The entire study population was divided in two groups; the first group included all the patients with IMHA selected from the *Pisa* hospital and the second group included all the patients with IMHA selected from the *Beit-Dagan* hospital. The groups were then compared using statistical analysis for the various parameters regarding: age, gender, breed , season of clinical manifestation, clinical presentation and clinicopathological findings.

A control population was selected from the *Pisa* hospital electronic database and will be referred to as *control group*. The control group includes all canine patients, treated between

May 1st, 2010 and January 31st 2016 at the *Pisa* hospital**.** It was then used as comparative database to assess factors regarding gender and breed predisposition in the *Pisa* study group (patients ill with IMHA). Unfortunately, for lack of data, it was impossible to make the same analysis for the *Beit-Dagan* study group.

The study population was divided into sub groups based on the season of clinical presentation. The division was made in consideration of climate differences between the two countries. The seasons were defined as: Winter – December to February, Spring- March to May, Summer- June to August in *Pisa* and June to September in *Beit-Dagan*, Fall-September to November in *Pisa* and October to November in *Beit-Dagan*.

A large database was created using Microsoft Excel. Statistical analysis of data was performed under Windows platform using *MEDCALC* software. For all continuous parameters, the normality of data distribution was evaluated by means of the D'agostino-Pearson test. Normally and non-normally distributed continuous parameters are reported as mean ± standard deviation (SD) and as median and range (minimum observation – maximum observation), respectively. Study groups included; the *Pisa* group, the *Beit-Dagan* group and the *Control* group. Comparison between the different groups was made by means of the Chi-squared test for normally distributed numeric parameters or Mann–Whitney test for non-normally distributed numeric parameters. Proportions of categorical variables expressed as a percentage were compared using a Chi-squared test for independent samples. For all tests applied, $P < 0.05$ was considered statistically significant. Karl Pearson correlation test was used to evaluate correlations between variables. Correlation coefficient values $|r =/> 0.3|$ were considered not significant, values between $|0.3|$ and $|0.5|$ were considered weakly correlated and values $|r =/> 0.5|$ were considered moderately correlated. Correlations were considered significant if P was < 0.05.

7.3 RESULTS

7.3.1 SIGNALMENT

AGE

In this study the age of first clinical presentation was taken in account in all cases, including cases that were presented to the hospitals during a relapse of the disease (n=4), using the documented history of each patient. The group was divided in four age categories and the mean and standard deviation calculated for each hospital and for the entire study group. Age

was normally distributed in both groups. Although minimal differences were visibly notable between groups they were not statistically significant (P >0.05) using a Chi-squared test.

Table 7.1 Age groups and descriptive statistics of age.

Age of first clinical manifestation (years):	Total	Beit Dagan	Pisa
0-1	1	1	0
2-4	13	7	6
5-7	13	5	8
8+	25	9	16
Mean ± SD	7.00±3.36	6.25± 3.47	7.53± 3.24
Variance	11.3	12.1	10.5
Nº dogs	52	22	30

GENDER

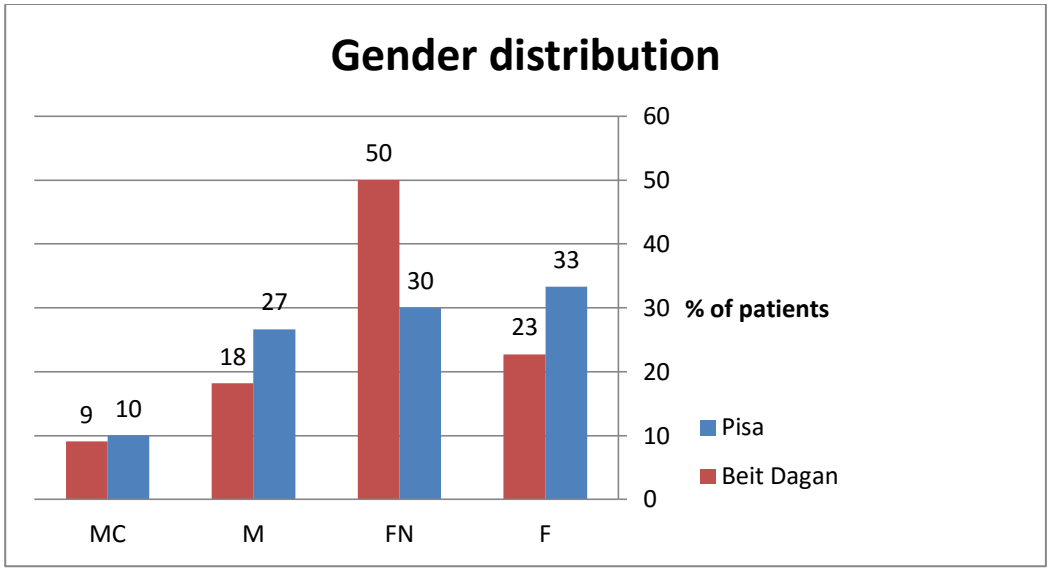
The entire study population was composed of 29% females (n=15), 38% neutered females (n=20), 23% males (n=12) and 10% castrated males (n=10).

There were no significant differences in gender representation between the two groups using the Chi-squared test (P >0.05).

Table 7.2 Gender distribution. F; female, FN; neutered female, M; male, MC; castrated male.

Gender	Pisa	Beit Dagan	Total population
F	10	5	10
FN	9	11	9
M	8	4	8
MC	3	2	3
total	30	22	30

Chart 7.1 Gender distribution of the entire study group.

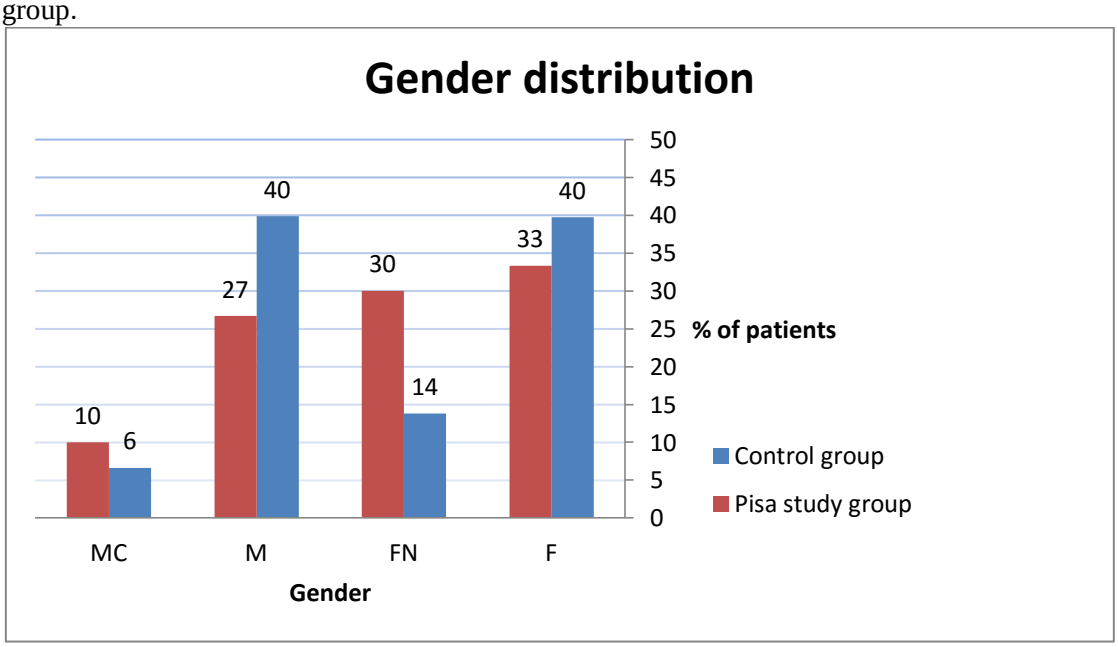


A comparison was made between the *control* group gender distribution and the *Pisa* group gender distribution. Neutered females were overrepresented in the *Pisa* study group when compared to the *control* group using a chi squared test for comparison of proportions for independent samples (P= 0.021). We didn't find any other significant differences regarding gender between the two groups.

Table 7.3 Gender distribution of control group and Pisa study group in terms of absolute numbers and percentiles.

Sex	Control group	%	Pisa study group	%	P=
F	7806	40	10	33	> 0.05
FN	2709	14	9	30	**0.021**
M	7831	40	8	27	> 0.05
MC	1301	6	3	10	> 0.05
Total	19647		30		

Chart 7.2 Gender distribution in *Pisa* study group compared to the gender distribution of the *control* group.



BREED

Both the Italian and the Israeli groups had a high prevalence of mixed breed patients and 54% of the study population is composed of mongrel dogs (n=25). There were no significant differences between *Pisa* and *Beit-Dagan* groups regarding breed (P>0.05) using a Chi-squared test. Labrador Retriever and Cocker Spaniel breeds were overrepresented in the entire study population, which included 4 patients of each of these breeds. Nonetheless, when we compared the *Pisa* study group to the *control* population, no significant difference was noted between the two groups regarding the Labrador breed, since it is overrepresented in the entire hospital population as well (P >0.05). On the contrary, Cocker Spaniels were overrepresented in the *Pisa* study group in comparison to the control population (P=0.025). Another breed, Maltese, was likewise more frequent in the study group when compared to the hospital population (P=0.012).

