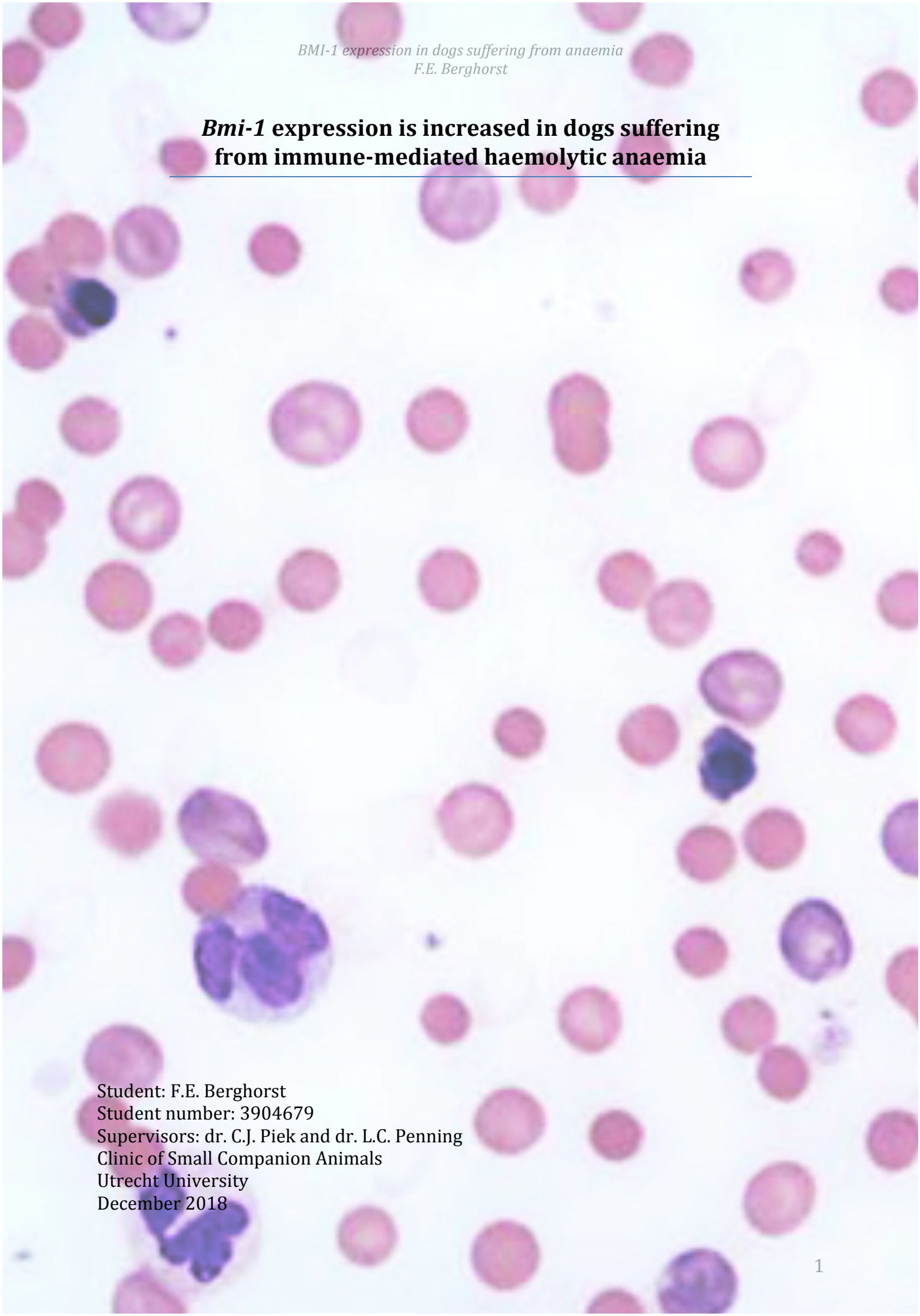
Bmi-1 expression is increased in dogs suffering from immune-mediated haemolytic anaemia



Student: F.E. Berghorst
Student number: 3904679
Supervisors: dr. C.J. Piek and dr. L.C. Penning
Clinic of Small Companion Animals
Utrecht University
December 2018

Abstract

Background – Immune-mediated haemolytic anaemia (IMHA) is one of the most common types of anaemia in dogs. In haematopoiesis, the protein BMI-1 determines postnatal self-renewal of haematopoietic stem cells (HSC).

Objectives – To provide new insights in haematopoiesis for dogs suffering from IMHA by investigating gene expression of *Bmi-1*.

Methods – Blood samples from 28 anaemic canine patients referred to the Department of Clinical Sciences of Companion Animals Utrecht University were analysed for *Bmi-1* expression levels, including upstream and downstream targets using quantitative PCR.

Results – *Bmi-1* expression shows 3-fold elevated levels in patients suffering from IMHA compared to healthy individuals. Upstream factors *Zeb1* and *E2f1* showed significant increase in mRNA levels in all anaemia cases, where *Zeb1* was upregulated in IMHA cases. Surprisingly, cell cycle inhibitors $p19^{Arf}$ and *p27*, often inhibited by BMI-1, showed 1.5-fold and 4.1-fold increase in comparison to control cases, respectively.

Conclusions – *Bmi-1* and *p27* showed contrasting results regarding patients suffering from IMHA. Further research should be directed at locating *p27* expression to either bone marrow or whole blood to clarify the event of apoptosis or haemolysis. This may elucidate the lack of a regenerative response in a lot of cases of IMHA. Interaction between BMI-1 and p27 and possible disturbance of this interaction in IMHA should also be examined. In this way, a role for dysfunctional haematopoiesis in pathophysiology of non-regenerative IMHA can be explored.

Contents

Introduction

Many haemolytic pathologies can exist in dogs, when regeneration of red blood cells does not meet the demand to restore destroyed cells. For immune-mediated disorders, immune mediated haemolytic anaemia (IMHA) is one of the most common types of anaemia in dogs. In IMHA, red blood cells are destroyed and removed from systemic circulation as a result of coating with immunoglobulins, complement, or both (Cohn 2015). When looking at blood cell production, haematopoiesis, the protein BMI-1 determines postnatal self-renewal of haematopoietic stem cells (HSC) (Park et al. 2003). However, its function in the maintenance and production of the erythroid lineage remains uncertain and is minimally investigated. Kim *et al.* showed the importance of BMI-1 in proliferation of extensively self-renewing erythroblasts, but the function in erythroid maturation is unclear (Kim et al. 2015). Moreover, the role of BMI-1 in canine blood disorders has not yet been described.

Haematopoiesis

Haematopoiesis in mammals represents the production of myeloid and lymphoid cells present in the blood. This process occurs in embryonic development and adult life, in the liver and bone marrow, respectively. The adult human produces an extraordinary amount of more than 2 million red blood cells every second (Kim et al. 2015). Starting at the level of pluripotent haematopoietic stem cells (HSC), the cells give rise to multi potential progenitors (MPP) and later to committed progenitors that give rise to one of the haematopoietic lineages. HSCs give rise to lymphoid stem cells that commit to lymphocytes and NK cells, MPPs that differentiate into platelets and white blood cells, or erythrocytes. Moreover, they can also establish self-renewal next to the multilineage differentiation (Hattangadi et al. 2011; Iwama et al. 2005). The earliest committed progenitors that will ultimately differentiate into erythrocytes are slowly proliferating burst-forming unit-erythroids (BFU-E) and rapidly proliferating colony-forming unit-erythroids (CFU-E). Undergoing different substantial steps like decrease in cell size, haemoglobinization and enucleation, proerythroblasts give rise to the cells that oxygenize our tissue, erythrocytes (Figure 1). Finally, erythrocytes are delivered to the bloodstream, where their progenitors reside in bone marrow (Hattangadi et al. 2011).

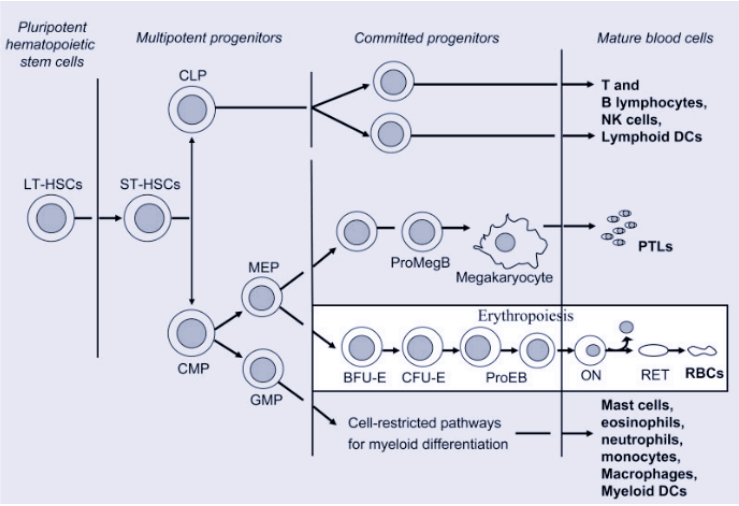


Figure 1 Schematic representation of haematopoiesis with highlighted erythropoiesis (from Tsiftsoglou 2009)

Many haemolytic pathologies can occur in dogs, where erythropoiesis does not meet the demand for new cells to supplement for the destroyed cells. The list of differential diagnoses is long, existing of immune-mediated haemolysis and non-immune-mediated haemolysis caused by different diseases or stimuli. For idiopathic immune-mediated causes, immune mediated haemolytic anaemia (IMHA) is one of the most common and most fatal types of anaemia in dogs (Balch & Mackin 2007a; Piek 2011).

IMHA

Pathophysiology

IMHA is caused by immune-mediated premature destruction of red blood cells, resulting in decrease of the total erythrocyte count. As a result of different stimuli or diseases, erythrocytes of all ages become the target of a type II hypersensitivity reaction (Balch & Mackin 2007a). The red blood cells may have a normal membrane surface or altered membrane-antigens due to disease, neoplasias or drugs. Different immunoglobulins bind the erythrocyte membrane and cause intravascular haemolysis, extravascular haemolysis and intravascular erythrocyte agglutination (Balch & Mackin 2007a). The level of erythrocyte-bound immunoglobulins alone does not have a relationship with the rate of haemolysis or severity of the anaemia, which describes that the disease has a complex pathogenesis involving complement and the different immunoglobulin classes (Barker et al. 1992).

In the case of immunoglobulins produced against a normal red blood cell membrane, the glycoprotein glycophorin is a common target. Normally, suppressor T cells inhibit this attack, but T cells of IMHA patients are believed to be poorly regulated or overruled by an overstimulated immune system. Whether this is genetically predisposed, is still under discussion (Balch & Mackin 2007a).

