Prognosis in canine idiopathic immune-mediated haemolytic anaemia

Christine J Piek

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Prognosis in canine idiopathic immune-mediated haemolytic anaemia

De prognose van idiopatische immuungemedieerde hemolytische anemie bij de hond

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van
de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van
het college voor promoties in het openbaar te verdedigen op dinsdag 30 augustus 2011
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door

Christina Jantine Piek

geboren op 9 juni 1966 te Hazerswoude

Promotor:	Prof. dr. J. Rothuizen
Co-promotoren:	Dr. E. Teske
	Dr. L.C. Penning

An die Musik

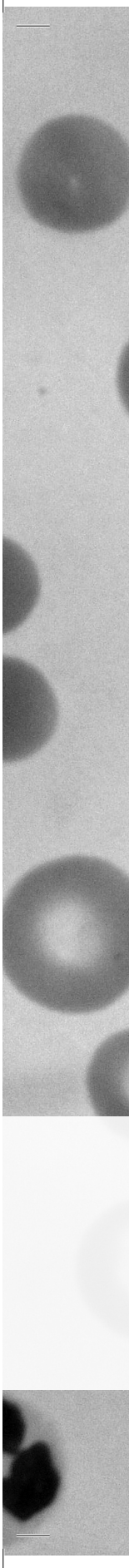
Du holde Kunst, in wieviel grauen Stunden,
Wo mich des Lebens wilder Kreis umstrickt,
Hast du mein Herz zu warmer Lieb' entzunden,
Hast mich in eine beßre Welt entrückt!

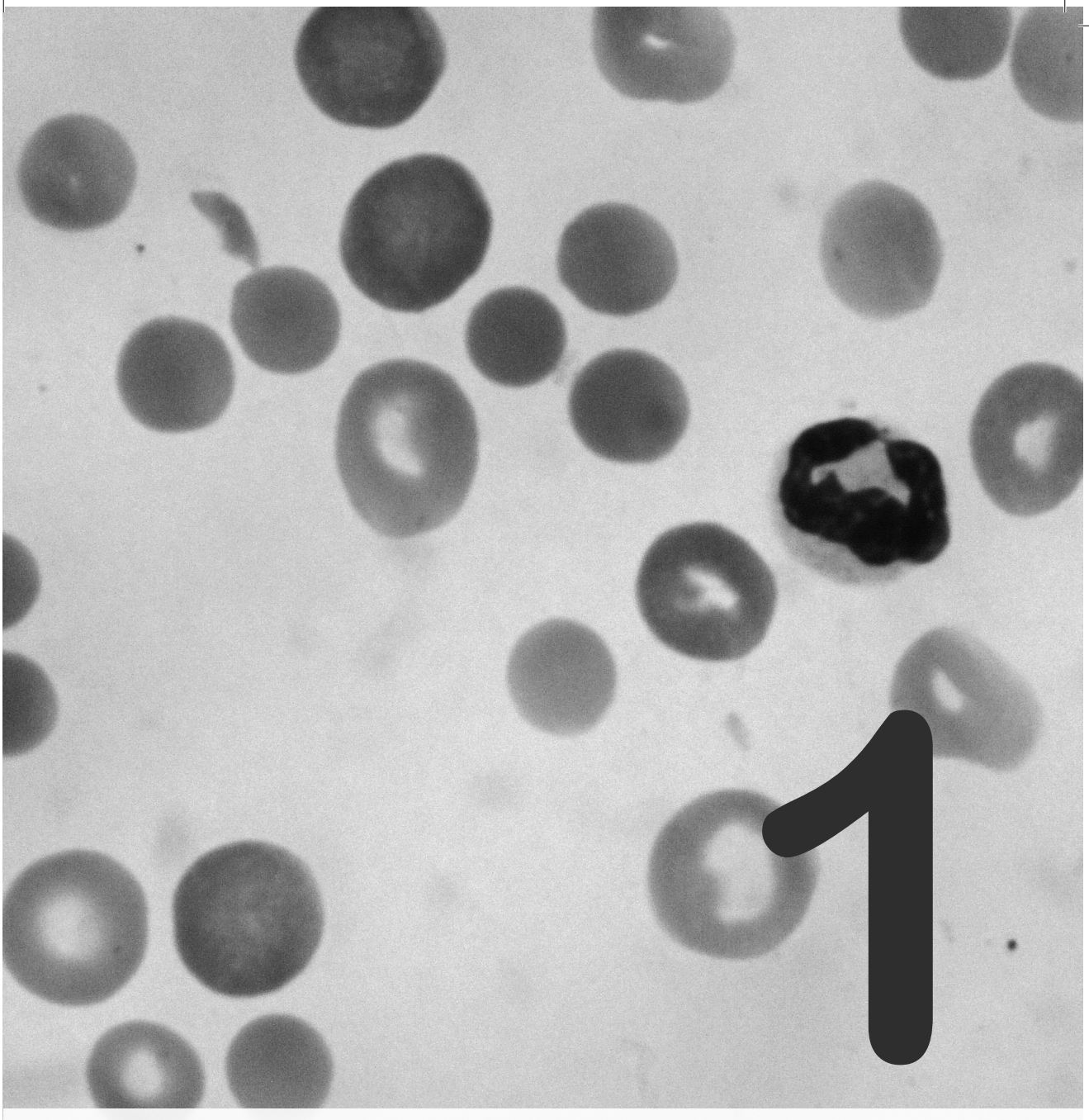
Oft hat ein Seufzer, deiner Harf' entflossen,
Ein süßer, heiliger Akkord von dir
Den Himmel beßrer Zeiten mir erschlossen,
Du holde Kunst, ich danke dir dafür!

Text: Franz von Schober
Musik: Franz Schubert

Voor Bep en Jan

Contents





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Introduction

Idiopathic immune-mediated haemolytic anaemia (IMHA), characterized by antibody-mediated red cell destruction, is recognized as one of the most frequently occurring immune-mediated diseases in the dog (1). Idiopathic IMHA is diagnosed based on a Coombs' positive haemolytic anaemia, the presence of spherocytes, and exclusion of infectious or neoplastic disorders, vaccination, and medication that can cause a secondary IMHA (2). The presentation of a dog diagnosed with idiopathic IMHA varies from a mild anaemia to a severe haemolytic crisis with a mortality of 20% - 70% that mainly occurs in the first 2 weeks post diagnosis (3-5). The central theme of this thesis is to analyze clinical, therapeutic, and pathophysiological factors that contribute to disease outcome in dogs with idiopathic IMHA.

Retrospective studies report a poor outcome in dogs with autoagglutination (4), non-regenerative or severe anaemia (6), thrombocytopenia (7), severe leucocytosis (5), high plasma bilirubin concentration (4, 6, 7), or increased prothrombin time (5). Not every dog diagnosed with idiopathic IMHA displays all these characteristics, however, and it is unclear if these characteristics are part of the process of antibody-mediated red cell destruction or that in fact they result from independent pathways or pathophysiological processes secondary to the haemolysis. The relationship between various characteristics and an event may be explored by statistical multivariate models that analyze the probability that death occurs in a specified time period. Lifelong immunosuppression has been recommended but evidence for this recommendation is lacking (2, 8). The scope of **Chapter 2** is to estimate survival time in dogs with idiopathic IMHA and to explore which clinical and laboratory characteristics determine the probability of survival and in addition to determine if immunosuppression for three months is sufficient to maintain remission of idiopathic IMHA.

Immunomodulation is the mainstay of treatment in IMHA and may be combined with whole blood or packed red cell transfusions and anticoagulation (2, 8-10). It has been suggested to combine glucocorticoids with other immunosuppressive agents of cytotoxic drugs if clinical condition worsens, if side effects of glucocorticoids are unacceptable, or as part of standard treatment protocols (2, 11, 12). Azathioprine, a thiopurine analog, is a cytotoxic drug that interferes with DNA synthesis by competition with adenosine (13). It has been reported to have a synergistic effect with prednisolone and if used in combination the prednisolone dose may be reduced (14). A beneficial effect of treatment with azathioprine in idiopathic IMHA has been reported in two retrospective studies (5, 15). However, the duration of azathioprine therapy in one study must be judged suboptimal since the clinical effect of azathioprine may be expected after at least 11 days of treatment (5, 14). In the other study the efficacy of azathioprine alone could not be determined since dogs were treated with cyclophosphamide on top of azathioprine and prednisolone (5). The scope of **Chapter 3** is therefore to assess the additional beneficial therapeutic effect of azathioprine compared to prednisolone therapy in dogs with idiopathic IMHA

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The laboratory diagnosis of IMHA rests upon the demonstration of an immune-mediated mechanism for the haemolysis (2). The direct agglutination test (DAT) demonstrates the presence of anti-erythrocyte antibodies by incubating a suspension of washed patient erythrocytes with polyvalent or monovalent antisera specific for dog immunoglobulin or complement (16). There is a lack of uniformity between laboratory procedures for the DAT (16). Instead of testing only a few dilutions testing of more dilutional steps in a microtitre tray has been advocated to result in less false negative results due to the prozone effect (16, 17). Incubation is generally performed at 37°C but some laboratories also incubate at 4°C, a procedure that is debated by others (2, 16, 18, 19). The monovalent DAT is suggested to have a higher sensitivity than the polyvalent DAT and the sensitivity and specificity of reagents from different manufacturers differ considerably (16, 20). A gel-based polyvalent DAT has been developed that offers the possibility to standardize anti-erythrocyte antibody testing between laboratories (21). The scope of **Chapter 4** is to assess the performance of this gel-based DAT in comparison to the traditional DAT based on erythrocyte agglutination and to assess the usefulness of this gel-test as a diagnostic tool in the diagnosis of IMHA.

Abundant evidence has been presented for a state of hypercoagulability during the hospitalization period in dogs with idiopathic IMHA (7, 22-25). Post-mortem examinations of dogs with idiopathic IMHA demonstrate the presence of thromboembolisms in many organs (7, 22, 26). Leucocytosis and a left shift are present in the majority of dogs with IMHA and increased leucocyte counts have been associated with moderate to marked tissue damage (5, 7, 15, 22, 26, 27). In dogs with IMHA changes in acute phase protein concentrations have been measured fitting the presence of an acute phase response (28-32). Interleukin-8 (Il-8) is one of the cytokines that is increased in the acute phase response. It is a major chemotaxin for leucocytes and orchestrates the margination and extravasation process of leucocytes through increases in selectin expression on endothelial cells (33, 34). Leucocytes, in particular monocytes, play an important role in thrombogenesis by producing tissue factor (TF) which initiates the extrinsic pathway of coagulation (35, 36). It was hypothesized that dogs with idiopathic IMHA have increased blood levels of IL-8 and TF. The scope of **Chapter 5** is to validate reference genes for future quantitative RT-PCR studies in idiopathic IMHA in canine whole blood. The scope of **Chapter 6** is to assess the contribution of whole blood gene expressions of TF and IL-8 to the inflammatory response and the coagulation abnormalities in dogs with idiopathic IMHA.

The mortality rate in canine idiopathic IMHA has not decreased since the earliest publications (3-5, 37, 38). Azathioprine, cyclophosphamide, other immunomodulators, and heparin are used often in the treatment of canine idiopathic IMHA despite the fact that evidence of their efficacy is lacking (5, 15, 39-45). The scope of **Chapter 7** is to provide clinicians with an overview of the current state of evidence in canine IMHA and to critically evaluate this evidence to identify possible reasons why research as yet has failed to improve outcome of canine idiopathic IMHA.

In summary, the research questions that are addressed in this thesis are the following:

1) What is the estimated survival time in dogs with idiopathic IMHA and which clinical and laboratory characteristics determine this outcome? In addition, is a protocol of 3 months immunosuppression sufficient to maintain remission of IMHA?
2) Is there an additional beneficial therapeutic effect in dogs with idiopathic IMHA of a protocol that includes azathioprine and prednisolone versus prednisolone alone?
3) How does a fast and simple to perform gel-based DAT perform in comparison to a traditional DAT and is such a test useful as a diagnostic tool in the diagnosis of IMHA?
4) Which reference genes are suitable for future quantitative RT-PCR studies into idiopathic IMHA in canine whole blood?
5) What is the contribution of whole blood gene expressions of TF and IL-8 to the inflammatory response and the coagulation abnormalities in dogs with idiopathic IMHA?
6) What is the current state of evidence in canine IMHA and why has research as yet failed to improve outcome of canine IMHA?

In **Chapter 8** the interrelationship of the results of the chapters 2 - 7 will be discussed.

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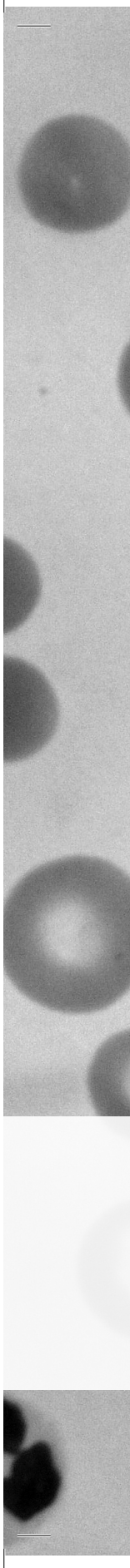

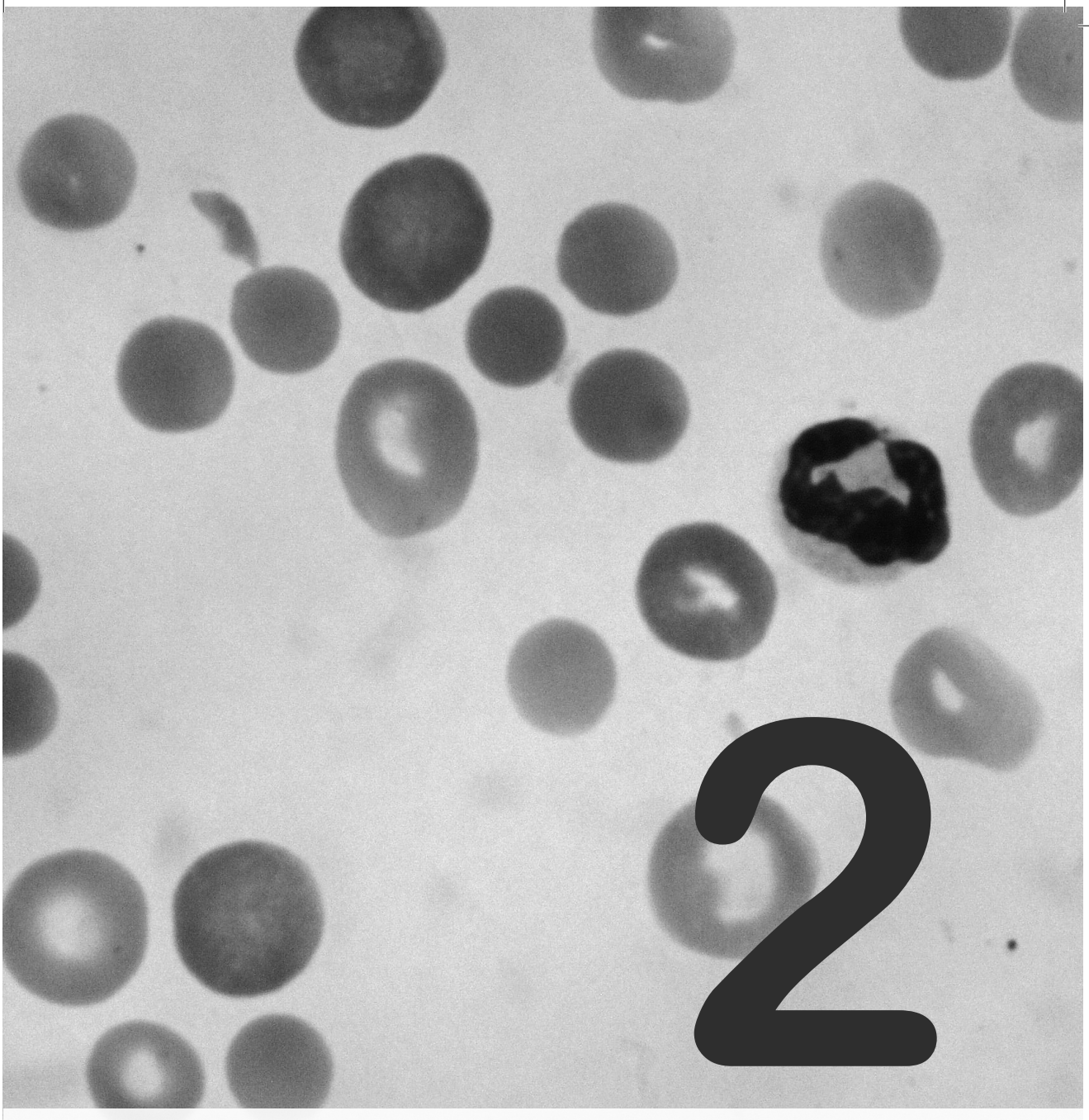

2

Idiopathic immune-mediated haemolytic anaemia: treatment outcome and prognostic factors in 149 dogs

CJ Piek, G Junius, A Dekker, E Schrauwen, RJ Slappendel, E Teske

J Vet Int Med. 2008;22(2):366-373

Abstract

Background - Canine idiopathic immune-mediated haemolytic anaemia (IMHA) is associated with a high mortality especially in the first 2 weeks after diagnosis despite treatment.

Objectives - To determine treatment outcome and identify prognostic variables in order to define areas of future research.

Methods – One hundred forty-nine dogs with haematocrit < 30% and either a positive Coombs' test or spherocytosis, and no evidence of disease that can trigger IMHA were included. In this retrospective cohort study all dogs were treated with prednisolone and azathioprine according to a standard protocol. Survival analysis was performed using the Kaplan-Meier method. Variables recorded at the time of diagnosis were tested as possible prognostic variables in a univariate and multivariate Cox proportional hazard model.

Results – The main predictors for mortality in dogs with idiopathic IMHA are the presence of increased plasma urea concentration, bands, thrombocytopenia and petechiae at the time of diagnosis. The estimated Kaplan Meier half-year survival was 72.6% (95% CI: 64.9 – 81.3%). Mortality occurred mostly within the first 2 weeks. Cox proportional hazards analysis indicated that increased plasma urea concentration, icterus and petechiae were the major independent predictors of mortality in the first 2 weeks. In most dogs that survived IMHA, a 3-month protocol of azathioprine with prednisolone maintained clinical remission. The estimated half-year survival for dogs that survived the first 2 weeks was 92.5% (95% CI: 86 – 99.3%).

Conclusions - If the dogs survived IMHA a 3 – month protocol of prednisolone and azathioprine was effective with regard to survival and clinical outcome. Future research should be directed at identifying whether thrombotic tendency in dogs with IMHA is the main contributor to the development of increased plasma urea concentration, icterus, thrombocytopenia and petechiae.

Introduction

In immune-mediated haemolytic anaemia (IMHA) red blood cells are destroyed as a consequence of anti-erythrocyte antibody production. IgM-mediated haemolysis is caused mainly by intravascular complement activation and subsequent intravascular haemolysis. This is in contrast with the IgG-mediated IMHA where haemolysis is mainly caused by macrophages in liver, spleen or both (1). IMHA may be primary or secondary in nature. Secondary IMHA occurs when an underlying disease such as neoplastic and chronic infectious diseases, exposure to drugs, toxins, and vaccines leads to attachment of immunoglobulins to erythrocytes (2, 3). Sixty to 75% of IMHA cases in dogs are thought to be primary or idiopathic in origin (2, 4).

Clinical signs of IMHA include lethargy, inappetence, pigmenturia, tachycardia, pale mucous membranes, and fever (2). Diagnosis is based on the demonstration of a haemolytic anaemia together with evidence of immune-mediated destruction of red blood cells. In some cases, the anti-erythrocyte antibodies lead to autoagglutination, and in other cases the presence of anti-erythrocyte antibodies can be confirmed by the Coombs' test (2, 5). Spherocytosis is considered to be a strong indicator of immune-mediated haemolysis (5). Spherocytes are formed when macrophages remove a portion of the erythrocyte membrane that is coated with antibody, complement, or both. An indirect way to detect spherocytosis is the osmotic red cell fragility test (5). Neither presence of spherocytes, positive Coombs' test, nor increase of the osmotic red cell fragility indicate whether the aetiology of the immune-mediated haemolysis is primary or secondary.

Previous studies have reported mortality rates up to 70% during the first 3 weeks of treatment (3, 4, 6). The presence of autoagglutination (3), the degree of reticulocytosis (7), severity of anaemia (7), thrombocytopenia (8), severe leucocytosis (6, 9), increase in bands (9), serum bilirubin concentration (3, 7, 8, 10), and increase in prothrombin time (PT) (6, 9) all were associated with poor outcome. Clinical decision-making in cases of idiopathic IMHA might be easier when the clinician is provided with factors that predict outcome. The objective of this study was to identify the prognostic variables that determine the outcome in dogs with idiopathic IMHA treated with prednisolone and azathioprine following a standard protocol. These factors might define areas of future research that will hopefully result in a better prognosis for these patients.

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Materials and Methods

Patients

Dogs included were referred to the Utrecht University Clinic of Companion Animals (UUCCA) between January 1st 1994 and December 31st 2000. Inclusion criteria were haematocrit < 30% and either a positive Coombs' test or presence of spherocytes in a blood smear. In addition, these dogs had been treated according to a standard immunosuppressive protocol and a complete medical record had to be present. Dogs were excluded if they had evidence of diseases that could induce IMHA such as neoplasia, medications and infectious diseases. As a result, dogs that had visited areas where ehrlichiosis and babesiosis are endemic were excluded unless serologic examination for *Ehrlichia canis* and *Babesia canis* or *gibsonii* was negative. Dogs with a travel history to countries in which the above infectious diseases are endemic within 3 weeks before diagnosis of idiopathic IMHA also were excluded. In addition, dogs that received immunosuppressive treatment for longer than 14 days before referral to the UUCCA were excluded.

Complete history and physical examination were recorded including age at time of first diagnosis of idiopathic IMHA, occurrence of generalized weakness, anorexia, vomiting, diarrhoea, and dark red urine as well as the presence of an increased rectal body temperature, pale mucous membranes, icterus, petechiae, lymphadenopathy, and cranial abdominal organomegaly. Additional diagnostics were performed when judged necessary by the attending clinician.

Laboratory tests

All tests were performed at the UUCCA. The following laboratory tests were performed on admission, and during return visits, approximately 4 and 10 weeks after starting the therapeutic protocol: Complete Blood Count (CBC), reticulocyte count, presence of spherocytes, Coombs' test and osmotic red cell fragility testing, and plasma urea and creatinine concentrations. Results of prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration were recorded when performed. PT and APTT were considered prolonged when they were increased at least 10 % above results obtained using normal pooled canine citrated plasma.

A monovalent direct Coombs' antiglobulin test was performed using anti-dog IgG (Central Blood Laboratory, Amsterdam, The Netherlands) and IgM (Nordic, Tilburg, The Netherlands) antibodies and a anti-dog complement antibody (Nordic, Tilburg, The Netherlands) for agglutination of the patients red cells as described before (5).

The osmotic fragility of the erythrocytes was determined as previously described (5). Plasma creatinine concentrations were corrected for body weight as described previously (11).

Therapy

Blood transfusions alone or in combination with IV fluid therapy were given when judged necessary by the attending clinician. The number of blood transfusions was noted. All clinic-owned donor dogs used in the study are DEA 1.1 and 1.2 negative. Whenever a client-owned donor dog was used or when a second transfusion was given cross matches were performed.

A standard protocol of immunosuppressive treatment consisting of a combination of prednisolone and azathioprine was instituted in all dogs. The *outcome category* (see below) was assessed at least once daily during the hospitalization period and during return visits that were scheduled 4 and 10 weeks after the start of therapy.

As long as the outcome category was *no effect* prednisolone (Alfasan International BV, Woerden, The Netherlands) was given in a dosage of 2 mg/kg/day PO. Dogs that were not able to take oral medication were treated with dexamethasone (0.5 – 1 mg/kg/day) IV or SC. As soon as the outcome was assessed as *improvement* prednisolone therapy was started a dosage of 2 mg/kg/day PO for 3 days, followed by 1.5 mg/kg/day PO for 7 days, 1 mg/kg/day PO for 10 days, 0.5 mg/kg/day PO for 14 days, after which the same dose was given on alternate days for 14 days, and subsequently tapered down to 0.25 mg/kg/day PO for 21 days. Azathioprine (Imuran, Glaxo-Wellcome, Zeist, The Netherlands) was started at a dosage of 2 mg/kg/day PO for dogs weighing < 20 kg. The daily azathioprine dose in dogs of 25, 30, 40, and 50 kg, was maximized at 45, 50, 60, and 70 mg, respectively. Azathioprine treatment was stopped 10 days after prednisolone treatment. If the outcome was assessed as *complete recovery* at the 4 or 10 week return visit the prednisolone therapy protocol as described above was followed. If a *relapse* was diagnosed at any time during this treatment the prednisolone therapy protocol was started from the beginning. If at the 4 or 10 week return visit the outcome was assessed as *improvement* and not yet as *complete recovery,* the duration of the interval at which prednisolone was tapered, as described above, was doubled.

Outcome

The response to therapy and its adverse effects were recorded from the medical record. The response was assessed at the first and second return visit. At both times the findings were compared to the last visit.

We defined 4 outcome categories. *Complete recovery* was defined as an increase in haematocrit to > 36%, a negative Coombs' test and an osmotic red cell fragility within normal reference range. The category *improvement* included dogs that experienced lesser increases in haematocrit, as well as dogs that had haematocrits > 36 % that still had a positive Coombs' test or an increased osmotic red cell fragility. *No effect* of therapy was defined as no increase in haematocrit. A *relapse* was defined as a decrease in haematocrit

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after an initial *improvement* or *complete recovery,* in combination with recurrence of a positive Coombs' test or increased red cell fragility. The time at which a *relapse* occurred was recorded from the medical record.