Table 7.4: Breed representation among study groups.

Breed	Pisa	Beit-Dagan	Total
mongrel	13	12	25
Cocker Spaniel	3	1	4
Labrador	2	2	4
Maltese	2	0	2
Miniature Poodle	2	1	3
Lagotto Romagnolo	1	0	1
American Staffordshire	1	0	1
Pug	1	0	1
Bernese	1	0	1
Springer Spaniel	1	0	1
Setter	1	0	1
Boxer	1	0	1
Breton	1	0	1
Collie	0	1	1
Australian Shepherd	0	1	1
German Shepherd	0	1	1
Sharpei	0	1	1
Japanese Spitz	0	1	1
Shih-tzu	0	1	1

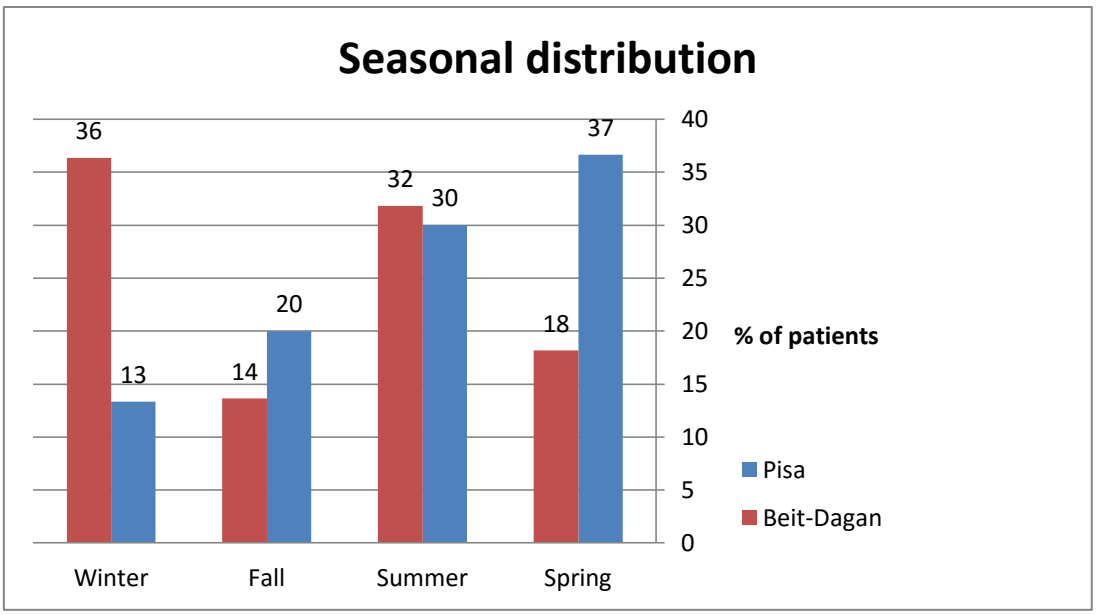
SEASON

Following the division of the study populations into sub-groups based on season of clinical presentation of the disease, a Chi-squared test was performed separately for each season to look for significant difference in seasonal occurrence between the two hospitals. No significant differences were found (P >0.05). Another Chi-squared test was performed in order to evaluate if significant differences exists in the occurrence of the disease between the different seasons. The test showed no increased occurrence in any of the seasons (P >0 .05).

Table 7.5 Season of clinical presentation.

Season	Pisa	Beit-Dagan	Total
Spring	11	4	15
Summer	9	7	16
Fall	6	3	9
Winter	4	8	12

Chart 7.3 Seasonal distribution**.**



7.3.2 CLINICAL FINDINGS

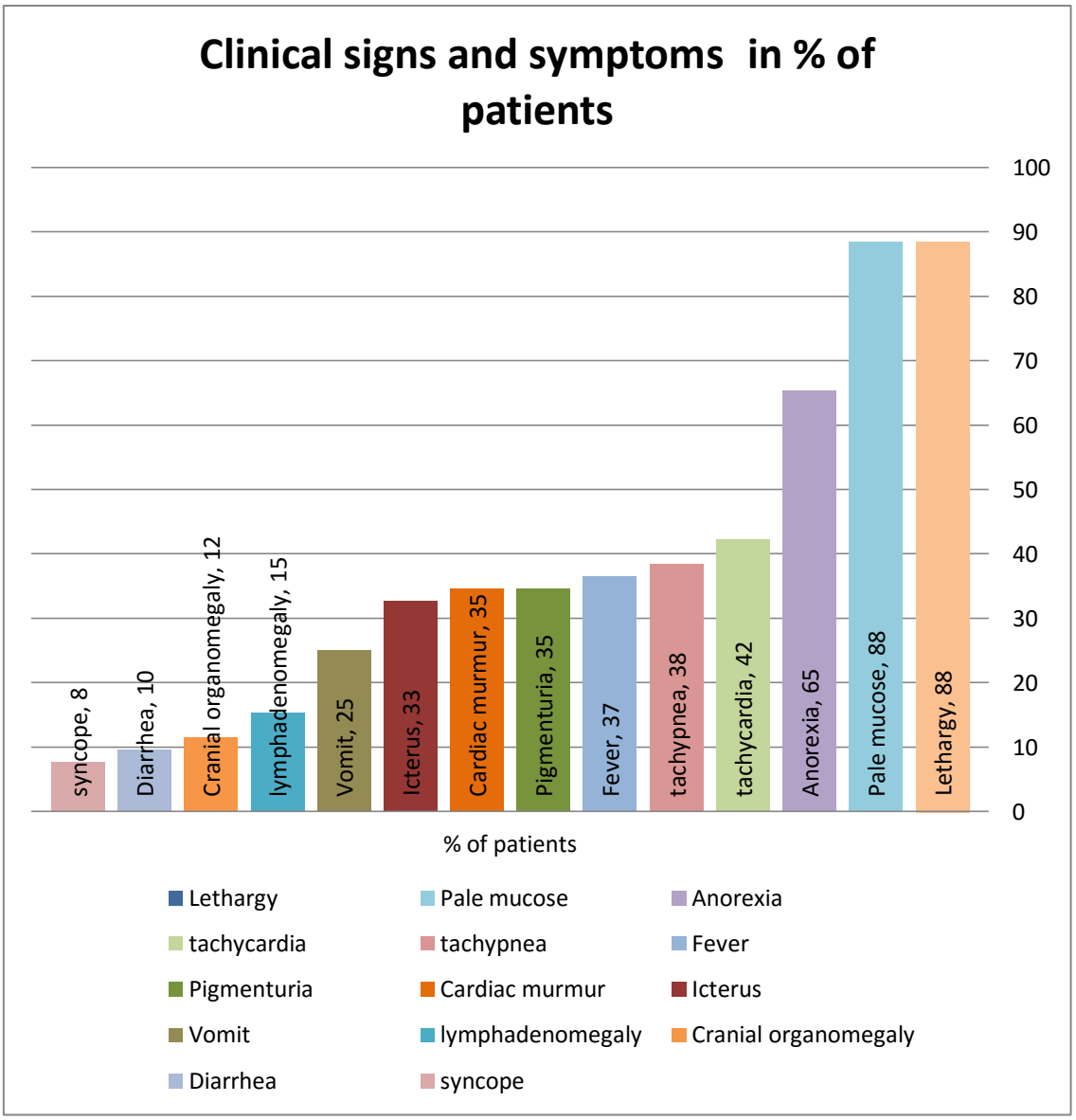
Clinical alterations reported in this study include alterations detected by owner- or better yet, symptoms, as well as clinical signs observed by clinician during the physical examination. Among dogs in this study group, the most common alterations detected by owners were lethargy reported in 88% of dogs (n=46), anorexia in 65% of dogs (n=34) and pigmenturia in 35% of dogs (n=18). Most common clinical signs revealed by clinicians were pale mucous membranes reported in 88% of dogs (n= 46), tachycardia in 42% of dogs (n=22) and tachypnea in 38% of dogs (n=20). A Chi-squared test for proportions was used and some differences were noted regarding the clinical alterations between the two hospitals. They are reported in the table 7.6.

Table 7.6 difference in symptoms and clinical signs between the two hospitals**.**

Symptom	Pisa (n patients)	Beit-Dagan (n patients)	P=	Total
Pale mucous membrane	26	20	> 0.05	46
Lethargy	25	21	> 0.05	46
Anorexia	16	18	> 0.05	34
Fever	12	7	> 0.05	19
Cardiac murmur	12	6	> 0.05	18
Pigmenturia	9	9	> 0.05	18
Tachycardia	8	14	**0.017**	22
Vomit	7	6	> 0.05	13
Icterus	6	11	**0.048**	17
Tachypnea	5	15	**0.001**	20
Lymphadenomegaly	4	4	> 0.05	8
Syncope	4	1	> 0.05	5
Cranial organomegaly	2	4	> 0.05	6
Diarrhea	2	3	> 0.05	5

Minor variation in the clinical presentation of the disease exists between the two groups. Symptoms which varied significantly were: increased respiratory rate and heart rate (P=0.001 and P=0.017, respectively). Another parameter that differed significantly was icterus (P=0.048), which is a qualitative measurement that will be confirmed by comparing the total bilirubin value discussed in the biochemistry section (7.3.3). Arithmetic means and standard deviation of body temperature were similar between the two hospitals (38.8±0.815 and 38.6±0.731 in *Pisa* and *Beit-Dagan* respectively) and a Chi-squared test performed on the data showed no significant difference (P>0.05) between the two hospitals.