IgM, IgG and IgA can all cause haemolysis by activation of the complement system or mononuclear phagocyte system (Figure 2) (McCullough 2003; Balch & Mackin 2007a).
IgM, a pentamer, agglutinates the red blood cells. If many IgM antibodies attach, complement factor C1 joins and the complement reaction is activated. A membrane attack complex of factors C8, C9, C5b, C6 and C7 perforates the cell membrane, leading to influx of extracellular fluid and intravascular haemolysis. In addition, binding of the C3b complex can enhance extravascular lysis as it is removed by the spleen and produces spherocytes. These spherocytes are fragile and can easily be destroyed as they pass through the liver or spleen. They are also removed by antibody mediated phagocytosis (McCullough 2003).
Attachment of IgG, a monomeric antibody, causes less severe extravascular haemolysis unless a large amount of antibody is present. Macrophages, mostly present in the liver and spleen, use an Fc receptor to bind the Fc component of the IgG antibody present on the erythrocyte (Balch & Mackin 2007a). This activation of the mononuclear phagocyte system results in complete erythrophagocytosis and destruction or formation of spherocytes by removing only part of the cell membrane (McAlees 2010).

Causes

IMHA can be divided in a primary or secondary type, depending on the presence of underlying disease. Primary IMHA is idiopathic and with 60-75% of the canine cases of immune-mediated anaemia the most common type of the disease (Piek et al. 2008). Autoantibodies against the dogs own erythrocyte membrane antigens are produced and the red blood cell is attacked and destroyed (Balch & Mackin 2007a).

Occurrence of idiopathic IMHA tends to have a breed predisposition, suggesting a genetic component is involved in the etiology. Dog leucocyte antigen (DLA) haplotypes differ between breeds and other genes within the major histocompatibility complex (MHC) region contribute to presence of auto reactive T cells in dogs with IMHA (Corato et al. 1997). This may be the explanation for the reported higher incidence of primary IMHA in Doberman Pinschers, Cocker Spaniels, Miniature Poodles, Irish Setters, Collies, English Springer Spaniels and Old English Sheepdogs (McCullough 2003).

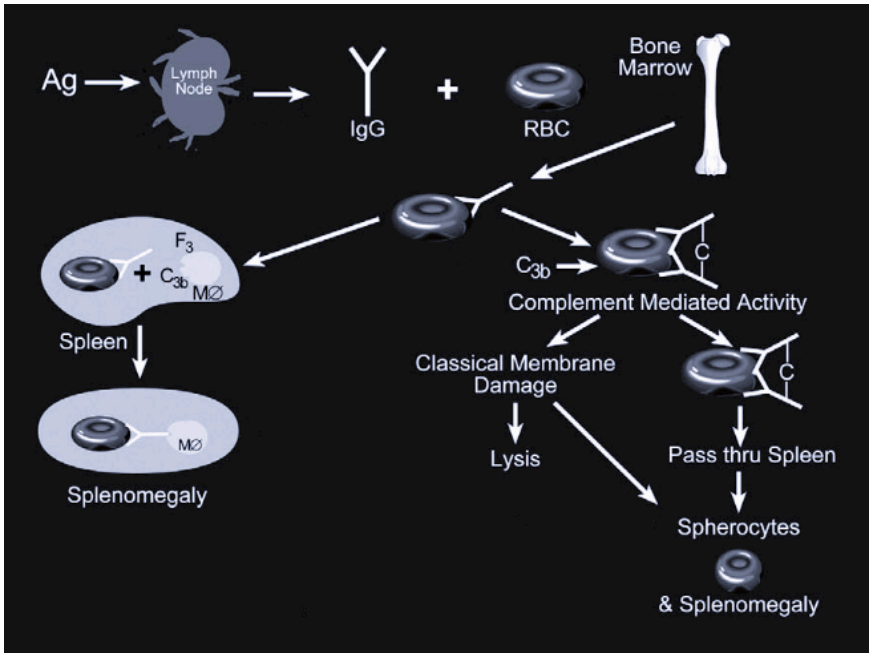


Figure 2 Schematic representation of the pathogenesis of canine IMHA (edited from McCullough 2003)

In secondary IMHA, underlying infectious or non-infectious diseases cause non-self antigens to attach to or become expressed in the normal erythrocyte cell membrane. Various parasitic infections, neoplasias, drugs and vaccines, intrinsic membrane defects or intoxications are reported to be possible stimuli for secondary IMHA (McCullough 2003; Piek 2011; Balch & Mackin 2007a). Most of these stimuli are confirmed in human literature, but have yet to be confirmed in dogs (C. J. Piek 2011). For example, drug-induced secondary IMHA may be caused by attachment of drug products to the membrane or stimulation of IgM production and subsequent binding of IgM-drug complex to the cell membrane (Balch & Mackin 2007a).

Clinical presentation
Idiopathic IMHA mostly occurs at the average age of 6 years old, whereas secondary IMHA can develop at any age depending on the underlying disease (Balch & Mackin 2007a). Young female spayed dogs are considered to be at a higher risk for primary IMHA (McCullough 2003). Patients present themselves with signs of haemolysis and can develop anaemia in a short period of 3 days. Lethargy, collapse, exercise intolerance, loss of appetite and anorexia are non-specific signs that can suggest anaemia (Balch & Mackin 2007a; Piek 2011). More specific physical signs of anaemia are tachycardia, tachypnoea, lymphadenopathy, steep pulse, pale mucous membranes, jaundice and systolic murmur. Around half of the patients have a fever.
Splenomegaly and hepatomegaly can be found in 40% of the dogs. As a result of haemolysis, haemoglobinaemia and pigmenturia (haemoglobinuria or bilirubinuria) occur. Haemoglobinaemia or haemoglobinuria only occur in the event of intravascular haemolysis, not extravascular haemolysis (Balch & Mackin 2007a; McCullough 2003; Piek 2011). Signs of severe thrombocytopenia such as petechiation occur when the patient has developed concurrent immune-mediated thrombocytopenia (ITP) or Evans syndrome, which happens in 2-5% of the cases (Piek 2011). With secondary IMHA, specific or non-specific signs due to the underlying condition have to be taken into account with examination. For a differential diagnosis, pure red cell aplasia (PRCA) and Evans syndrome may present themselves in a similar way as idiopathic IMHA (Piek 2011).

Diagnosis
For primary IMHA, multiple criteria for diagnosis exist, as there are no pathognomonic findings for the condition. In whole blood, anaemia with a haematocrit <25-30% should be followed up by further examination for IMHA. Spherocytosis can occur in some rare diseases such as hereditary spherocytic disorders, but is generally accepted pathognomonic for IMHA (Piek 2011).
Absolute reticulocyte count and corrected reticulocyte percentage are required for the determination of a regenerative or non-regenerative anaemia. This way, distinction between haemolytic anaemia and blood loss (both regenerative) and destructive processes within the bone marrow or attack of precursors (non-regenerative) is possible (McCullough 2003).
Persistent agglutination of red blood cells can be seen in positive saline agglutination tests by macroscopic or microscopic examination. This test distinguishes autoagglutination as a result of anti-erythrocyte antibodies, showing persistent agglutination, and rouleaux formation due to non-immunological reasons, showing destruction of the rouleaux (Piek 2011). Dogs with low titres of antibodies can be examined with the Coombs' test. The direct Coombs' test can reveal anti-erythrocyte antibodies and is used as main diagnostic test for IMHA (Balch & Mackin 2007a). Direct flow cytometry shows a new sensitive, objective and quick results diagnosing IMHA in dogs. Nevertheless, the Coombs' test is used as a routine test in the clinic. Bone marrow aspirates are used as well to determine any destruction processes or tumours (McCullough 2003).

For secondary IMHA, identification of parasites in blood smears or PCR testing for parasite DNA is an important part of diagnosis. Also, diagnostic imaging can reveal masses in thorax or abdomen (Balch & Mackin 2007a). Distinction between primary and secondary IMHA is crucial for a suiting and effective treatment. However, spherocytosis, autoagglutination and positive Coombs' tests do not distinguish primary and secondary IMHA. Thorough examination is needed to find and identify underlying conditions that cause secondary IMHA (Balch & Mackin 2007a).

Treatment

Treatment of IMHA is still being reviewed extensively, as mortality remains high. The majority of the patients require hospitalization to monitor and treat the haemolysis and subsequent anaemia. Treatment consists of stabilization and immunomodulation (Piek 2011; Balch & Mackin 2007b).

Supportive therapy is important in the first place as the average patient presents with a severe anaemia that needs to be treated to maintain tissue oxygenation. Blood transfusions are often applied when the patient shows signs of haemolytic crisis (Balch & Mackin 2007b) In addition to blood transfusion, oxygen and fluid therapy are recommended to maintain tissue oxygenation, volume and acid-base homeostasis (Balch & Mackin 2007b).

Immunomodulation plays a key role in pharmacologic treatment of IMHA, as its aim is to suppress immunoglobulin production and decrease erythrophagocytosis. Glucocorticoids such as prednisone and dexamethasone are thought to be effective on their own, with side effects taken for granted (Swann & Skelly 2013). Among other immunosuppressive agents, azathioprine and cyclosporine are administered when glucocorticoids fail to reduce haemolysis or induce unacceptable side effects. Both drugs suppress T cell function in order to reduce erythrophagocytosis. Nevertheless, no clinical evidence has been published to guide the use of azathioprine and cyclosporine, and no solid scientific evidence is available that supports its effectivity in idiopathic IMHA (Swann & Skelly 2013; Balch & Mackin 2007b).

Prognosis

Prognosis of patients with IMHA remains reserved as mortality can be as high as 80%, and most deaths take place within two weeks after diagnosis. If there is a response to treatment, this takes place within two weeks, but relapse takes place in 15% of the dogs (Balch & Mackin 2007b). The presence of thromboembolisms is the most substantial complication that causes death, and can be stimulated by different events. The hypercoagulable state, thrombocytopenia, hypoalbuminemia, glucocorticoid therapy and blood transfusions can all contribute to the development of these thromboembolisms (Piek 2011).

Next to the function of spherocytosis in diagnosis of IMHA, it is suggested spherocytosis actually indicates a better prognosis. Spherocytes are mainly the result of IgG-mediated attack of the red blood cell, considered less severe compared to IgM-mediated attack. Partial phagocytosis and less severe haemolysis in IgG-mediated IMHA results in a more effective and positive therapy outcome (Piek 2011). In addition, presence of jaundice, high urea or creatinine plasma concentration, neutrophilia, monocytosis, thrombocytopenia and prolonged activated partial thromboplastin time (APTT) are associated with death as a result of IMHA (Piek et al. 2008; Piek 2011; McCullough 2003).