The haematocrit, the corrected reticulocyte percentage, the platelet count, results of the Coombs' test, and the osmotic fragility of the erythrocytes were recorded from the medical records at the times of the first and second return visits and when a relapse occurred. Vomiting and diarrhoea were divided into 3 categories: absent, < 2 days, or > 2 days.

Survival was determined at the last date of contact with the owner by the attending clinician or by 1 of the investigators (GJ) at the time of the study. The outcome was divided into 4 categories: death caused by IMHA, death by another cause, alive but still on immunosuppressive treatment, and alive without treatment.

Data analysis

Statistical analysis of the data was performed using S-plus statistical package (S-PLUS 6.1, Insightful Corporation, Seattle, US). The data set was split into a group that was in the study for ≤ 14 days and a group that was in the study > 14 days. All variables that were measured at the time of diagnosis were compared between groups. Comparison between groups for categorical variables was performed with the Fisher's exact test. Continuous variables were compared between groups using the Wilcoxon rank-sum test. $P < 0.05$ was considered significant.

Survival analysis was performed for both groups as well as for the entire data set. The endpoint of the study was death caused by IMHA. Dogs that were alive at the end of the study and dogs that died by a cause other than IMHA were censored. Survival curves were drawn with the Kaplan-Meier method.

All variables recorded at the date of first diagnosis were evaluated as possible prognostic indicators in a univariate Cox proportional hazard model except the variable breed. Variables significant at the $P < 0.15$ level in the univariate model were analyzed in a multivariate model allowing for interaction among variables. Multivariate analysis was performed by forward stepwise selection using a $P < 0.05$ in the likelihood ratio test as a criterion for inclusion. Compliance with the proportional hazard assumption was tested graphically by plotting the Schoenfeld residuals against time.

Results

Patients

One hundred ninety-seven dogs were selected from the laboratory database of the UUCCA. Seventy-nine of these dogs had both a positive Coombs' test and spherocytes, 109 dogs only a positive Coombs' test, and 9 dogs only spherocytes. Forty-three dogs were excluded because they were positive for at least one of the exclusion criteria.

As a result, 149 dogs were included in the study. Twenty-six dogs were crossbreeds, 11 were English cocker spaniels, 9 old English sheepdogs, 8 dachshunds, and 7 were Labrador retrievers. Each of the following breeds was represented by 5 dogs: Bouvier des Flandres, German shepherd dog, Jack Russell Terrier, and Maltese. The remaining 68 dogs were of other breeds. Sixty-one of the 149 dogs were males (46 intact, 15 castrated) and 88 were female dogs (51 intact, 37 neutered). Median age at the time of first diagnosis of idiopathic IMHA was 5 years (range, 13 weeks – 13 years).

The median duration of clinical signs preceding the first diagnosis of idiopathic IMHA was 6 days (range, 1 – 131 days). One hundred and forty-four dogs had a history of weakness, 119 dogs were anorectic, in 66 dogs dark red urine was noticed, 44 dogs had vomited, 23 dogs had a period of diarrhoea, 16 dogs were dyspnoeic, and in 15 dogs signs of haemorrhagic diathesis were seen. In five dogs the history suggested the occurrence of syncopes. Physical examination disclosed pale mucous membranes in 146 dogs, 69 dogs had an increased rectal body temperature, 57 dogs had icterus, 51 dogs had cranial abdominal organomegaly, 16 dogs had generalized lymphadenopathy, and in 8 dogs petechiae were seen.

Laboratory tests

The laboratory examination results of the 149 dogs at the time of diagnosis are listed in Table 1. Six dogs had leucopenia, 23 had normal leucocyte counts, and 119 dogs had leucocytosis (missing data = 1). A left shift with > 0.3 X 10^9/l band neutrophils was seen in 117 dogs (missing data = 1).

Thirty-seven dogs had a platelet count < 50 X 10^9/l, 82 dogs had a platelet count < 150 X10^9/l, 9 dogs had a normal platelet count and 12 dogs had a platelet count > 400 X 10^9/l (missing data = 9). Spherocytes were seen in 77 dogs.

The PT and APTT were determined in 98 dogs. The PT was prolonged in 45 dogs and the APTT in 66 dogs, respectively. The fibrinogen concentration was determined in 96 dogs. Seventeen dogs had hypofibrinogenemia, 46 dogs had normofibrinogenemia, and 33 dogs had a fibrinogen concentration > 5 g/l. The Coombs' test was positive in 143 dogs. Fourteen dogs were positive for IgM, 27 dogs for IgG, 15 dogs for the combination of IgG and IgM, 4 dogs for IgM and complement, 27 dogs for IgG and complement, and 55 dogs for the combination of IgG, IgM and complement. The osmotic red cell fragility test was positive in

117 of the 142 dogs in which it was performed. Spherocytes were present in all 6 dogs in which the Coombs' test was negative or not performed.

Table 1. Laboratory results at the time of diagnosis in 149 dogs with IMHA.

	Median	Range	n	Reference
Haematocrit (%)	13	4 - 27	149	42 - 57
Corrected reticulocytes (%)	2.7	0.01 – 19	147	< 2
Leucocytes (x 10^9/l)	27.9	2.1 – 130	148	5.9 – 13.8
Band neutrophils (x 10^9/l)	1.4	0 - 22.1	148	0 – 0.3
Thrombocytes (x 10^9/l)	122	0 – 958	140	150 – 400
Urea (mmol/l)	7.6	2.9 - 69.5	123	3.0 – 12.5
Creatinine corrected for body weight (µmol/l)	42	0 – 652	112	< 50
PT (seconds)	8	6 – 12	98	7 ± 1
PT patient minus PT control (seconds)	0	0 - 5	98	
APTT (seconds)	16	11 – 98	98	14 ± 1
APTT patient minus APTT control (seconds)	3	0 - 83	98	
Fibrinogen (g/l)	3.9	0.6 - 13.8	96	2 – 5
Osmotic red cell fragility (mOsm/l)	238	120 – 317	139	< 162

PT, prothrombin time; APTT, activated partial thromboplastin time

Therapy

Blood transfusions were given to 98 dogs: only once in 78 dogs, twice in 18 dogs, 3 times in 1 dog and 4 times in another dog. The median haematocrit in dogs that received 1 blood transfusion was 12% (range, 4 – 21%). The median haematocrit in dogs that did not receive a blood transfusion was 18% (range, 10 – 27%).

All dogs were treated with prednisolone and azathioprine according to the protocol described above. Dogs that were not able to take oral medication were treated with dexamethasone IV or SC for 1 or 2 days before the protocol was started. The median duration of treatment in the whole dataset was 59 days (range, 0 – 622 days; n = 92) for prednisolone and 53 days (range, 0 – 622 days; n = 84) for azathioprine, respectively. The median duration of prednisolone therapy and azathioprine in dogs that survived for more than 14 days was 69 (range, 2 – 622; n = 96) and 83 (range, 2 – 622) days, respectively. Figure 1 shows duration of prednisolone therapy in dogs that survived IMHA.

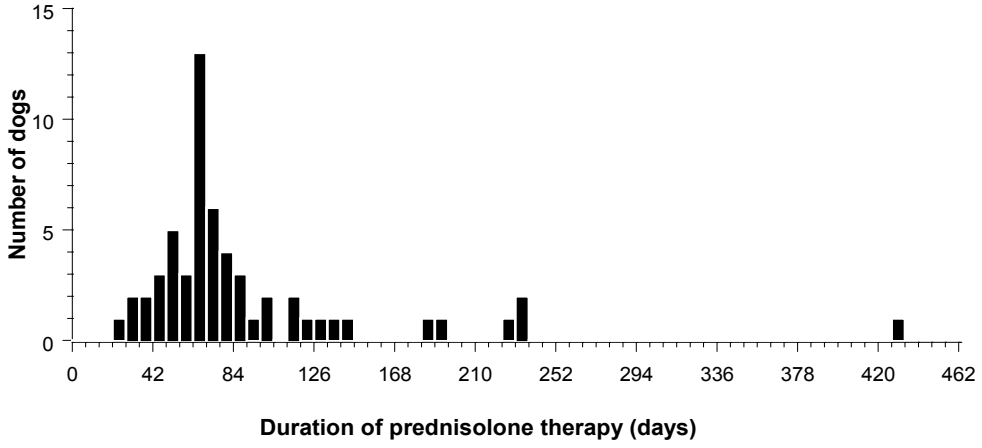

Figure 1. Duration of prednisolone therapy in the 96 dogs in this study that were treated according to a standard protocol of prednisolone and azathioprine and did not die because of idiopathic IMHA.

Treatment outcome

Details of response and occurrence of adverse effects are presented only for the dogs that were re-examined during return visits at the UUCCA and not for dogs for which follow-up was performed by the referring veterinarians. The first return visit after the diagnosis of IMHA occurred after a median duration of 25 days (range, 2 – 83 days) in 93 dogs and 66 of these dogs had a second return visit after a median duration of 77 days (range, 21 – 399 days). Results for haematocrit, reticulocyte percentage, platelet count, Coombs' test, as well as osmotic red cell fragility testing at the time of the first and second return visits are presented in Table 2. Therapy had no effect in 3 dogs, 87 dogs improved, and 5 dogs completely recovered by the first control visit. Of the 66 dogs that were examined for a second time 4 dogs had a relapse, 42 dogs were still considered improved, and an additional 20 dogs were completely recovered.

A relapse was diagnosed in 18 dogs after a median of 112 days (range, 32 – 1757 days). The median haematocrit at the time of the relapse was 28% (range, 6 - 44%). The median reticulocyte percentage was 6.1% (range, 0.1 – 22%). The median platelet count was 132 x 10^9/l (range, 0 – 382 x 10^9/l). The Coombs' test was positive in 10 of 14 dogs in which it was performed. The osmotic red cell fragility was increased in 11 of 12 dogs in which this test was performed.

The presence of adverse effects was recorded during the whole period that treatment with prednisolone and azathioprine was continued. Vomiting was absent in 75 dogs, present for < 2 days in 5 dogs, and present for > 2 days in 7 dogs. Diarrhoea was absent in 72 dogs, present for < 2 days in 5 dogs, and present for > 2 days in 9 dogs. Twelve dogs had leucopenia (< 5 x 10^9/l) and 12 dogs had thrombocytopenia (< 100 x 10^9/l).

Survival data are presented for all 149 dogs in the study. The last date of contact with the owner occurred after a median of 46 days (range, 0 – 2026 days). The estimated survival rate for the first 14 days of the study was 78.5% (95% CI: 71.9 – 85.6%). At this time, 30 deaths due to IMHA had occurred and 23 dogs were censored because they were lost for follow-up. The estimated half-year survival for the whole group was 72.6% (95% CI: 64.9 – 81.3%) (Fig 2). The estimated half-year survival rate for the 96 dogs that survived the first 14 days was 92.5% (95% CI: 86 – 99.3%).

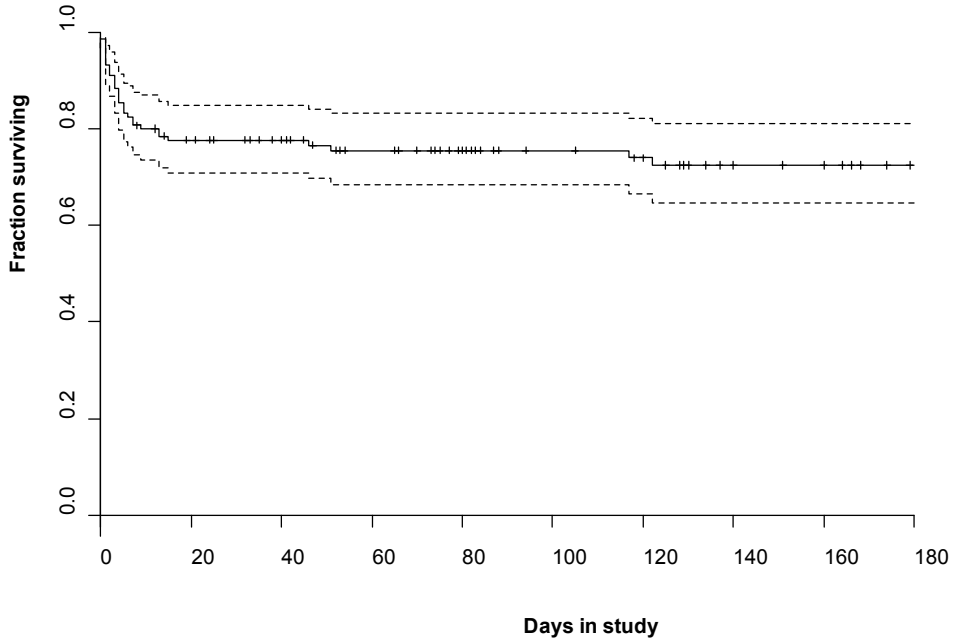

Figure 2. Estimated Kaplan-Meier half-year survival times for 149 dogs with idiopathic IMHA treated according to a standard protocol of prednisolone and azathioprine.

Table 2. Laboratory results in 93 dogs with IMHA which were re-examined during treatment.

	First control visit[1]			Second control visit[1]		
	Median	**Range**	**n**	**Median**	**Range**	**n**
Haematocrit (%)	35	6 - 50	93	40	11 - 54	66
Reticulocytes (%)	0.9	0.1 - 28	87	0.8	0.1 - 12	60
Thrombocytes (x 10^9/l)	402	8 - 986	88	278	3 - 834	63
		# Positive	**n**		**# Positive**	**n**
Coombs' test		16	85		7	60
Osmotic red cell fragility		62	82		30	58

1. First control visit took place after a median of 25 days (range 2 – 83 days) and the second control visit after a median of 77 days (range 21 – 399 days) after diagnosis.

Data analysis

The age at the time of diagnosis and the time that clinical signs started were significantly (P = 0.0168 and P = 0.0174, respectively) lower in the group surviving > 14 days (median, 5.5 years: range, 19 weeks – 12 years) than in the group surviving ≤ 14 days (median, 7 years; range, 14 weeks – 13 years). Plasma urea concentration was significantly (P = 0.0229) lower in the group surviving > 14 days (median 7.1 mmol/l, range 2.9 – 61 mmol/l) than in the group surviving ≤ 14 days (median, 8.9 mmol/l: range, 3.8 – 70.0 mmol/l). Increased osmotic red cell fragility was noted significantly (P = 0.0126) more often in the group that survived > 14 days (79/89) than in the group that survived < 14 days (38/53).

Compliance with the proportional hazard assumption was valid for all variables that had a p < 0.15 in the univariate analysis. Results for the univariate analysis of the whole dataset are shown in Table 3. Receiving a blood transfusion and the number of transfusions received both were significant negative predictors for survival in the univariate analysis. Duration of treatment did not significantly affect survival (Hazard Ratio (HR) 0.000247, 95% CI 0.995 – 1.01; P = 0.92). As blood transfusions and duration of treatment are not prognostic criteria at the time of diagnosis they were not put into the multivariate model.

Multivariate analysis of the entire data set indicated that urea (HR 2.85, 95% CI 1.69 – 4.80), bands (HR 1.11, 95% CI 1.02 – 1.20), petechiae (HR 4.01, 95% CI 1.19 – 13.54), and platelet count (HR 0.71, 95% CI: 0.56 – 0.91), were positive predictors of death. For the dogs surviving ≤ 14 days the presence of icterus (HR 4.61, 95% CI 1.53 – 13.89), petechiae (HR 11.31, 95% CI 2.56 – 49.99), and urea (HR 2.51, 95% CI 1.51 – 4.17) were all positive predictors of death. For dogs surviving > 14 days, an increase in leucocyte count (HR 0.37, 95% CI: 0.18 – 0.76) was associated with decreased risk of death and the presence of fever (HR 11.56, 95% CI 1.35 – 98.66) was associated with increased risk of death (Table 4).

Table 3. Univariate Cox proportional hazards results for risk of death in 149 dogs with idiopathic IMHA which were included in the multivariate analysis.

	HR	n[1]	95% CI	P
Fibrinogen (g/l)	0.77	96	0.65 – 0.93	0.00146*
Urea (20 mmol/l)	2.11	123	1.41 - 3.15	0.00285*
Creatinine corrected for body weight (50 μmol/l)	1.27	112	1.12 – 1.43	0.00466*
PT patient minus PT control (sec)	1.44	98	1.14 – 1.82	0.00559*
Thrombocytes (50 x 10^9/l)	0.82	140	0.69 – 0.97	0.00671*
Osmotic red cell fragility (mOsm/l)	0.37	142	0.18 – 0.75	0.0107*
APTT patient minus APTT control (sec)	1.03	98	1.01 – 1.06	0.0235*
Spherocytes	0.48	149	0.25 – 0.92	0.0247*
Icterus	2	149	1.07 – 3.77	0.0321*
IgM (titer of highest positive dilution)	1.05	148	1.0 – 1.11	0.0405*
Petechiae	2.51	149	0.89 – 7.13	0.124

1. Number of dogs in which the parameter was determined for which the Cox proportional hazards model calculated the HR.

CI, confidence interval; HR, hazard ratio; PT, prothrombin time; APTT, activated partial thromboplastin time; IgM, immunoglobulin M.

Table 4. Multivariate Cox proportional hazards results for risk of death in short and long-term survivor dogs with idiopathic IMHA.

	HR	95% CI	P
Entire dataset (n = 115, 34 observations deleted due to missing values)			
Urea (20 mmol/l)	2.85	1.69 – 4.80	0.0038
Thrombocytes (50 x 10^9/l)	0.71	0.56 – 0.91	0.0005
Bands (x 10^9/l)	1.11	1.02 – 1.20	0.0007
Petechiae	4.01	1.19 – 13.54	0.046
Dogs surviving < 14 days (n = 47, 6 observations deleted due to missing values)			
Urea (20 mmol/l)	2.51	1.51 – 4.17	0.0005
Icterus	4.61	1.53 – 13.89	0.0024
Petechiae	11.31	2.56 – 49.99	0.0027
Dogs surviving > 14 days (n = 96)			
Fever	11.56	1.35 – 98.66	0.0042
Leukocytes (10 x 10^9/l)	0.37	0.18 – 0.76	0.0018

CI, confidence interval; HR, hazard ratio

Discussion

In this study, dogs with idiopathic IMHA that had been treated according to a protocol of prednisolone and azathioprine were investigated with the objective to determine the treatment outcome and to identify prognostic variables in order to define areas of future research. It is difficult to compare study outcomes of canine IMHA with respect to survival, outcome and efficacy of therapy, because study populations are small, composition of study groups differs among clinics, and usually more than one treatment protocol is used within a study (6, 7, 9, 10, 12, 13). We present here a large cohort of dogs with IMHA that were uniformly treated. This provides statistical power to the analysis as well as the opportunity to report on the duration of immunosuppressive therapy that was necessary to maintain clinical remission of IMHA.

The estimated Kaplan-Meier half-year survival time for all 149 dogs in our study was 72.5%. Judging from the accompanying 95% confidence interval (65 – 81%) this survival time is comparable to those reported by others (9). Most reports only describe the immunosuppressive protocol that was used in the first weeks of therapy. Clinical studies that report on effectiveness of long-term treatment are lacking (14). Our protocol incorporates guidelines to adjust the therapy to clinical outcome. As a result, duration of therapy differed among dogs (Figure 1), but did not significantly influence survival in the dogs that survived the first 14 days. The estimated half year survival time in this subgroup of the dataset was 92.5% (95% CI: 86 – 99.3%) and 87 of 95 dogs that were examined at the first control visit 25 days after the diagnosis were categorized as *improved* (Table 2). As can be seen in Figure 1, most dogs were treated for 3 months and only in a small number of dogs, was prednisolone therapy necessary > 6 months. Because adverse effects of prednisolone occurred in virtually every dog, only adverse effects of azathioprine were monitored. The thrombocytopenia that was seen in 12 dogs during treatment could have been a result of idiopathic IMHA but was more likely due to bone marrow toxicity of azathioprine therapy as concurrent leucopenia also was present (2, 15). We can conclude from these findings that the described combination protocol of prednisolone and azathioprine was effective with regard to survival and clinical outcome, and duration of therapy of approximately 3 months was sufficient in successfully treated dogs.

Multivariate analysis (Table 4) indicated an increase in urea concentration, an increase in bands, a decrease in thrombocytes and the presence of petechiae at the time of diagnosis as the major individual negative prognostic indicators. With the exception of an increase in plasma urea concentration and petechiae that to our knowledge have not been reported before these variables overlap with prognostic factors found by others (8, 9). To appreciate the effect of an individual variable on survival, the HR's have been given for clinically relevant changes (Table 4). The presence of more than one of these prognostic variables increases the

death risk by the product of their respective HR's. For example, the presence of petechiae in a patient increases the death risk to die due to IMHA by a factor 4.011, but when present in combination with a increase of plasma urea concentration of 20 mmol/l and a decrease in thrombocytes of 50 x 10^9/l it confers an estimated death risk that is 16 times higher as in a patient that has no increase in plasma urea concentration, no petechiae and normal thrombocyte count.

The mortality associated with idiopathic IMHA ranges between 29 and 70% (3, 7, 8, 10, 13). As has been reported by others, in this study a large part of the overall mortality due to IMHA was concentrated in the first 2 weeks after diagnosis (7, 10, 12). The fact that the proportional hazard assumption was valid for all variables that were introduced into the multivariate analysis does not exclude the possibility that the influence of these variables on the outcome of treatment is different over time. For this reason, the data set was split into a group surviving ≤ 14 days and dogs that survived > 14 days. The initial steep descent of the Kaplan-Meier curve in the first 14 days in this and in other studies (3, 6, 16) suggested this time span as the logical cut-off.

The multivariate model that best predicted death in the dogs that survived < 14 days had an increased plasma urea concentration and the presence of icterus and petechiae in it as main determinants (Table 4). Petechiae has the highest HR, both in the whole data set, and in the group that survived < 14 days. Petechiae can be the result of immune-mediated destruction of platelets which in combination with IMHA has been associated with a poorer outcome than immune-mediated thrombocytopenia or IMHA alone (4). The fact that both thrombocytopenia and petechiae were major independent variables in the multivariate analysis suggests that they are the result of independent pathophysiologic pathways. Severe thrombocytopenia (8, 17) and hyperbilirubinemia (8) have been shown to be risk factors for the presence of thromboembolism in dogs with idiopathic IMHA. Abnormalities in PT and APTT and other coagulation parameters may be indicative of diffuse intravascular coagulation (DIC) or result from thromboembolism and are found often in dogs with idiopathic IMHA (6, 8, 17-19). Post mortem examination of dogs with IMHA revealed pulmonary thromboembolism in 10 – 80% of cases as well as thromboembolisms in other organs (17, 20). Evidence for the presence of prothrombotic tendencies also has been reported in prospective studies that show the presence of haemostatic abnormalities suggestive of DIC (19, 21) and activation of platelets in dogs with IMHA (22). For these reasons, we hypothesize that prothrombotic tendencies in IMHA play an important role in the development of both petechiae and thrombocytopenia.

Icterus in IMHA can be explained by increased bilirubin production and a moderate decrease in hepatic bilirubin clearance most likely due to impaired liver function resulting from centrolobular necrosis due to hypoxia (23). Increased plasma urea concentration in IMHA most likely results from hypoxia-induced renal injury, concurrent thromboembolic

renal disease, and pre-renal factors (24). The severity of anaemia has been suggested as a negative prognostic indicator (7), however, it was not identified in other studies (6, 9, 25) and also was not found in the univariate or multivariate analysis in this study. Therefore, we hypothesize that activation of coagulation during IMHA is an important pathway that contributes to the development of an increased plasma urea concentration and icterus. Future studies should focus on examining the pathogenesis of thrombotic tendencies in dogs with IMHA. This approach will help to develop diagnostic markers as well as more effective treatment protocols.

Leucocytosis, including a left shift, is a common laboratory finding associated with IMHA (3, 6, 8, 10) (Table 1). The presence of leucocytosis with a left shift has been associated with, increasingly severe, histopathological lesions (25). One study reported leucocytosis as a negative prognostic indicator, without having evaluated the contribution of a left shift (10). In our study, a left shift predicted death in the entire data set (Table 4). However, once dogs survived the first 14 days, an increase in leucocytes (Table 4) at the time of diagnosis was associated with a decreased death risk. From this observation, it can be concluded that the left shift most likely is associated with increased risk of death rather than the magnitude of the leucocyte response. In an inflammatory response, extravasation of leucocytes is followed in time firstly by increased release and then increased production of leucocytes by the bone marrow (26). The left shift that precedes a leucocytosis reflects both the magnitude and acuteness of onset of the inflammatory response (26).

Fever at the time of diagnosis increased the risk of death in long-term survivors (Table 4). Cytokines such as tumor necrosis factor alpha, interleukin-1 and interleukin 6 released by phagocytes act as endogenous pyrogens and mobilize neutrophils that results in the left shift and leucocytosis, as explained above (26). Fever and leucocytes are the major indepent variables in the multivariate model that describes survival in dogs that survived the first 14 days and are not part of the univariate and multivariate models that describe the whole dataset containing plasma urea concentration, thrombocytes, left shift and petechiae, nor of model for the subset of dogs that survived < 14 days that contains plasma urea concentration, icterus and petechiae. This suggests that the influence of thrombotic tendencies on survival mainly affects the first 2 weeks and the consequences of the inflammatory response still exert their influence if dogs survive these 2 weeks.