Chart 7.4 clinical signs and symptoms for entire study group.



Correlation analysis preformed using the Karl-Pearson correlation coefficient found a moderate correlation between few symptoms: Icterus and pigmenturia (r =0.508, P= 0.001). Tachycardia and tachypnea were moderately correlated as well (r = 0.557, P= 0.0001).

7.3.3 CLINICOPATHOLOGICAL ALTERATIONS

ERYTHROCYTES

Table 7.7 comparison of erythrocyte parameters between the two hospitals.

Parameter	Pisa	Range (min-max)	median	Beit-Dagan	Range (min-max)	Median	P=
RBC M\μL	n=30	0.93-5.48	2.00	n=21	0.57-4.18	2.0	>0.05
Hct %	n=30	8.7-30.1	17.2	n=22	4.8 - 31.6	15.3	>0.05
HGB g\dL	n=30	2.2-12.5	4.7	n=21	2.1 -10.8	5.0	>0.05
MCV fL	n=30	61.8-110.9	81.3	n=21	61.8 – 84.0	75.5	**0.004**
MCH pG	n=30	19.3-29.4	24.4	n=21	19.1-61.7	25.8	>0.05
MCHC g\dL	n=30	22.5-36.0	30.3	n=21	24.2-73.2	34.0	**0.001**
RDW %	n=30	14.1-38.7	21.4	n=21	10.0-42.0	18.6	**0.018**

Mann-Whitney tests were used for comparison of data for all parameters showed in table 7.7. Three erythrocyte indexes were significantly different between the two hospitals: Mean corpuscular volume (P=0.004), mean corpuscular hemoglobin concentration (P=0.001) and red blood cell distribution width (P=0.018). Reviewing the data we noticed that one dog from the *Pisa* group had significantly higher MCV (110.9) that could alter the calculation. Nonetheless, repetition of the statistic test after disregarding the extreme value yield similar results (P= 0.014). It appears that the *Pisa* group had indeed significantly larger erythrocytes in comparison with the *Beit-Dagan* group.

Erythrograms of dogs with immune mediated hemolytic anemia are usually characterized by a highly regenerative, macrocytic, hypochromic anemia. In our study 67% (35/52) of dogs suffered from severe anemia defined by a hematocrit value equal or lower then 20. 33% (17/52) of dogs had moderate anemia at time of blood sampling, with hematocrit values of less than 30%. Classification of anemia was performed for all patients.

Table 7.8 Anemia classification comparison between hospitals.

Type of anemia	Pisa	Beit-Dagan	P=
macrocytic-hypochromic	19	3	**0.001**
macrocytic-normochromic	7	7	>0.05
nomocytic-normochromic	3	2	>0.05
normocytic-hypochromic	1	1	>0.05
normocytic-hyperchromic	0	2	>0.05
macrocytic-hyperchromic	0	6	**0.005**

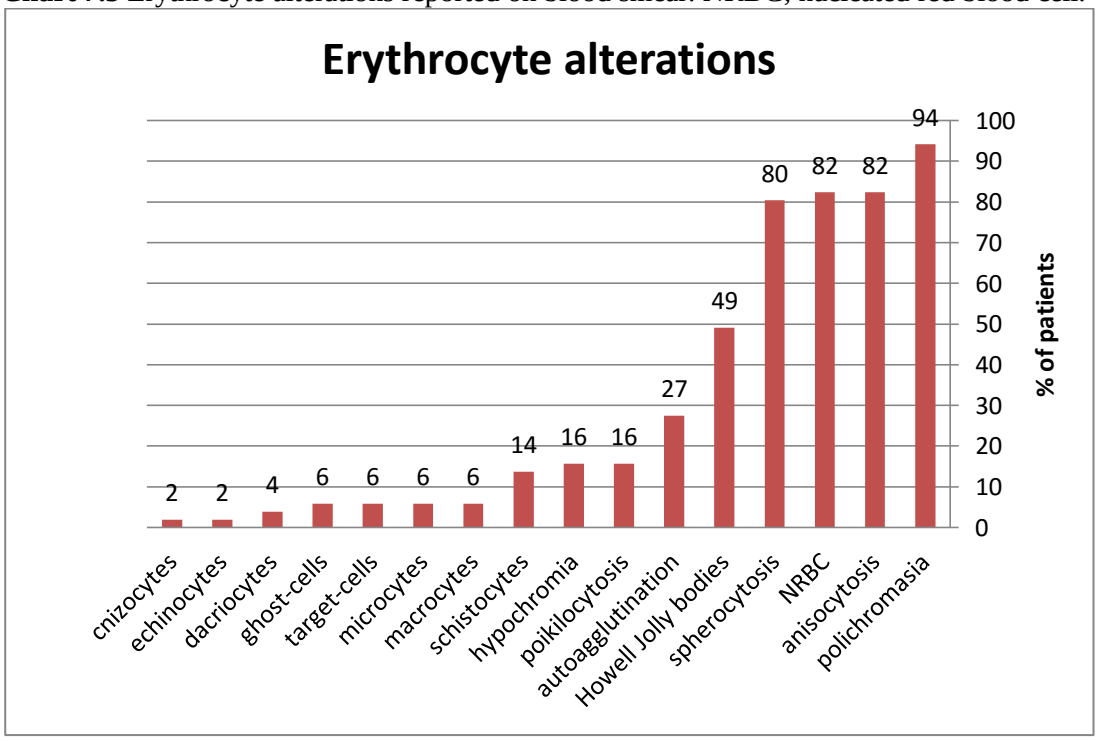
As seen in table 7.8, nineteen out of thirty patients in the *Pisa* group had macrocytic-hypochromic anemia. The *Beit-Dagan* group was more variable in terms of anemia classification and had two types of anemia represented more frequently; macrocytic-normochromic (7/21) and macrocytic-hyperchromic (6/21). Significant differences between groups were calculated using a Chi-squared test for proportions. We found significant difference in the proportions of the following types of anemia: macrocytic-hyperchromic (P=0.001) and macrocytic-hyperchromic (P=0.005).

Table 7.9 Summery of entire study group anemia classification.

Type of anemia	normochromic	hypochromic	"hyperchromic"/hemoglobinemia
normocytic	5	2	2
microcytic	0	0	0
macrocytic	14	22	6

Out of fifty-two dogs with IMHA in this study, 43% (22/51) had macrocytic-hypochromic anemia, 27% (14/51) had macrocytic-normochromic anemia, 12% (6/51) had macrocytic-hyperchromic anemia, 10% (5/51) had normocytic-normochromic anemia, 4% (2/51) had normocytic-hypochromic anemia and 4%(2/51) had normocytic-hyperchromic anemia. RBC indexes of one dog weren't available.

Chart 7.5 Erythrocyte alterations reported on blood smear. NRBC; nucleated red blood cell.



Blood smear evaluation was performed in fifty-one out of fifty-two patients. The most frequent erythrocyte morphological alteration in this study was polychromasia in 94% of dogs (48/51) followed by anisocytosis (42/51) and nucleated RBCs (42/51) in 82% of dogs. Spherocytosis (80%) and Howell-Jolly bodies (49%) were frequently observed as well (41/51 and 25/51 dogs respectively). A few other morphological abnormalities were observed with a lower frequency and they were: microagglutination in 27% of dogs (14/51), hypochromia and poikilocytosis in 15% of dogs (8/51), schistocytes in 14% of dogs (7/51), microcytes, macrocytes, ghost-cells and target-cells in 6% of dogs (3/51), dacriocytes in 4% of dogs (2/51), cnizocytes and echinocytes in 2% of dogs (1/51).