In general, IMHA is characterized as a regenerative anaemia, but up to 33% of dogs suffering from IMHA lack a regenerative response of the bone marrow. Absence of this regeneration can be due to the 4-5 days needed to develop a response in peracute cases, bone marrow suppression or infiltration due to an underlying disease, or destruction of progenitors. For immune-mediated haemolysis of progenitor cells in the bone marrow, like pure red cell aplasia (PRCA) characterized by erythroid aplasia or precursor directed immune-mediated anaemia (PIMA) characterized by erythroid hyperplasia, response to therapy can be very ineffective (Stokol et al. 2000; Weiss 2008). As investigation in IMHA mainly focuses on pathophysiology regarding haemolysis, ideas about a role and cause of dysfunctional haematopoiesis in the pathophysiology of IMHA remains unexplored. It was recently shown that a specific protein, BMI-1, plays a role in haematopoietic stem cell self-renewal and erythroid development. BMI-1 could therefore possibly elucidate a share for dysfunctional haematopoiesis and subsequent lack of a regenerative response in IMHA pathophysiology.

BMI-1

The Polycomb Group Complex (PGC)

Regulation of haematopoiesis is performed at different levels, from stem cell to fully differentiated erythrocytes. An important protein complex functioning in stem cell proliferation and survival, is the polycomb group complex (PGC). Acting in neuronal, neoplastic and haematopoietic stem cell proliferation, the complex is highly evolutionary conserved (Iwama et al. 2005; Park et al. 2003; Gao et al. 2015). The PGC functions mainly in maintaining and promoting gene repression, forming ubiquitin ligase complexes to perform chromatin modification on cell fate determination genes. In this way, it manages lineage commitment choices during development and differentiation in multiple tissues (Gao et al. 2015; Iwama et al. 2005). The PGC contains two functional units, polycomb repressive complex 1 and 2 (PRC 1 and 2). Cooperating in their actions, PRC2 establishes the repression by chromatin modification, whereas PRC1 maintains this repression (Iwama et al. 2005).
An important component of the PGC and PRC1 is B-cell specific Moloney MLV insertion site-1 (BMI-1). This protein supports stem cell self-renewal in different tissues, and is a well-known oncogene as overexpression is associated with uncontrolled proliferation and prevention of differentiation in many cancers (Kim et al. 2015).

Function in haematopoiesis

In haematopoiesis, BMI-1 enables self-renewal of HSCs via multiple routes. Among all components of the PRC1, loss of *Bmi-1* results in a profound defect in self-renewal of these stem cells. Additionally, *Bmi-1* overexpression induces enhancement of HSC repopulation *in vivo*, confirming the central role of BMI-1 in HSC self-renewal. This advocates that BMI-1 acts as a core component of the PCG, either recruiting essential molecules or providing a docking site for epigenetic proteins (Kim et al. 2015; Iwama et al. 2005).
Investigation by Park *et al*. and Iwama *et al*. shows no change in fetal liver HSCs in *Bmi-1* knockout mice, but postnatal HSC numbers show distinct reduction, supporting the idea of postnatal functioning for BMI-1 (Park et al. 2003; Iwama et al. 2005). This postnatal self-renewal results in asymmetric cell division, producing a permanent HSC pool.
Both myeloid and lymphoid cell lineages can appear from this HSC pool. Without BMI-1, cellular senescence is not repressed, the HSC pool cannot be maintained and the process of differentiation stagnates. This leads to progressive pancytopenia (Park et al. 2004;

Park et al. 2003). In addition to expression in HSCs, *Bmi-1* was also recently shown to be upregulated in both limited and extensively self-renewing erythroblasts. Moreover, BMI-1 overexpression induces extensive ex vivo self-renewal of adult erythroblasts, normally only capable of limited self-renewal. This suggests a key role of BMI-1 in the ability of self-renewal in erythroid progenitors (Kim et al. 2015). Gao *et al*., showing a role for BMI-1 in erythrocyte differentiation via ribosome biogenesis, supported this finding. As BMI-1 associates with ribosomal proteins, it facilitates differentiation in erythroid precursors. In BMI-1 knockout erythroid progenitors, dysfunction of the ribosome activates the p53 pathway. This can be caused via inhibition of $p19^{Arf}$ that represses p53 via Mdm2 or binding of ribosomal proteins to Mdm2 (Gao et al. 2015).

Signalling pathways
Regulation of the expression of *Bmi-1* is performed by multiple transcription factors. Zeb1, E2F1, Twist1 and c-Myc stimulate *Bmi-1* expression, where Mel18 and Nanog inhibit this event (Figure 3). Twist1, E2F1 and ZEB1 regulate expression of *Bmi-1* in a direct or indirect manner. Twist1 and E2F1 directly stimulate transcription and expression of *Bmi-1*, whereas ZEB1 stimulates *Bmi-1* expression via inhibition of microRNA 200 (Sahasrabuddhe 2016; Liuq et al. 2014; Nowak et al. 2006).

BMI-1 acts on different signalling pathways, each regulating different elements of cell cycle control. The Ink4a/Arf locus is one of the main targets, repressed by BMI-1. The Ink4a/Arf locus encodes $p16^{Ink4a}$ and $p19^{Arf}$, a cyclin-dependent kinase inhibitor (CKI) and tumour repressor, respectively (Figure 3). The locus consists of cell-cycle inhibitory proteins, thus inducing senescence, growth arrest and apoptosis when activated (Iwama et al. 2005; Jacobs et al. 1999).
Inactivation of *Bmi-1* promotes cellular senescence due to derepression of the Ink4a/Arf locus and consequent $p16^{Ink4a}$ activation. G1 to S-phase transition is inhibited this way, leading to senescence. Moreover, $p19^{Arf}$ expression as a result of BMI-1 inactivation induces p53 action, leading to apoptosis and cell cycle arrest. Overexpression of *Bmi-1* leads to inhibition of Ink4a/Arf signalling and promotes tumorigenesis by repression of cell cycle checkpoints p16 and p21. In addition, repression of $p19^{Arf}$ and its downstream target p53 in *Bmi-1* overexpression is seen as the main initiation and support of tumorigenesis (Sahasrabuddhe 2016; Iwama et al. 2005). In self-renewing erythroblasts, BMI-1 may function independently of the $p16^{Ink4a}$/ $p19^{Arf}$ repression seen in erythroid maturation (Kim et al. 2015).
In addition, Park et al. and Kim et al. suggest members of the Hox gene family as potential targets of BMI-1. Regulating cell fate decisions of HSCs, Hoxa9 specifically shows an increase in expression in BMI-1-knockout mice (Park et al. 2003; Kim et al. 2015).
Furthermore, BMI-1 promotes self-renewal of stem cells and thus symmetric division of HSCs by upregulating the expression of transcription factors Sox2 and KLF4. Protection against oxidative stress and DNA damage response pathways are managed by BMI-1 as well (Moon et al. 2011; Sahasrabuddhe 2016).

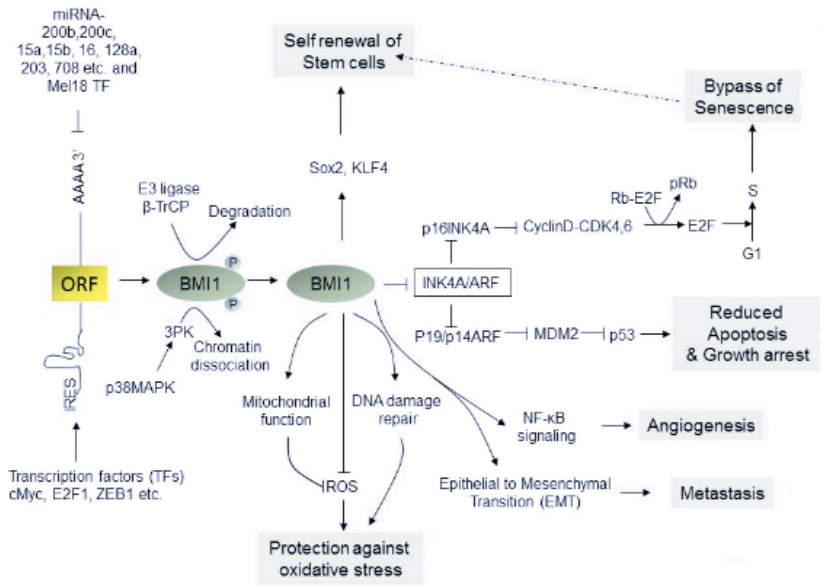


Figure 3 Upstream and downstream signaling pathways of BMI-1 in normal cellular physiology (edited from Sahasrabuddhe 2016)

Haematologic malignancies

With a function in stem cell self-renewal, dysfunction of *Bmi-1* is associated with types of cancer in different tissues. Uncontrolled proliferation and self-renewal of haematopoietic cells and cancer stem cells lead to malignancies from both myeloid as well as lymphoid lineages. For example, acute myeloid leukaemia, acute lymphoblastic leukaemia and lymphomas are all correlated with overexpression of *Bmi-1* (Sahasrabuddhe 2016; Kim et al. 2015). Also, BMI-1 shows an essential role for the maintenance and self-renewal of acute myeloid leukaemia stem cells (Lessard & Sauvageau 2003). Future investigations are focussed on proving BMI-1 as a biomarker or therapeutic target in these and other malignancies (Sahasrabuddhe 2016; Kim et al. 2015).

Aim of the study

Bmi-1 function in maintenance of stem cells and self-renewal in lymphoid lineages has been studied (Iwama et al. 2005; Sahasrabuddhe 2016; Rizo et al. 2008; Oguro et al. 2010), but recently evidence for *Bmi-1* functioning in differentiation of erythroid cells has been described (Gao et al. 2015; Kim et al. 2015). Function in erythroid maturation remains unclear, and the role of *Bmi-1* in canine blood disorders has not yet been described. In IMHA, extensive research has been performed on the destruction of red blood cells, however possible dysfunction of haematopoiesis remains unexplored. As up to 33% of the canine IMHA patients lack a regenerative bone marrow response, investigation in haematopoiesis is needed. To elucidate the role of BMI-1 in IMHA, blood samples of dogs suffering from anaemia will be analysed on expression of *Bmi-1* and possible up- and downstream targets.