The presence of spherocytes was associated with increased survival both in this study and in an independent data set of 143 dogs with idiopathic IMHA (unpublished data, Slappendel & Teske). A positive IgM titer was associated with a negative effect on survival in this study (Table 3). Haemolysis in IMHA results from either IgM-mediated intravascular complement activation or IgG-mediated erythrophagocytosis that leads to spherocyte formation and decreased osmotic red cell fragility (1). The opposite survival effect of having spherocytes versus an IgM titer might be explained by the following factors. Firstly, IgM-mediated

haemolysis has been reported to be more severe than IgG-mediated haemolysis (1). Secondly, glucocorticoid therapy is more effective in reducing the clearance of IgG-coated cells (1). Because spherocytes are mainly the result of partial phagocytosis of IgG-sensitized red cells, both the fact that haemolysis is less severe and the therapy protocol more effective might explain the more favourable outcome in these dogs.

From this retrospective cohort study, it can be concluded that the main predictors of mortality in dogs with idiopathic IMHA are the presence of increased plasma urea concentration, bands, thrombocytopenia and petechiae at the time of diagnosis. The estimated Kaplan Meier half-year survival was 72.6% (95% CI: 64.9 – 81.3%). Mortality occurred mostly within the first 2 weeks. Cox proportional hazards' analysis revealed increased plasma urea concentration, icterus and petechiae as major independent predictors of mortality in the first 2 weeks. In most dogs, a 3-month protocol of azathioprine with prednisolone maintained clinical remission. The estimated half-year survival for dogs that survived the first 2 weeks was 92.5% (95% CI: 86 – 99.3%). Future research should be directed at determining if thrombotic tendency in dogs with IMHA is the main contributor to the development of increased plasma urea concentration, icterus, thrombocytopenia and petechiae.

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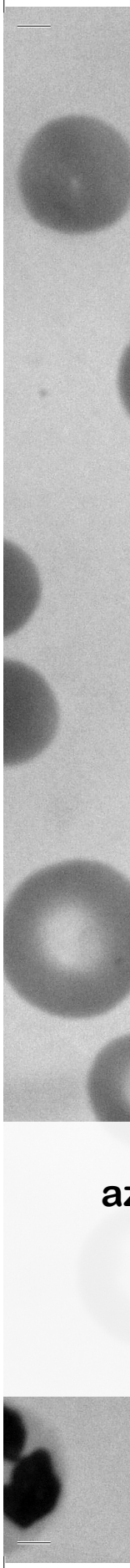

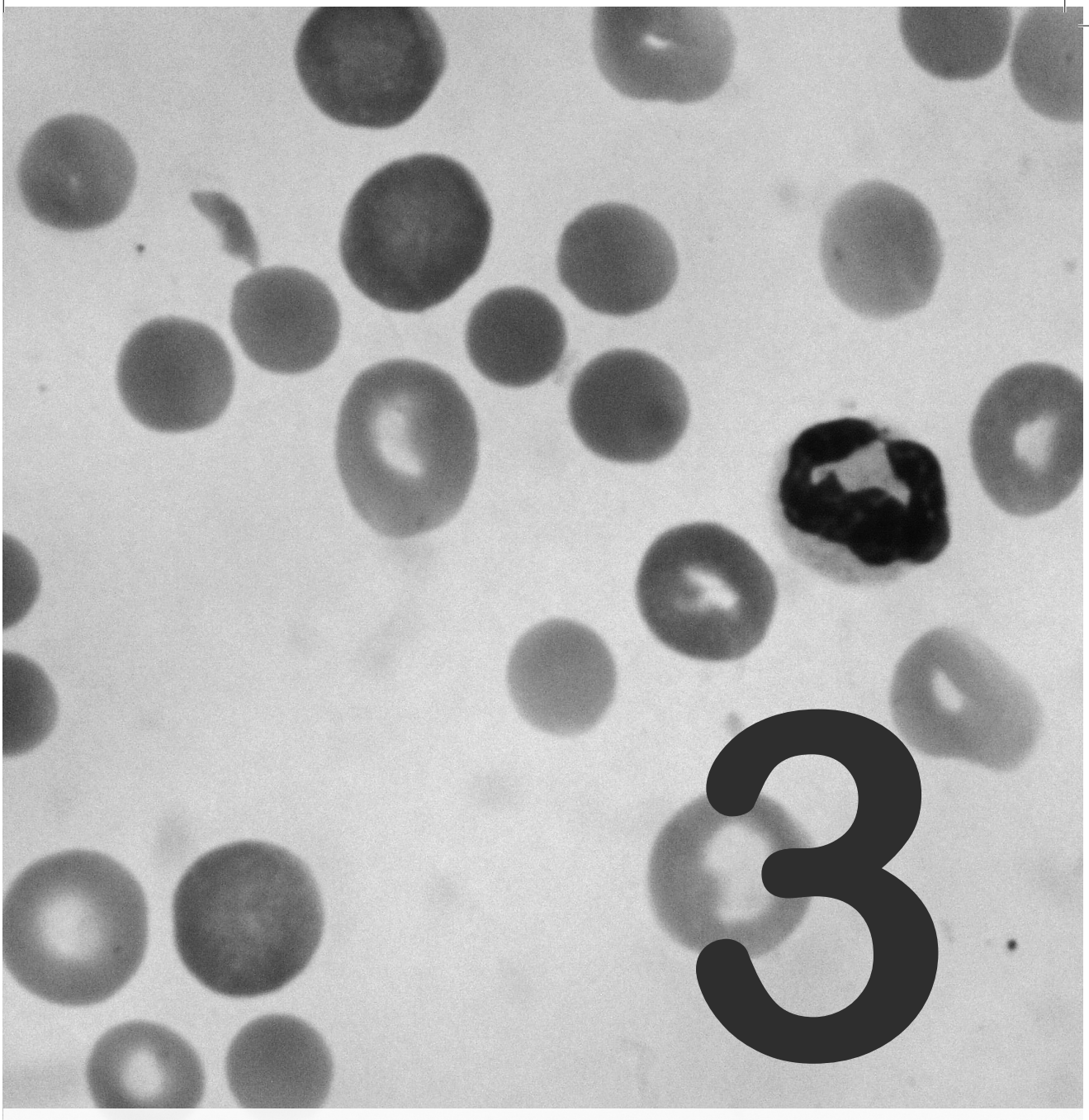

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Lack of evidence of a beneficial effect of azathioprine in dogs treated with prednisolone for idiopathic immune-mediated haemolytic anaemia: a retrospective cohort study.

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Abstract

Background - Azathioprine is used as an immunosuppressant in canine immune-mediated haemolytic anaemia (IMHA), but this potentially toxic and carcinogenic drug has not been proven to be beneficial.

Objectives -The aim of this study was to determine the difference in outcome and survival of dogs with idiopathic IMHA treated with a protocol that included azathioprine and prednisolone versus a protocol that included prednisolone alone.

Methods - The study included 222 dogs with a haematocrit lower than 0.30 L/L and either a positive Coombs' test or spherocytosis and no evidence of diseases that could trigger IMHA. The clinical and laboratory data at the time of diagnosis and the response to therapy and survival were compared in dogs treated according to the prednisolone and azathioprine protocol (AP protocol; n = 149) and dogs treated according to the prednisolone protocol (P protocol; n = 73).

Results - At study entry, the two groups were comparable, except that thrombocyte counts were significantly lower and clinical signs had been present significantly longer in the AP protocol group. No significant difference in survival was found between the two groups: the 1-year survival was 64% (95% CI 54 – 77%) in the P protocol group and 69% (95% CI 59–80%) in the AP protocol group, respectively.

Conclusions - Azathioprine would appear not to be beneficial as standard treatment for all cases of IMHA; however, a blinded, randomized clinical trial is needed to establish whether outcome is different with the two treatment protocols.

Background

Glucocorticoids are the main component of the immunosuppressive treatment of canine idiopathic immune-mediated haemolytic anaemia (IMHA) (1), but cytotoxic drugs such as azathioprine are advised for severe disease, such as cases with intravascular haemolysis or autoagglutination, and if the disease is refractory to glucocorticoids alone (1-3). A few studies have evaluated the effect of azathioprine in canine IMHA and reported conflicting results, ranging from a possible beneficial effect to no effect (4-7). Cytotoxic drugs can have potentially severe side effects, such as bone marrow suppression (8) and gastrointestinal disturbances, and long-term adverse effects of cytotoxic drugs in humans and animals include neoplasms, leukaemia, and testicular and ovarian dysfunction (9). Similar problems have been reported in people working with these agents (9). Azathioprine is recognized by the International Agency for Research on Cancer as being a possible or probable cancer-causing agent (9).

We previously reported on the treatment of dogs with idiopathic IMHA with an immunosuppressive protocol consisting of azathioprine and prednisolone, at that time the standard treatment in our clinic (10). The lack of evidence of a beneficial effect of azathioprine in canine idiopathic IMHA combined with concerns about the safety of the drug with regard to animal owners, animal caretakers, and veterinarians prompted the question whether the use of azathioprine is justified in dogs with idiopathic IMHA. The standard treatment protocol was revised from one including combined therapy with azathioprine and prednisolone to one including prednisolone monotherapy. After several years, we now have sufficient documented cases to compare the efficacy of the two protocols. The aim of this study was to determine whether there are differences in the outcome and survival of dogs with idiopathic IMHA treated according to a protocol including azathioprine and prednisolone (AP) versus a protocol including prednisolone alone (P).

Methods

Patients

The dogs had been referred to the Utrecht University Clinic of Companion Animals (UUCCA) from 1st January 1994 to 31st December 2005. Inclusion criteria were a haematocrit <0.30 L/L and either a positive Coombs' test or the presence of spherocytes in a blood smear. All dogs had been treated according to either a standard immunosuppressive protocol consisting of azathioprine and prednisolone (1st January 1994 until 31st December 2000) or a protocol consisting of prednisolone alone (1st January 2002 until 31st December 2005). A complete medical record had to be present.

Dogs were excluded if they had evidence of diseases that could induce IMHA, such as neoplasia, medications, and infectious diseases. As a result, dogs that had visited areas where ehrlichiosis and babesiosis are endemic within 3 weeks of the diagnosis of idiopathic IMHA were excluded unless serologic examination for *Ehrlichia canis* and *Babesia canis* or *B. gibsonii* was negative. Dogs that had received immunosuppressive treatment for longer than 14 days before referral to the UUCCA were excluded. Dogs that were referred between 31st December 2000 and 1st January 2002 were excluded to avoid possible selection bias, since in that period both treatment protocols were used.

Breed, sex, complete history, and physical examination were recorded, including age at time of first diagnosis of idiopathic IMHA, anorexia, vomiting, diarrhea and dark red urine, as well as the presence of an increased rectal temperature, pale mucous membranes, icterus, and petechiae. Additional diagnostics were performed if judged necessary by the attending clinician.

Laboratory tests

All tests at the time of diagnosis were performed at the UUCCA. The outcomes of the following laboratory tests performed on admission were retrieved from the medical records: complete blood count (CBC), reticulocyte count, presence of spherocytes (confirmed by one of the authors, CP or ET), Coombs' test, osmotic red cell fragility, and plasma urea and creatinine concentrations. Data that were available for a subset of the patients only were not recorded. During return visits, approximately 4 and 10 weeks after start of treatment, CBC, reticulocyte count, Coombs' test and osmotic red cell fragility were determined. A monovalent direct Coombs' antiglobulin test 11 was performed using anti-dog IgG (Central Blood Laboratory, Nordic), anti-dog IgM antibodies (Nordic) and, before January 2005, an anti-dog complement antibody (Nordic), for agglutination of the patients' red cells; results were reported as negative, weakly positive, or positive. The osmotic fragility of erythrocytes was determined as previously described (11). Plasma creatinine concentrations were corrected for body weight (12).

The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration were recorded when measured (Option 4, BioMerieux). PT and APTT were considered prolonged if they were increased at least 10% above levels measured in normal pooled canine citrated plasma. From March 2003 onward, coagulation profiles were determined with the Thrombolyser Compact-X (Trinity Biotech PLC) and new reference values were established for this system. From March 2003 onward, the haematology analyzer was changed from the combination of the ABX Helios and ABX Helios 5 Diff (ABX International) to the ADVIA 120 (Siemens Medical Solutions Diagnostics) and reference values were established for this system.

Therapy

Blood transfusions, alone or in combination with IV fluid therapy, were given if considered necessary by the attending clinician. A transfusion consisted of the equivalent of 450 mL of donor blood. In dogs weighing less than 10 kg, a lower transfusion volume was given, with a maximum of 40 mL/kg. Depending on availability, either packed red blood cells or fresh whole blood was used. The number of blood transfusions was noted. All clinic-owned donor dogs used in the study were DEA 1.1 and 1.2 negative. No cross-match was performed in the case of first blood transfusion. Only in the case of client-owned donor dogs or if a second transfusion was given, were crossmatches performed.

Two treatment protocols were used. A standard protocol consisting of a combination of prednisolone and azathioprine (AP protocol) was instituted in dogs that were treated before 31st December 2000. From 1st January 2002 onward, the standard protocol for dogs with idiopathic IMHA contained prednisolone only (P protocol). In both protocols prednisolone was given following the same schedule. The response category (see below) was assessed at least once daily during hospitalization and during return visits that were scheduled 4 and 10 weeks after the start of therapy. As long as the response category was no change, prednisolone (Alfasan) was given in a dosage of 2 mg/kg/day PO. Dogs that were not able to take oral medication were hospitalized and treated with dexamethasone (0.5–1 mg/kg/day) IV or SC. Once the response had improved, prednisolone therapy was started at a dosage of 2 mg/kg/day PO for 3 days, followed by 1.5 mg/kg/day PO for 7 days, 1 mg/kg/day PO for 10 days, 0.5 mg/kg/day PO for 14 days, after which the same dose was given on alternate days for 14 days and subsequently tapered down to 0.25 mg/kg/day PO for 21 days. If the outcome was assessed as complete recovery at the 4- or 10-week return visit, the prednisolone therapy protocol described above was followed. If relapse occurred at any time during this treatment, the prednisolone therapy protocol was started from the beginning. If at the 4 or 10 week return visit the treatment response was assessed as improved but there was no complete recovery, the duration of the intervals during which prednisolone was tapered, as described above, was doubled. Additionally, in the AP protocol, dogs were treated with azathioprine (Imuran, Glaxo–Wellcome) at a dosage of 2 mg/kg/day PO for dogs weighing <20 kg. The daily azathioprine dose in dogs of 25, 30, 40 and 50 kg was maximized at 45, 50, 60 and 70 mg, respectively. Azathioprine treatment was stopped 10 days after prednisolone treatment.

Outcome

The response to therapy was retrieved from the medical record. The response was assessed at the actual time of the first and second return visits. At both times the findings were compared to those of the last visit. We defined four response categories. Complete recovery was defined as an increase in the haematocrit to >0.36 L/L, a negative Coombs' test, and

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

an osmotic red cell fragility within the reference range. Improvement was defined as a modest increase in the haematocrit or an increase to >0.36 L/L but with a positive Coombs' test or an increased osmotic red cell fragility. No response was defined as no increase in the haematocrit. And relapse was defined as a decrease in the haematocrit after an initial improvement or complete recovery in combination with the recurrence of a positive Coombs' test or increased red cell fragility. The time at which a relapse occurred was retrieved from the medical record.

Survival was determined by telephone contact with the owner by one of the investigators (GJ for the AP protocol group and ES for the P protocol group). If we were unsuccessful in contacting the owner, the last date of contact was recorded from the file. The outcome at the last date of contact was divided into three categories: death due to IMHA, death due to another cause, and alive with or without treatment.

Statistical analysis

Statistical analysis of the data was performed using S-plus statistical package (Insightful Corporation). Comparisons between the two protocol groups were made assuming no difference between the treatment groups. Non-parametric tests were used when the data were not normally distributed (Chi-square or Fisher's exact test for categorical variables and Two-sample Wilcoxon rank-sum test for continuous variables).

As described above, minor changes were made in the laboratory procedures for PT, APTT, fibrinogen, and thrombocytes during the period that dogs were entered in the P protocol group. The results for these variables in the P protocol group were split into two groups, before and after the change, and handled as individual groups during the statistical analysis. Comparisons between the AP protocol group and both subgroups of the P protocol groups were made using the Kruskall-Wallis χ^2 test. In case of multiple testing, Bonferroni correction was performed.

Survival analysis was performed for the AP protocol group and the P protocol group. The end point was death due to IMHA. Dogs that were alive at the end of the study or had died of other causes were censored. Survival curves were drawn with the Kaplan Meier method. The treatment protocol used and the variables recorded at the date of first diagnosis, with the exception of the variable breed, were evaluated in a univariate Cox proportional hazard model and used to generate hazard ratios (HRs). The variable treatment protocol and the variables significant at the $P<0.20$ level in the univariate analysis were introduced in a multivariate model, allowing for interaction between variables. Multivariate analysis was performed by forward stepwise selection using a probability of $P<0.05$ in the likelihood ratio test as a criterion for inclusion. Compliance with the proportional hazards assumption was tested graphically by plotting the Schoenfeld residuals against time.

Results

Clinical characteristics of dogs

Of 108 dogs eligible for the P protocol, 32 met exclusion criteria and 3 further dogs were excluded because they switched from the P protocol to the AP protocol, leaving 73 dogs in the P protocol group. Of the 32 dogs excluded, 6 had been treated with glucocorticoids for more than 14 days, 5 had been diagnosed with babesiosis and /or ehrlichiosis (n=2) or had visited an endemic area (n=3), 8 had concurrent inflammatory disease (pneumonia n=2, mesenteric lymphadenitis n=1, gastroenteritis n=3, dermatitis n=1, necrotizing inflammation tail n=1), 9 had neoplasia (carcinoma n=3, haematopoietic tumours n=5, haemangiosarcoma n=1), and 3 had a concurrent immune-mediated disease (hypothyroidism n=1, SLE n=1, allergic dermatitis n=1). The owner of 1 dog decided not to start treatment.

Of 197 dogs eligible for the AP protocol, 48 met exclusion criteria, leaving 149 dogs for inclusion in the AP protocol group, as described earlier (10). Of the 48 dogs excluded, 10 dogs had been diagnosed with babesiosis and /or ehrlichiosis (n=3) or had visited an endemic area (n=7), 6 had concurrent lung disease, 4 had neoplasia (spleen n=2, mediastinum n=1, heart n=1), 17 had haematopoietic tumors (myeloid leukemia n=2, malignant lymphoma n=8, malignant histiocytosis n=5, haemangiosarcoma n=2), 2 had SLE, and 1 had renal disease. Three dogs were treated with medications that can trigger IMHA, the owner of 1 dog chose not to start treatment, and data were incomplete for 4 dogs.

To exclude concomitant disease, the 73 dogs in the P protocol group underwent additional investigations, namely, thoracic radiography (n=33), abdominal ultrasound (n=52), cytological investigations (spleen n=22, liver n=31, lymph nodes n=13, bone marrow n=31, skin nodules n=4), pathological examination (liver n=2, spleen n=1, intestinal biopsies n=2), gastroduodenoscopy (n=2), laparotomy (n=1), electrocardiography (n=1), and bacteriological investigations (n=2). For the same reason, the 149 dogs included in the AP protocol group also underwent additional investigations, namely, thoracic radiography (n=19), abdominal ultrasound (n=69), cytological examination (spleen n=9, liver n= 3, lymph nodes n=4, bone marrow n=17, skin nodules n=1), pathological examination (liver n=4, intestinal biopsies n=2), gastroduodenoscopy (n=2) dogs, explorative laparotomy (n=1), electrocardiography (n=2). Cytology of the spleen showed that the dogs in both groups had extramedullary erythropoiesis in the spleen and liver, and that most dogs had steroid-induced hepatopathy. There was no evidence of concomitant disease in any of the dogs.

The characteristics of the AP protocol group have been described earlier (10). Of the 73 dogs that were included in the P protocol group, 12 dogs were crossbreeds, 5 were Maltese terriers, 4 were Jack Russell terriers, 4 were Labrador retrievers, 4 were Flat coated retrievers, 3 were English Springer spaniels, 3 were Dachshunds, 3 were Cairn terriers, 2 were Shetland sheepdogs, and 2 were Appenzeller Sennen dogs, respectively; the remaining 31 dogs were

single dogs of other breeds. Twenty-seven of the 73 dogs were males (22 intact, 5 castrated) and 46 were females (24 intact, 22 castrated). In the AP protocol group, 61 were male (46 intact, 15 castrated) and 88 were female (51 intact, 37 castrated). There was no significant difference in the number of male and female dogs in the two groups (P=0.77), or in the number of intact and neutered male and female dogs (P=0.67). Median body weight at the time of diagnosis was 15.8 kg (range 2.5–45 kg) in the P protocol group and 18.7 kg (range 2.5–48.5 kg) in the AP protocol group (P=0.44).

The median age at diagnosis of IMHA was 4.6 years (range 0.4 - 12.7 years) in the P protocol group and 5.7 years (range 0.3–13.9 years) in the AP protocol group; this difference was not statistically significant (P=0.09). The median duration of clinical signs prior to diagnosis of idiopathic IMHA was 3 days (range 0–141 days) in the P protocol group and 6 (range 0–131 days) in the AP protocol group; this difference was significantly significant (P=0.015). Anaemia and clinical signs consistent with a tentative diagnosis of IMHA were documented by the referring veterinarian in 4 of 222 dogs 127, 128, 131, and 141 days before the diagnosis of idiopathic IMHA in our clinic.

There was no significant difference in the occurrence of anorexia, vomiting, diarrhoea, red urine, dyspnoea, fever, pale mucous membranes, icterus, or petechiae between the treatment groups. Information from the history and the physical examination, and the results of laboratory investigations at the time of diagnosis of IMHA are presented in Table 1. Thrombocyte counts were significantly lower in the AP protocol group than in the P protocol group at the time of diagnosis (P=0.00001), but none of the other laboratory variables were significantly different at the time of diagnosis. The laboratory procedures for determining PT, APTT, fibrinogen, and thrombocytes changed during the study, but this led to significant differences in the results for PT and fibrinogen only. The mean PT before and after the change was 7.9 (n=111) and 7.4 (n=21) seconds (median 8 and 6.9 seconds), respectively, and the mean fibrinogen concentration before and after the change was 4.6 (n=109) and 7.7 (n=27) mg/L (median 4 and 7.5 mg/L), respectively. Only the PT and fibrinogen data for P protocol patients that entered the study before the change in laboratory procedures are included in Table 1 and were used in the univariate analysis. There were no significant differences in PT and fibrinogen levels between the two groups.

Therapy

Blood transfusions were given to 56 of 73 (76%) dogs in the P protocol group (once in 45 dogs, twice in 10 dogs, and three times in 1 dog) and in 98 of 149 (66%) dogs in the AP protocol group (once in 78 dogs, twice in 18 dogs, three times in 1 dog, and four times in 1 dog). The difference in transfusion requirement between the two groups was not statistically significant (P=0.61). The median duration of prednisolone therapy was 68 days (range 0–936; n=64) in the P protocol group and 59 days (range 0–622; n=92) in

the AP protocol group; the difference in treatment duration was not statistically significant (P=0.20). In the AP protocol group, the median duration of azathioprine therapy was 53 days (range 0–622; n=83).

Outcome

Dogs in the P protocol group made the first return visit to the clinic after a median of 17 days (range 0–141; n=43) and the second return visit after a median of 56 days (range 21–171; n=25). Dogs in the AP protocol group made the first return visit to the clinic after a median of 25 days (range 2–83; n=95) and the second return visit after a median of 77 days (range 21–399; n=69). The first and second return visits were significantly earlier in the P protocol group than in the AP protocol group (P=0.001 and 0.003, respectively, Table 2). Apart from a significantly higher reticulocyte count in the P protocol group at the first return visit (P=0.027), there were no significant differences in haematocrit, thrombocytes, and red cell osmotic fragility between the two groups at either visit (Table 2). Eight dogs in the P protocol group relapsed after a median of 63 days (range 7–581 days), as did 17 dogs in the AP protocol group after a median of 112 days (range 32–1757 days). There was no significant difference in the time to relapse (P=0.3) or in the number of relapses (P=0.9) between the two groups.