Fig 7. 1 Nucleated red blood cell

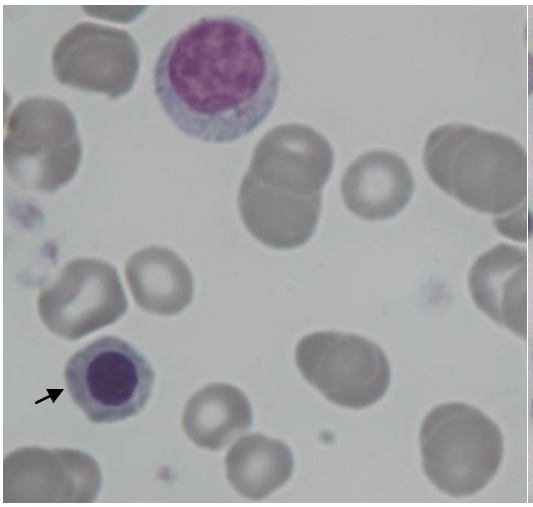


Fig 7.2 Anisocytosis. High arrow: microcyte, low arrow: spherocyte

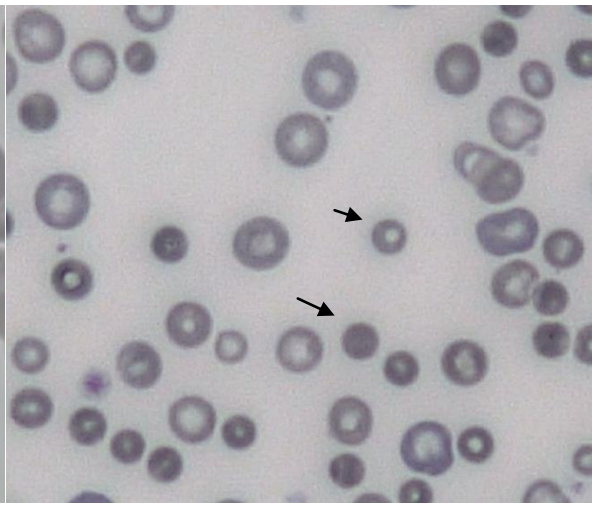


Fig 7.3.Howell-Jolly body

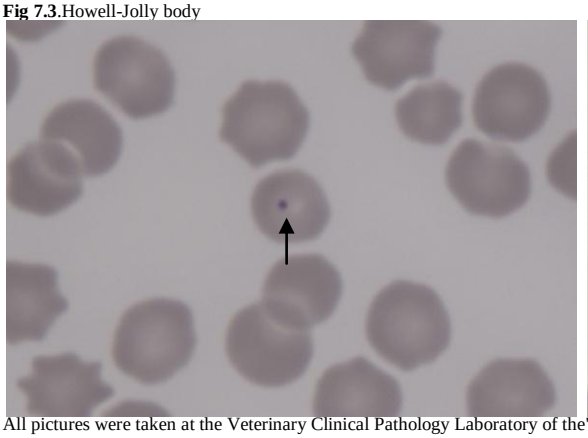
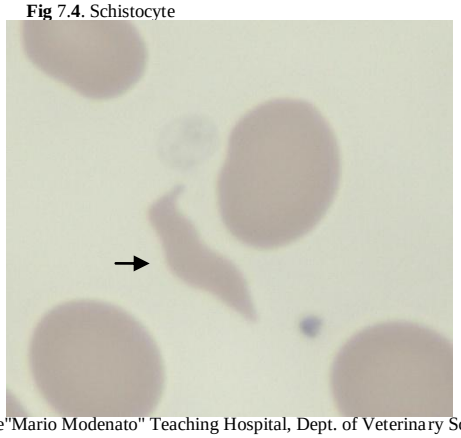


Fig 7.4. Schistocyte



All pictures were taken at the Veterinary Clinical Pathology Laboratory of the"Mario Modenato" Teaching Hospital, Dept. of Veterinary Sciences, Pisa University

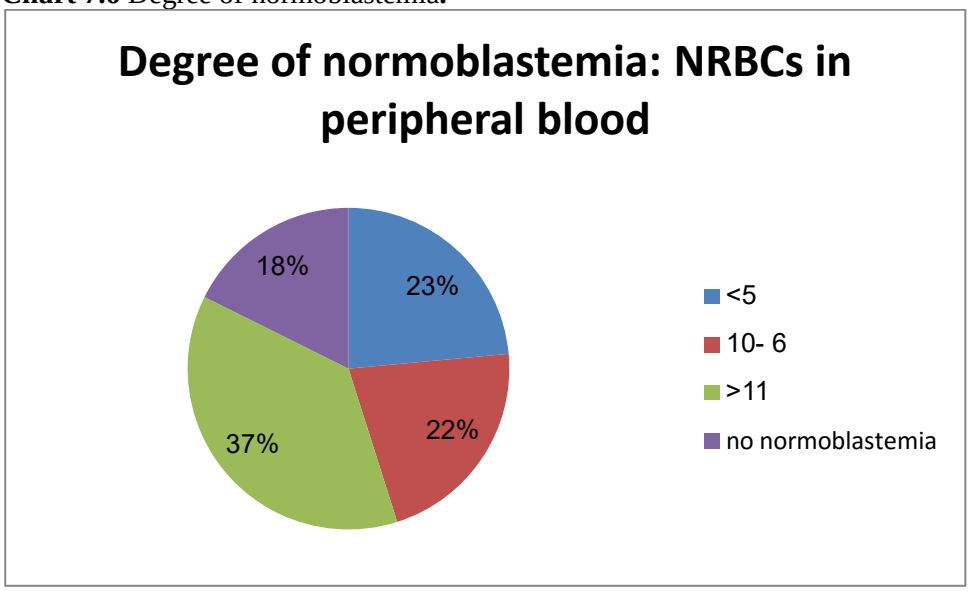
In this study 82% of dogs had NRBCs (42/51) in peripheral blood. The level of normoblastemia was divided in three categories based on number of NRBCs counted per 100 WBC: 1-5, 6-10 and more than 11.

Table 7.10 Degree of normoblastemia and its distribution in the study groups.

NRBC:	Pisa	Beit Dagan	Total	P=
>5	5	4	12	>0.05
6 -10	8	6	11	>0.05
>11	14	5	19	>0.05
no normoblastemia	3	6	9	>0.05

As seen in table 7.10, no significant differences regarding normoblastemia were found between hospitals, using the Chi- squared test.

Chart 7.6 Degree of normoblastemia.



18% of dogs (9/51) had no normoblastemia, 24% (12/51) had five or less NRBCs in the blood smear, 22% (11/51) had between six to ten NRBCs in blood smear and 37% (19/51) had more than eleven NRBCs in blood smear.

The presence of NRBCs was weakly but significantly correlated to the presence of macrothrombocytes (r=0.32, P=0.024), as well as to the presence of band neutrophils (r=0.32, P=0.024).

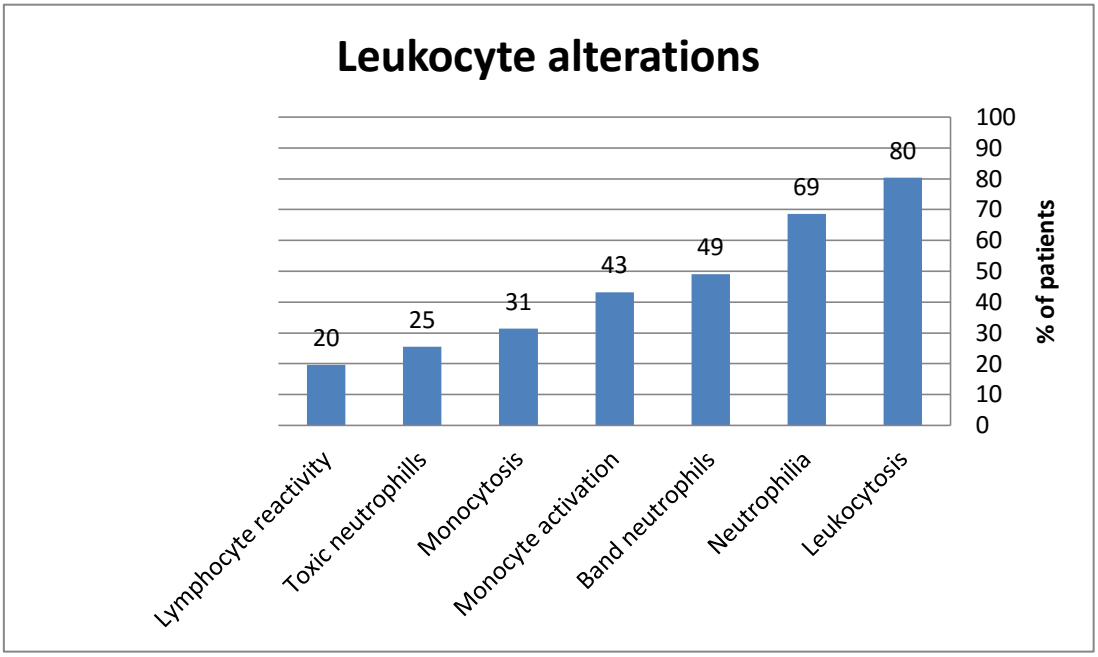
LEUKOCYTES

Table 7.11 comparison of leukocyte parameters between the two hospitals.