Materials and methods

All procedures were approved and performed according to standards of the Ethical Committee of Animal Experimentation of Utrecht University as required under Dutch legislation.

Patient selection and blood sample collection
Dogs used in the experiment were all referred to the Department of Clinical Sciences of Companion Animals Utrecht University, between June and November 2018. All patients with haematocrit values < 0.30 L/L were included in the experiment. Patients were divided in 4 groups with following classification: IMHA (%retic <1.5)(n=10), non-regenerative anaemia (%retic <1.5) (n=7), regenerative anaemia (%retic >1.5)(n=7) or pancytopenia (n=3). For patients in the IMHA group, diagnosis with immune-mediated destruction of mature erythrocytes, or erythrocyte precursors in the case of PRCA or PIMA was confirmed with bone marrow aspiration or blood tests. Bone marrow function is intact in these patients. Patients suffering from a non-regenerative anaemia without underlying immune-mediated cause, suffer from bone marrow dysfunction, resulting in anaemia caused by decreased production. Regenerative anaemia can be caused by chronic haemorrhage or chronic haemolysis, in combination with functioning bone marrow. Blood samples of patients with unclear or unrelated pathologies were omitted in the analysis. The control group consisted of blood donors referred to the Department of Clinical Sciences of Companion Animals Utrecht University (n=7).
In the study, 27 individual patients represented 16 different breeds and 3 mixed breed dogs. The average age of the dogs was 6.2 years old and there were 10 castrated females, 4 females, 6 castrated males and 7 males. Table 1 describes case details and further individual pathology related to anaemia. Second or more following samples were available for 4 dogs.
Blood samples were taken from animals in consultation or admitted to the intensive care unit of the clinic. Blood was collected as surplus EDTA anticoagulated blood (0.5-1mL), secured in 0.5mL RNA*later* (Ambion, Applied Biosystems, Foster City, California, USA) and stored at 4°C.

Experimental design
Whole blood samples of all 5 groups (n=46) were compared regarding *Bmi-1* expression and expression of downstream factors *Sox2, Klf4, Oct4, p53, p27, p19^{Arf}* and upstream factors *E2f1, Zeb1* and *Twist1*. Blood of patients with regenerative anaemia were initially used as positive control, samples of patients with pancytopenia were used as negative control.

Table 1 Classification and case details of patients used in this experiment

Classification	Patient no.	Breed	Age	Sex	Haematocrit (L/L)	Absolute reticulocyte count (10^9/L)	Concurrent disease	Bone marrow biopsy	Pathogenesis
IMHA (non-regenerative)	1	French Bulldog	7Y	F cas	0,146	28,4	PIMA	Shows bone marrow rich in cells with complete lymphoid and myeloid lineage, and a regenerative myeloid aspect. No hints for malignancies	Destruction of erythroid precursors and possibly mature cells in circulation
	2	Labrador Retriever	2Y	F cas	0,286	125,9	IMHA	Shows bone marrow rich in cells, adequate number of megakaryocytes, complete myeloid and lymphoid lineage and slight predominance of myeloid lineage. No erythrophagocytosis	Destruction of mature erythrocytes in circulation
	3	Dachshund	11Y	F cas	0,193	31,7	PIMA	Shows bone marrow with poor cell count, mostly blood. A predominance of myeloid precursors	Anaemia as a result of destruction of erythroid precursors
	4	Labrador Retriever	3Y	F	0,325	46,1	IMHA	Shows bone marrow rich in cells, extensive myeloid regeneration	Anaemia as a result of destruction of erythrocytes
	5	Mixed breed	8Y	F cas	0,293	109,4	IMHA		Destruction of mature erythrocytes in circulation
	6	Labrador Retriever	3Y	F	0,240	9,8	PRCA	Shows bone marrow rich in blood, a single megakaryocyte and complete lymphoid and myeloid lineage. Extensive regeneration of the myeloid lineage, lymphoid lineage is underrepresented. No presence of parasites	Destruction of marrow precursors and possible mature cells in circulation.
	7	Cairn Terrier	5Y	F	0,172	27,1	PCRA	Shows bone marrow rich in blood, adequate number of megakaryocytes. Slight predominance of a complete myeloid lineage, small numbers of lymphoid lineage. No presence of parasites	Destruction of marrow precursors and possible mature cells in circulation.
	8	Basset Fauve de Bretagne	9Y	F cas	0,133	13,5	IMHA	Shows bone marrow rich in cells, adequate number of megakaryocytes. Complete myeloid and lymphoid lineage, with overrepresented regenerative myeloid lineage and some erythrophagocytosis	Destruction of mature erythrocytes in circulation.
	10	Chihuahua	9Y	M	0,334 (after blood transfusion)	83,6	PIMA	Shows bone marrow with low cell count, no megakaryocytes but predominantly present platelet aggregation. Complete myeloid lineage with some early-stage erythroid cells. Estimated ME ratio 1:1, erythroid hypoplasia. No presence of lymphocytes, plasma cells or infectious agents	Destruction of erythroid precursors.

Non-regenerative anaemia	11	Dalmatian	8Y	F cas	0,227	100,3	Trauma		Acute blood loss
	12	Dachshund	8Y	F cas	0,138	50,1	Idiopathic		
	13	Chihuahua	1Y	M	0,142	12,6	Haemothorax		Acute blood loss
	14	Bullmastiff	5Y	F	0,149	12,8	Chronic kidney disease		Lack of erythropoietin as a result of renal failure
	15	Shih Tzu	2Y	M	0,123	20,2	Chronic kidney disease	Shows complete myeloid lineage and adequate number of megakaryocytes. Some erythroid progenitors, mostly early-stage. Some plasma cells. Erythroid hypoplasia	Lack of erythropoietin as a result of renal failure
	16	American Staffordshire Terrier	12Y	M	0,256	48,6	Multicentric B-cell lymphoma		Immune-mediated haemolytic anaemia as a result of paraneoplastic syndrome
	17	Bernese Mountain Dog	4Y	M cas	0,144	30,3	Systemic immune-mediated disease	Shows bone marrow with high cell count, many megakaryocytes. Lack of myeloid and predominance of lymphoid lineage. No erythrophagocytosis	Haemolytic anaemia as a result of immune-mediated pathology
Regenerative anaemia	18	Maltese	13Y	M	0,156	211,1	Lymphoma		Immune mediated anaemia as a result of paraneoplastic syndrome
	19	Australian Cattle Dog	7Y	M	0,256	140,5	PRCA	Shows bone marrow rich in cells, adequate number of megakaryocytes. Extensive myeloid regeneration	Destruction of marrow precursors and possible mature cells in circulation
	20	Cocker Spaniel	6Y	M cas	0,218	292,0	IMHA		Anaemia as a result of destruction of erythrocytes
	21	Shih Tzu	6Y	M cas	0,154	195,4	Idiopathic		
	22	Bernese Mountain Dog	6Y	F cas	0,202	339,1	Malign histiocytosis		Immune-mediated haemolytic anaemia as a result of paraneoplastic syndrome
	23	Chihuahua	4Y	M cas	0,177	222,4	Trauma		Blood loss
	24	Mixed breed	11Y	F cas	0,250	511,8	Neoplastic metastases in respiratory system		Chronic blood loss from gastrointestinal and respiratory system
Pancytopenia	25	Jack Russell	10Y	M	0,296	60,1	Multiple	Shows predominance of	Myelophtisis as

		Terrier		cas			myeloma	plasma cells	a result of myeloma, affecting haematopoiesis
	26	Yorkshire Terrier	6Y	M cas	0,249	20,3	Ehrlichiosis	Shows bone marrow with low cell count, slight presence of myeloid lineage. Predominantly erythroblast/rubriblast. A single fibroblast Associated with end-stage Ehrlichiosis. No hints for haematopoietic neoplasias.	Infection of haematopoietic cells and bone marrow failure as a result of chronic infection
	27	Mixed breed	2Y	M cas	0,280	16,3	Leishmaniasis		Destruction of erythrocytes as a result of immune complex binding

Sample testing and RNA isolation
All blood samples were analysed performing a complete blood count with a haematology analyser (ADVIA® 2120i Hematology System, Siemens healthcare) to measure haematocrit, white blood cell count and reticulocyte percentages. Total RNA was extracted from the blood samples using the QIAamp RNA Blood Mini Kit reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Science, IJsselstein, The Netherlands). Total RNA was stored at -20°C.

Primer design and testing
Selection of target genes was based on previously described data. Reported downstream targets of BMI-1 in human and veterinary research are *Sox2, Klf4, Oct4, p53, p27* and $p19^{Arf}$ (Moon et al. 2011; Kim et al. 2015; Sahasrabuddhe 2016; Gao et al. 2015; Iwama et al. 2005; Lessard & Sauvageau 2003; Schuringa & Vellenga 2010). Upstream targets of BMI-1 are *E2f1, Twist1* and *Zeb1* (Sahasrabuddhe 2016; Liuq et al. 2014; Wu 2011; Nowak et al. 2006).
Primers for the targets were designed using known canine sequences. Primers for $p19^{Arf}$ were designed with Perlprimer (http://perlprimer.sourceforge.net), based upon known human sequences and then blasted for known canine sequences (Han et al. 2017). qPCR products for $p19^{Arf}$ were sequenced and confirmed by blasting. Optimal melting temperatures were determined running a temperature gradient on 16-fold dilution series. Sequencing of the amplicon confirmed the specificity of the qPCR reaction. Primers for *p16* were designed based on human sequences and blasted for canine sequences, but failed to give an optimized product.

Quantitative PCR
Complementary DNA (cDNA) synthesis and quantitative PCR (qPCR) was performed following MIQE guidelines (Bustin et al. 2010). Complementary DNA was designed using Bio-Rad iScript™ cDNA Synthesis Kit, containing oligodT and random hexamer primers. cDNA was synthesized from 500 ng of total RNA according to the manufacturer's instructions (iScript™, Bio-Rad, Veenendaal, The Netherlands).

qPCR was performed with DNA-binding SYBR green using the BioRad CFX384 Touch Real-Time PCR Detection System (BioRad, Hertfordshire, United Kingdom) according to the manufacturers instructions. Each qPCR reaction was performed in duplo with six microliter of cDNA. Optimal Tm for all primers was determined in advance (table 2). Reference genes for qPCR were selected based upon known stable expression in haematopoietic cells or whole blood. Independent from changes in leukocyte count, *HNRNPH, GUSB* and *SRPR* show stable expression in blood samples (Piek et al. 2011). Three other reference genes, *HPRT, B2M* and *GADPH*, representing cellular and ribosomal gene products were also selected to use an optimal number of used reference genes (Brinkhof et al. 2006).