Table 1. Clinical signs and laboratory results for dogs in the AP protocol group and the P protocol group at time of diagnosis

	Azathioprine- Prednisolone Protocol				Prednisolone Protocol			
Clinical signs	**Present**	**Absent**	**n[a]**		**Present**	**Absent**	**n**	**P[b]**
Anorexia	119	30	149		55	17	72	0.55
Vomiting	44	105	149		27	46	73	0.26
Diarrhoea	23	126	149		17	56	73	0.15
Dyspnoea	16	133	149		4	69	73	0.20
Fever	69	80	149		33	38	72	0.98
Pale mucous membranes	146	3	149		72	1	73	0.74
Icterus	57	92	149		27	46	73	0.86
Petechiae	8	141	149		1	72	73	0.16
Red urine	47	102	149		17	55	72	0.22
Laboratory results	**Median**	**Range**	**n**	**Reference**	**Median**	**Range**	**n**	**P[b]**
Haematocrit (%)	13	4–27	149	42–57	12	5–26	73	0.36
Reticulocytes (%)	8	0.1–90	147	<2	5.8	0.1–51.5	64	0.29
Corrected reticulocytes (%)	2.7	0.01–19.2	147	<2	2	0.02–14.3	64	0.11
Osmotic red cell fragility (mOsm/l)	238	120–317	139	<162	250	131–317	28	0.28
Leucocytes (x 10^9/l)	27.9	2.1–130	148	5.9–13.8	21.7	5.7–78.6	68	0.30
Band neutrophils (x10^9/l)	1.4	0–22.1	148	0–0.3	1	0–21.9	67	0.08
Urea (mmol/l)	7.6	2.9–69.5	123	3.0–12.5	7.4	2.3–49.5	45	0.80
Creatinine (µmol/l)	41.8	0.4–652	112	<50	41.2	6.5–228.4	48	0.16
PT[c] (seconds)	8	6–12	98	7 ± 1	7	7–10	13	0.74
APTT[d] (seconds)	19.8	11–98	98	14 ± 1	17.4	9.1–64.8	37	0.98
Fibrinogen (g/l)	3.9	0.6–13.8	96	2–5	5	2.3–8.2	13	0.11
Thrombocytes (x 10^9/l)	122	0–958	140	150–400	230	9–1079	65	0.00001*
Duration of clinical signs (days)	6	0–141	149		3	0–131	73	0.015*

[a] Number of dogs in which the parameter was determined.
[b] *P* = *P* value
[c] PT = Prothrombin time
[d] APTT = Activated partial thromboplastin time

[d] APTT = Activated partial thromboplastin time

Table 2. Results for laboratory tests and response categories for dogs with idiopathic IMHA treated according to the AP protocol (n = 149) or the P protocol (n = 73) group at the time of the first and second return visits and relapse

	Azathioprine-Prednisolone Protocol				Prednisolone Protocol			
First return visit	**Median**	**Range**	**n**	**Reference**	**Median**	**Range**	**n**	**P[b]**
Haematocrit (%)	35	6 - 50	93	42–57	36	12–47	41	0.5
Thrombocytes (x 10^9/l)	402	8-986	88	150–400	421	20–1555	29	0.48
Reticulocytes (%)	0.9	0.1-28	87	<2	2	0.2–6.4	34	0.027*
Osmotic red cell fragility (mOsm/l)	176	136-258	82	<162	164	125–247	24	0.06
Time after diagnosis[g] (days)	25	2-83	95		17	0–141	43	0.001*
Response category[a]	**No. dogs**		**n**		**No. dogs**		**n**	**P**
No response[c]	3		95		1		26	
Improvement[d]	87		95		25		26	
Complete recovery[e]	5		95		0		26	0.92
Second return visit	**Median**	**Range**	**n**	**Reference**	**Median**	**Range**	**n**	**P**
Haematocrit (%)	40	11–54	66	42–57	42	18–51	24	0.38
Thrombocytes (x 10^9/l)	278	3–834	63	150–400	339	12–636	17	0.73
Reticulocytes (%)	0.8	0.1–12	60	<2	1	0.3–9.7	18	0.39
Osmotic red cell fragility (mOsm/l)	163	133–238	58	<162	165	142–206	11	0.40
Time after diagnosis[g] (days)	77	21–399	69		56	21–171	25	0.0032*
Response category[a]	**No. dogs**		**n**		**No. dogs**		**n**	**P**
No response	0		62		0		13	
Improvement	42		62		12		13	
Complete recovery	20		62		1		13	0.12
Relapse[f]	**Median**	**Range**	**n**	**Reference**	**Median**	**Range**	**n**	**P**
Haematocrit (%)	28	6–44	17	42–57	31	10–41	5	0.96
Thrombocytes (x 10^9/l)	132	0–382	15	150–400	327	228–426	2	0.15
Reticulocytes (%)	2.5	0.1–22	14	<2	0.3	0.2–30.5	3	0.9
Osmotic red cell fragility (mOsm/L)	212	159–274	12	<162	221	187–254	2	0.65
Time after diagnosis[g] (days)	112	32–1757	21		63	7–581	8	0.27

[a] Number of dogs counted in each response category.
[b] *P* = P value
[c] No effect of therapy was defined as no increase in the haematocrit.
[d] Improvement was defined as an increase in haematocrit to <0.36 L/L, or a haematocrit >0.36 l/l but with a positive Coombs' test or an increased osmotic red cell fragility.
[e] Complete recovery was defined as an increase in the haematocrit to >0.36 l/l, a negative Coombs' test, and an osmotic red cell fragility within the reference range.
[f] Relapse was defined as a decrease in the haematocrit after an initial improvement or complete recovery in combination with the recurrence of a positive Coombs' test or increased red cell fragility.
[g] The times of the first and second return visits or when a relapse occurred were retrieved from the medical record.

R39 R38 R37 R36 R35 R34 R33 R32 R31 R30 R29 R28 R27 R26 R25 R24 R23 R22 R21 R20 R19 R18 R17 R16 R15 R14 R13 R12 R11 R10 R9 R8 R7 R6 R5 R4 R3 R2 R1

Analysis of survival

The 1-year survival was 64 % (95% CI, 54–77%) in the P protocol group and 69% (95% CI, 59–80%) in the AP protocol group, respectively (Fig. 1); this difference in survival was not statistically significant (P=0.65). The difference in survival at 14 days, 6 months, and 1 year was 1.7 % (95% CI, minus 10 – 14%), 6.4% (95% CI, minus 7.4 – 20%), and 6.4% (minus 11 – 19.9%), respectively. At 1 year, 25 dogs had died of IMHA and 13 dogs were censored (1 had died of other causes, 9 were alive with treatment, and 3 were alive without treatment) in the P protocol group, and 36 dogs had died of IMHA and 106 dogs were censored (3 had died of other causes, 74 were alive with treatment, and 26 were alive without treatment) in the AP protocol group. Survival among dogs that lived longer than 14 days (n=151) was not significantly different between the two groups (P=0.649).

The proportional hazards assumption was valid for all variables that were significant in univariate and multivariate analyses. Results of the univariate analysis of the pooled data from the two groups with P <0.20 and the variable treatment (P=0.65) are presented in Table 3. The best multivariate model included plasma urea concentration (HR=2.56; 95% CI, 1.73–3.79; n=164) and icterus (HR=2.94; 95% CI, 1.60–5.42; n=164) as negative predictors of death, and spherocytes (HR=0.38; 95% CI, 0.2–0.72; n=164) as a positive predictor of death. The HRs were calculated for clinically relevant intervals.

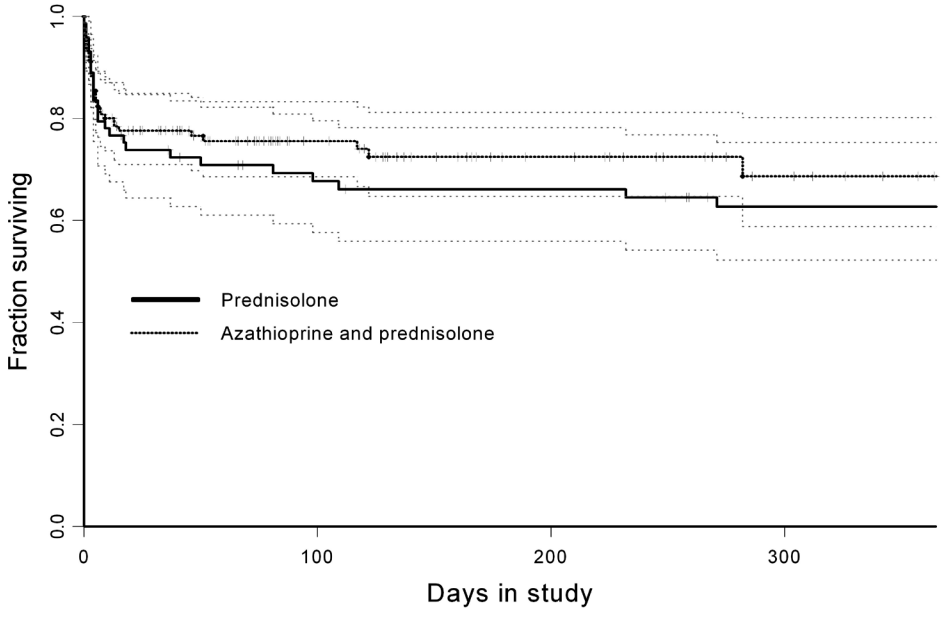

Figure 1. Kaplan-Meier survival curve for dogs with idiopathic IMHA treated according to the AP protocol (n = 149) or the P protocol (n = 73).

Table 3. Univariate and multivariate Cox proportional hazards results for risk of death in 222 dogs with idiopathic IMHA for variables determined at time of first diagnosis with P <0.20 and the variable "treatment protocol"

		Univariate analysis		
Variable[a]	**Hazard ratio**	**n[b]**	**95% CI**	**P[c]**
Icterus	2.47	222	1.52 - 4	0.0003*
Urea (20 mmol/l)	2.22	168	1.55 - 3.19	0.0004*
Creatinine [d] (50 µmol/l)	1.28	160	1.15 - 1.42	0.0012*
Red cell osmotic fragility	0.43	181	0.22 - 0.82	0.018*
Thrombocytes (50 x 10^9/l)	0.90	205	0.82 - 0.99	0.0184*
Age (years)	1.09	221	1.01 - 1.17	0.0266*
APTT[e] (seconds)	1.03	135	1 - 1.05	0.0445*
Spherocytes	0.66	215	0.40 - 1.11	0.115
Haematocrit (%)	0.0173	222	0.00011 - 2.77	0.109
Treatment protocol	1.12	222	0.68 - 1.86	0.65
		Multivariate analysis		
Urea (20 mmol/L)	2.56	164	1.73 - 3.79	0.0001*
Icterus	2.94	164	1.60 - 5.42	0.0005*
Spherocytes	0.38	164	0.2 - 0.72	0.0023*

[a] Variables were entered in the Cox proportional hazards model either as a factor or as a continuous variable, in which case the hazard ratio was calculated for the interval that is given in the table.
[b] Number of dogs is given in which the parameter was determined for which the Cox proportional hazards model calculated the hazard ratio.
[c] P = P value
[d] Plasma creatinine concentrations were corrected for body weight (12)
[e] APTT = Activated partial thromboplastin time

Table 4. Life table and estimated survival results for the AP protocol group (n=149) and P protocol group (n=73) at 14 days, 6 months, and 1 year after the date of diagnosis of idiopathic IMHA and at the time of the scheduled first visit (28 days) and second control visit (70 days)

Azathioprine-Prednisolone Protocol					
Time (days)	**# at risk[a]**	**Cumulative # events[b]**	**Cumulative # censored[c]**	**Estimated survival**	**95% CI Interval**
14	98	30	21	0.785	0.719 – 0.856
28	61	31	57	0.776	0.710 - 0.849
70	67	32	50	0.756	0.686 – 0.833
182	31	35	86	0.725	0.647 – 0.812
365	12	36	106	0.685	0.588 – 0.802
Prednisolone Protocol					
14	56	17	0	0.767	0.676 – 0.870
28	53	19	1	0.738	0.644 – 0.847
70	46	21	8	0.709	0.611 – 0.822
182	42	24	11	0.661	0.559 – 0.782
365	36	26	18	0.643	0.539 – 0.767

[a] Number of dogs alive.
[b] Cumulative number of dogs in each protocol group that died of idiopathic IMHA.
[c] Cumulative number of dogs in each protocol group that were censored.

Discussion

The aim of this study was to determine whether treatment according to a protocol including azathioprine and prednisolone (AP) compared with a protocol including prednisolone (P) alone leads to differences in outcome and survival in dogs with idiopathic IMHA. There were no significant differences between the treatment groups in the duration of immunosuppressive therapy, number of blood transfusions, survival (Fig. 1), or treatment response.

Thrombocyte counts at the time of diagnosis were significantly lower in the AP protocol group than in the P protocol group. Although a low thrombocyte count has a negative influence, mainly on short-term survival (10, 13), it is unlikely that the lower thrombocyte count in the AP protocol group masked a potential beneficial effect of azathioprine for a number of reasons. Firstly, the most likely explanation for the low thrombocyte count in dogs with IMHA is the decrease over time due to both immune-mediated destruction and thrombotic tendencies (13-16). The median duration of clinical signs prior to diagnosis of idiopathic IMHA was the longest in the AP protocol group, which might explain the lower

thrombocyte count in this group. Studies of IMHA show survival curves with similar slopes, with most deaths occurring in the first 2 weeks after diagnosis, despite differences in severity of clinical disease (4, 5, 17). This suggests that recovery from the acute IMHA crisis and the associated pathology, such as thrombosis and disseminated intravascular coagulation, takes about 2 weeks. Indeed, at the first return visit thrombocyte counts were no longer different between the two groups (Table 2). We have previously found that thrombocytopenia at the start of therapy decreases short-term survival but not long-term survival (10). On the basis of this, it is unlikely that the difference in thrombocyte count at the time of diagnosis had an effect on long-term outcome. Secondly, it is debatable whether azathioprine has a clinical effect within 2 weeks of therapy initiation in dogs. Although azathioprine decreased the lymphocyte blastogenic response in dogs after 7 days of treatment (18), it induced significant changes in immunoglobulin levels and lymphocyte numbers only after 2 weeks of treatment (19). Thirdly, the best multivariate Cox proportional hazards model (Table 3) contained urea plasma concentration, icterus, and spherocytes as significant predictors of death due to IMHA. Neither inclusion of the variable "treatment protocol" nor "thrombocytes" improved this model significantly. For these reasons, we conclude that the difference in thrombocyte count between the two protocol groups at the start of the study is not likely to have had an influence on long-term survival.

One of the drawbacks of using historical controls is that time-related differences in the study population or in the treatment received, other than the effect of azathioprine, might confound the effect of treatment. Because inclusion criteria were unchanged throughout the trial and based on the results of objective quantitative laboratory tests, it is unlikely that this led to unrecognized inclusion bias. To minimize exclusion bias based on time-related differences in the diagnostic work-up, at the time of the study each individual case was evaluated by one of the authors to ensure that causes of secondary IMHA had been appropriately excluded. Although the addition of azathioprine was the only identifiable difference in treatment between the two groups, it cannot be excluded that supportive care in the intensive care unit had improved during the trial. In our institute, contrary to what is advocated by others (7), it is not routine practice to use antithrombotics or anticoagulants that might otherwise have influenced outcome.

Although there were no treatment-related differences in treatment response and survival, there were some differences between the laboratory results at the time of first and second return visit. The reticulocyte count in the P protocol group was significantly higher, which, although modest, might indicate that the red cell regeneration response was still active, because the first return visit in the P protocol group was 6 days earlier than that of the AP protocol group. Alternatively, it might reflect azathioprine-induced bone marrow suppression in the AP protocol group (8). Azathioprine related side effects were noted in 12 of 149 (8.1%) dogs in the AP protocol group (10). This seems a less likely explanation,

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

however, since haematocrit, leucocyte, and thrombocyte counts at the time of first return visit were not significantly different between the two treatment groups. Eighteen dogs in our study developed idiopathic IMHA before the 1 year of age, in contrast with previous reports describing an onset only after the first year of age (3-5, 17, 20). The clinical and laboratory findings and survival of these 18 dogs were not significantly different from those of the other 204 dogs in this study (data not shown).

Azathioprine is listed as a human carcinogen (9), and for this reason its use should be restricted in veterinary medicine to indications for which an evidence-based effect has been demonstrated, or to studies seeking to prove its beneficial effect (9). While there was no significant difference in 1-year survival between the two groups, the confidence interval included both a 20% superior survival and a 11% lower survival. Given the limitations that are inherent to a retrospective study, this potential difference in outcome might be regarded as clinically significant. A randomized placebo-controlled study is necessary to estimate the true effect size of the AP protocol for the treatment of canine IMHA. The estimated effect size in this study can be used for sample size calculations (21). However, given the side effects in dogs and the carcinogenicity in humans, we feel that the findings of this study do not justify the use of azathioprine in each IMHA patient. Given that at least 95% of the dogs in both protocol groups were classified as improved or completely recovered at the first control visit a median of 25 days after the start of therapy (Table 2), we suggest that the addition of azathioprine to the prednisolone protocol should be considered if there is no, or an inadequate, response to prednisolone after 2–3 weeks of treatment, provided the guidelines for adjustment of the prednisolone treatment have been followed.

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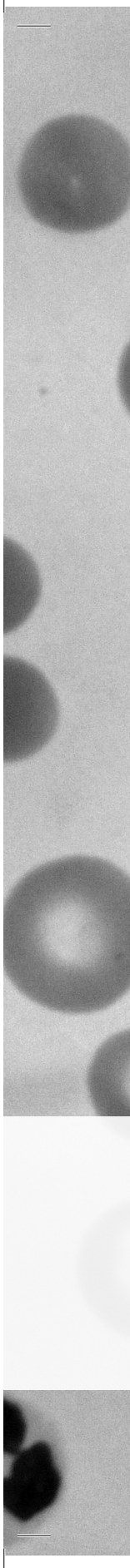

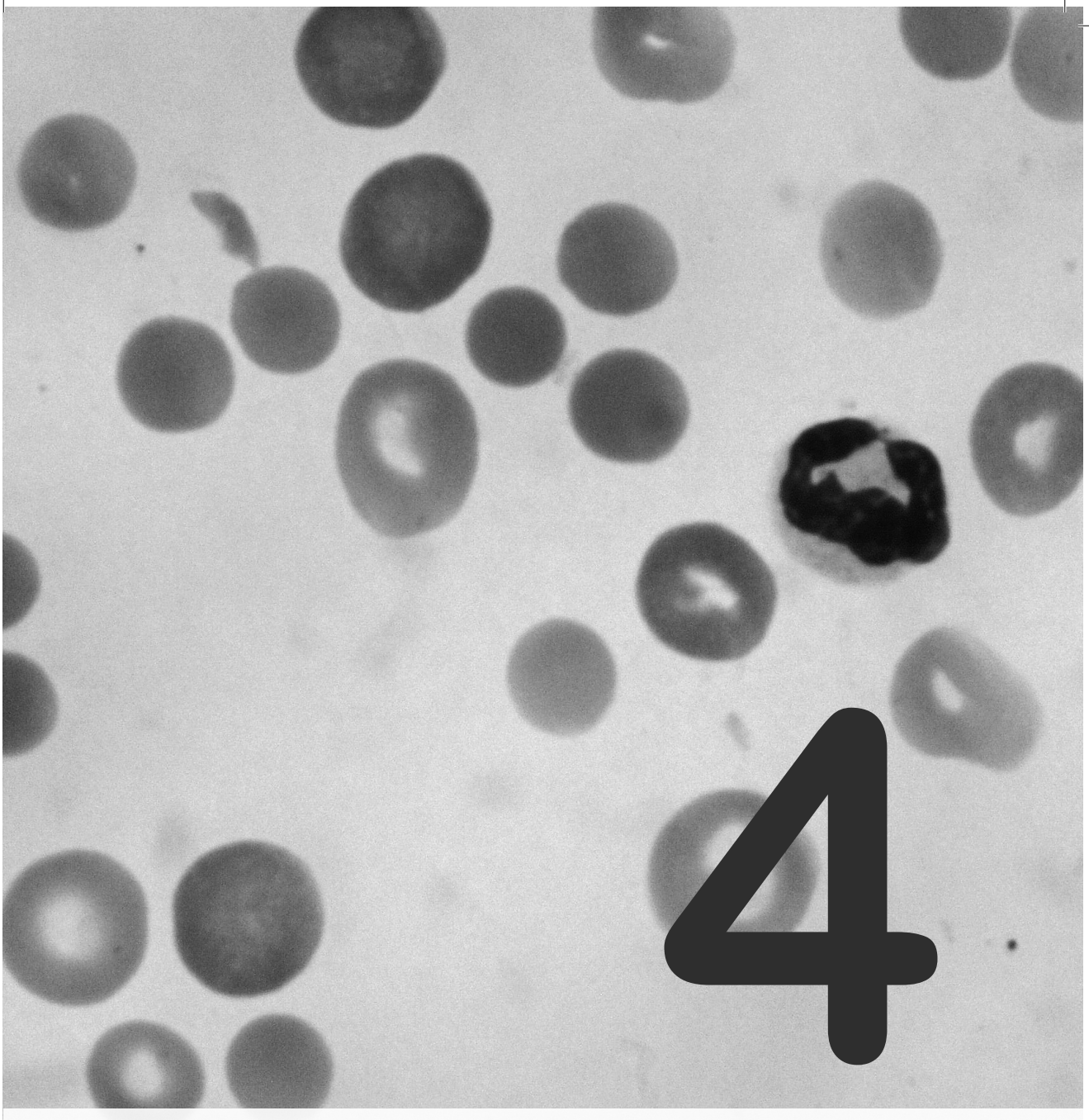

4

Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia

CJ Piek, E Teske, MW van Leeuwen, MJ Day

Abstract

Background – The direct agglutination test (DAT) is the accepted method to confirm the presence of anti-erythrocyte antibodies. A gel-based DAT has been developed that offers the possibility of standardisation of anti-erythrocyte antibody testing.

Objectives - To compare a gel-based test with the traditional DAT for the diagnosis of immune-mediated haemolytic anaemia (IMHA).

Methods - Canine (n=247) and feline (n=74) blood samples were submitted for DAT testing to two laboratories. A subset of canine samples was categorized as having idiopathic IMHA, secondary IMHA, or no IMHA.

Results - The kappa values for agreement between the tests were in one laboratory 0.86 for canine and 0.58 for feline samples, and in the other 0.48 for canine samples. The lower agreement in the second laboratory was caused by a high number of positive canine DATs for which the gel test was negative. This group included significantly more dogs with secondary IMHA.

Conclusions - The gel test might be used as a screening test for idiopathic IMHA and is less often positive in secondary IMHA than the DAT.

Introduction

Immune-mediated haemolytic anaemia (IMHA) is caused by the binding of antibodies to the surface of red blood cells (RBCs). The production of such antibodies can be a primary autoimmune phenomenon or be associated with underlying neoplasia, chronic infections, inflammatory disease or be triggered by exposure to drugs or vaccines (secondary IMHA; (1). The criteria used to define IMHA in dogs and cats vary between different studies; however, it is generally accepted that a positive direct agglutination test (DAT), marked spherocytosis or true autoagglutination are three hallmarks of canine IMHA. At least one of these changes must be present in a patient with haemolytic anaemia to warrant a diagnosis of IMHA (2).
The DAT demonstrates the presence of anti-erythrocyte antibodies by incubating a suspension of washed patient erythrocytes with polyvalent or monovalent antisera specific for immunoglobulin or complement. More recently, a gel-based test has been developed (Diamed, Cressier, Switzerland) (3). The gel test is fast and easy to perform and has the potential for in-house use. A whole-blood sample may be used (instead of washed and resuspended RBCs) and the test uses a smaller blood volume than that required for the DAT (4). Moreover, this test offers the possibility of standardizing anti-erythrocyte antibody testing between laboratories. The aims of the present study were: (1) to perform a comparison between the feline and canine gel test with the traditional DAT in a two-centre study, and (2) to assess the usefulness of the gel test as a diagnostic tool in IMHA in dogs.

Materials & Methods

Study design and definition of sample material and patients

The comparison of the gel test with the DAT was performed on samples from referral patients and samples sent by private practitioners submitted to two centres, the Utrecht University Veterinary Diagnostic Laboratory (UVDL) and the Bristol Clinical Immunology Diagnostic laboratory (BCIDL).
In the UVDL, 126 canine samples with a haematocrit (Ht) below the lowest end of the reference range (0.42 l/l) were included in the study. In the BCIDL, 74 feline and 121 canine samples were included in the study without restriction on the Ht.
The assessment of the usefulness of the gel test as a diagnostic tool for IMHA was performed on cases from the UVDL only. For this, all dogs that had either a positive gel test or DAT were categorized clinically as having: (1) idiopathic IMHA, (2) secondary IMHA, or (3) anaemia that was not immune-mediated (no IMHA).
The inclusion criteria for idiopathic IMHA were: (1) acute onset anaemia, (2) Ht below 0.35 l/l, (3) a positive DAT or spherocytosis. In cases where an underlying disorder or recognized

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

trigger factor (e.g. infection, neoplasia or administration of drugs) was identified the dog was classified as having secondary IMHA. In order to make this classification, information available at the time of presentation as well as follow-up and post-mortem results were reviewed.

Laboratory tests

Gel test

The gel test (Diamed, Cressier, Switzerland) was performed according to the manufacturers' instructions. The test is presented in the form of a small plastic card within which there are six columnar tubes with an overlying reservoir of wider diameter. The tube contains a gel matrix impregnated with rabbit polyvalent DAT-reagent specific for either dog or cat. The cards used by the two centres were the same, but the UVDL was also supplied with separate negative control cards that comprised tubes containing only the gel matrix without antibody. Using the diluent supplied by the manufacturer a 0.8% RBC suspension was prepared and 50 µl of this suspension was pipetted into the reservoir above the gel. In the BCIDL the patient RBC were first washed twice in phosphate buffered saline (PBS; pH 7.4, 0.01M) and then resuspended in the diluent. The card was centrifuged in a purpose-designed centrifuge supplied by the manufacturer that runs at a set centrifugal force for a defined time period. Following centrifugation, the tubes were examined to determine the distribution of the red cells. In a negative test the cells were pelleted at the bottom of the tube. Where cells were distributed within the cell matrix the test result was recorded as weak positive. Where cells were retained at the top of gel the test was reported as positive.

DAT

In both laboratories a similar DAT protocol using was used as described previously (5, 6). Erythrocytes from each dog were washed three times in PBS and resuspended at 5 % in PBS. All reagents were titrated in PBS across the rows of a microtitre tray. An equal volume of the 5% erythrocyte suspension was added to each well and to control wells containing PBS alone. The microtitre trays were incubated until erythrocytes in the control wells had settled. Agglutination was assessed visually, and the titre was recorded as the reciprocal of the last dilution of each antiserum giving a positive agglutination reaction. The DAT was considered positive if at least one of the reagents resulted in a positive titre and the control wells showed no agglutination.

UVDL: The test was performed with monovalent canine Coombs' reagents: rabbit anti-dog IgG (Fc), anti-dog IgG (H+ L), and anti-dog IgM (Fc) antibodies (Nordic Laboratories, the Netherlands). Tests with anti-IgG reagents were performed at 37°C and tests with anti-IgM at 4°C. A titre ≥ 16 was considered positive with any antibody.