Parameter (10^3/µL)	Pisa	Range (min-max)	median	Beit-Dagan	Range (min-max)	Median	P=
WBC	n=30	7.7 - 52.6	20.6	n=21	3.6 - 59.9	23.74	>0.05
Neutrophils	n=30	4.8-43.5	16.04	n=15	2.82 - 43.2	15.04	>0.05
Band Neutrophils	n=30	0 - 4.97	0.49	NA	NA	NA	-
Lymphocytes	n=30	0 - 4.1	1.00	n=19	0.27 - 14.80	3.93	0.001
Monocyte	n=30	0 - 7.5	2.11	n=19	0.29-5.49	1.17	>0.05
Eosinophils	n=30	0- 0.26	0.01	n=15	0 - 0.46	0.10	>0.05

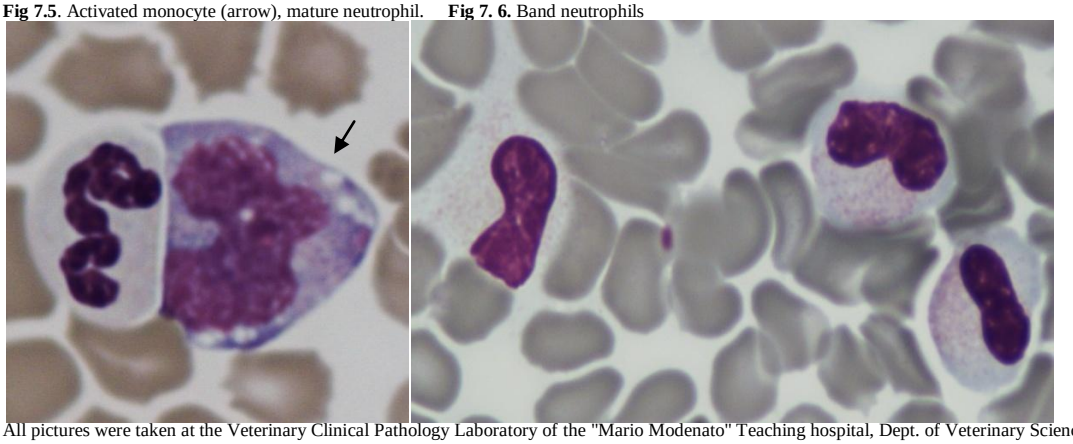
Mann-Whitney tests were used for data comparison for all parameters specified in table 7.11. Significant difference was found in the lymphocyte count between the two hospitals (P=0.001).

Chart 7.7 Leukocyte alterations**.**



Leukocytosis was found in 80% (41/51) of dogs in this study, and was in 69% (35/51) of cases, neutrophilic. Band neutrophils were reported in 49% (25/51) of cases. Monocytosis was seen in 31% (16/51) of cases. Monocyte activation, Neutrophilic toxicity and lymphocyte reactivity were reported in 43% (22/51), 25% (13/51) and 20% (10/51) of cases, respectively.

Fig 7.5. Activated monocyte (arrow), mature neutrophil. **Fig 7. 6.** Band neutrophils



All pictures were taken at the Veterinary Clinical Pathology Laboratory of the "Mario Modenato" Teaching hospital, Dept. of Veterinary Sciences, Pisa University.

PLATELETS

Table 7.12 Platelet count.

Parameter (10^3/μL)	Pisa	range(min -max)	median	Beit-Dagan	range(min-max)	median	P=
PLT	n= 30	1 - 655	189	n=21	0 - 651	160	>0.05

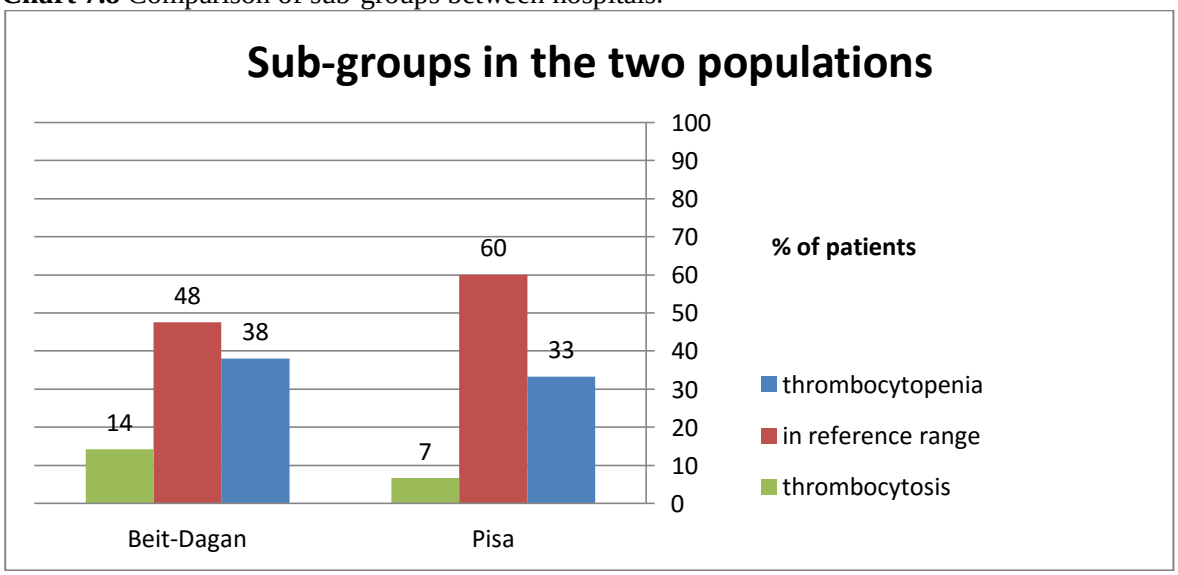
Platelets quantitative values in both hospitals were non parametric in distribution and therefore a Mann-Whitney test was used to compare these values. No significant difference was found regarding numbers of platelets between the two hospitals (P>0.05).

The groups were then divided to three sub-groups based on number of platelets: dogs with platelet count lower then reference range (thrombocytopenia); dogs with platelet count within reference ranges; dogs with platelet count higher than the reference range (thrombocytosis). Absolute numbers of platelets were also verified by the blood smear assessment of platelet and the presence or absence of platelet aggregates.

Table 7.13 Sub-groups of platelet quantities.

Platelet count	Pisa	Beit-Dagan	Total	P=
thrombocytopenia	10	8	18	>0.05
In the reference range	18	10	28	>0.05
thrombocytosis	2	3	5	>0.05

Chart 7.8 Comparison of sub-groups between hospitals.

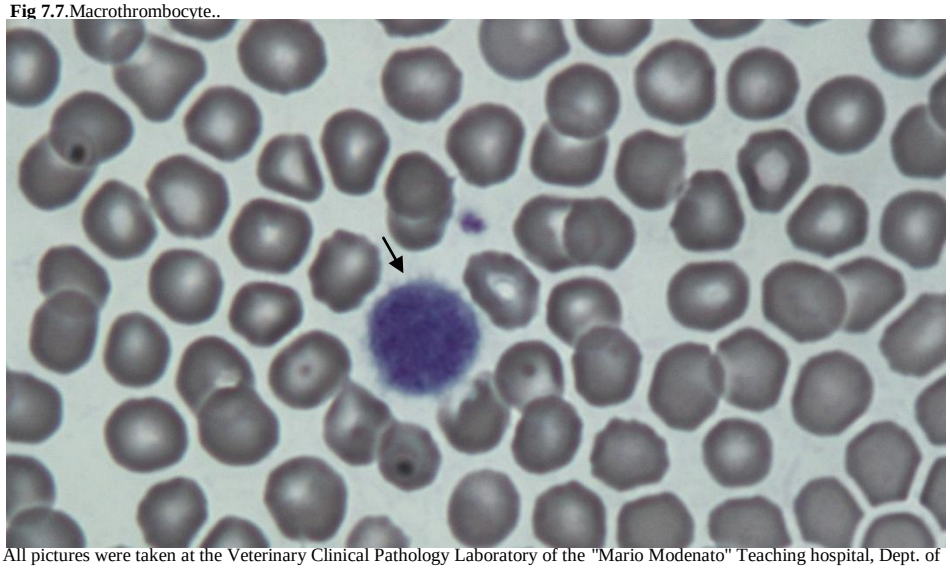


A Chi-squared test of proportions was used to compare sub-groups between the two hospitals. No significant differences were found between the two hospitals ($p>0.05$).

55% (28/51) of dogs had a normal platelets, 35% (18/51) were thrombocytopenic and 10% (5/51) had thrombocytosis.

Thrombocyte alterations were assessed in peripheral blood smear. 45% (23/51) of patients had lager then normal platelets (macro PLT) reported on blood smear. 2% (1/51) of patients had activated platelets reported on blood smear. Macrothrombocytes were not correlated with thrombocytopenia using a Pearson correlation test ($r= -0.001$, $P=0.946$)

Fig 7.7.Macrothrombocyte..



All pictures were taken at the Veterinary Clinical Pathology Laboratory of the "Mario Modenato" Teaching hospital, Dept. of Veterinary Sciences, Pisa University

BIOCHEMISTRY PROFILE

Table 7.14 Comparison of biochemistry parameters between the two hospitals.

Parameter	Pisa	range (min-max)	Median	Beit-Dagan	range (min-max)	Median	P=
TP	n=28	4.9-8.7	7.00	n=21	3.9-7.7	6.30	**0.02**
ALB	n=28	2.0-4.5	3.19	n=21	1.7-4.4	3.10	>0.05
Bilirubin	n=28	0.19-9.2	0.80	n=19	0.01-56.64	1.40	>0.05
C-reactive protein	n=23	0-10.3	1.40	NA	NA	NA	NA

Mann-Whitney tests were used to compare data from both hospitals. Significant difference was found in the total protein (TP) measurements (P= 0.02). Hyperproteinemia was present in 14% (4/28) of dogs in the *Pisa* group and 5% (1/21) of dogs from the *Beit-Dagan* group.