Optimal T_m values varied from 56°C for *HPRT* to 67°C for *p53*. Amplification efficiency calculations from all standard curves were between 80.1 and 110.1%. Table 2 shows primer sequences, optimal melting temperatures and relation to *Bmi-1* of the target genes and references genes. Reactions were performed using 10 min denaturation at 95°C, followed by 40 cycles of 15s at 95°C, 30s at Tm, and 20s at 72°C.

Table 2 Primer sequences and melting temperatures for qPCR amplification used in this study

Gene	Primer sequences (‘5-‘3)	T_m (°C)	Relation with BMI-1	
Bmi-1		63		
Forward	TGGACTGACAAATGCTGGAGAACT			
Reverse	AGGGAACTGAGGATGAGGAGACTG			
Sox2		61	Downstream	
Forward	AACCCCAAGATGCACAACTC			
Reverse	CGGGGCCGGTATTTATAATC			
Klf4		65,5	Downstream	
Forward	TACCCAAATGACACTTTGCG			
Reverse	ATCACAGTGGTAGGGTTTCTC			
Oct4		65,5	Downstream	
Forward	ACGATCAAGCAGTGACTATTCG			
Reverse	GAGGGACTGAGGAGTAGAGCGT			
p53		65.5	Downstream	
Forward	GCCCCTCCTAGCATCTCATC			
Reverse	GGCTCATAAGGCACCACCACAC			
p27		63	Downstream	
Forward	AAGACTGATGCGCCCGA			
Reverse	AGGAGAGGAATCGTCGGTC			
p19^{Arf}		59	Downstream	(Han et al. 2017)
Forward	AGCTGGTGCATCCCGACG			
Reverse	GGGGCTGGCACCTTGCTT			
E2f1		58	Upstream	
Forward	CCACCTGATCCATATCTGCAC			
Reverse	GTCTGCGATGCTACGAAGGT			
Twist1		62	Upstream	
Forward	GAGACCTAGATGTCATTGTTCC			
Reverse	ATGTAGAGGTGTGAGGATGG			
Zeb1		59	Upstream	
Forward	CAAATGGGAATCAGGAGGA			
Reverse	TGGGTGGTGTAGAATCAG			
Reference genes				
HNRNPH		61		
Forward	CTCACTATGATCCACCACG			
Reverse	TAGCCTCCATAACCTCCAC			
GUSB		62		
Forward	AGACGCTTCCAAGTACCCC			
Reverse	AGGTGTGGTGTAGAGGAGCAC			
SRPR		62		
Forward	GCTTCAGGATCTGGACTGC			
Reverse	GTTCCCTTGGTAGCACTGG			
HPRT		58		
Forward	AGCTTGCTGGTGAAAAGGAC			
Reverse	TTATAGTCAAGGGCATATCC			
B2M		62		
Forward	TCCTCATCCTCCTCGCT			
Reverse	TTCTCTGCTGGGTGTCG			
GAPDH		58		
Forward	TGTCCCCACCCCAATGTATC			
Reverse	CTCCGATGCCTGCTTCACTACCTT			

Analysis of gene expression
Analysis of qPCR results was performed with CFX Manager 3.1 software (BioRad, Veenendaal, The Netherlands) based on average Cq from the duplos. Mean Cqs of the duplo samples were corrected for geometric mean Cq of the reference gene expression. *HNRNPH*, *SRPR* and *GADPH* were used for this correction. The other three reference genes did not follow the trendline or showed too little expression in the samples (Figure 4).

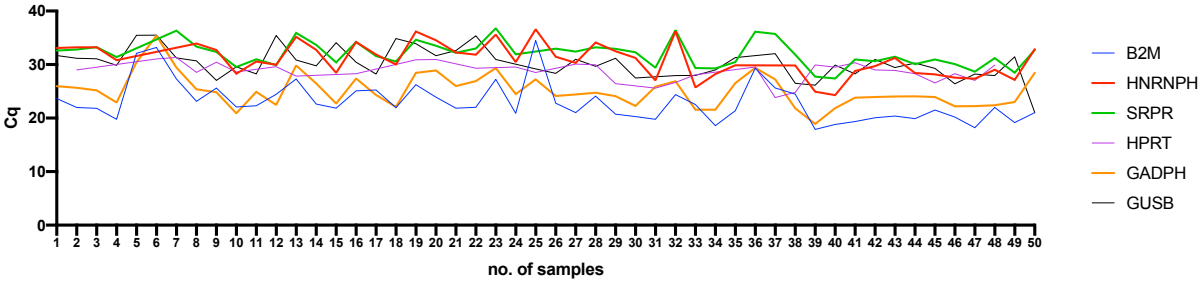


Figure 4 Trendlines of reference gene expression

Data was analysed for statistical significance using SPSS Statistics, performing Mann-Whitney U tests as the data do not have a normal distribution.
As bone marrow functions normally in IMHA patients, and their anaemia may become regenerative over time, these patients share pathology with individuals suffering from regenerative anaemia without underlying immune-mediated cause. For this reason, groups of IMHA patients and regenerative anaemia were combined for further analysis of qPCR products in figure 6 and 7 (Figure 6e and 7b).

Results

Bmi-1 shows elevated levels in patients suffering from IMHA
It was recently discovered that *Bmi-1* expression positively regulates self-renewal of HSCs and negatively influences erythroid differentiation (Kim et al. 2015). To explore the action of *Bmi-1* in canine patients suffering from haematologic disorders, qPCR on leukocytes originating from whole blood samples was performed to compare mRNA expression levels of *Bmi-1* between patients suffering from IMHA, non-regenerative anaemia, regenerative anaemia and healthy individuals.
Bmi-1 showed significantly higher levels in all three groups of patients with anaemia, compared to control (Figure 5). Expression levels were significantly higher in patients suffering from IMHA and non-regenerative anaemia, compared to control (Figure 6a-d).
When taken together, IMHA patients and individuals suffering from a regenerative anaemia, *Bmi-1* expression reached significantly higher levels than the control group (Figure 6e). This group of IMHA and regenerative anaemia patients showed 3-fold increase in *Bmi-1* expression (Figure 7b), where patients with a non-regenerative anaemia showed 3.3-fold increase in *Bmi-1* expression (Figure 7a).

Increased expression of upstream factors Zeb1 and E2f1 in anaemia
To substantiate the increased *Bmi-1* expression in 19 of 27 dogs, mRNA levels of upstream factors were examined. When looking at *Zeb1* and *E2f1* as upstream factors of *Bmi-1*, higher expression levels are reached in all three groups with anaemia compared to control as did the *Bmi-1* expression in all anaemia groups (Figure 5). mRNA levels of *Zeb1* show significant 4-fold increase in IMHA and regenerative anaemia compared to the control group (Figure 7b).
Surprisingly, this was not the case for *E2f1* expression. None of the anaemia groups reached significantly higher levels in comparison with the control group, regardless of 3 to 8-fold increase in mRNA levels for IMHA and regenerative groups together and non-regenerative anaemia, respectively (Figure 5 and 6e).

Oct4 and Klf4 as downstream targets of BMI-1
Changes in *Oct4* and *Klf4* show little significance (Figure 5), but 2.5 to 3.2-fold upregulation, respectively, when IMHA and regenerative anaemia are compared with the control group (Figure 7b). Overall expression levels were very low, in line with their role in stem cell pluripotency (Moon et al. 2011).

Erythroid differentiation and apoptotic markers $p19^{Arf}$ and p53
BMI-1 regulates stem cell self-renewal and erythroid differentiation via multiple cyclin-dependent kinase inhibitors (CDKIs), one of them being the Ink4a/Arf locus, which encodes $p16^{Ink4a}$ and $p19^{Arf}$. $p19^{Arf}$ repression by action of BMI-1 in HSCs prevents growth arrest and apoptosis by p53 to maintain the HSC pool (Park et al. 2003). Expression of $p19^{Arf}$ in erythroblasts stimulates terminal erythroid differentiation by stimulating p53 and interfering with G_0 to G_1-phase of the cell cycle (Sahasrabuddhe 2016; Iwama et al. 2005; Han et al. 2017).