BCIDL: The test was performed using a polyvalent canine Coombs' reagent (raised in rabbit) with specificity for canine IgG, IgM and complement C3 (Nordic Laboratories) and monovalent rabbit anti-dog IgG (Fc), rabbit anti-dog IgM (Fc) and goat anti-dog C3 (Nordic Laboratories). All reagents were pre-absorbed against a pool of normal canine erythrocytes. The test was performed in duplicate plates, one of which was incubated at 4°C and the other at 37°C. A titre $\geq$ 20 was considered positive.

The feline DAT was performed at both temperatures with three species-specific antisera: polyvalent feline Coombs' reagent (ICN Pharmaceuticals, United Kingdom), rabbit anti-cat IgG (Fc) and rabbit anti-cat IgM (Fc) (Nordic Laboratories) (7).

Haematology

UVDL: The ADVIA 120 (Siemens Medical Solutions, Diagnostics, USA) was used to determine Ht, total white blood cell count (WBC), differential WBC counts, platelet count and reticulocyte count. All blood smears were examined for the presence of spherocytes at the time of this study by MWvL.

BCIDL: Haematology results were available for samples that had been submitted for concurrent haematological examination and DAT testing. A Cell Dyn 3700 analyzer (Abbot, USA) was used to determine Ht, WBC, differential WBC count, platelet and reticulocyte counts. These analyses were performed by the Langford Veterinary Services Diagnostic Laboratories.

Statistics

The results of the gel test were compared with the DAT by plotting the log titres of the DAT against the gel test results and determining correlation by ANOVA. Since in the UVDL only samples with a Ht below the reference range were tested, we created for comparative purposes a subset of 48 canine BCIDL samples with Ht below the reference range used by the BCIDL (0.35 – 0.55 l/l). The percentage agreement between the gel test and the DAT beyond chance, kappa, was calculated with Win Episcope 2 (http://www.clive.ed.ac.uk/winepiscope). To test whether the kappa was significantly different from 0, a one sided Z-test was used.

A comparison of the number of gel test and DAT outcomes, pattern of reactivity, and the titre in the DAT for dogs in the three clinical categories was made with Fisher's exact test. All statistical analyses were performed in R (http://www.r-project.org). $P < 0.05$ was considered significant.

Results

Samples

Thirty two of the 126 UVDL dogs were classified as having idiopathic IMHA, 31 as secondary IMHA, and 63 as no IMHA. The underlying disorders in the dogs with secondary IMHA included neoplastic disease (six haemopoietic tumours, one cutaneous mast cell tumour and two abdominal masses), parasitic infections (one *Babesia canis* and one *Ehrlichia canis*), bacterial infections (two urogenital tract infections, two cases of bacterial lymphadenitis), immune-mediated disorders (two idiopathic inflammatory bowel disease, one immune-mediated thrombocytopenia, one systemic lupus erythematosus, four pure red cell aplasia and three bone marrow dysplasia), central neurological disease (two dogs), and in three dogs no definitive diagnosis was made.

Of the 32 dogs classified as having idiopathic IMHA, 20 animals were still alive at least 6 months after the last sample was entered into the study. Eleven dogs died due to IMHA within the first 2 weeks after diagnosis. In these dogs extensive diagnostic testing did not reveal an underlying disorder, nor did the post-mortem examination performed in two of these cases. All but six of the dogs with idiopathic IMHA had a regenerative anaemia at the time of diagnosis (corrected reticulocyte count $\geq$ 1.5 %; range 0.4 – 9.6 %); one of these dogs died but the other five developed a regenerative anaemia during the first week of treatment.

Performance of the Gel Test versus the DAT

The log titres of the UVDL DAT were significantly associated with a negative result in the gel test, versus the combined weak positive and positive gel test results. For the BCIDL data there was a significant association between the log titres of the DAT and all three outcomes of the gel test (data not shown). Kappa statistics were performed using both the DAT and gel test results as binary data. The comparative analysis of the two test methods for canine samples for the two laboratories is summarized in Table 1.

Table 1. Results of Gel test versus the DAT as performed in the UVDL and BCIDL.

Gel test	UVDL canine DAT			BCIDL canine DAT			BCIDL feline DAT			BCIDL Canine DAT Ht < 0.35 l/l		
	-	+	Total	-	+	Total	-	+	Total	-	+	Total
Negative	52	26	78	74	0	74	46	11	57	36	0	36
Positive	7	41	48	8	39	47	2	15	17	1	11	12
Total	59	67	126	81	40	121	48	26	74	37	11	48
Kappa	0.48*			0.86*			0.58*			0.94*		

* $P < 0.0001$; Tested against H_0 that all observed agreement is based on chance

Pattern of Isotype Reactivity in DAT versus the Gel Test Outcome

The pattern of antibody reactivity in the DAT in relation to the gel test outcome is shown in Figures 1, 2 and 3.

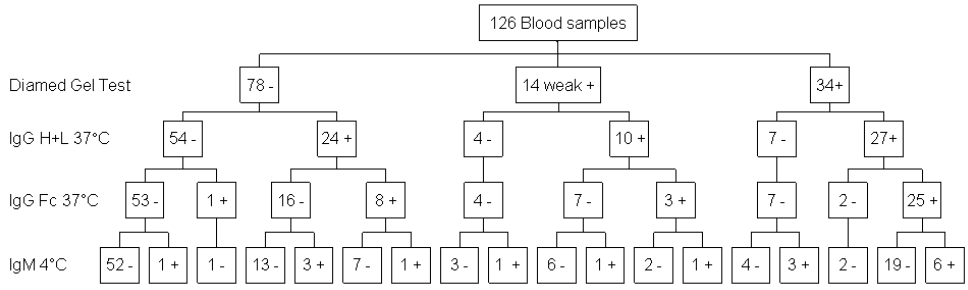

Figure 1. DAT antibody isotype reactivity pattern for 126 canine blood samples tested in the UVDL.

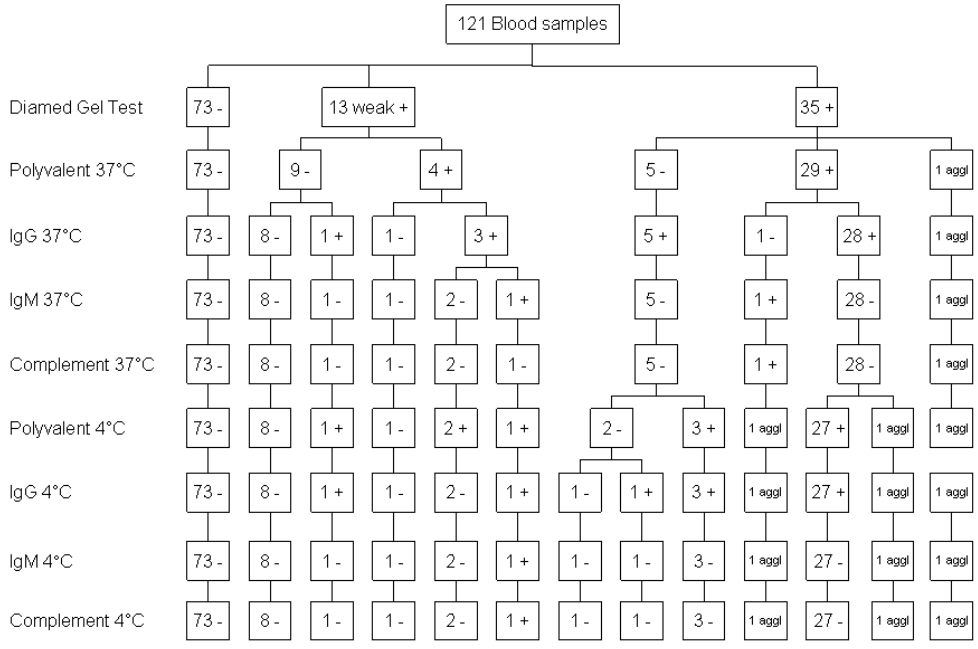

Figure 2. DAT antibody isotype reactivity pattern for 121 canine blood samples tested in the BCIDL.

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

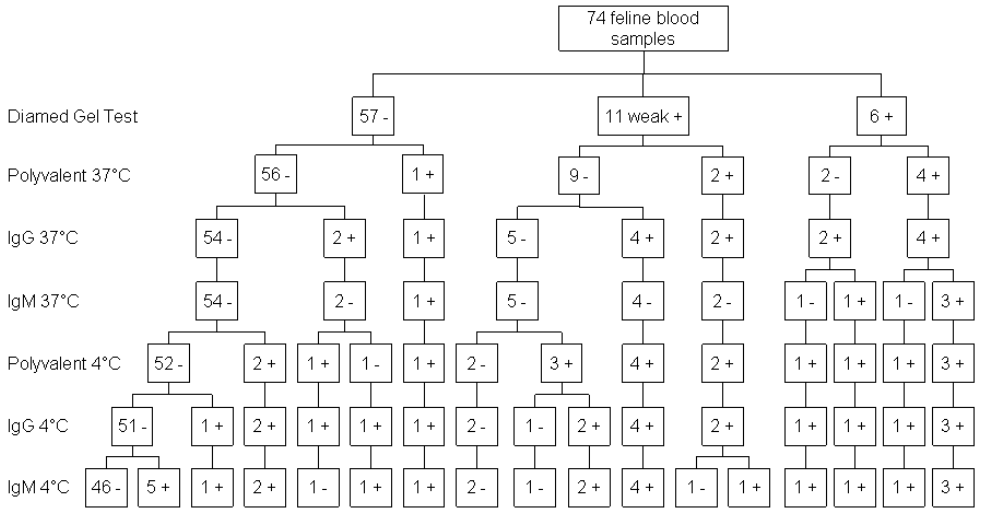

Figure 3. DAT antibody isotype reactivity pattern for 74 feline blood samples tested in the BCIDL.

Relation with Clinical Classification

The difference in distribution of the clinical outcome over the DAT and gel test results was significantly different ($P < 0.0001$) (Figure 4). Of the 52 dogs negative for both the gel test and the DAT, 51/52 (98%) did not have IMHA. Only one of these dogs (2%) had idiopathic IMHA. The gel test and the DAT in this dog were performed one day after the start of immunosuppressive treatment.

A prozone phenomenon was noted in 17 of the 126 UVDL canine samples. In all but one of these 17 samples the DAT titre was positive. In 5 of the 17 dogs with prozones, the titre of the prozone was ≥16 (the cut-off titre at which the UVDL DAT is regarded as positive). Three of these five dogs had idiopathic IMHA and two had secondary IMHA. Of the 17 dogs with prozone effects, nine had idiopathic IMHA and the other eight had secondary IMHA.

The number of DAT reaction patterns in the dogs with idiopathic IMHA was significantly different from dogs with secondary IMHA ($P< 0.0001$) and no IMHA ($P< 0.0001$), and also for the comparison of dogs with secondary IMHA versus dogs without IMHA ($P < 0.0001$) (Table 2). Samples from dogs with idiopathic IMHA more often achieved higher titres than those from dogs with secondary IMHA, but this was not statistically significant ($P =0.34$) (Table 3).

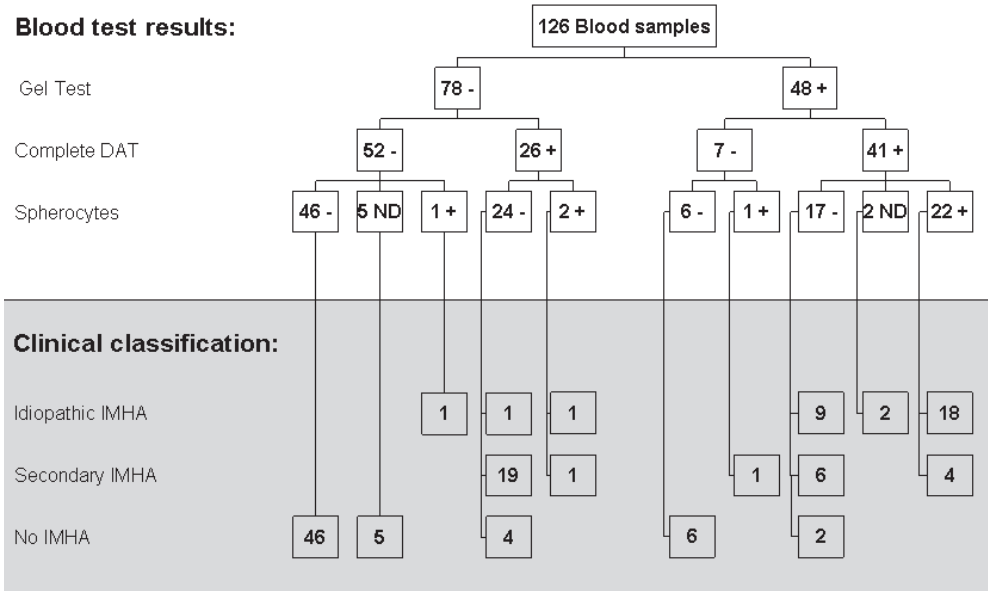

Figure 4. Gel Test, DAT and spherocytosis in dogs with idiopathic IMHA, secondary IMHA and no IMHA.

Table 2. UVDL DAT reactivity patterns in dogs having idiopathic IMHA, secondary IMHA, and no IMHA.

IgG H+L 37°C	IgG Fc 37°C	IgM 4°C	Idiopathic IMHA	Secondary IMHA	No IMHA
+	+	-	20	7	1
+	-	-	4	12	5
+	-	+	0	4	0
+	+	+	4	4	0
-	+	-	0	1	0
-	+	+	0	0	0
-	-	+	3	2	0
-	-	-	1	1	57
		Total	32	31	63

Table 3. Number of dogs for each titre reached in the UVDL complete DAT for dogs having idiopathic IMHA, secondary IMHA, and no IMHA.

	Highest titre reached in the complete DAT														
	0	2	4	8	16	32	64	92	128	256	512	1024	2048	4096	Total
Idiopathic IMHA	1	0	0	0	5	3	2	0	4	7	1	0	3	6	32
Secondary IMHA	0	0	1	0	7	4	6	1	3	2	1	2	2	3	31
No IMHA	44	8	0	5	1	2	1	0	1	0	0	1	0	0	63
Total	45	8	1	5	13	9	9	1	8	9	2	3	5	9	126

Discussion

The DAT is an accepted method of detection of anti-erythrocyte antibodies and an essential part of the diagnosis of IMHA (2). The first aim of this study was to compare the results obtained in the DAT versus the gel test. The high number of samples, 247 canine and 74 feline samples, creates a relatively powerful study, as for such comparative investigations 40 samples has been described as sufficient (8). Analysis of the correlation between the log titres of the DAT results and the ordinal gel test results revealed that the latter can be interpreted as binary test results. Therefore kappa statistics were chosen as the appropriate method to compare the two tests in the absence of a "gold standard" for the diagnosis of IMHA (8, 9). The kappa values for the comparison of the canine BCIDL (0.86) as well as the kappa of 0.94 for the BCIDL canine samples with Ht below 0.35% can be interpreted as "almost perfect agreement" and the kappa for the feline BCIDL samples (0.58) and kappa for the UVLD canine samples (0.48) as "moderate agreement" using the classification of Landis and Koch (9).

The use of an extended DAT protocol such as performed by both laboratories in this study in their diagnostic support for a university referral hospital has been questioned for samples derived from first opinion clinical practice (10). A still unresolved issue is the use of multiple dilutions of antisera to prevent false negative DAT outcomes due to the prozone effect (5, 11). Five of 17 UVDL samples had prozones with a titre around the cut-off value defined for the DAT and would have been missed if higher dilutions of antiserum had not been used.

A second issue concerns the inclusion of multiple monovalent reagents to increase the sensitivity of the DAT (5, 12, 13). Indeed in the BCIDL canine samples, six positive DATs would have been missed if the test had relied on polyvalent antisera only. This finding corroborates a previous study by this laboratory in which 11 of 77 dogs with IMHA would have had a negative DAT had the extended test protocol not been used (5).

The third issue related to the DAT is the clinical relevance of performing an extended DAT with full determination of the pattern of antibody reactivity instead of use of a polyvalent reagent only, such as in the gel test. In human IMHA the antibody reactivity pattern is of diagnostic relevance (14). In dogs, however, the clinical revelance remains debated. C3b and IgM reactivity have been reported with higher frequency in secondary IMHA (5, 6) and IgM reactivity at 4°C was also more often seen in the dogs with secondary IMHA in the present study (Table 2). The clinical significance of cold agglutinating antibodies is also widely debated. There is a belief that they are less significant as they are rarely active at body temperature (2), but few studies have investigated the complete temperature gradient of the reactivity of these immunoglobulins.

Despite the fact that the DAT is an accepted test for the diagnosis of IMHA (2), it is by no means internationally standardized. To realise such standardisation between laboratories

will be very difficult, if not impossible. A commercially produced test, such as the gel test, might be better suited to this task. Therefore, in addition to the comparative study of the two diagnostic procedures, the second goal of the present study was to investigate the value of the gel test as a diagnostic tool for IMHA. In the absence of a gold standard we defined clinical categories of idiopathic IMHA, secondary IMHA and no IMHA on the basis of a combination of diagnostic criteria present at clinical presentation and as used by others (2, 15-20) Additionally, these case definitions included follow-up and pathology data in order to prevent misclassification. It can be argued that this clinical classification is based on subjective criteria; however, until better methods have been found, it is consistent with current clinical practice.

The gel test identified fewer positive samples than the DAT despite the fact that in essence it is also a test reliant on the use of a polyvalent antiserum (Figure 4). 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. This observation contrasts, however, with the findings in human samples where the micro-column gel test is found to be more sensitive than the DAT (21, 22).

Overall, in the present study there was a good agreement between the results of the DAT and the gel test. Additionally, the gel test performed well in identifying dogs with idiopathic IMHA, but missed most of the samples that were from dogs with secondary IMHA. From a laboratory viewpoint these samples also differed in having lower titres in the DAT, more frequent IgM positivity and almost no spherocytosis. The gel test offers an appealing option as an initial screening test for idiopathic IMHA and a much-needed means of standardizing anti-erythrocyte antibody testing.

Acknowledgements

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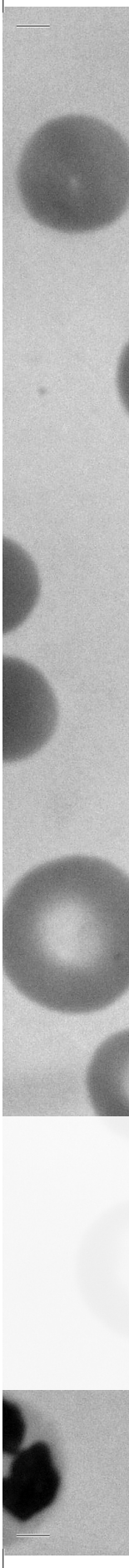

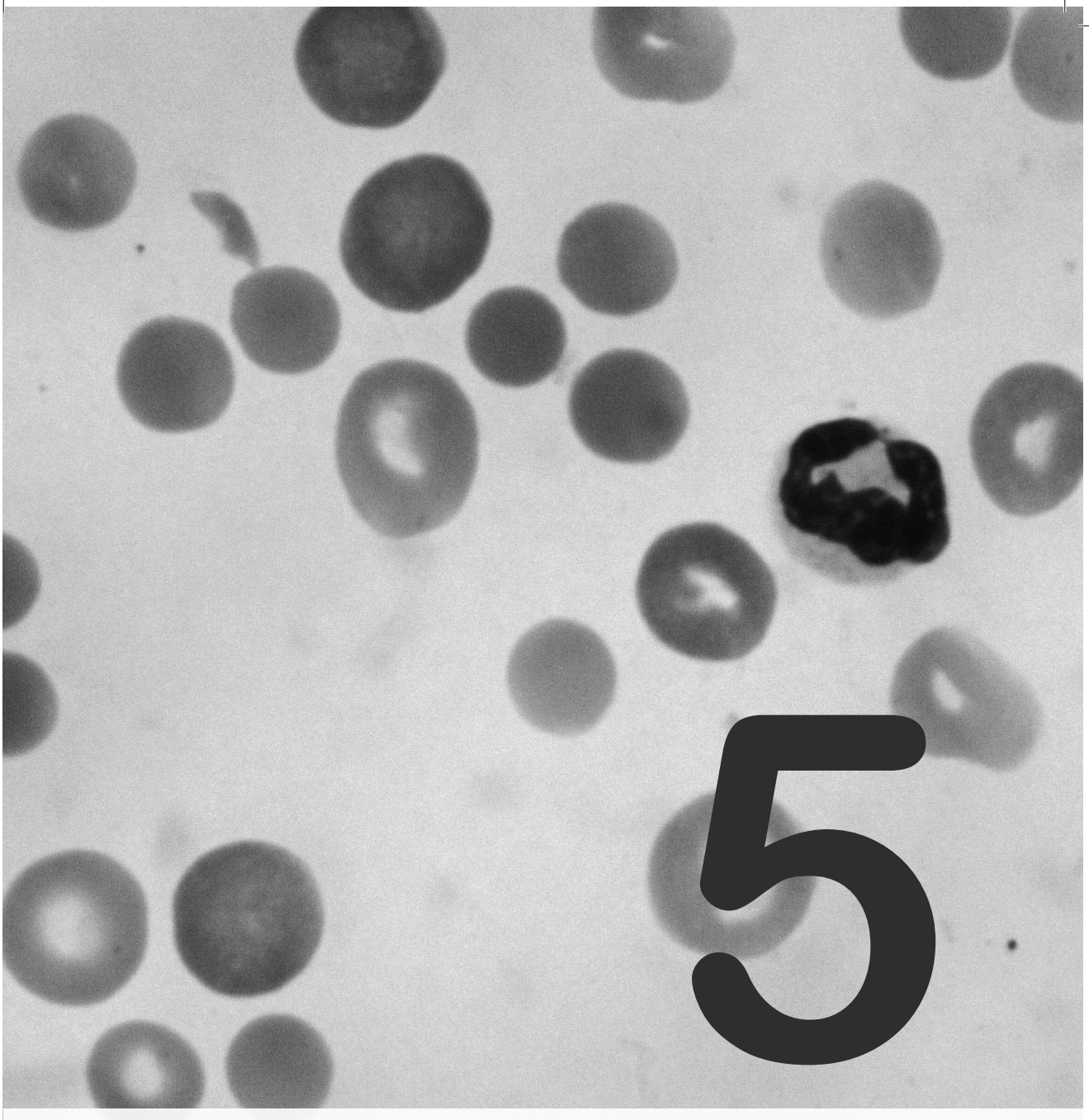

5

Leucocyte count affects expression of reference genes in canine whole blood samples

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BMC Res Notes. 2011;4:36

Abstract

Background - The dog is frequently used as a model for haematologic human diseases.

Objectives - To investigate the suitability of nine potential reference genes for quantitative RT-PCR studies in canine whole blood.

Methods - The expression of these genes was measured in whole blood samples of 263 individual dogs, representing 73 different breeds and a group of 40 mixed breed dogs, categorized into healthy dogs and dogs with internal and haematological diseases, and dogs that underwent a surgical procedure.

Results - GeNorm analysis revealed that a combination of 5 to 6 of the most stably expressed genes constituted a stable normalising factor. Evaluation of the expression revealed different ranking of reference genes in Normfinder and GeNorm. The disease category and the white blood cell count significantly affected reference gene expression.

Conclusions - The discrepancy between the ranking of reference genes in this study by Normfinder and Genorm can be explained by differences between the experimental groups such as "disease category" and "WBC count". This stresses the importance of assessing the expression stability of potential reference genes for gene experiments in canine whole blood anew for each specific experimental condition.

Introduction

The dog is frequently used as an experimental model for haematologic human diseases (1). The use of dogs can be explained by the fact that the dog offers a variety of spontaneous and experimental models of haematologic diseases. Recent examples are the use of canine haemophilia A (2) and B models (3, 4), and the Canine Leucocyte Adhesion Deficiency model (CLAD) (5, 6) in gene therapy experiments (2-8), and pharmacological experiments in leucopenic dogs (7) and in dogs with CLAD (8). The larger size of dogs compared to small rodent models allows similar surgical procedures in humans as in dogs, and permits in most cases adequate acquisition of diagnostic samples. The dog has been a longstanding model for bone marrow and more recently for stem cell transplantations (9, 10). Anticoagulant therapy has been tested extensively in canine cardiac surgery models (11, 12). Also the pathogenesis and therapy of acquired disorders of haemostasis such as disseminated intravascular coagulation (13), thrombosis (14, 15), and haemolytic uremic syndrome (16) have been investigated in canine models.

A disadvantage of the canine model compared to human or small rodent models is the limited availability of antibodies against canine intra- and extracellular proteins such as CD markers. At least 350 CD markers are defined in humans (17, 18), while in the first and to date only workshop on canine leucocyte antigens only 127 antibodies were investigated (19). A more recent study tested cross species reactivity with commercially available anti human CD molecules against canine leucocytes, erythrocytes and platelets and identified only a limited number of 51 cross reacting mAbs (20). In contrast to the limited knowledge of canine CD markers, the canine genome has been sequenced in total (21). Therefore most molecular tools can be readily applied in dog research. Real-time quantitative reverse transcriptase PCR (Q-PCR) offers an accurate and sensitive alternative to quantification of gene expression (22) and for that reason is well suited to study biological processes and has also many practical clinical applications. Q-PCR has already been shown to be a valuable adjunct in immunophenotyping and the quantification of residual disease in leukemia (23-26).