7.4 DISCUSSION

General Considerations

First, it is essential to mention a potential bias risk in selection of subjects. Study exclusion criteria indicated elimination of cases with positive serology to infectious disease that could have caused the hemolytic crisis. Four suspected cases were excluded due to seropositivity to infectious disease despite the fact that the test is not 100% specific [136]. Furthermore, positive serology in endemic areas doesn't necessarily indicate an active infection[137,138]. One case of a 6-month-old puppy with IMHA was included in the study. The age of presentation did not fit the usual IMHA patient profile, and yet diagnostic procedures including serology and PCR were negative and could not identify a primary cause for the disease. The case was included in the study despite the fact that neither of the tests were 100% sensitive [136,139].

Data used for this study was collected from a computerized database in *Pisa* and from clinical charts in and *Beit-Dagan*. Since the charts were hand-written in most cases by veterinary students, they were more difficult to interpret and collect into homogeneous, empirical data. Information was more easily retrieved from the *Pisa* computerized database primarily because it was more systematic and uniform.

Diagnostic work-up varied between hospitals. The most significant difference was the lack of a manual WBC differential count in the *Beit-Dagan* cases. A manual differential WBC count is one of the vital elements of a blood smear evaluation and is preformed routinely for almost every blood sample in the *Pisa* Clinical Pathology Laboratory. The manual differential count was made for all cases from the *Pisa* group and was considered in all cases to be a more accurate quantification of the various leukocyte populations.

Some biochemistry parameters were tested for in *Pisa* but not in *Beit-Dagan*. One of them is the C-reactive protein (mg/dL); an important acute-phase inflammatory marker that might be used as for long-term monitoring of IMHA patients[37]. A definitive diagnosis of IMHA was made in *Pisa* by means of flow-cytometry to detect antibodies anti-RBC on the surface of erythrocytes (n=22). At *Beit-Dagan*, a definitive diagnosis of IMHA was made by means of osmotic fragility test (n=13), which confirmed the presence of spherocytes, in-slide saline auto agglutination test (n=4), detection of spherocytes or micro-agglutination in blood smears. The cost-effective value of flow cytometry for diagnosis of IMHA is debatable. According to some authors [15,34] the presence of spherocytes or saline-persistent auto agglutination together with matching clinicopathological alterations is enough to make a diagnosis of IMHA. On the other hand, other authors [6,8,50] claim that either a Coombs' test or a flow-cytometry are necessary in order to have a definitive diagnosis. A correct diagnosis is important in order to effectively treat affected dogs and avoid treating unaffected dogs with potentially harmful drugs[48]. In this study we have two veterinary teaching facilities that represent two different diagnostic approaches. Whether one approach surpasses the other in terms of treatment efficiency or cost-effective value is yet to be determined by further research.

Signalment

Age of first clinical presentation of the disease was similar in both hospitals and no significant difference was noted (P>0.05). The arithmetic mean and standard deviation of age in the entire study population (*Pisa* and *Beit-Dagan*) was 7.00±3.36. Both groups had a similar (P>0.05) gender distribution. The entire study population was composed of 29% females, 38% neutered females, 23% males and 10% castrated males. Neutered females were slightly overrepresented in our study. Compared with the *control* group, the *Pisa* group had a significantly greater proportion of neutered females (P=0.021). Study population was quite variable in terms of breed and 19 different breeds were represented. No significant differences existed between the two hospitals (P>0.05) in terms of breed representation. 54% of dogs in this study were mixed-breed dogs. The breeds most represented were Cocker Spaniel and Labrador retriever with 4 subjects of each of these breeds in the entire study population. However, when a comparison of proportions was made between the *Pisa* study group and the *control* group we found no significant difference in proportions regarding the Labrador Retriever breed (P>0.05). Apparently this breed is very popular in Italy and is overrepresented in the *control* group as well. Cocker-Spaniels were indeed overrepresented, even when compared to the *control* group (P=0.025). Furthermore, the Maltese breed was also significantly overrepresented when compared to the *control* group.

Canine immune mediated hemolytic anemia is described in the literature as a disease of middle-aged dogs (6-8 years)[2,17,20], with a higher prevalence among females[17] and mainly neutered females[17,20]. The Cocker-Spaniel breed is often reported as overrepresented in IMHA studies[6,17,19,20,140] and some authors claim it to have a genetic predisposition to the disease[18]. The Maltese breed is likewise commonly reported in studies of IMHA [2,43,125,132,141]. Our findings in this study are in agreement with findings of recent studies regarding signalment and patient profiling.

Seasonality

The question whether IMHA is a seasonal disease has been frequently debated. Some authors have described an increased occurrence of the disease during the warm season [11,20,30,31]. Others claim that no seasonal variation exists [6,15]. In our study, two groups from different climate settings were compared. No significant difference was found between the groups ($P>0.05$) and no season was particularly represented in our study ($P>0.05$). Increased exposure to infectious agents in certain seasons might be the cause of the seasonal variation reported by several authors[34]; some dogs with tick borne disease might be under-diagnosed due to the hyper-acute nature of these diseases. We suspected that in Israel, the disease would be more frequent during the warm season since it is considered to be endemic for some tick borne diseases [142]. Possibly our sample size ($n=22$) is not large enough to demonstrate such seasonal predisposition.

Clinical findings

Symptoms reported in this study were collected from owners by veterinary students or clinicians. Clinical signs were collected by a clinician or a student with further confirmation by a clinician. The term "pigmenturia" was used as a substitute for red, dark red, orange or brown urine reported by owners since retrospectively it is impossible to know with certainty if the color alteration was due to hemoglobinuria, bilirubinuria, excessive presence of urobilinogen or erythrocytes in the urine.

Some variations were found between the clinical presentation in *Pisa* and the one in *Beit-Dagan*. Animals presented to the *Beit-Dagan* hospital were more frequently tachypnoic and tachycardic (P=0.001 and P=0.017) than those presented to the *Pisa* hospital. The two clinical signs were also moderately correlated to each other (r = 0.55, P= 0.001). One possible hypothesis is that the difference is due to weather conditions which differ greatly between the two countries. Central Israel has a very warm climate throughout 7 months of the year that could explain why more dogs are presented with tachypnea and tachycardia [143,144]. However, when correlations were calculated between symptoms, no correlation was detected between fever and rapid respiratory or heart rate. Furthermore, the arithmetic means of body temperature from each of the groups were very similar (38.8 Cº and 38.6 Cº in *Pisa* and *Beit-Dagan*, respectively) and no significant difference was noted (P>0.05). Another hypothesis is that dogs from the *Beit-Dagan* group were more frequently or more severely hypoxic in comparison to dogs from the *Pisa* group and therefore had a more rapid respiratory rates[145]. The latter hypothesis cannot be confirmed since an arterial blood gas analysis is indicated in order to associate tachypnea to hypoxia and was not preformed for the majority of patients. Tachypnea has also been described in dogs suffering from pulmonary thromboembolism [146]. The involvement of a thrombo-embolic event in tachypnoic patients with IMHA is possible, but a necropsy is warranted in order to confirm this hypothesis.

Patients from the *Beit-Dagan* group were also more frequently icteric than the ones from the *Pisa* group (P=0.048). Icterus or jaundice is the clinical manifestation of high serum bilirubin concentration and is visible in mucous membranes, skin and sclera of dogs in which levels of serum bilirubin exceed 1.5 mg/dL. When a hemolytic process is present, the amount of bilirubin in circulation exceeds the hepatocytes' capacity to perform glucuronide conjugation and secretion into the intestinal tract. Jaundice in IMHA dogs is often related to a more severe and acute intravascular hemolysis[6] rather than the more common extra vascular hemolytic form of the disease. It is possible that more patients from the *Beit-Dagan* group had an acute onset of the disease. Icterus was moderately correlated to the symptom pigmenturia (r =0.51, P= 0.001). The latter could be explained by the renal excretion of excess bilirubin found in the blood prior to its clinical manifestation as jaundice.

The most common symptoms in this study were lethargy (n=46), anorexia (n=34) and pigmenturia (n=18). Most common clinical signs were pale mucous membranes (n= 46), tachycardia (n=22) and tachypnea (n=20). Other frequent findings included: fever (n=19), cardiac murmur (n=18), icterus (n=17) and vomit (n=13). All the above mentioned symptoms and signs have been commonly reported in the literature [2,6,17,20]. Less frequent clinical findings in our study were lymphadenomegaly (n=8), cranial organomegaly (n=6), syncope (n=5) and diarrhea (n=5). These symptoms have been reported less commonly in the literature as well [2,8,147].

Clinicopathological alterations

Clinicopathological alterations evaluated in this study included: erythrocyte, leukocyte and platelet parameters and a blood smear evaluation. Additionally, a few selected biochemistry parameters that were previously reported by some authors as significant for the assessment of disease gravity and prognosis[2,17,20,24] were evaluated as well.