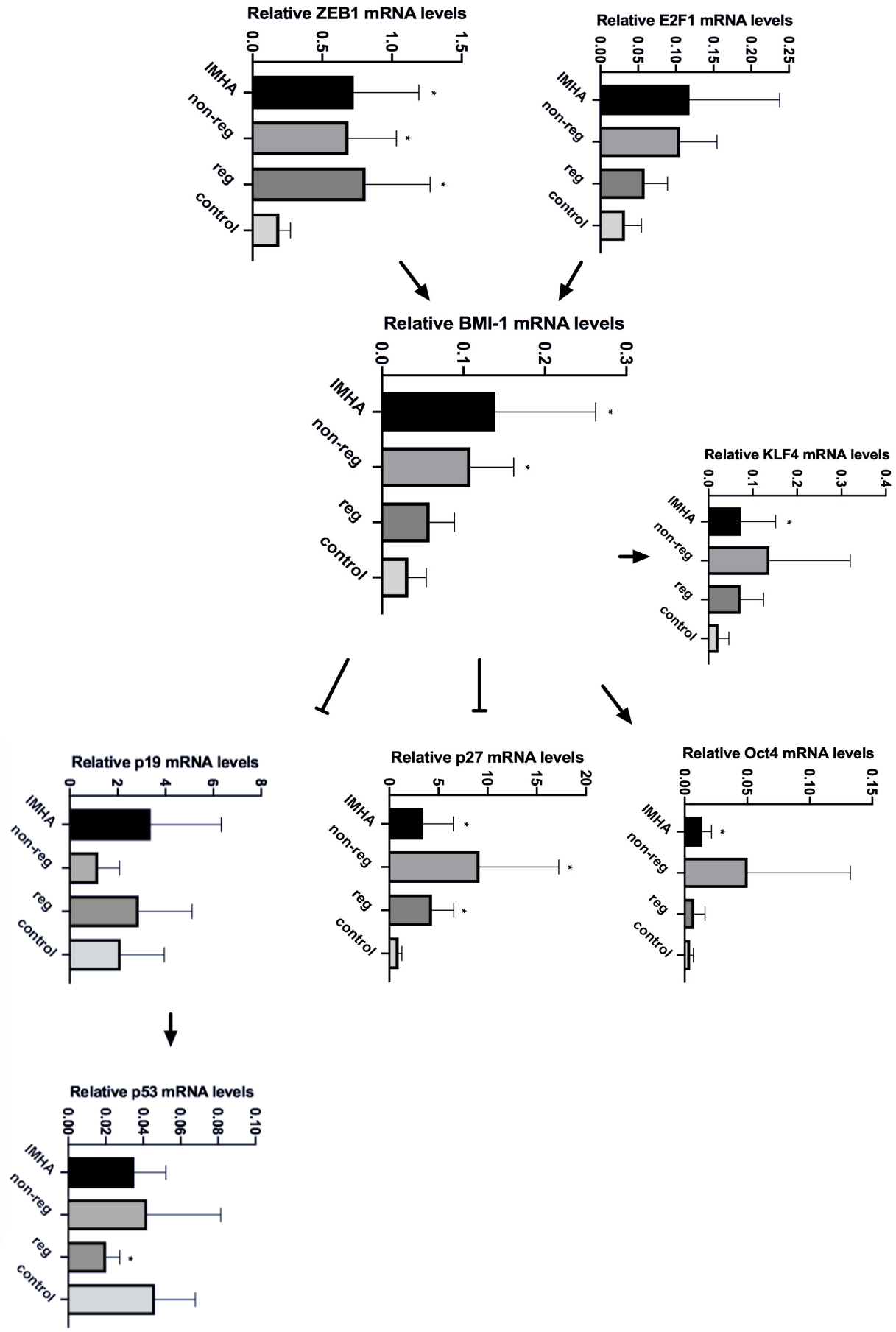


Figure 5 qPCR results showing relative mRNA levels of *Bmi-1*, upstream and downstream genes in schematic representation according to the expected cellular BMI-1 signalling pathway. Arrows indicate stimulating effect, while capped lines indicate an inhibiting effect. Data represent mean + SD of all samples. Dogs with IMHA (n=10), non-regenerative anaemia (n=7), regenerative anaemia (n=7) and control (n=7) were compared. (* compared to control, p<0.05)

To evaluate action of BMI-1 on downstream targets in anaemic patients, qPCR for *p19^{Arf}* and *p53* was performed on whole blood samples. For *p19^{Arf}* expression, none of the anaemia groups reached significant differences compared to the control group (Figure 5), but expression in IMHA reached levels increased 1.5-fold in comparison with control groups and a 2.6 fold increase compared with *p19^{Arf}* expression in non-regenerative anaemia (Figure 7). Expression of *p53* remained low in all conditions, and showed no significant changes for IMHA compared to control (Figure 5 and 6). Levels of mRNA expression were all decreased in comparison with the control group (Figure 7).

Erythroid differentiation by p27

To explore a second downstream target of BMI-1, *p27* mRNA expression levels were analysed. p27 interferes with cell cycle progression in G_1 to S-phase during erythroid differentiation (Zhang et al. 2010; Tamir et al. 2000). *p27* shows significant increase in groups suffering of anaemia compared to the control group (Figure 5 and 6). mRNA levels show a 9.7-fold increase in non-regenerative anaemia compared to control. The group suffering from IMHA and regenerative anaemia showed 4.1-fold increase of *p27* mRNA levels compared to control (Figure 7).

Blood samples of patients with a pancytopenia (n=3) did not give any detectable qPCR results and this groups was therefore omitted in the results. In patients that supplied more than one blood sample due to multiple referrals to the clinic, qPCR analysis of gene expression of *Bmi-1* and target genes for group analysis was only performed on the sample linked to the first visit. Measurements on multiple samples of one individual suffering from one pathologic process may affect the outcome of significant differences between patient groups, a second reason to omit them from the analysis.

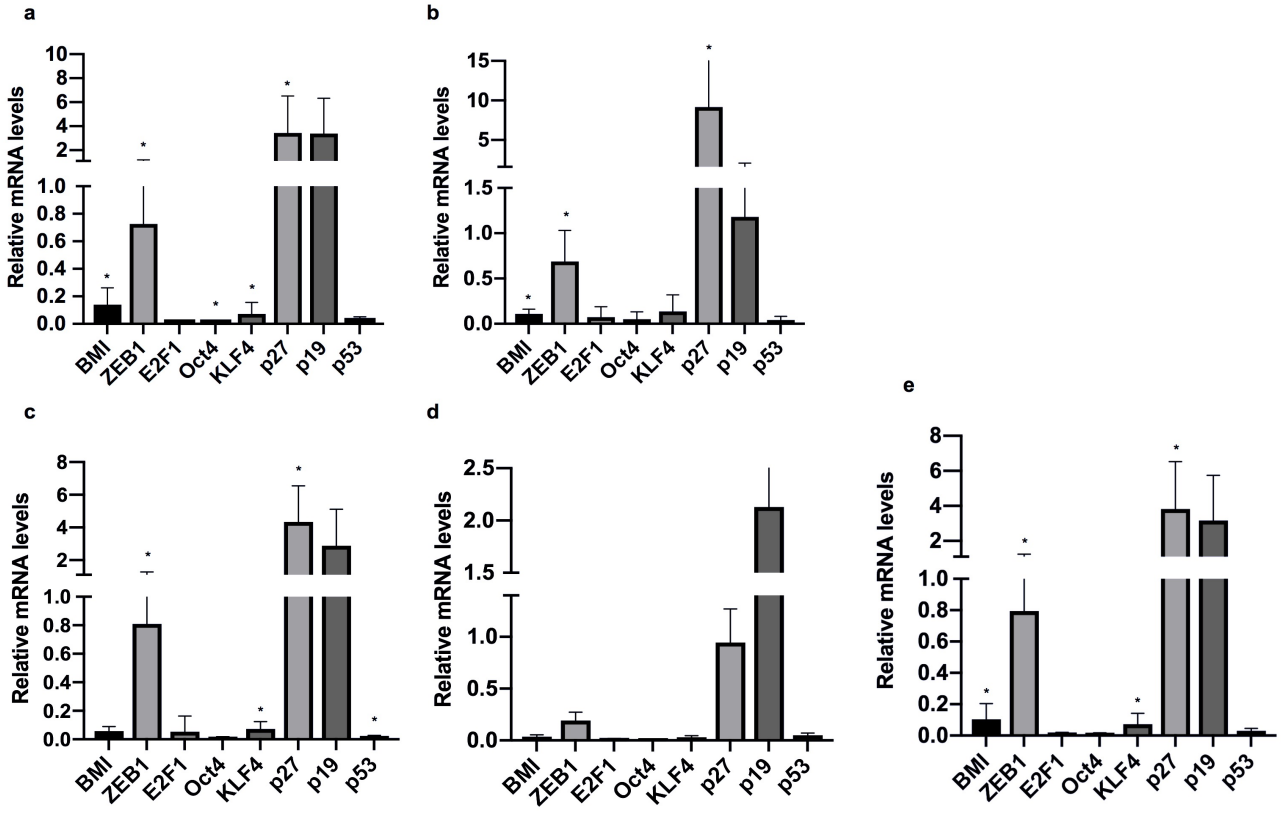


Figure 6 qPCR results showing expression for *Bmi-1*, upstream and downstream genes comparing all four patient groups a) IMHA (n=10) b) non-regenerative anaemia (n=7) c) regenerative anaemia (n=7) d) control group (n=7) and e) IMHA and regenerative anaemia combined (n=17). Data represent mean + SD of all samples. (* compared to control, p<0.05)

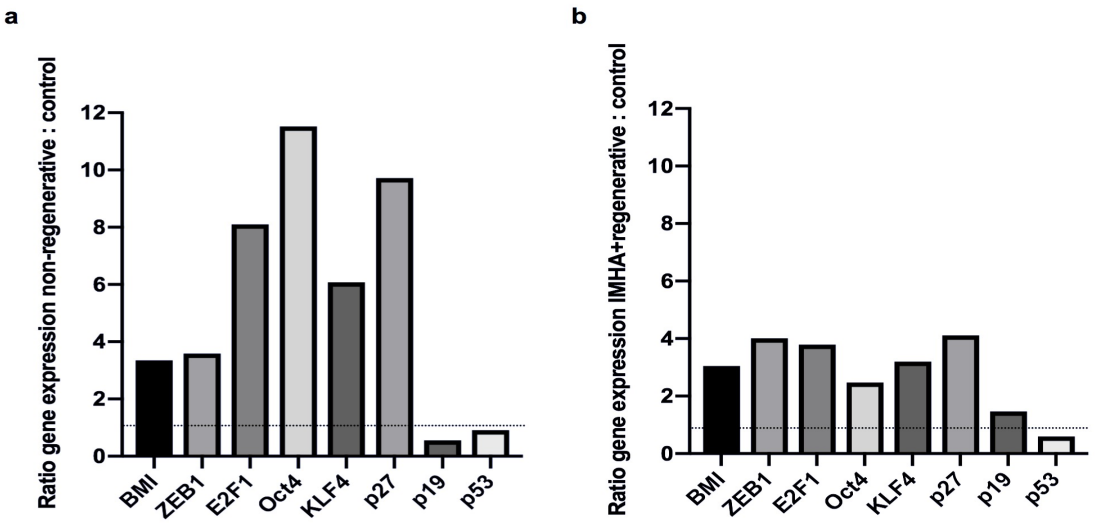


Figure 7 Ratios of qPCR results showing expression for *Bmi-1*, upstream and downstream genes a) ratios of gene expression of non-regenerative anaemia (n=7) : control b) ratios of gene expression of IMHA combined with regenerative anaemia (n=17) : control. Dotted lines indicate level 1 for the control group

Gene expression over time in individual cases

To explore *Bmi-1* expression over time in cases of anaemia, blood samples of four dogs referred to the clinic for three or more times were analysed with qPCR (Table 3 and Figure 7). Three of the patients suffer from non-regenerative IMHA, one from a regenerative IMHA. For patient 4, the anaemia shifts from regenerative to non-regenerative in between week 1 and 5 (Table 3 and Figure 7d).

Clinical status of the patients correlates with increase of decrease in haematocrit. Results show dropping *Bmi-1* mRNA expression levels in correlation with increasing haematocrit in all cases. This also applies for *Bmi-1* levels after blood transfusion. *Bmi-1* levels do not have a correlation with reticulocyte counts (Table 3).

Expression of upstream transcription factor *Zeb1* parallels *Bmi-1* in all cases (Figure 7a-d).

Expression of downstream and apoptotic factor $p19^{Arf}$ decreases after every blood transfusion and generally mirrors haematocrit changes. The factor shows no correlation with *Bmi-1* expression in patient 1 and 3. $p19^{Arf}$ levels parallel *Bmi-1* expression in patient 2 and 4 (Figure 7a-d).