Multiple variables need to be controlled when performing a Q-PCR, such as the quality of RNA after isolation, the input amount and quality of mRNA and cDNA reaction efficacy, efficiency of the enzymatic reactions, and cell to cell variability in translational activity. One of the solutions to control for the internal variation that affect the outcome of the Q-PCR reaction is the use of reference genes as an internal standard (22, 27). Reference genes are selected based on the supposition that their expression is stable in all cells regardless of the tissue or individual (28). It has been proven, however, that many genes essential for basic cellular mechanisms and hitherto thought to have a stable expression throughout the organism actually did not comply with this assumption (29-35). Therefore, it is essential

that the assumption of stable expression of potential reference genes is verified for each experimental set up (28, 36-38).
In this study we investigated the suitability of nine frequently used reference genes in Q-PCR for the use as reference genes in a quantitative real-time PCR in canine whole blood and the influence of dog breed, sex, disease category and disease duration on the Cq of these genes was assessed.

Methods

Blood sample collection

Between September 2007 and October 2008 canine blood samples (n = 263) were taken from dogs submitted to the intensive care unit of the small animal hospital of the Veterinary Faculty of the Utrecht University (The Netherlands) from healthy control dogs (n = 6; group A) and dogs categorized into three disease groups. Group B (n = 85) had surgery within the preceding 24 hours, group C (n = 107) were dogs with miscellaneous internal diseases, and group D (n = 65) had haematologic disease (disseminated intravascular coagulation (n = 27), systemic inflammatory disease (n = 24), and immune-mediated haemolysis (n = 14).
The 263 dogs represented 73 different breeds and a group of mixed breed dogs (n = 40). Breeds that were represented by at least 5 dogs were the Labrador retriever (n = 30), Golden retriever (n = 18), Jack Russell terrier (n = 10), (Bordeaux dog (n = 9), Dachshund n = 9), Boxer (n = 7), German shepherd and German pointer (n = 6), and the Bernese mountain dog, Beagle, English Cocker spaniel, and the Bearded collie were all represented by 5 dogs. There were 42 female dogs, 91 castrated female dogs, 78 males, and 47 castrated male dogs. Of 3 dogs the sex was not noted in the file. The mean age of the dogs was 6.5 years (range 12 weeks to 14 years, SD 3.5 years).
Two milliliters of EDTA-anticoagulated blood were collected from each dog on the day of admittance and during the period the dog was hospitalized consecutive samples were taken at least 24 hours apart.
From 99 of the dogs, a second sample was available (37 of group B, 30 of group C, 32 of group D), and in, respectively, 34 dogs a third (10 of group B, 6 of group C, 18 of group D), and in 13 dogs a fourth sample (4 of group B, 3 of group C, 6 of group D) was available.
All procedures were approved by and performed according to the ethical committee as required under Dutch legislation.

RNA isolation and cDNA synthesis

In view of the large sample number but small samples size, the RT-reaction was performed only once. The MIQE guidelines, however, suggest to perform it twice (39, 40). From each dog duplo samples were prepared by mixing 0.5 ml EDTA-anticoagulated blood with 1.3 ml RNA*later* (Ambion, Applied Biosystems, Foster City, California, USA). Samples were stored at -20°C. Total RNA was extracted from the samples using the RiboPure ^{tm} –Blood kit reagent (Ambion, Applied Biosystems, Foster City, USA) according to the manufacturer's instructions including a DNAse treatment to destroy contaminating genomic DNA and minimize the effect of pseudogenes. The RNA concentration was determined spectrophotometrically by the NANOdrop 1000 (Isogen Life Science, IJsselstein, The Netherlands). Bio-Rad iScript, containing both oligodT and random hexamer primers, was used to synthesize cDNA from 1µg of total RNA according to the manufacturer's instructions (iSCRIPT, Bio-Rad, Veenendaal, The Netherlands).

Primer design and testing

The selection and testing of candidate reference genes was based on gene targets that have already been used in human and veterinary research, and have been previously reported (41, 42). Nine genes representing various biological processes (GAPDH, SRPR, HPRT, B2M, GUSB, HNRNPH, RPL8, RPS5, RPS19) were selected as candidate reference genes. Their full names, GenBank accession numbers, and location in the canine chromosome are given in Table 1. The primers that were used, location of these primers within the gene, and the length of the resulting amplicon are given in Table 2. Primers were developed based upon known canine sequences (Ensembl, www.ensembl.ort and GenBank, www.ncbi.nih.gov/genbank/index.html). The primers were designed with Oligo Explorer 1.1 (www.genelink.com/tools/gl-downloads.asp). The specificity and uniqueness of each primer were verified with the Basic Local Alignment Search tool expecting return of Genbank accession numbers of candidate reference genes only (www.genelink.com/tools/gl-downloads.asp). All primer pairs, except for GAPDH, were intron spanning. The PCR reaction was optimized for the primers. Optimal T_m values ranged from 55 °C for RPL8 to 62.5 °C for RPS5 (Table 2). Amplification efficiency calculations from all standard curves were between 93.9 and 106.7%. All no template controls were negative.

Table 1. Abbreviations, GenBank Accession numbers, names, and chromosomal location of canine candidate reference genes evaluated.

Gene	GenBank Accession No.	Name	Chromosomal location in *Canis familiaris*
RPS19	XM_533657	Ribosomal protein S19	Chromosome 1
RPL8	XM_532360	Ribosomal protein L8	Chromosome 13
RPS5	XM_533568	Ribosomal protein S5	Chromosome 1
GUSB	NM_001003191	Beta-Glucuronidase	Chromosome 6
B2M	XM_535458	Beta-2-microglobulin	Chromosome 30
HNRNPH	XM_538576	Heterogeneous nuclear ribonucleoprotein H	Chromosome 11
HPRT	AY283372	Hypoxanthine phosphoribosyltransferase 1	Chromosome X
GAPDH	NM_001003142	Glyceraldehyde-3-phosphate dehydrogenase	Chromosome X
SRPR	X03184	Signal recognition particle receptor	Chromosome 5

Table 2. Primer sequences, exon locations, amplicon size, and optimal melting temperature of canine candidate reference genes.

Gene	Forward 5′ → 3′*	Exon(s)	Reverse 5′ → 3′	Exon(s)	Product length (bp)	T_m (°C)
RPS19	CCTTCCTCAAAAA/GTCTGGG	2/3	GTTCTCATCGTAGGGAGCAAG	3	95	61.0
RPL8	CCATGAAT/CCTGTGGAGC	4/5	GTAGAGGGTTTGCCGATG	5	64	55.0
RPS5	TCACTGGTGAG/AACCCCCT	2/3	CCTGATTCACACGGCGTAG	3	141	62.5
GUSB	AGACGCTTCCAA/GTACCCC	3/4	AGGTGTGGTGTAGAGGAGCAC	4	103	62.0
B2M	TCCTCATCCTCCTCGCT	1	TTCTCTGCTGGGTGTCG	2	85†	61.2
HNRNPH	CTCACTATGATCCACCACG	5	TAGCCTCCATAAC/CTCCAC	5/6	151	61.2
HPRT	AG/CTTGCTGGTGAAAAGGAC	5/6	TTATAGTCAAGGGCATATCC	7	114‡	56.0
GAPDH	TGTCCCCACCCCCAATGTATC	2	CTCCGATGCCTGCTTCACTACCTT	2	100	58.0
SRPR	GCTTCAGGATCTGGACTGC	5/6	GTTCCCTTGGTAGCACTGG	6	81	61.2

* If a primer is located on two exons, the junctions are shown with a dividing forward slash (/).
† Genomic product size would be approximately 3.6 kb.
‡ Genomic product size would be approximately 300 bp.

Quantitative PCR

Q-PCR was done with the DNA-binding SYBR green using the BioRad iCycler MyiQ Real-Time PCR Detection System (BioRad, Hertfordshire, United Kingdom) according to the manufacturer's instructions. Primers (Eurogentec, Maastricht, The Netherlands) had a final concentration of 400 nM each. One microliter of cDNA was used per Q-PCR reaction. Optimal T_m was determined previously (41, 42). Reactions with a T_m less than 58 °C started with 5 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 30 s at T_m, and 30 s at 72 °C.

This reaction was continued by a melting curve, stepwise increasing temperature each 15 s by 0.5 °C, ranging from 60 to 95 °C. In case the T_m was 58 °C or higher, the elongation step at 72 °C was omitted and T_m remained 30 s. Analysis of Q-PCR results were performed with iQ^{tm}5 software (Biorad, Veenendaal, The Netherlands) based on the mean Cq obtained from the duplo of each Q-PCR reaction.

Analysis gene expression

Firstly, the influence of experimental condition such as disease category and duration, sex, leucocyte count on potential reference gene expression were determined. For each potential reference gene a comparison of the mean Cq values obtained at the first sampling for the disease groups A, B, C, and D, and sex was performed using the ANOVA. To determine if the differences in Cq's for the nine potential reference genes were due to changes in expression levels over time an ANOVA was used. Using a forward selection process, two explanatory variables, "dog" and "sample number", were introduced as factors in the ANOVA. The result variable was the observed Cq value. The resulting models were compared using the likelihood ratio test.

The mean Cq values for dogs with a leucocyte count within the reference range (4.5 – 14.6 x 10^9/l) were compared to mean Cq's of dogs with a leucocyte count above 30 x 10^9/l, which can be considered a clinically relevant leucocytosis. If a significant difference was observed, a pair wise comparison was made using the T-test with Holmes correction for multiple comparisons. Secondly, a linear mixed effects model was used to assess the significance as well as the magnitude of the effect of leucocyte count on Cq per dog, with the mean Cq as response variable, the natural logarithm of the "leucocyte count" as explanatory variable, and the "dog" as random effect. Similarly, a linear mixed effects model was used to determine if the leucocytes count changed over time per dog. An ANOVA was used to compare the leucocyte counts in the disease groups A, B, C and D. A linear model was used to examine the relationship of the Cq with the variables "disease category" and the natural logarithm of the "leucocyte count".

All statistical analyses were performed in R (www.r-project.org). P below 0.05 was considered significant in all analyses.

To determine the ranking of best performing reference genes in whole blood the stability of expression of the candidate reference genes was calculated using the GeNorm (27) and Normfinder (43) algorithm software. The gene expression stability calculations in this study were performed on the first sample that was taken when the dog entered the study.

In Genorm the expression ratio for each pair of candidate reference genes is calculated for the data array of all samples and $\log_2$-transformed. "M" is the arithmetic mean of the pair wise variation measured as the standard deviation of thus obtained values. A low "M" indicates little variation in expression of the two genes. Then the optimal number of control genes for

normalisation is determined. Firstly, the normalisation factor is calculated based on the two reference genes with the lowest "M"-values. Secondly, the contribution of an additional candidate reference gene to the variance of the normalisation factor ratios is calculated by the stepwise introduction of the reference genes following the earlier established ranking order of their "M"-values.
Shortly, Normfinder makes use of a mathematical model to describe the expression values measured by RT-PCR, separate analysis of the sample subgroups, and estimation of both the intra- and the intergroup expression variation, and finally calculates a candidate gene "Stability Value."

Results

Expression of candidate reference genes

The range and median Cq values of the first sample that was taken in the dogs in the disease groups A, B, C and D (described above) are depicted in Figure 1. There was a significant difference between the mean Cq's measured in groups B and C for RPL8, RPS19, B2M and HNRNPH, the differences being 0.35, 0.39, 0.44, and 0.35 Cq, respectively. The difference between groups B and D for B2M was 0.51 Cq, and between A and C for GAPDH it was 1.1 Cq (Figure 1). "Sample number" did not significantly determine the Cq, except for SRPR ($P = 0.013$), nor did "sex" and "breed".
Next the leucocyte count was examined. The leucocyte counts of disease group A was within the reference range (median 8.6, range 6.6 – 12.5 x 10^9/l). The leucocyte counts of disease groups B (median 15.9, range 3.8 – 107.8 x 10^9/l) and C (median 16.8, range 2.1 – 44.6 x 10^9/l) were statistically significant from group D (median 22.6, range 4.8 – 175.9 x 10^9/l) ($P = 1.9 \times 10^{-7}$ and 7.8×10^{-6} respectively). The linear mixed effects model revealed that "leucocyte count" did not significantly change between sequential samples taken during the course of the disease.

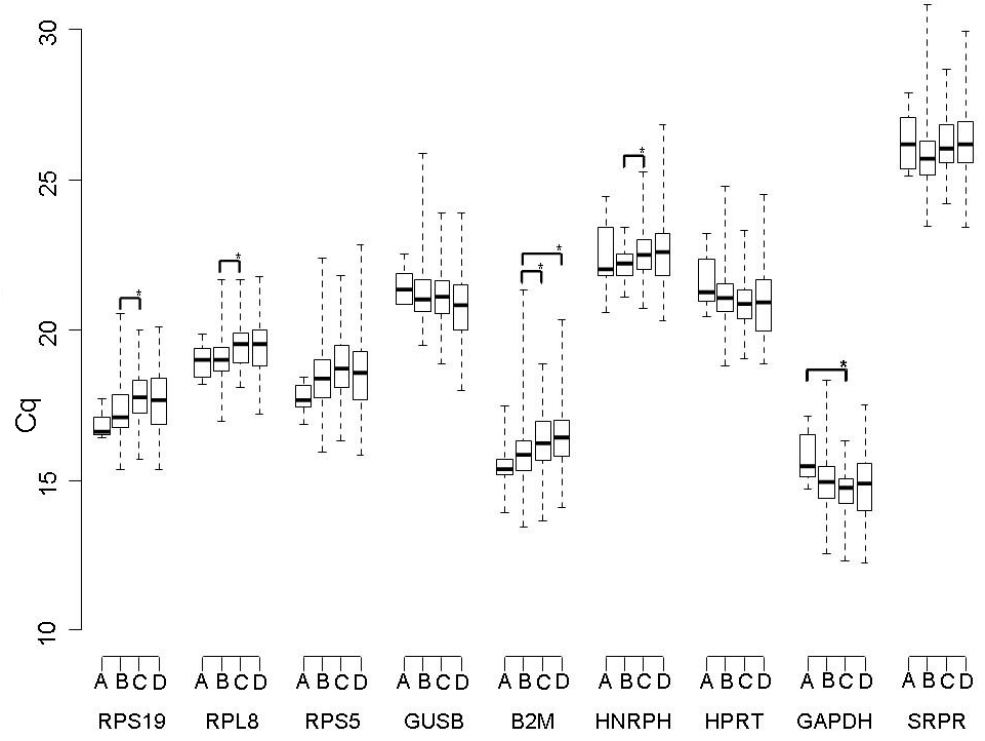

Figure 1. Real-time PCR cycle threshold numbers (Cq values) for nine potential reference genes in 4 disease categories (n=263).

Legend:

Real-time PCR cycle threshold numbers (Cq values) are plotted for nine potential reference genes. Group A included 6 healthy dogs, group B 85 dogs within 24 hours after a surgical procedure, group C 107 dogs with miscellaneous internal diseases, and group D 65 dogs with haematologic diseases. Statistically significant differences between mean Cq of the disease categories are depicted.

The boxes represent the two middle quartiles with medians. Whiskers delineate the range.

The linear mixed effects model that included only "leucocyte count" as explanatory variable for the Cq was not significant for SRPR, HNRNPH, and GUSB. The other 6 potential reference genes (B2M, RPL8, RPS19, RPS5, GAPDH, and HPRT) had significant changes in Cq, ranging from -0.87 to 1.28 for an a ten fold increase in leucocyte count. A significant difference between the Cq's of dogs with a leucocyte count within the reference range and dogs with a leucocyte count above 30 x 10^9/l was found for RPS19, RPL8, RPS5, B2M, and HPRT. Additionally, in this analysis, GAPDH, was identified as the fourth of the nine reference genes that was not significantly influenced by leucocyte count (Table 3).

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

Table 3. Relation of Cq and white blood cell count (WBC).

Gene	Cq change for 10 fold increase in WBC count	P	WBC within reference range		WBC > 30 x 10^9/l		Difference	P
			Mean Cq	SD	Mean Cq	SD		
RPS19	0.92	0	17.35	0.82	17.90	1.31	0.56	0.002
RPL8	0.52	0.006	19.20	0.67	19.52	1.14	0.32	0.026
RPS5	1.06	0	18.30	0.86	19.01	1.61	0.71	0.0009
GUSB	- 0.07	0.75	21.09	0.97	21.08	1.47	-0.01	0.93
B2M	1.28	0	15.85	0.86	16.58	1.74	0.73	0.0006
HNRNPH	- 0.09	0.68	22.40	0.86	22.20	1.02	-0.20	0.19
HPRT	- 0.87	0	21.14	0.93	20.71	1.03	-0.43	0.01
GAPDH	- 0.51	0.023	14.87	0.97	14.80	0.96	-0.07	0.38
SRPR	0.10	0.70	18.30	1.09	19.01	1.51	0.03	0.91

Change in Cq is given for a ten fold increase in WBC count, mean Cq and SD for dogs with WBC count in the reference range and for dogs with a leucocytosis with a WBC > 30 x 10^9/l.

The linear model that included both "leucocyte count" and the "disease category" as explanatory variables for the Cq was statistically significant for both RPS5 and B2M. "Disease category" was the statistically significant factor determining Cq in the case of SRPR, HNRNPH, GUSB and GAPDH and "leucocyte count" in the case of RPS19, RPL8, and HPRT.

In order to identify the genes that had the least variable expression, expression stability was evaluated using GeNorm and Normfinder software analysis. The pair wise variation between the normalisation factors calculated by GeNorm steadily decreased after inclusion of the fourth additional reference gene and falls below the cut-off of 0.15 that is suggested by the GeNorm programme after adding the fifth gene (27) (Figure 2). The ranking of the potential reference genes by GeNorm and Normfinder is given in Table 4.

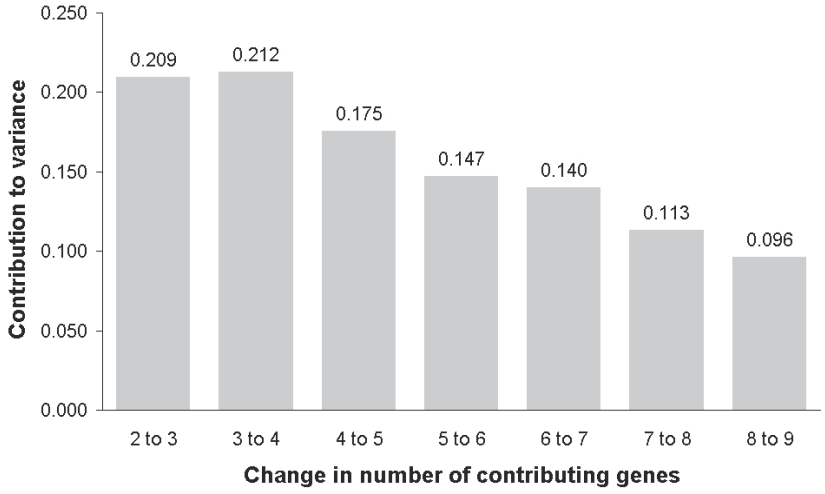

Figure 2. Pair wise variations between 2 sequential normalisation factors including an increasing number of potential reference genes.

Legend:

To determine the optimal number of reference genes, first the geometric mean of the expression of the previously ranked genes was calculated and then pair wise variations between sequential normalisation factors were calculated. Using the cut-off recommended by GeNorm of 0.15 the optimal number of reference genes for the data set in this study would be at least 5.

Table 4. Ranking of potential reference genes according to their expression stability by GeNorm and Normfinder.

GeNorm	Stability measure*	Normfinder	Stability Value*	Normfinder	Stability Value*
	M	Disease category		WBC quartiles	
RPL8 and RPS19	0.55	RPL8	0.16	RPL8	0.09
		HNRNPH	0.17	HNRNPH	0.14
RPS5	0.64	SRPR	0.19	GUSB	0.14
GUSB	0.78	GUSB	0.26	SRPR	0.16
B2M	0.87	HPRT	0.26	GAPDH	0.17
HNRNPH	0.93	RSP19	0.26	RSP19	0.17
HPRT	0.99	B2M	0.28	RPS5	0.21
GAPDH	1.01	RPS5	0.32	HPRT	0.21
SRPR	1.03	GAPDH	0.35	B2M	0.21

*Genes are ranked according to decreasing expression stability.

Discussion

Studying gene expression by the sensitive, specific, and accurate technique of quantitative RT-PCR has become increasingly important in biomedical research. The goal of this study was to select reference genes that can be used as a normaliser when studying gene transcription in canine blood cells. Nine genes that are either conventionally used as reference genes or were shown to have a stable expression in haematopoietic cells or whole blood were chosen as potential candidate reference genes in this study (36, 41, 42, 44, 45) (Table 1). Even genes that regulate basic cellular tasks have been shown to be regulated (29-35, 46). To exclude that the expression of the potential reference genes was influenced by the experimental conditions in our study we investigated the effect of several parameters such as disease category, disease duration, and leucocyte count. Additionally, two software algorithms, respectively, Normfinder (43) and GeNorm (27), were used to calculate gene expression stability and help select the combination of reference genes that provides the most stable normaliser for a specific experimental situation.

Whole blood RNA originating from all cells present in the peripheral blood, as opposed to RNA derived from a cell sorting procedure, was used for the reverse transcriptase reaction in this study. To correct for the leucocyte count reaction was performed on a fixed amount of starting RNA. The influence of a disproportionate increase of a subset of the leucocytes on reference gene expression is not countered by this. This disadvantage has to be weighed against the advantage of being able to investigate simultaneously the expression of multiple genes originating from distinct cell types. And, additionally, against the fact that cell sorting procedures have been shown to affect gene expression. Five to nine fold up regulation of cytokine expressions were seen after density gradient separation of leucocytes (47).

Several conditions that might effect gene expression were examined in this study. Figure 1 reveals significant increases in Cq between the disease groups for RPS19, RPL8, B2M, HNRNPH, and GAPDH, The maximum increase is seen in case of B2M between groups B and D (0.51 Cq). These differences between the disease groups can be contributed mainly to the disease condition as opposed to disease duration since the Cq was not significantly different between the sequential samples taken during the disease period in a subset of the dogs. The leucocyte count gradually increases comparing the disease groups, revealing a significant difference between group B (median leucocyte count 15.9 x 10^9/l) versus C (median leucocyte count 16.8 x 10^9/l) and D (median leucocyte count 22.6 x 10^9/l), respectively. The changes in Cq associated with leucocyte count had a similar direction as the Cq changes in the disease categories (Figure 1). This suggested that "leucocyte count" might a major factor explaining the directional change in Cq. The linear model examining the influence of "disease category" and "leucocyte count" revealed that this was the case for RPS19 and RPL8. RPS5 and B2M were best explained by the linear model containing both parameters,

however. The reference genes that were not significantly influenced by the WBC count were GUSB, HNRNPH and SRPR (Table 3).

B2M has shown a highly variable expression in several tissues other than whole blood (41, 42, 44, 48) but had a stable expression in one study where human leucocytes from 13 healthy donors were examined (27). B2M also had stable expression in a large study where 526 human whole blood samples representing healthy individuals and 6 disease groups (49). The influence of leucocyte count on B2M expression was not examined in both these studies. B2M encodes for beta-2-microglobulin which is part of the canine MHC I molecule and abundantly expressed on haematopoietic cells. The decrease in B2M expression associated with increases in leucocyte count in this study might reflect both a decrease in induced expression, and a shift in leucocyte subsets displaying different MHC class I receptor densities.

The selection of one, or a set of, potential reference genes for a future experiment depends besides practical points such as available sample sizes and costs mainly on stability of expression in the experimental samples. In this study we evaluated the expression stability with Normfinder and GeNorm. Both software algorithms are frequently used and freely available but have a different working rationale. Normfinder selects out of a set of potential reference genes one single, or the pair of, best performing reference genes that show the least variation between and within experimental groups. The focus on the detection of directional changes in reference gene expression due to differences between the experimental groups is the major difference with GeNorm that focuses on pair wise comparisons of reference gene expression in the experimental samples and is therefore less apt to identify coregulated genes (50). Since WBC count and disease category had a statistically significant effect on potential reference gene expression, it is not surprising that the ranking provided by Normfinder and GeNorm differed. Among the genes ranked highest by Normfinder were the genes that were not significantly influenced by the WBC count (GUSB, HNRNPH and SRPR, Table 3).

Contrastingly, GeNorm ranked RPS8, RPS19, and RPS5 highest. Similarly, RPL8 had the best Stability Value in Normfinder, but both RSP19 and RSP5 were ranked at the low end of the list (Table 4). An explanation might be that these three genes are all coding for ribosomal proteins which are likely to be coregulated. Despite the fact that they have less variation in expression as pointed out by GeNorm, the directional difference in expression of these coregulated reference genes, will potentially decrease the sensitivity of detecting changes in expression of the genes of interest in an experiment (51).

The discrepancy between the ranking of reference genes in this study by Normfinder and Genorm can be explained by differences between the experimental groups such as "disease category" and "WBC count". These results reveal that experimental conditions can result in unforeseen group wise up regulation or down regulation of reference genes that otherwise may have a stable expression when the whole dataset is considered. Minor group specific

directional changes in reference gene expression might obscure changes in candidate gene expression between groups. The results of this study emphasize that it is prudent to assess each new data set specifically for changes in reference gene expression due to the experimental conditions even when reference genes are chosen that were previously shown to have a stable expression.

List of abbreviations

B2M, beta-2-Microglobulin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GUSB, beta-Glucuronidase; HNRNPH, Heterogeneous nuclear ribonucleoprotein H; HPRT, Hypoxanthine phosphoribosyltransferase; RPL8, Ribosomal protein L8; RPS5, Ribosomal protein S5; RPS19, Ribosomal protein S19; SRPR, Signal recognition particle receptor.