Some differences were noted between the two hospitals regarding erythrocyte parameters. MCV, MCHC and RDW indexes differed significantly (P=0.004, 0.001 and 0.018, respectively). The MCV value is a measurement of the average volume of a patient's RBCs. Larger than normal RBCs are considered to be a sign of regeneration since the "younger" cells, known as polychromatophils, are bigger then mature erythrocytes and therefore increase the average size measurement. Furthermore, the common phenomenon of autoagglutination of RBCs in the course of a hemolytic crisis can potentially increase MCV values since the agglutinated erythrocytes can be counted by the automated analyzers as one large erythrocyte. MCV values were significantly higher in the *Pisa* group. A low MCHC value indicates lower concentration of hemoglobin inside the cells and is a typical observation in regenerative anemia. MCHC values were significantly lower in the *Pisa* group. The difference between hospitals could in fact indicate that some of the patients in the *Beit-Dagan* group were presented to the hospital in a pre - regenerative phase of IMHA. Interestingly, 8/21 dogs in the *Beit-Dagan* group had high MCHC values – which is considered to be a pre-analytical error since the erythrocyte cannot "over-saturate" with hemoglobin. This error usually indicates biological or mechanical hemolysis, and in the case of IMHA dogs could be an indication of intravascular hemolysis mediated by the immune system.

RDW value reported by the automated analyzers reflects the level of variability in terms of size among the erythrocyte population in the blood. As mentioned before, younger erythrocytes are bigger then mature erythrocytes. The presence of many younger erythrocytes can increase the RDW value and is an indication, in many cases, of erythrocyte regeneration. RDW values are particularly high in IMHA patients due to the significant size difference between spherocytes and polychromatophils.

Regeneration of RBCs starts at three days after onset of hemolysis and peaks at seven days after it[148]. It is therefore possible to assume that the *Beit-Dagan* group is characterized by a more acute onset of the disease and consequently shows less signs of regeneration.

As previously mentioned, classification of anemia was performed for all patients in which RBC indexes were available (51/52). Some differences were noted regarding anemia classification proportions between the two hospitals. The *Pisa* group had significantly (P=0.001) more patients with macrocytic-hypochromic anemia. The latter suggests a more frequent regenerative response in that group[5,148]. The *Beit-Dagan* group had significantly more (P=0.005) patients with macrocytic-hyperchromic anemia. The latter suggests a more frequent intra-vascular hemolytic process among patients from the *Beit-Dagan* group.

In this study 67% of dogs suffered from severe anemia defined by a hematocrit value equal or lower then 20 and 33% of dogs had moderate anemia defined by a hematocrit value of less than 30. Classification of anemia revealed that 43% of dogs had macrocytic-hypochromic anemia, 27% of dogs had macrocytic-normochromic anemia, 12% of dogs had macrocytic-hyperchromic anemia, 10% of dogs had normocytic-normochromic anemia, 4% of dogs had normocytic-hypochromic anemia and 4% of dogs had normocytic-hyperchromic anemia. RBC indexes of one dog were not available.

A recent review of the diagnostic approach to anemia in the dog and the cat [148] referred to hemolytic anemia as initially pre-regenerative (normocytic and normochromic). This is followed by a phase in which the anemia shift into the macrocytic hypochromic pattern, when reticulocytosis becomes relevant. In addition, a regenerative anemia may have enough macrocytosis to increase the MCV value, but not enough hypochromic RBCs to decrease the MCHC value out of the reference interval [5] resulting in a macrocytic-normochromic anemia. All phases of regenerative response to hemolytic anemia described by the literature were represented by our study population. Furthermore, a few patients exhibited hematologic signs of hemoglobinemia, which was previously described as a characteristic element of the acute onset of IMHA[6]. It is also possible that in *Pisa*, where blood sampling is preformed primarily by clinicians, the event of mechanical hemolysis due to difficulties in venipuncture is less common.

The interpretation of erythrocyte indexes should always be made together with a blood smear evaluation, since they are easily altered by large numbers of abnormal RBCs. Dogs with IMHA often have morphologic abnormalities that can only be identified by examining the patient's blood smear.

The most frequently observed erythrocyte morphologic alteration in our study was polychromasia followed by anisocytosis (94% and 82%, respectively). These alterations confirm the data received from the automated analyzers. Polychromasia and anisocytosis indicate that a population of young RBCs, called polychromatophils is present in the blood. Polychromatophils correspond frequently to reticulocytes and indicate an increased release of those cells from the bone marrow or from extra-medullary hemopoietic organs [5]. Howell-Jolly bodies (HJB) are small round nuclear residues commonly found in RBCs in regenerative anemia. In this study, 49% of patients had HJB in peripheral blood. These findings are consistent with the definition of a highly regenerative anemia reported in other IMHA studies[6].

Nucleated RBC's were found in 82% of blood smears. The presence of these nucleated erythroid cells in peripheral blood can also be referred to as normoblastemia. NRBCs are not normally present in the peripheral blood of healthy adult dogs except in very low percentages. No explanation is available on the mechanism that prevents these cells from reaching the peripheral circulation. The most probable hypothesis is that, possibly as a result of the presence of the nucleus, NRBCs are not deformable enough to pass the bone marrow sinus endothelium and enter into the lumen of blood vessels. Therefore, an elevated NRBC count in the peripheral blood may indicate disruption of the blood-bone marrow barrier or, alternatively , that extra-medullary haematopoiesis has been activated in response to anemia[149]. The trigger for this response is believed to be hypoxia[150]. Hypoxic erythropoietin-induced compensatory erythropoiesis has been reported in human patients with cardiopulmonary disorders, such as pulmonary emboli and may indicate unfavorable prognosis[150]. In this study, 18% of dogs had no normoblastemia, 24% had five or less NRBC's in blood smear, 22% had between six to ten NRBCs in blood smear and 37% had more than eleven NRBCs in blood smear. Normoblastemia has been frequently reported in dogs with IMHA[6], yet its prognostic value for these patients is still unclear.

Correlations between NRBCs in peripheral blood and other types of cells have been investigated in this study. We found a weak but significant correlation between NRBCs and macrothrombocytes (r=0.32, P=0.024) and band neutrophils (r=0.32, P=0.024). This three cell types have been previously reported in humans and dogs in which an extra-medullary hematopoietic mechanism has been activated[150–152]. The combination of these three alterations was present in 13 dogs (25%) that took part in this study. The significance of this observation and its relation to extra-medullary hematopoiesis in IMHA dog cannot be confirmed without a fine needle aspiration (FNA) or hepatic and splenic biopsy and therefore further study of the subject is warranted.

Spherocytosis and autoagglutination are the most frequently reported RBC morphologic alterations in patients with IMHA[6,11,20,34,92,140]. Reported percentage of spherocytosis in IMHA study groups vary from 70 to 80 percent in most studies[11,140]. In the present study, 80% of patients had spherocytosis. Autoagglutination was present in 27% of our patients. Other authors have reported greater percentages of autoagglutination in IMHA studies[15,140] (60-87%).

Other morphologic abnormalities found in this study include: hypochromia and poikilocytosis (16% of dogs), schistocytes (14% of dogs), microcytes, macrocytes, ghost-cells and target-cells (6% of dogs) and dacriocytes (4% of dogs). Hypochromia is the visual confirmation of a low MCHC value indicated by the automated analyzers. It is often correlated to iron deficiency anemia[5], but in our case reflects the fact that the iron reservoir is decreasing and so the concentration of HB in the cells is lower than normal. The cells therefore appear more transparent under the microscope. Poikilocytosis is a non specific term for variation in RBC shape and should be further classified as to the type of shape change present. Schistocytes or schizocytes are RBC fragmentations that may appear in the blood when RBCs are forced through altered vascular channels or as a result of turbulent blood flow [5]. They have also been associated with disseminated intravascular coagulation (DIC) [5] ,thrombotic thrombocytopenic purpura in humans[153] and , microangiopatic hemolytic anemia in children [154] as well as in dogs [1]. The presence of RBC fragments in blood of IMHA dogs could be the result of DIC, which is a common complication of the disease. This alteration has been mentioned sporadically in IMHA dogs; according to Duncan and Prasse (2011) the presence of schistocytes suggests that excessive phagocytosis of erythrocytes is present[1] . The clinical application of this observation and its correlation to DIC or pulmonary thromboembolism is yet to be determined. Other alterations, which had lower frequencies among this study group (less than 10% of dogs) are considered arbitrary and their relevance to the disease in inconclusive.

As mentioned before, differential leukocyte counts were performed manually in *Pisa* and using automated analyzers in *Beit-Dagan*. The *Beit-Dagan* population had significantly more lymphocytes than the *Pisa* population (p=0.001). It is possible that the lymphocyte count was falsely increased by high numbers of NRBCs. Falsely increased lymphocyte counts due to normoblastemia is a common occurrence in the Pisa University Veterinary Clinical Pathology Laboratory and therefore a manual WBC count is performed for almost every blood sample (unless instructed otherwise).