The second apoptotic factor, p27, does not show any correlation in expression with BMI-1 in all four patients. *p27* levels drop after blood transfusion in all cases, but show no correlation with haematocrit (Figure 7a-d). Upstream and target genes of BMI-1 that did not show any significant change in expression level over time, are not depicted.

Table 3 Case details and relative expression of *Bmi-1, Zeb1, p27* and *p19* in individual patients over time

Patient	Time (weeks)	Haematocrit (L/L)	Absolute reticulocyte count (10^9/L)	Relative expression *Bmi-1*	*Zeb1*	*p27*	*p19*
1	0	0,241	28,4	0,2405089963	0,6172664699	4,024593369	5,541475596
	1	0,201	7,4	0,2016365932	0,7721274893	7,43714582	4,66218791
	2	0,447	12.6	0,4469035079	1,40852425	10,91847327	2,202848971
	4	0,043	72,3	0,04316373012	0,7282975908	4,271331559	1,564689457
2	0	0,133	13,5	0,1138735326	0,4248674525	2,695700455	10,59516117
	1	0,289	10,2	0,04204665421	0,3304350179	2,257562767	3,790210463
	3	0,158	19,6	0,05681237225	0,4033153283	1,081991137	2,862071774
3	0	0,193	31,7	0,1347964285	0,3382661319	2,073493639	0,35398094
	3	0,384	32,6	0,1388299302	0,9209336779	1,745992779	3,709830064
	9	0,241	28,5	0,02303983853	0,1451999573	0,6932537051	2,963822549
4	0	0,256	140,5	-	-	6,508797002	-
	1	0,248	129,3	0,2816387061	1,367262367	10,72110008	1,639844972
	5	0,364	18,5	0,02493609847	0,3132213672	1,691913497	0,3697167844

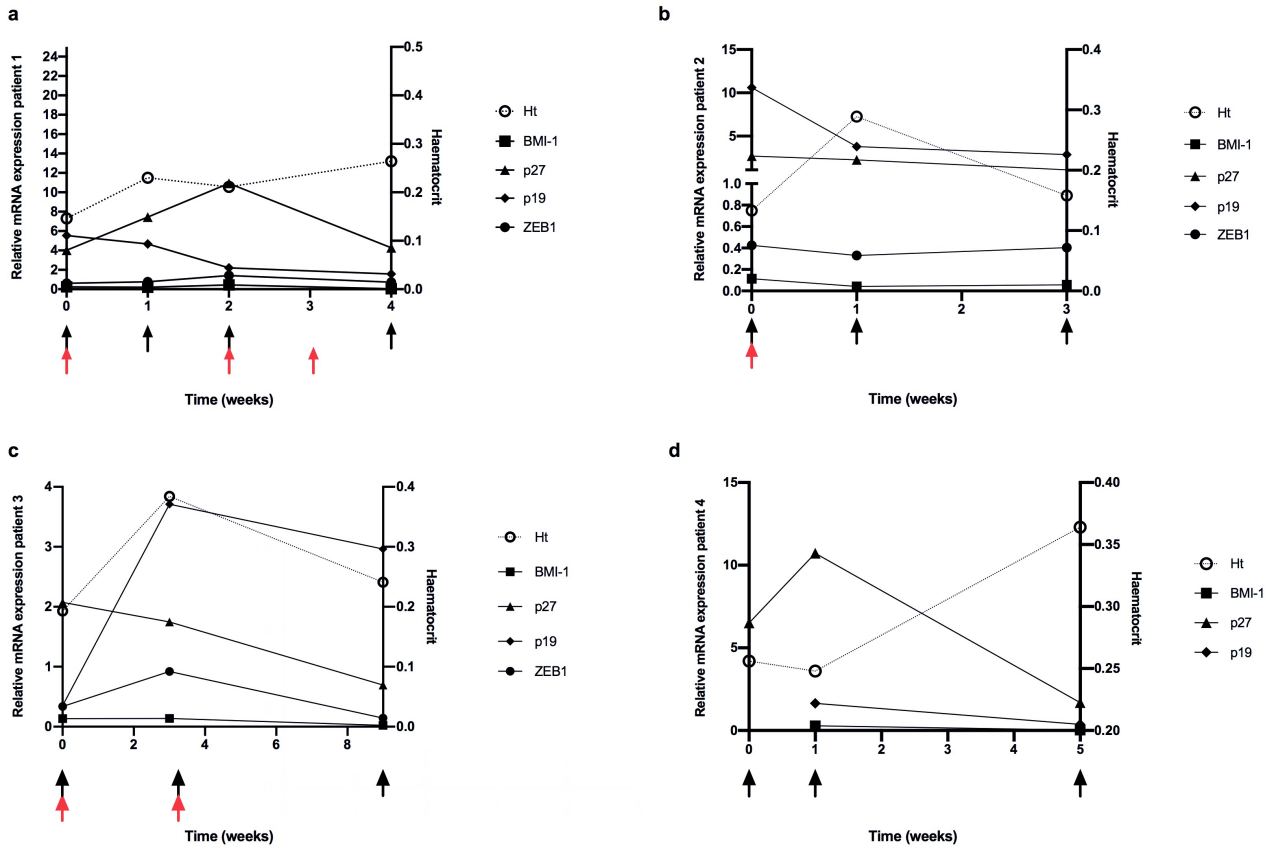


Figure 7 Data for individual patients showing haematocrit and qPCR results for gene expression of *Bmi-1*, *p27*, *p19*^{Arf} and *Zeb1* over time. Figure a) b) and c) show results for patients suffering from IMHA, d) shows a patient suffering from regenerative anaemia. Black arrows indicate day of blood draw. In the case of blood transfusion, red arrows indicate this moment.

Discussion

Polycomb group protein BMI-1 has been of broad and current interest, leading to elucidating its key role in stem cell self-renewal of neuronal and haematopoietic tissue and development of neoplasias. Recently, Kim *et al.* and Gao *et al.* showed a new niche of BMI-1; erythroid development (Kim et al. 2015; Gao et al. 2015). As the role of BMI-1 in canine blood disorders has not yet been investigated, the objective of this study was to find a role for and possible dysfunction of BMI-1 in haematopoiesis for patients suffering of IMHA. Furthermore, this can also suggest possible pathophysiologic processes for IMHA lying in haematopoiesis instead of haemolysis, leading to a lack of the regenerative response of the bone marrow as a complication of the disease. Because little is known about the function and signalling of BMI-1 in haematopoiesis, upstream and downstream targets were also analysed.
Until now, BMI-1 functioning in erythroid lineage differentiation and HSC self-renewal had only been investigated in human and mice. This study provides some insight in functioning of this process in dogs. To elucidate the expression of the different genes, canine patients suffering from IMHA were compared with other cases suffering from either non-regenerative or regenerative anaemia by performing qPCR on leukocytes originating from whole blood samples.
Here, higher *Bmi-1* expression was found in all cases of anaemia, with highest expression levels in patients suffering from IMHA. Therefore, problems in haematopoiesis due to inadequate regulation of *Bmi-1* expression in IMHA pathophysiology are not expected.
Surprisingly high levels of $p19^{Arf}$ and *p27* were found in all cases of anaemia, with a remarkable increase in expression of *p27*. This indicates apoptotic or differentiation events in the erythroid lineage, depending on the site of expression.

To provide more reliable and statistically significant outcomes concerning this study, larger groups of patients need to be examined. Analysis of individual cases will also benefit from larger patient groups. Use of a suboptimal number of reference genes was due to unsatisfying results of three of the six selected genes. For further research, the use of at least five reference genes will decrease the contribution to variance between the samples. Furthermore, patient classification based on microscopic examination of bone marrow biopsies instead of whole blood reticulocyte counts will give a more reliable categorization. Concerning gene expression analysis, performing qPCR on bone marrow instead of whole blood increases the chance to find more relevant levels of gene expression of *Bmi-1* and target genes. For *Bmi-1* and target genes, expression is limited to cells that reside in the bone marrow. Nevertheless, $p19^{Arf}$ and p27 are expressed in erythroblasts, circulating in the bloodstream. Next to expression in erythroblasts, these apoptotic markers may be expressed in tissue suffering from hypoxia as a result of anaemia. qPCR analysis of whole blood may therefore pick up expression profiles of whole blood components, including factors that might originate from said tissue. While mRNA will be degraded in the circulation, this event may interfere with drawing hard conclusions regarding BMI-1 downstream targets.

High levels in *Bmi-1* expression in patients suffering from IMHA can be explained by the need for erythroid production following from haemolysis, stimulating stem cell self-renewal.
While many studies advocate inhibitory functioning of BMI-1 on p27, in neoplasias (Kim et al. 2017; Zheng et al. 2014), but also healthy bone marrow (Cheng et al. 2000), our results show contrasting high mRNA levels of *p27*. Also, *p19^{Arf}* is shown to be inhibited by BMI-1 in normal cellular physiology (Sahasrabuddhe 2016; Moon et al. 2011) and in the erythroid lineage (Oguro et al. 2010; Gao et al. 2015; Park et al. 2003).
For p19^{Arf} and p27, the main regulatory function resides in progenitor cell differentiation through cell cycle inhibition. Being components of two different CDKI families, their actions differ slightly. For their site of action, it is suggested that p19^{Arf} is mainly expressed in HSCs, while p27 blocks the G_1 to S phase of the cell cycle in more mature progenitor cells (Hao et al. 2016)
As a member of the INK4A/Arf locus, p19^{Arf} facilitates cell cycle exit and subsequent depletion of the HSC pool. The protein shows a role in erythroid differentiation, and its action depends on the site of expression. It was shown in humans, p19^{Arf} sequesters Mdm2, resulting in p53 mediated cell cycle arrest and apoptosis when expressed in HSCs. In contrast, p19^{Arf} also stimulates the HSP70-GATA1 pathway, resulting in erythroid differentiation when expressed in erythroblasts (Han et al. 2017)(Park et al. 2003)(Avgustinova & Benitah 2016).
p27 is suggested to serve the same purpose in differentiation via inhibition of CDK2 in erythroblasts, but without associated impact on the HSC pool (Cheng et al. 2000). The CDKI regulates progenitor proliferation and pool size by stimulating cell cycle exit in erythroid differentiation, independent from p53. Correspondingly, levels of expression of *p27* show elevation when differentiation occurs in the erythroid lineage (Hsieh et al. 2000). *p27* knockout mice do not have altered number or self-renewal regarding HSCs, but progenitors show more proliferation and a bigger pool size, supporting the thought of a role for the protein in progenitors rather than HSCs. Despite this, *p27* expression is found in HSCs, so functioning in HSCs cannot be excluded (Hao et al. 2016).