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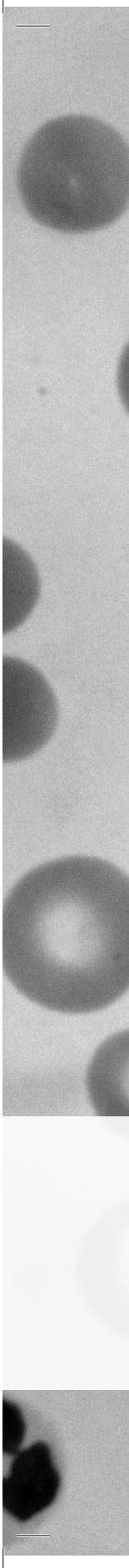

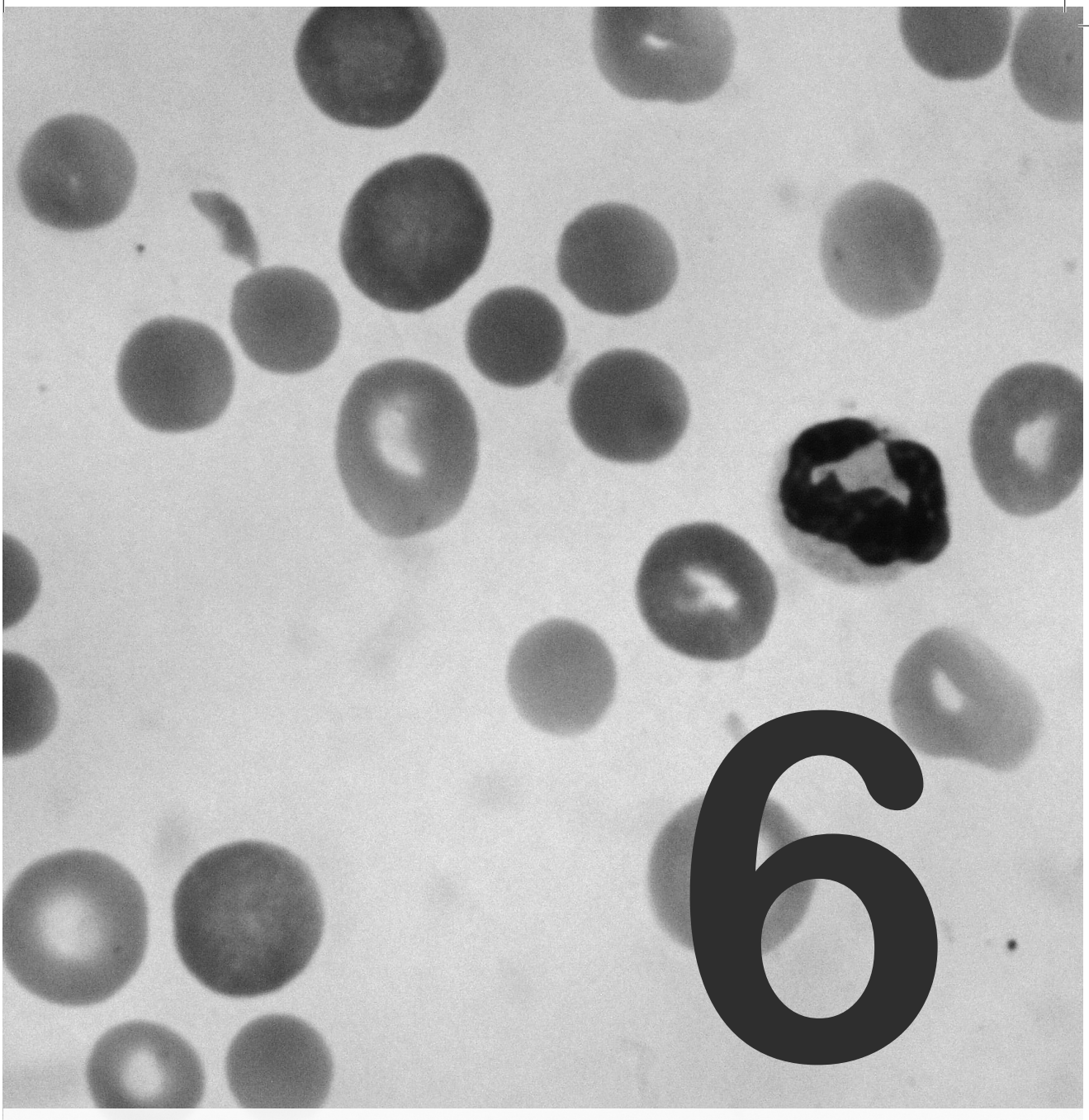

6

High intravascular tissue factor but low IL-8 gene expressions in dogs with immune-mediated haemolytic anaemia

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Abstract

Background - A high mortality occurs in dogs with idiopathic immune-mediated haemolytic anaemia (IMHA) during the first 2 weeks after the diagnosis.

Objectives - To investigate the inflammatory response and coagulation abnormalities in dogs with IMHA in relation to the prognosis and to establish the contribution of whole blood Tissue Factor (TF) and interleukin-8 gene expressions.

Methods - Gene expressions in dogs with IMHA were compared to healthy dogs, dogs with DIC, dogs with sepsis, and in two groups of dogs that underwent intensive care treatment but had no evidence for either DIC or sepsis.

Results - The whole blood TF and IL-8 expressions were up regulated in all non-IMHA groups. Similarly, the TF expression in IMHA dogs was high, but the intravascular IL-8 expression was not increased. The dogs with IMHA had a pronounced inflammatory response that included a high WBC, left shift and monocytosis in comparison to the other disease groups. Coagulation factor activities in IMHA dogs were decreased fitting consumptive coagulopathy and the acute phase proteins FVIII and fibrinogen were increased. The platelet parameters suggested platelet activation and high platelet turnover in IMHA dogs. The model that best explained mortality contained monocytosis, increased activated partial thromboplastin time and elevated plasma creatinine.

Conclusions - Whole blood TF gene expression is up regulated and may contribute to consumptive coagulopathy in dogs with IMHA. The concomitant low whole blood IL-8 expression suggests another route for increased TF expression than the NF-kB signalling pathway in monocytes. Increased TF expression by activated platelets is an alternative explanation and should be investigated.

Introduction

Canine idiopathic immune-mediated haemolytic anaemia (IMHA) is associated with a high mortality that mainly occurs in the first 2 weeks of the disease (1). This mortality is determined mostly by the presence of icterus (1-3) increases in urea and/or creatinine (1), leucocytosis (1, 4, 5), a left shift (1, 4) increase in coagulation times and/or decrease in fibrinogen concentration (1, 6, 7) and thrombocytopenia (1, 4, 6, 8). Abundant evidence has been presented for a state of hypercoagulability during the hospitalization period in dogs with IMHA (8-12). Apart from being a prognostic factor, leucocytosis and left shift occur commonly in dogs with IMHA (1, 9) and has been associated moderate to marked tissue damage (13). The leucocyte counts are intriguingly high in dogs with IMHA in comparison to dogs with sepsis (14).

IL-8 is a major chemotaxin that guides the margination process of leucocytes through increases in selectin expression (15). The dominant receptor for L-selectin on leucocytes is P-selectin glycoprotein ligand 1 (PSGL-1). PSGL-1 binds also to P- and E-selectin which is present on inflamed endothelium, and to P-selectin on activated platelets (15). At the location of an acute inflammation endothelial cells and leucocytes present increasing amounts of PSGL-1 thus optimizing the margination and extravasation process (16). Neutrophilia develops fast in inflammatory processes. In a canine experimental model of inflammation neutrophilia occurred within hours of the inflammatory stimulus, and was followed by a left shift within the first 24 hrs (17) due to depletion of the post-mitotic pool in the bone marrow (17-19).

The current cell based model of coagulation states that intravascular Tissue Factor (TF) formation initiates the intrinsic pathway of coagulation in absence of substantial epithelial cell damage which explains the link between inflammation and coagulation. In sepsis, inflammatory mediators such as LPS and TNF have been shown to induce TF expression on blood cells, particularly monocytes (20, 21). This occurs mainly through the NF-kB signaling pathway and leads to increases in other cytokines such as IL-8. Neutrophils (22) and platelets (23) have been reported as sources of TF as well. The role of platelets may be as part of leucocyte-platelet aggregates (21).

The goal of this study was to assess the contribution of blood borne TF expression to coagulation and inflammation in dogs with IMHA. It was hypothesized that expression of TF and IL-8 in dogs with idiopathic IMHA, dogs with sepsis, and dogs with DIC is increased in blood cells. Dogs with sepsis and dogs with DIC served as positive controls for up regulated expression. Basal, low expressions were assumed in healthy dogs. In addition two additional groups were added in order to quantitatively assess up regulation of gene expression that was not caused by sepsis or DIC, but due to systemic illness, surgery, or patient care related issues such as intravenous catheters.

Materials and methods

Experimental groups

TF and IL-8 expressions were measured in 6 groups; a group of healthy dogs (A) and five groups that were selected from dogs admitted to the intensive care unit of the small animal hospital of the Veterinary Faculty of the Utrecht University between September 2007 and October 2008. Selection criteria were based on diagnostic criteria in combination with the presence or absence of diffuse intravascular coagulation (DIC) or sepsis. DIC was defined as the presence of at least 2 of the following criteria: thrombocytopenia, abnormal PT and or APTT, hypofibrinogenemia, low plasma AT activity, or increased D-dimer concentrations (24). Sepsis was defined as cytologic, histologic or microbiological confirmation of infection combined with 2 or more of the following criteria: hypo- (< 37.8°C) or hyperthermia (39.4°C), tachycardia (>140/minute), tachypnea (>20/minute) or leucopenia (< 6 x 10^9/L), leucocytosis (> 16 x 10^9/L), or > 3% bands (25).

Group B had systemic disease, and group C underwent surgery necessitating intensive care treatment. Neither of the dogs in groups A, B, and C was allowed to have DIC or sepsis. Group D dogs had cytological of histological evidence for metastasized neoplasia and DIC. Group F dogs had sepsis. Group F, dogs with idiopathic IMHA had a Ht < 0.30 l/l, in combination with a positive Direct Agglutination Test (DAT) or spherocytosis (> 5 spherocytes per 100 objective oil immersion field) (26), and underlying disorders known to trigger IMHA were excluded (27). All procedures were approved by and performed according to the ethical committee as required under Dutch legislation.

Laboratory tests

Blood samples (1.8 ml sodium citrate 3.8%, 2 ml EDTA) were collected once very 24 hours. Complete blood counts (CBC), platelet parameters, coagulation profile were determined within 24 hrs in all dogs. The ADVIA 120 (Siemens Medical Solutions, Diagnostics, Deerfield, USA) was used to determine the CBC including haematocrit (Ht), white blood cell count (WBC) and differentiation, platelet count (PLT), and platelet parameters (PDW, PCT, MPV, MPM, MPC). Automated PT, APTT, fibrinogen, determination of coagulations factors activities, antithrombin, and protein C were performed with a coagulation analyzer (Thrombolyzer Compact X, BioMérieux, Holliston, USA). For semi-quantitative determination of D-dimers, a latex agglutination test (Minutex D-dimer Latex, Biopool, Leiden, Netherlands) was used (28).

The following tests were performed in the dogs with IMHA only. Coagulometric tests were used to determine the activity of specific coagulation factors in the collected plasma samples (29), the DAT, and examination of the blood smear for spherocytosis. In most cases determination of D-dimers and coagulation factor activities (FII, FV, FVII, FVIII, FIX, FX, and

FXI) were not performed immediately after blood collection. In those cases citrated plasma was stored at -70°C until measurements were performed, with a maximum storage period of 6 months.
The DAT protocol was used as described previously (30), using monovalent canine Coombs' reagents: rabbit anti-dog IgG (Fc), anti-dog IgG (H+ L) at 37°C, and anti-dog IgM (Fc) antibodies (Nordic Laboratories, Tilburg, the Netherlands) at 4°C.

RNA isolation and cDNA synthesis

In view of the large sample number but small samples size, the RT-reaction was performed only once. The MIQE guidelines, however, suggest to perform it twice (31). From each dog duplo samples were prepared by mixing 0.5 ml EDTA-anticoagulated blood with 1.3 ml RNA*later* (Ambion, Applied Biosystems, Foster City, California, USA). Samples were stored at -20°C. Total RNA was extracted from the samples using the RiboPure ^{tm} –Blood kit reagent (Ambion, Applied Biosystems, Foster City, USA) according to the manufacturer's instructions including a DNAse treatment to destroy contaminating genomic DNA and minimize the effect of pseudogenes. The RNA concentration was determined spectrophotometrically by the NANOdrop 1000 (Isogen Life Science, IJsselstein, The Netherlands). Bio-Rad iScript, containing both oligodT and random hexamer primers, was used to synthesize cDNA from 1µg of total RNA according to the manufacturer's instructions (iSCRIPT, Bio-Rad, Veenendaal, The Netherlands) (32).

Primer design and testing

Nine candidate reference genes representing cellular and ribosomal gene products (GAPDH, SRPR, HPRT, B2M, GUSB, HNRNPH, resp RPL8, RPS5, RPS19) were selected as candidate reference genes (32). Primers for the target genes, TF and and Il-8 were developed based upon known canine sequences (Ensembl, www.ensembl.ort; GenBank, www.ncbi.nih.gov/genbank/index.html). Relevant details of the primers are depicted in Table 1. The primers were designed with Oligo Explorer 1.1 (www.genelink.com/tools/gl-downloads.asp). The specificity and uniqueness of each primer were verified with the Basic Local Alignment Search tool (www.ncbi.nlm.nih.gov/blast) expecting return of Genbank accession numbers of candidate reference genes only. Sequences of the amplicon confirmed the specific genes. Amplification efficiency calculations from all standard curves were between 95 and 108%. All no template controls were negative, as were the minus-RT controls.
The gene expression stability calculations in this study were performed on the first sample that was taken when the dog entered the study. To determine the ranking of best performing reference genes in whole blood the stability of expression of the candidate reference genes was calculated using the GeNorm (33) and Normfinder (34) algorithm software.

Table 1. Primer characteristics of TF and IL-8.

Target genes	Primer	Sequence (5' → 3')	Gene bank accession number	Product Length (bp)
Tissue Factor	Forward Reverse	CACAGACACGGAGTGTGACCTC CCCCAGAGTAGTCABTBBCATC	NM_001024640	107
IL-8	Forward Reverse	CTBTTBCTCTCTTGGCAGC GGGATGGAAAGGTGTGGAG	XM_850481	122

Quantitative PCR

Q-PCR was done with the DNA-binding SYBR green using the BioRad iCycler MyiQ Real-Time PCR Detection System (BioRad, Hertfordshire, United Kingdom) according to the manufacturer's instructions. Primers (Eurogentec, Maastricht, The Netherlands) had a final concentration of 400 nM each. One microliter of cDNA was used per Q-PCR reaction. Optimal T_m for the reference genes was determined previously (35, 36). The optimal T_m for TF was 68°C, and for IL-8 63.3 °C. Reactions with a T_m less than 58°C started with 5 min at 95°C, followed by 40 cycles of 20 s at 95°C, 30 s at T_m, and 30 s at 72°C. This reaction was continued by a melting curve, stepwise increasing temperature each 15 s by 0.5°C, ranging from 60 to 95°C. In case the T_m was 58°C or higher, the elongation step at 72°C was omitted and T_m remained 30 s. Analysis of Q-PCR results were performed with iQ^{tm}5 software (Biorad, Veenendaal, The Netherlands) based on the mean Cq obtained from the duplo of each Q-PCR reaction (32).

Statistics

All data were visually examined for normality of distribution before statistical analysis. If data were not normally distributed they were logarithmically transformed or non-parametric tests were used. Comparisons of the results for the laboratory parameters and the normalised expression of the TF and IL-8 expressions between experimental groups and between consecutive samples was performed with Kruskal-Wallis. Additional pair wise comparisons between groups were made with the Wilcoxon's signed rank test using Holmes' correction for multiple testing. These comparisons were repeated after standardisation of the relative TF expression for the monocyte count since TF in peripheral blood is mainly expressed by monocytes. The relationship between laboratory variables and logarithm of the normalised relative expressions of TF and IL-8 in the experimental groups in the samples of the day of admittance to the intensive care unit were examined with linear regression analysis. The variables that were significant at the $P < 0.20$ level were introduced in a multivariate regression model allowing for interaction between the variables using a forward stepwise selection process. The resulting models were compared using the likelihood ratio test.

Survival analysis was performed in the dogs with idiopathic IMHA. The end point was death caused by IMHA. Dogs that were alive at the end of the study or died by a cause other

than IMHA were censored. Survival curves were drawn with the Kaplan Meier method. The variables recorded at the date of first diagnosis, with the exception of the variable breed, were evaluated in a univariate Cox's proportional hazard model and used to generate hazard ratios (HRs). The 95% CI intervals of the HR's are based on the Likelihood ratio test applying Firth's method (37). The variables significant at the P <0.20 level in the univariate analysis were introduced in a multivariate model allowing for interaction between variables. Multivariate analysis was performed by forward stepwise selection using a probability of P <0.05 in the likelihood ratio test as a criterion for inclusion. Compliance with the proportional hazards assumption was tested graphically by plotting the Schoenfeld residuals against time. For group F, the dogs with IMHA, a uni- and multivariate linear regression was performed for clinical characteristics, CBC results, coagulation test and specific coagulation factor activity results on respectively, the log normalised TF and IL-8 expressions. All statistical analyses were performed in R (www.r-project.org). $P < 0.05$ was considered significant in all analyses.

Results

Characteristics of the experimental groups

The laboratory characteristics of the 6 groups (A-F, n=77) are depicted in Table 2.

The definitive diagnoses for group B (n= 8) with systemic diseases were renal insufficiency (n=3), hypoadrenocorticism (n=2), idiopathic pericardial effusion (n=1), chronic active hepatitis (n=1), and traumatic epistaxis (n=1). Surgical procedures in surgical group C (n=11) were gastropexy (n=5), pericardial resection (n=1), total ablation auris and bullectomy (n=1), thoracotomy (n=1), ovariohysterectomy (n=1), pancreatic surgery for insulinoma (n=1), and enterotomy (n=1). The definitive diagnoses for the neoplasia's in group D (n=12) were gastric carcinoma (n=3), gastric leiomyosarcoma (n=1), splenic haemangiosarcoma (n=3), malignant lymphoma (n=1), fibrosarcoma of the liver (n=1), renal carcinoma (n=1), intestinal adenocarcinoma (n=1), and in one dog extensive lungmetastases were seen, however, the primary tumour was not identified. In group E sepsis (n=16) was caused by septic peritonitis (n=11), pyometra (n=1), pyothorax (n=1), pneumonia (n=1), abdominal abcesses (n=1), and valvular endocarditis (n=1).

Table 2. Laboratory parameters in healthy dogs and 5 disease groups.

	Healthy dogs A (n = 6)	**Internal disease B** (n = 8)	**Surgery C** (n = 11)	**DIC D** (n = 12)	**Sepsis E** (n = 16)	**IMHA F** (n = 24)	**P**
Parameter (Reference)	Median Range	Median Range	Median Range	Median Range	Median Range	Median Range	
Ht (l/l) (0.42 – 0.61)	0.50 0.41 – 0.54	0.29 0.22 – 0.52	0.38 0.23 – 0.46	0.27 0.08 – 0.51	0.32 0.22 – 0.44	0.16 0.06 – 0.34	A, E * ABCE, F **
WBC (x 10^9/l) (2.9 – 11)	8.6 6.6 – 12.5	10.4 5.6 – 15.2	15.6 10.8 – 34.9	19.4 7.6 – 37.9	21.8 4.8 – 39.0	33.5 5.5 – 86.8	B, E * B, F **
Lymphocytes (x 10^9/l) (0.8 – 4.7)	2.1 1.5 – 4.2	1.4 1.2 – 4.5	1.7 0.8 – 2.7	2.6 0.6 – 7.8	1.5 0.4 – 5.2	3.2 0.0 – 12.2	-
Monocytes (x 10^9/l) (0 – 0.9)	0.5 0.4 – 0.9	0.5 0.2 – 10.8	0.8 0.1 – 1.4	0.8 0.5 – 2.7	1.3 0.1 – 4.4	2.1 0.4 – 6.9	B, F *
Bands (x 10^9/l) (0 – 0.3)	0 0 – 0	0 0 – 0	0 0 – 1.8	0 0 – 1.3	0 0 – 0.6	1.3 0 – 9	AD, F * BCE, F **
PLT(x 10^9/l) (144 – 603)	251 145 – 385	286 198 – 570	255 166 – 453	72 22 – 137	225 99 – 691	200 24 - 652	A, D * BCEF, D **
PDW (%) (54.2 – 73.3)	68.3 67.3 – 70.1	65.7 53.4 – 70.3	59.8 54.2 – 79.6	70.2 17.6 – 83.7	68.4 60.7 – 77.4	68.8 59.0 – 87.9	-
Pct (0.002 – 0.004)	0.003 0.002 – 0.004	0.003 0.002 – 0.006	0.002 0.002 – 0.005	0.001 0.0 – 0.002	0.003 0.001 – 0.008	0.004 0.0 – 0.013	BCE, D* D, F **
MPV (fl) (6.8 – 13.4)	11.6 10.4 – 12.0	9.5 9.1 – 11.9	10.0 8.2 – 13.5	15.8 8.3 – 22.7	12.0 10.1 – 16.5	19.4 13.3 – 28.6	A,F / B,E * BCE, F **
MPM (pg) (1.42 – 2.46)	2.16 1.99 – 2.43	1.97 1.59 – 2.10	2.19 1.72 – 2.69	2.57 1.70 – 3.69	2.23 1.93 – 2.97	2.93 2.18 – 3.83	BCE, F **
MPC (g/l) (162 – 261)	229 224 – 234	230 188 – 240	234 211 – 247	225 134 – 250	216 183 – 237	214 144 – 243	C, F *
PT (sec) (6.7 – 9.5)	6.9 5.0 – 9.1	7.5 6.0 – 8.8	7.6 6.8 – 11.3	8.6 6.1 – 66.4	8.1 6.3 – 13.2	6.5 5.0 – 11.8	-
APTT (sec) (10 – 17.2)	14.2 12.4 – 17.4	16.3 13.8 – 18.4	16.3 14.3 – 18.9	17.5 12.9 – 44.2	17.5 13.4 – 27.2	16.6 11.2 – 58.0	-
Fibrinogen (g/l) (1.0 – 2.8)	2.7 0.8 – 2.9	2.4 1.7 – 18.5	5.7 1.8 – 9.3	2.0 0.5 – 4.3	5.1 1.7 – 8.9	7.7 1.1 – 13.1	D, E * D, F **
D-dimers (1-5) (< 2)	2 2 – 5	2.5 1 – 5	3 2 – 4	5 2 – 5	4 1 – 5	3.5 1 – 5	-
Antithrombin (%) (87 – 140)	131 103 – 149	111 106 – 130	107 84 – 208	84 54 – 128	101 35 – 160	153 61 - 244	-

Significant difference between the groups A - F are given in the last column: - $P > 0.05$; * $P < 0.05$; ** $P < 0.01$.

Group F, 24 dogs with idiopathic IMHA, consisted of 13 female dogs (10/13 castrated) and 11 male dogs (2/11 castrated) with a median age of 6 years (range, 0.85 – 12.4; SD = 3.1). The median duration of clinical signs was 3 days (range, 1 – 8; SD = 2). Seven dogs had macroscopic haemoglobinuria, 13 dogs were icteric. The DAT was positive in 21 dogs, and 18 dogs had spherocytosis. The median plasma creatinine and urea concentrations were, respectively, 65.5 µmol (range 47 – 200; SD = 32) and 7.6 mmol/l (range 3.1 – 32; SD = 6.6). The activity of all coagulation factors (n=13) was decreased except FVIII and FIX (Figure 1). Evidence for thromboembolisms in the splenic veins was documented in 3 dogs by ultrasonographic examination.

Significant differences between the experimental groups were found with respect to several parameters (Table 2). Dogs with IMHA had the lowest Ht and the highest WBC, bands and monocyte counts. Dogs with neoplasia had the lowest PLT and Pct. Pct was highest for dogs with IMHA. Dogs with IMHA had the highest MPV and MPM, and lowest MPC. Fibrinogen was lowest in the neoplasia group and highest in the IMHA dogs.

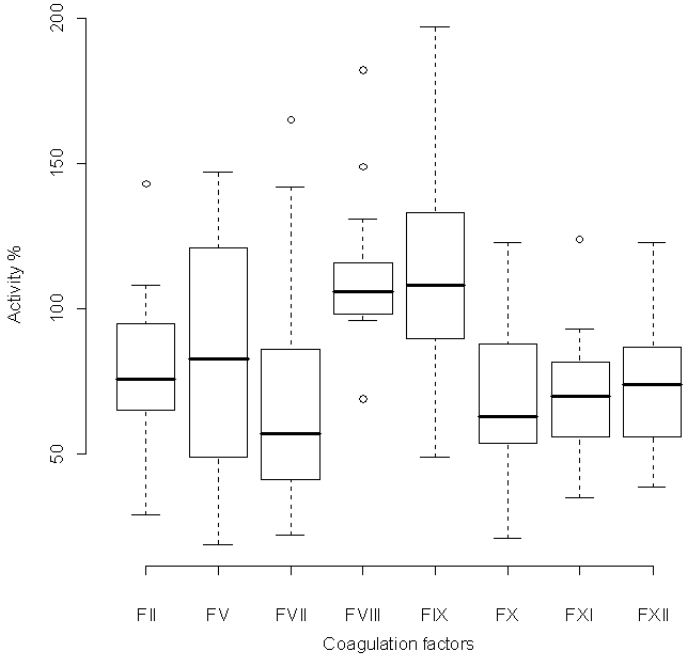

Figure 1. Coagulation factor activity in dogs with idiopathic IMHA.