The majority of our study population had leukocytosis at time of diagnosis (80%). Medians from both hospitals were well beyond reference intervals in both hospitals. Some dogs were presented with extremely increased leukocyte counts with maximum values of up to 59.9 and 52.6 (WBCx10^3\ ųL) in *Beit-Dagan* and *Pisa* respectively. This dramatic increase in WBC is defined by some authors as "Leukemoid Reaction" and has been previously described in IMHA patients[5,155]. The combination of the latter reaction along with the presence of NRBCs in peripheral blood is defined as "Leukoerythroblastic reaction" and may or may not include immature forms of neutrophils ("band" neutrophils)[156,157]. Leukocytosis was most often neutrophilic (69%) and was accompanied by a left shift in 49% of cases. Monocytosis was also observed, but less frequently (31%). These findings are consistent with previously described leukogramms of IMHA dogs[6,15,17,34,82,140]. Activated monocytes in blood smears of dogs are a frequent observation at the Pisa University Veterinary Clinical Pathology Laboratory. In addition to their phagocytic activity, monocytes secrete a variety of cytokines that are involved in hematopoiesis and modulation of inflammatory response. The term "activated monocytes" refers to cytoplasmatic vacuolation of monocytes in fresh blood smears[155]. In our study group we found microscopic evidence of monocyte activation in 43% of cases. Monocyte activation has been reported in people with sickle cell anemia[158]. In dogs, this finding may indicate a state of inflammation, but it scarcely correlates to any specific disease[155]. Neutrophilic toxicity and lymphocyte reactivity were reported in 25% and 20% of cases respectively. The presence of toxic neutrophils in non-infective disease has been reported previously in canine IMHA[159,160], among other non infective pathologies. Reactive lymphocytes are considered to be a sign that a strong immune stimulation is present. The latter has been attributed mainly to viral and chronic disease, but also to auto-immune disease[159].

Platelet counts were compared between the two hospitals, and no significant differences were found (P>0.05). As mentioned previously, three sub-groups based on the number of platelets were formed: dogs with platelet counts lower than reference range (thrombocytopenia); dogs with platelet counts within reference ranges; dogs with platelet counts higher than the reference range (thrombocytosis). Absolute numbers of platelets were also verified by the blood smear assessment of platelets and the presence or absence of platelet aggregates. No significant differences were found between the hospitals in terms of sub-group distribution (P>0.05). The majority of dogs (55%) had a normal platelet count, 35% were thrombocytopenic and 10% had thrombocytosis. Thrombocytopenia in IMHA patients is often mentioned in the literature, since the combined immune destruction of platelets and RBCs (also known as Evans Syndrome) is fairly common in dogs [6,8,20,34,82], as well as in humans[161]. Evans syndrome is correlated with a worse prognosis and increased chances of death [2,17,162] and occurs in 20-45% of IMHA cases[2,8]. Our study confirms the latter finding, with 35% of patients in the thrombocytopenic sub-group. Thrombocytosis can be the result of splenic contraction due to epinephrine release, immunosuppressive treatment and nonspecific bone marrow stimulation (as occurs in anemia). It was reported previously in correlation with extravascular hemolysis [1]. In our study, five dogs had thrombocytosis at time of blood sample and it is considered an occasional finding with little or no clinical significance.

Platelet (PLT) morphology was evaluated in peripheral blood smears. Platelets larger than normal (macrothrombocytes) were seen in 45% of blood smears. Platelet activation was only reported once in our study (2%). In IMHA-affected patients, platelets are a younger population recently mobilized from bone marrow[36]. Young platelets are larger and more metabolically and functionally active, and normally are sequestered in the spleen[36]. Another hypothesis that was previously discussed is the activation of an extra-medullary hematopoietic mechanism, causing release of larger than normal platelets from hemopoietic tissue [150–152] . Macrothrombocytopenia is a hereditary dysplasia in certain breeds (e.g. Cavalier King Charles Spaniel)[163], but these breeds were not represented in our study. It has been suggested that in IMHA-affected dogs, splenic contraction, associated with anemia or corticosteroid administration, could reduce platelet sequestration and allow these young metabolically active platelets to circulate. Regardless of the cause, the presence of macrothrombocytes in addition to activated platelets indicates that platelets may be more likely to contribute to prothrombotic events in dogs with IMHA[36]. Platelet activation in our study refers to the morphology of the platelets seen in blood smear. Activated PLTs appear to have cytoplasmatic prolongations and can be the result of a laborious venipuncture. To truly detect platelet activation, an evaluation of cell membrane expression of P-selectin using flow cytometry is required[36,164].

A few biochemistry parameters were selected and analyzed. Selection of parameters was based on prognosis indicators identified by recent veterinary hematology research[77]. C-reactive protein was selected for its possible use in monitoring response to treatment in IMHA patients.

A significant difference was noted between hospitals regarding total protein (TP) quantification (P=0.02), but not albumin quantification (P>0.05). An increase in plasma protein concentration may be present with intravascular hemolysis, resulting in artificial hyperchromasia that interferes with the automated reading of the analyte[1]. Surprisingly, the median of the *Pisa* group was significantly higher, although the latter group had less reported cases of artifactual hyperchromasia. Additionally, calculations of correlation between the two parameters yield no evidence of significant covariance between them (r=-0.14, P=0.341). It is therefore possible to assume that, since the albumin measurements did not vary significantly, the nature of the difference might be the plasmatic globulin concentration. Gamma or Immuno-globulin concentration tends to increase with infection or inflammation that causes a strong antigenic stimulation[155,165]. Furthermore, Acute phase proteins such as serum amyloid A, C- reactive protein and fibrinogen increase within 24-48 hours following tissue damage or inflammation[159,166]. These plasmatic elements may be the origin of a relative increased in TP among the *Pisa* group. Nonetheless, in order to properly study protein alterations a serum protein electrophoresis (SPE) is required. SPE was not performed in the majority of IMHA cases presented to the veterinary hospitals and was not evaluated in this study. Hyperproteinemia was present in 14% of dogs in the *Pisa* group and 5% of dogs from the *Beit-Dagan* group. This alteration has been formerly discussed by some authors[76,77,166], and can be used along with other parameters to predict the outcome of the disease [76,77].

Increased bilirubin was frequently reported in both hospitals. Medians were increased three and seven times higher in comparison to the upper reference value in *Pisa* and *Beit-Dagan*, respectively. Although no significant differences were noted between hospitals (P>0.05), the median in *Beit-Dagan* was distinctively higher and may justify the increased number of patients presented with icterus. Pre-hepatic, hemolysis induced hyperbilirubinemia is very commonly reported in IMHA patients[2,11,20,77] and as discussed formerly can be used along with other parameters to predict the outcome of the disease[77,167]. Measurement of C - reactive protein (CRP) was only available for the *Pisa* group. A recent study investigated serum concentrations of CRP in thirty dogs with IMHA. The authors found that all dogs with IMHA had CRP values higher than the reference interval at time of presentation [37]. In the present study, 78% (18/23) of dogs that were tested for CRP serum concentrations had values higher than reference range.

7.5 CONCLUSIONS

This retrospective study of 52 dogs with primary IMHA confirms the findings previously described by the literature regarding clinical and clinicopathological alterations of this complex disease.

Reviewing the results, it is possible to draw some conclusions about the nature of the two groups collected. The *Beit-Dagan* group was characterized by a more severe and acute onset of IMHA, with more evidence of intravascular hemolysis and a slightly lower Hct median. Some dogs in this group were presented in a pre-regenerative phase of IMHA, a fact that may suggest a more acute presentation of the disease. The *Pisa* group was characterized by less acute onsets and more cases were referred to the hospital for the purpose of specialist consulting. We had the clinical impression that mortality rates in *Pisa* were lower, but the latter cannot be confirmed since many cases were lost in follow-up in both hospitals.

In this study, the vast variety of morphological anomalies of cellular components in the blood of IMHA patients was thoroughly investigated. A few questions arise from our work that could form the basis for future research. The combination of NRBCs, band neutrophils and macrothrombocytes and its relation to extra-medullary erythropoiesis should be investigated using hepatosplenic cytology. The presence of schistocytes in IMHA patients' blood smears and the possibility of using them as biomarkers for DIC or pulmonary thromboembolism should be investigated using advanced diagnostic imaging techniques or post-mortem autopsies. The involvement of macrothrombocytes in the mechanisms that cause hyper - coagulative state of IMHA patients should be evaluated using thromboelastography.

In conclusion, the blood smear evaluation is an important instrument in the hands of the internal clinician and may, in many cases help guide our clinical and therapeutical decision making. In the case of immune mediated hemolytic anemia the blood smear evaluation reveals the battle-field in which the pathologic process is taking place and may contribute important information regarding coagulopathies and host response to the immune disorder.

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APPENDIX. 1: **Reference intervals.**

Parameter	Pisa	Beit-Dagan
RBC 106 /µL	5.65 - 8.87	5.7 - 8.8
Htc%	37.3 - 61.7	37.1 - 57.0
HGB g/dL	13.3 - 20.5	12.9 - 18.4
MCV fL	61.6 - 73.5	58.8 - 71.2
MCH pG	21.2 - 25.9	20.5 - 24.2
MCHC g/dL	32.0 - 39.7	31.0 - 35.7
RDW %	13.6 - 21.7	11.9 - 14.5
PLT K/µL	148 - 484	143 - 400
WBC × 103/ µL	5.06 - 16.76	5.02 - 13.9
Neut × 103/ µL	2.96 - 11.64	3.9 - 8.0
Lymph× 103/ µL	0.7 - 5.1	1.3 - 4.1
Mono× 103/ µL	0.2 - 1.7	3.3 - 10.3
Eosi× 103/ µL	0.1 - 1.35	0 - 0.6
TPP g/dL	5.8 - 7.8	5.4 - 7.6
Alb g/dL	2.6 - 4.1	3.0 - 4.4
Tbil mg/dL	0.07 - 0.30	0.0 - 0.2
CRP mg/dL	0 - 0.3	-