Where cases of IMHA show aberrant expression of *Bmi-1* expression levels in blood, suggesting HSC pool maintenance, it is expected that production of the erythroid lineage as a result of *Bmi-1* expression is functioning without any problems. Nevertheless, a lot of patients suffering from IMHA show a lack of a regenerative response in peripheral blood. This may suggest high rates of apoptosis in erythroid progenitor cells in the bone marrow.
High expression of *Bmi-1* also implies lower levels of these downstream targets *p27* and *p19^{Arf}*, while qPCR outcomes suggest the opposite. To investigate these contrasting results, and make distinction between occurring apoptosis or differentiation due to these proteins, expression of *p27* and *p19^{Arf}* in HSCs needs to be compared with these expression profiles in more mature progenitor cells. When comparing bone marrow with whole blood samples in healthy dogs as well as IMHA patients, distinction can be made in *p27* expression. Performing western blots for BMI-1 and p27 can validate actual protein synthesis. To confirm the event of apoptosis and associate this with localization in bone marrow or whole blood, western blots for caspases and apoptotic DNA fragments can be performed (Budihardjo et al. 1999). This may elucidate the hypothesised role of p27 in erythroid apoptosis and subsequent possible malfunction of haematopoiesis downstream of BMI-1 in patients suffering from a non-regenerative IMHA.

Acknowledgement

I want to thank Dr. C.J. Piek and Dr. L.C. Penning for the opportunity to write my research thesis at the department of Clinical Sciences of Companion Animals and re-introducing me to the diverse world of research. I felt very comfortable and learned a lot, maybe even developed enthusiasm for doing research. I would also like to thank them for the brainstorm sessions, reviewing and all the other time they put into facilitating and guiding my research.

Monique van Wolferen taught me the ins and outs of lab work with regard to RNA isolation and qPCR. Thank you for your time and patience!

I would also like to thank Martin van Leeuwen of the *Universitair Veterinair Diagnostisch Laboratorium* for processing all the blood samples.

References

Avgustinova, A. & Benitah, S.A., 2016. Epigenetic control of adult stem cell function. *Nature Reviews Molecular Cell Biology* 17(10), pp.643–658

Balch, A. & Mackin, A., 2007. Canine immune-mediated hemolytic anemia: pathophysiology, clinical signs, and diagnosis. *Compendium (Yardley, PA)* 29(4), pp.217–225

Balch, A. & Mackin, A., 2007. Canine Immune-Mediated Hemolytic Anemia: Treatment and Prognosis. *Compendium (Yardley, PA)* 29, pp.230–238

Barker, R.N. et al., 1992. Autoimmune haemolysis in the dog: Relationship between anaemia and the levels of red blood cell bound immunoglobulins and complement measured by an enzyme-linked antiglobulin test. *Veterinary Immunology and Immunopathology* 34, pp.1–20

Brinkhof, B. et al., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Analytical Biochemistry* 356(1), pp.36–43

Budihardjo, I. et al., 1999. Biochemical Pathways of Caspase Activation during Apoptosis. *Annual Review of Cell and Developmental Biology* 15, pp.269-90

Bustin, S.A. et al., 2010. MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology* 11(74)

Chen, F. et al., 2011. Knockdown of BMI-1 causes cell-cycle arrest and derepresses p16INK4a, HOXA9 and HOXC13 mRNA expression in HeLa cells. *Medical Oncology* 28, pp.1201-1209

Cheng, T. et al., 2000. Stem cell repopulation efficiency but not pool size is governed by p27(kip1). *Nature Medicine* 6(11), pp.1235–1240

Cohn, L.A., 2015. *Chapter 110- Acute Hemolytic Disorders in Small Animal Critical Care Medicine* second., Elsevier Saunders

Corato, A. et al., 1997. Proliferative responses of peripheral blood mononuclear cells from normal dogs and dogs with autoimmune haemolytic anaemia to red blood cell antigens. *Veterinary Immunology and Immunopathology* 59(3–4), pp.191–204

Gao, R. et al., 2015. Bmi1 promotes erythroid development through regulating ribosome biogenesis. *Stem Cells* 33(3), pp.925–938

Han, X. et al., 2017. Unexpected role for p19NK4d in posttranscriptional regulation of GATA1 and modulation of human terminal erythropoiesis. *Blood* 129(2), pp.226-237

Hao, S., Chen, C. & Cheng, T., 2016. Cell cycle regulation of hematopoietic stem or progenitor cells. *International Journal of Hematology* 103(5), pp.487–497

Hattangadi, S.M. et al., 2011. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood* 118(24), pp.6258–6268.

Hsieh, F.F. et al., 2000. Cell cycle exit during terminal erythroid differentiation is associated with accumulation of p27(Kip1) and inactivation of cdk2 kinase. *Blood* 96(8), pp.2746–54

Iwama, A. et al., 2004. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* 21, pp.843-851

Iwama, A. et al., 2005. Epigenetic regulation of hematopoietic stem cell self-renewal by polycomb group genes. *International journal of hematology* 81(4), pp.294–300

Jacobs, J.L. et al., 1999. The oncogene and Polycombgroup gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 397, pp. 164-168

Kim, A.R. et al., 2015. Bmi-1 Regulates Extensive Erythroid Self-Renewal. *Stem Cell Reports* 4(6), pp.995–1003

Kim, B.R., Kwon, Y. & Rho, S.B., 2017. BMI-1 interacts with sMEK1 and inactivates sMEK1-induced apoptotic cell death. *Oncology Reports* 37(1), pp.579–586

Lessard, J. & Sauvageau, G., 2003. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* 423(6937), pp.255–260

Liu, J. et al., 2009. Bmi1 regulates mitochondrial function and the DNA damage response pathway. *Nature* 459, pp.387–393

Liuq, Y. et al., 2014. The ZEB1 transcription factor acts in a negative feedback loop with miR200 downstream of ras and Rb1 to regulate Bmi1 expression. *Journal of Biological Chemistry* 289(7), pp.4116–4125

McAlees, T.J., 2010. Immune-mediated haemolytic anaemia in 110 dogs in Victoria, Australia. *Australian Veterinary Journal* 88, pp.25-28

McCullough, S., 2003. Immune-mediated hemolytic anemia: Understanding the nemesis. *Veterinary Clinics of North America - Small Animal Practice* 33(6), pp.1295–1315

Moon, J.H. et al., 2011. Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1. *Cell Research* 21(9), pp.1305–1315

Nowak, K. et al., 2006. BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. *Nucleic Acids Research* 34(6), pp.1745–1754

Oguro, H. et al., 2010. Poised Lineage Specification in Multipotential Hematopoietic Stem and Progenitor Cells by the Polycomb Protein Bmi1. *Cell Stem Cell* 6, pp.279-286

Park, I.K. et al., 2003. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423(6937), pp.302–305

Park, I.K., Morrison, S.J. & Clarke, M.F., 2004. Bmi1, stem cells, and senescence regulation. *Journal of Clinical Investigation* 113(2), pp.175–179

Piek, C., 2011. *Prognosis in canine idiopathic immune-mediated haemolytic anaemia*. Faculty of Veterinary Medicine, Utrecht University

Piek, C.J. et al., 2011. Leukocyte count affects expression of reference genes in canine whole blood samples. *BMC Research Notes* 4(36)

Piek, C.J. et al., 2008. Idiopathic immune-mediated hemolytic anemia: Treatment outcome and prognostic factors in 149 dogs. *Journal of Veterinary Internal Medicine* 22, pp.366-373

Piek, C.J., 2011. Canine idiopathic immune-mediated haemolytic anaemia: A review with recommendations for future research. *Veterinary Quarterly* 31, pp.129–141

Raaphorst, F.M., 2003. Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene Bmi-1. *Trends in Immunology* 24, pp.522–524

Rizo, A. et al., 2008. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111(5), pp.2621–2630

Sahasrabuddhe, A.A., 2016. BMI1: A Biomarker of Hematologic Malignancies. *Biomarkers in cancer* 8, pp.65–75

Schuringa, J.J. & Vellenga, E., 2010. Role of the polycomb group gene BMI1 in normal and leukemic hematopoietic stem and progenitor cells. *Current Opinion in Hematology* 17(4), pp.294–299

Stokol, T., Blue, J.T. & French, T.W., 2000. Idiopathic pure red cell aplasia and nonregenerative immune-mediated anemia in dogs: 43 cases (1988–1999). *Journal of the American Veterinary Medical Association* 216(9), pp.1429–1436

Swann, J.W. & Skelly, B.J., 2013. Systematic Review of Evidence Relating to the Treatment of Immune-Mediated Hemolytic Anemia in Dogs. *Journal of Veterinary Internal Medicine* 27, pp.1-9

Swann, J.W. & Skelly, B.J., 2015. Systematic Review of Prognostic Factors for Mortality in Dogs with Immune-mediated Hemolytic Anemia. *Journal of Veterinary Internal Medicine* 29, pp.7–13

Tamir, A. et al., 2000. Stem cell factor inhibits erythroid differentiation by modulating the activity of G1-cyclin-dependent kinase complexes: a role for p27 in erythroid differentiation coupled G1 arrest. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* 11(5), pp.269–277

Tsiftsoglou, A.S., Vizirianakis, I.S. & Strouboulis, J., 2009. Erythropoiesis: Model systems, molecular regulators, and developmental programs. *IUBMB Life* 61(8), pp.800-830

Valent, P. et al., 2018. Normal and pathological erythropoiesis in adults: from gene regulation to targeted treatment concepts. *Haematologica* 103(10), pp.1593–1603

Weiss, D.J., 2008. Bone Marrow Pathology in Dogs and Cats with Non-Regenerative Immune-Mediated Haemolytic Anaemia and Pure Red Cell Aplasia. *Journal of Comparative Pathology* 138(1), pp.46–53

Wu, K.J., 2011. Direct activation of Bmi1 by Twist1: implications in cancer stemness, epithelial-mesenchymal transition, and clinical significance. *Chang Gung Med J* 34(3), pp.229–238

Zhang, H.W. et al., 2010. Defects in mesenchymal stem cell self-renewal and cell fate determination lead to an osteopenic phenotype in Bmi-1 null mice. *Journal of Bone and Mineral Research* 25(3), pp.640–652

Zheng, X. et al., 2014. Bmi-1-shRNA Inhibits the Proliferation of Lung Adenocarcinoma Cells by Blocking the G1/S Phase Through Decreasing Cyclin D1 and Increasing p21/p27 Levels. *Nucleic Acid Therapeutics*, 24(3) pp.210–216