Coagulation factor activity is depicted in dogs with IMHA on the day of admittance to the intensive care unit. The boxes represent the two middle quartiles with medians. Whiskers delineate the range. Circles depict outliers.

Real-time qPCR

The gene expression of TF and IL-8 were examined using qPCR and 9 different potential reference genes. Previously we showed that the expression of these genes in canine whole blood was subject to regulation that depended both on clinical class and leucocyte count (32). In GeNorm a combination of 7 genes (RPL8, RPS19, RPS5, GUSB, HNRPH, GAPDH and B2M) was suggested as the best choice for normalisation. The calculations with Normfinder demonstrated that SRPR, or the combination of HPRT and RPL8 had the most stable expression between the six groups. Since the ribosomal genes RPL8, RPS19 and RPS5 are most likely co-regulated we decided on the combination of RPL8, GUSB, GAPDH, HNRPH, B2M and SRPR as reference genes. Because of the higher leucocyte count in group F, the dogs with IMHA, the best set of reference genes was calculated anew following the same procedure. This resulted in the same combination of the 6 references.

TF and IL-8 expressions

There is a tendency, however, for TF expression to rise in groups B and C in comparison to groups A and B, with a further increase in groups D, E, and F (Figure 2). IL-8 expression was significantly increased in groups C and D in comparison to A and in group F in comparison to B, C, D, and E. The normalised relative expressions of TF and IL-8 after correction for the percentage of monocytes revealed no relevant differences from these results (data not shown).

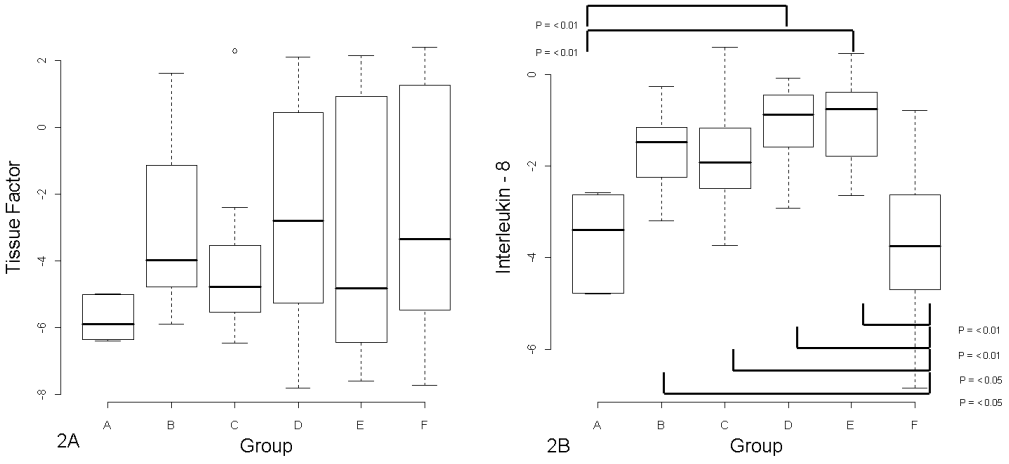

Figure 2. TF and IL-8 expression in whole blood.

Figure 2A depicts the natural logarithm of the normalised TF expression and Figure 2B depicts the natural logarithm of the normalised IL-8 expression on day of admittance to ICU. The boxes represent the two middle quartiles with medians. Whiskers delineate the range. Circles depict outliers.

Linear regression of TF and IL-8 expressions in all dogs

Next the association of TF and IL-8 expression with clinical parameters (WBC, Ht, monocyte count, PDW, PLT, MPV, PCT, MPM, APTT, PT, fibrinogen, D-dimers and AT) was assessed for all dogs in the study using regression analysis. An increase in TF expression was significantly associated with an increase in PDW. The group effect of dogs with IMHA on IL-8 expression was negative in contrast with the effect of the other disease groups and PLT had a negative effect on IL-8 expression (Table 3).

Table 3. Regression analysis for TF and IL-8 expressions.

Univariate linear regression model with P < 0.20							
	TF				**IL-8**		
Variable	**coefficient**	**SE**	**P**	**Variable**	**coefficient**	**SE**	**P**
PDW	0.09	0.03	< 0.01	Group	NA	NA	<0.001
Ht	-5.27	3.06	0.09	PCT	-323.65	75.54	<0.001
				PLT	-0.004	0.001	<0.01
				MPM	-0.95	0.34	<0.01
				Bands	-0.28	0.11	0.01
				MPV	-0.09	0.04	0.03
				Antithrombine	-0.01	0.00	0.03
				Fibrinogen	-0.10	0.06	0.08
				PT	0.04	0.03	0.11
				WBC	-0.02	0.01	0.18
Multivariate linear regression model							
Intercept	-9.16	2.28	<0.001	Intercept	-2.66	0.64	<0.001
PDW	0.09	0.03	<0.001	Group B	2.10	0.71	<0.01
				Group C	1.82	0.67	<0.01
				Group D	1.78	0.70	0.01
				Group E	2.56	0.64	<0.001
				Group F	-0.09	0.62	0.88
				PLT	-0.003	0.001	<0.01

Cox proportional hazards analysis in dogs with idiopathic IMHA

A Kaplan-Meier curve was drawn for the dogs with IMHA (Figure 3). The half year survival was 75% (95% CI: 59.5 – 94.5%). At 6 months 6 of 24 dogs had died of IMHA (all within the first 19 days). Two dogs were censored after respectively 9 and 72 days because they were lost for follow-up. The median time that dogs were in the study was 345 days (range 1 – 880 days). The proportional hazards assumption was valid for all variables that were significant in univariate and multivariate analysis. The results of the univariate analysis with P <0.20 are presented in Table 2. The TF and IL-8 expression had no significant association with death (TF, P = 0.458; maximum TF expression, P = 0.338; IL-8, P = 0.736). The best multivariate model included monocyte count (HR = 2.32; 95% CI: 1.34 – 6.05; n = 21) and APTT (HR = 1.12; 95% CI: 1.03 – 1.26, n = 21), and creatinine (HR = 1.15; 95% CI: 1 – 1.35); n = 21) as predictors of death (Table 4).

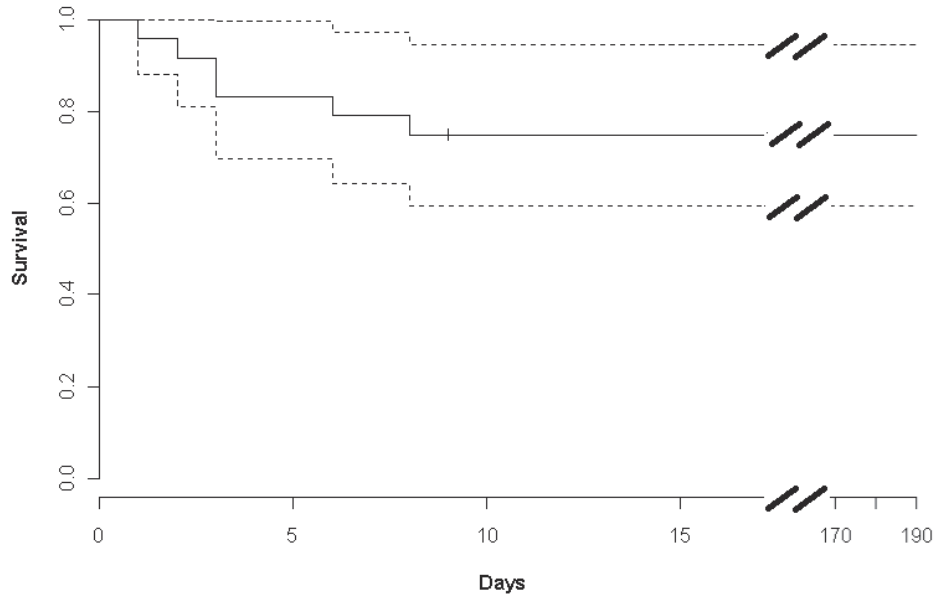

Figure 3. Kaplan Meier survival curve in dogs (n = 24) with idiopathic IMHA.
Solid line depicts the estimated Kaplan-Meier survival rate. Dashed lines depict the 95% CI. The half-year survival was 75% (95% CI: 59.5 – 94.5). The vertical stripe depicts a censored dog. The parallel slashes depict an axis gap.

Table 4. Cox's proportional hazards analysis in dogs with idiopathic IMHA.

Univariate Cox's proportional hazards model P < 0.20				
Variable	**Hazard ratio**	**95% CI**	**P**	**n**
Monocyte count (0.1 x 10^9/l)	1.06	1.02 - 1.12	0.002	24
APTT (seconds)	1.10	1.04 - 1.18	0.003	21
Urea (20 mmol/l)	17.2	1.77 - 189	0.017	24
Creatinine (20 µmol/l)	1.73	1.09 - 3.38	0.025	22
Hemoglobinuria	5.39	1.18 - 31.3	0.030	23
Leukocyte count (20 x 10^9/l)	2.95	1.07 - 8.65	0.036	24
Icterus	5.19	1.06 - 50.7	0.042	23
D-dimers (positive)	7.53	0.89 - 983	0.069	19
Bands (x 10^9/l)	1.22	0.91 - 1.55	0.159	20
Fibrinogen (g/l)	0.88	0.71 - 1.05	0.159	22
Spherocytes	3.04	0.60 - 30.63	0.195	24
Multivariate Cox's proportional hazards model				
Monocyte count (10^9/l)	2.32	1.34 - 6.05	0.001	21
APTT (seconds)	1.12	1.03 - 1.26	0.007	21
Creatinine (20 µmol/l)	1.15	1.00 - 1.35	0.058	21

Linear regression of TF and IL-8 expressions in dogs with IMHA

The best multivariate model that explained normalised relative TF expression in group the IMHA dogs, contained the variables MPM, urea, FII, MPV, icterus, bands, and FIX (Table 5). For IL-8 expression none of the variables reached significance in the univariate regression analysis.

Table 5. Regression analysis for TF expression in dogs with idiopathic IMHA.

Univariate analysis P < 0.20				
Variable	**beta**	**SD**	**n**	**P**
MPM (pg)	-3.65	1.34	23	0.02
F X (%)	-0.07	0.02	13	0.02
Icterus	-3.45	1.33	23	0.02
Urea (mmol/l)	-0.26	0.01	24	0.02
F II (%)	-0.06	0.03	13	0.03
F VII (%)	-0.04	0.018	13	0.04
MPV (fl)	-0.27	0.13	23	0.04
F XII (%)	-0.06	0.03	13	0.07
F IX (%)	-0.04	0.02	13	0.10
Reticulocytes (%)	-0.16	0.10	21	0.11
Antithrombine (%)	-0.01	0.01	18	0.14
F V (%)	-0.03	0.02	13	0.14
Kreatinine (µmol/l)	-0.04	0.03	22	0.15
Bands (x 10^9/l)	-0.45	0.33	20	0.19
PDW (%)	0.08	0.06	23	0.19
Multivariate analysis				
Intercept	20.20	0.35	13	0.01
MPM (pg)	-10.18	0.24	13	0.02
Urea (mmol/l)	-0.36	0.09	13	0.01
F II (%)	-0.04	0.04	13	0.05
MPV (fl)	0.50	0.02	13	0.02
Icterus	1.16	0.09	13	0.05
Bands (x 10^9/l)	-0.20	0.01	13	0.04
F IX (%)	0.01	0.002	13	0.13

Discussion

In this study we explored the possibility that the inflammatory response and hypercoagulability in dogs with idiopathic IMHA are caused by up regulation of intravascular TF and IL-8 expression. Gene expressions in dogs with IMHA were compared to healthy dogs, dogs with DIC, dogs with sepsis, and in addition two groups of dogs that underwent intensive care treatment but had no evidence for either DIC or sepsis. The dogs with IMHA had a marked inflammatory response in comparison to the other disease groups (Table 2). The

intravascular TF and IL-8 expressions were up regulated in all disease groups, except in the IMHA dogs where the IL-8 expression was low (Figure 2). The leucocytosis, monocytosis and left shift in the IMHA dogs suggest a high neutrophil turn over and predicted mortality (1) (Table 2 and 4). Severe post-mortem lesions in dogs with IMHA in another study correlated with moderate to marked leucocytosis (13).

IL-8 is a major leucocyte chemotaxin (38). IL-8 can be expressed by endothelial cells, fibroblasts, monocytes (38, 39) and neutrophils (40). In this study IL-8 presentation by the endothelial cells rather than by intravascular leucocytes may have been the driving force behind the leucocytosis (41, 42). Endothelial cells respond to acute hypoxia by an increase in P-selectin and an increase in transcription of IL-8 (43). Acute onset anaemia such as in the IMHA dogs that developed a mean Ht of 0.16 l/l within a median of 3 days leads to severe tissue hypoxemia (44, 45). The liver is an organ that is especially sensitive to hypoxia due to its partly venous blood supply. Icterus was seen in 13 of 24 IMHA dogs and associated with a negative outcome (Table 4). Hypoxia is also the leading force in trapping blood monocytes in tissues and converting them into macrophages (46, 47). Macrophages which are abundant in the liver respond to hypoxia by production of IL-1 and TNF and, in turn, induce IL-8 production. IL-8 is internalized by the endothelial cell and presented on the luminal side of post capillary venules (41). In combination, these mechanisms result in an increased stickiness of the endothelial cells and explain the marked inflammatory response in the dogs with hypoxia due to IMHA.

At least 3 of the dogs with IMHA in this study had documented splenic thromboembolism. Evidence for hypercoagulability in the dogs with IMHA has been reported by others (8-12, 48). The lowest MPC was reached in the dogs with IMHA and the dogs with DIC. Decreases in MPC occur due to platelet activation (49-51) and were useful to detect activated platelets in dogs (52). Interference of storage time with these results cannot be excluded since it has been shown that storage at room temperature over 16 hours leads to significant decreases in MPC (53). Large platelets, characterized by an increases in MPV and MPM are associated with an increase in platelet production rate (49, 51, 54) and with increased haemostatic capacity (49). Dogs with IMHA had a MPV and MPM that was similar to the DIC group, but significantly higher than in the other disease groups. Interference of storage time is not likely since significant increase in MPV an MPM were measured only after respectively 48 and 64 hours of storage at room temperature (53) and all samples in this study were measured within 48 hours. The high Pct, large range of PLT, and high PDW, which correlates linearly with MPV (51), are suggestive of high thrombocyte proliferation.

Increases in PT, APTT were seen in dogs with IMHA, dogs with sepsis, and in dogs with DIC. The decrease in the activities of almost all coagulation factor activities is additional evidence for the presence of a consumptive coagulopathy in the dogs with IMHA. The contrastingly high concentrations of FVIII and FIX may have been due to the presence of activated

coagulation factors which can lead to wide variations in one-stage assays such as used in this study (55). The high FVIII in combination with high fibrinogen concentrations rather than the expected decrease due to DIC in the dogs with IMHA may also be contributed to the fact that both are acute phase proteins (45). The best explanation for the platelet parameter and coagulation parameter results in the dogs with IMHA is activation and consumption of platelets and coagulation factors compensated by increased platelet production. The association of PDW with TF expression in all dogs (Table 3) and the association of decreases in MPV and MPM with TF production in the dogs with IMHA (Table 5) suggest a role for platelets in TF expression.

Monocytosis was identified as an independent prognostic factor in this study (Table 4). This is supported by a recent study that measured increased concentrations of IL-15, IL-18, GM-CSF, and MCP-1, which are all produced by cells of the monocyte lineage, in non-survivors of canine IMHA, and additionally, IL-18 and MCP-1 were identified as independent prognostic factors (56). MCP-1, but also IL-8, are chemotactic peptides released during an acute phase response (57, 58). Changes in acute phase protein concentrations have also been reported in dogs with IMHA (59-61). The leucocytosis may have been part of the acute phase response, but neutrophilia and monocytosis in dogs with IMHA may also be explained by the effect of glucocorticoid therapy. This seems unlikely, however, since in that case lymphopenia would have been expected (Table 1) (62).

In this study we choose to measure gene expressions in whole blood rather than in isolated cellular fractions because of the advantageous small sample size needed for qPCR and to prohibit up regulation of cytokine expressions caused by isolation procedures of leucocytes (63). The consequence is that the cellular origin of the TF and IL-8 expressions remains unknown. Leucocytes and platelets of the central rather than the marginal pool were the main contributors to the mRNA that was isolated. The high TF expression in the disease groups in comparison to the healthy dogs may be explained by activation of the NF-kB signalling pathway in monocytes. In that case, however, up regulation of IL-8 expression would be expected in the IMHA dogs as well. Another cellular source of TF in the IMHA dogs may be platelets which play a role by inducing endothelial TF expression through CD40L-CD40 interactions (64, 65), platelet- monocyte interactions (65), platelet-neutrophil interactions (65), and by platelet TF expression (23). Presence of TF mRNA has been demonstrated in monocytes (66), endothelial cells (67), neutrophils (22), and platelets (23), although the latter three sources have been debated and, to our knowledge, not been examined in dogs (66). Of these, increased platelet TF expression presents an attractive explanatory model and should be determined in further studies.

List of abbreviations

APTT, Activated Partial Thromboplastin Time; B2M, beta-2-Microglobulin; DAT, Direct Agglutination Test; DIC, Disseminated Intravascular Coagulation; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor; GUSB, beta-Glucuronidase; Ht, Haematocrit; HNRNPH, Heterogeneous nuclear ribonucleoprotein H; HPRT, Hypoxanthine phosphoribosyltransferase; HR, Hazard Ratio; IL-8, Interleukin 8; IMHA, Immune Mediated Haemolytic Anaemia; LPS, Lipo Poly Saccharides; MPC, Mean Platelet Content; MPM, Mean Platelet Mass; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; Pct, Plateletcrit; PSGL-1, P-Selectin Protein Ligand 1; PT, Prothrombin Time; RPL8, Ribosomal protein L8; RPS5, Ribosomal protein S5; RPS19, Ribosomal protein S19; SD, Standard Deviation; SRPR, Signal recognition particle receptor; TF, Tissue Factor; TNF, Tumour Necrosis Factor; WBC, White Blood Cell Count;

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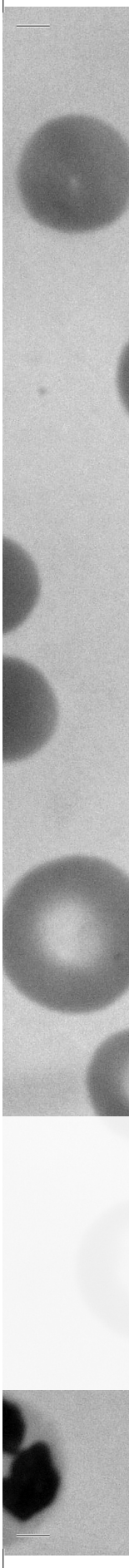

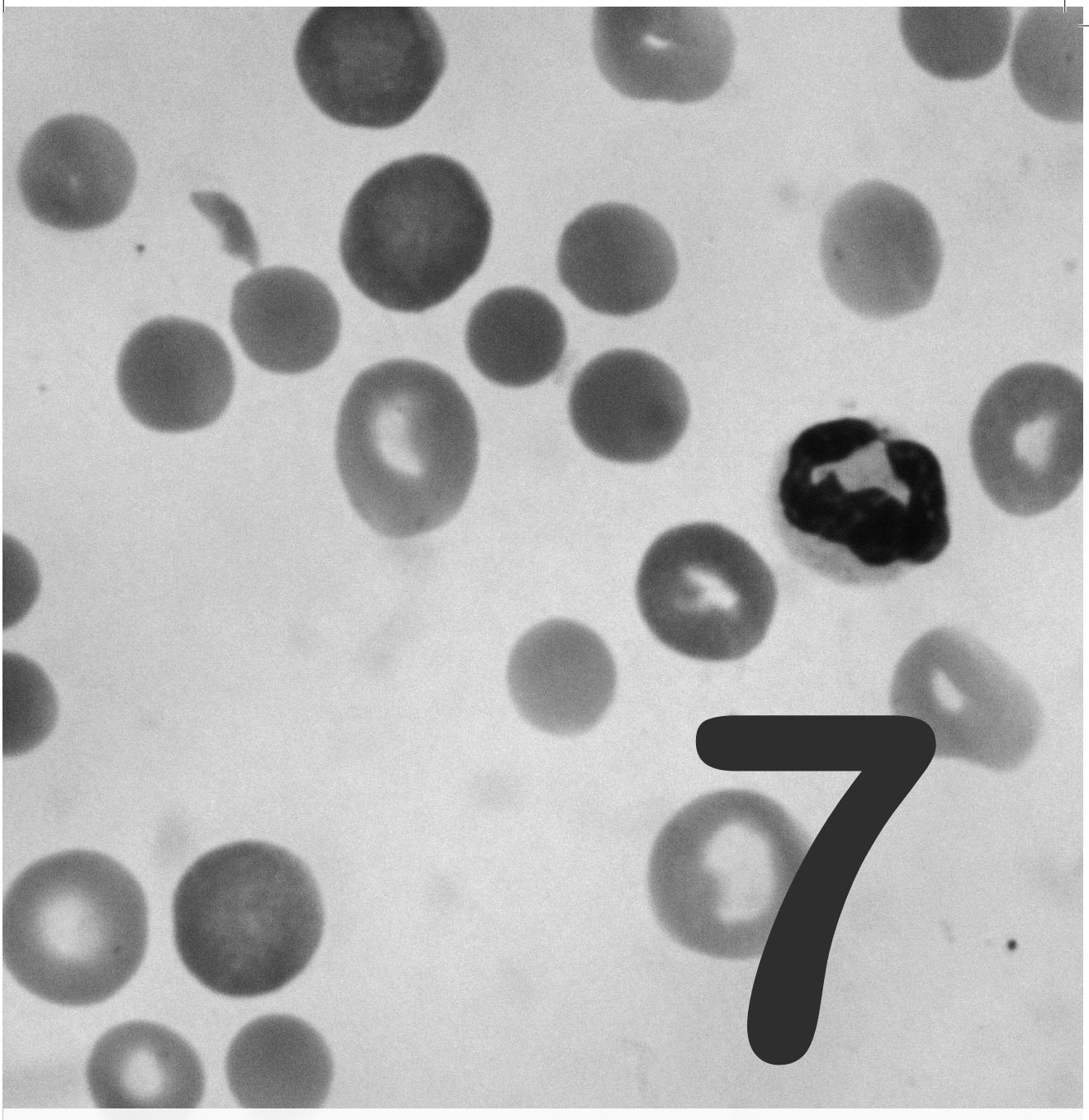

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Prognosis of canine idiopathic immune-mediated haemolytic anaemia: a review with recommendations for future research

CJ Piek

Vet Q. accepted (based on this manuscript)

Abstract

Background - Idiopathic immune-mediated haemolytic anaemia (IMHA) is one of the most common immune-mediated diseases of dogs.

Objectives - To review current knowledge of canine IMHA on aetiology, clinical presentation, diagnosis, complications, and treatment, in an attempt to establish why its outcome is still so poor.

Methods - A Pubmed search using the search terms "canine immune-mediated hemolytic anemia".

Results - Clinical signs of anaemia develop within 3 days and dogs present with a median haematocrit of 13%, leucocytosis, a left shift, and reticulocytosis; coagulation test results support the presence of disseminated intravascular coagulation. About 50% of dogs die in the first 2 weeks after presentation, and analysis of risk factors suggests that mortality is associated with hypercoagulability, inflammatory response, and liver and kidney failure. A positive direct agglutination test, spherocytosis, and true autoagglutination are widely accepted tests to demonstrate anti-erythrocyte antibodies, but are not yet standardized. To date, there is no evidence to support the efficacy of immunomodulators in addition to corticosteroids in the treatment of IMHA.

Conclusions - Despite numerous investigations, the prognosis of IMHA remains dismal. There is an urgent need to validate and standardize diagnostic tests and criteria, and clinical trials might benefit from stratifying dogs by mortality risk. Analysis of samples from well-defined cases of canine IMHA might provide insight into the aetiology and pathophysiology of IMHA.

Introduction

The publication of the properties of antihuman antisera in the detection of sensitized erythrocytes by the veterinarian Dr. Robert Coombs (1) in 1947 was the foundation for the development of the Direct Agglutination Test (DAT) that carries his name. Other laboratory characteristics of acquired haemolytic anaemia's such as autoagglutination, spherocytosis, and increased osmotic fragility had been recognized already, but were thought to be specific for congenital haemolytic anaemia's. The DAT clearly identified an immune-mediated pathogenesis in patients which displayed the characteristic signs of haemolysis (2). Since then, the DAT plays a pivotal role in the diagnosis of immune-mediated haemolytic anaemia. Idiopathic immune-mediated haemolytic anaemia (IMHA) is one of the most common immune-mediated diseases of dogs (3, 4). The incidence was estimated as 0.2% in a case-load study of a general veterinary university hospital (5). The first case series of 19 dogs with IMHA was described in the 1960s (6). Clinical signs were identical to today: pallor (n =19), icterus (n=8), and splenomegaly (n=10). Six of these cases had petechiae, and may have had immune-mediated thrombocytopenia as well. Anti-erythrocyte antibodies were demonstrated with the DAT in all dogs. The dogs were treated with prednisolone. Six of the 19 dogs died during the initial haemolytic episode, and a further 5 during recurrences (6). Despite numerous studies since then, the mortality of IMHA remains high (7-12).
The aim of this article is to review current knowledge of canine IMHA, its diagnosis and treatment, with a view to understanding why the prognosis remains so poor. The review concludes with recommendations to optimize future research.

Methods

A Pubmed search (http://www.ncbi.nlm.nih.gov/sites/entrez) using the search terms "canine immune-mediated hemolytic anemia" was performed. The focus was on aetiology, clinical signs, diagnosis, risk factors, and evaluation of treatment in canine IMHA. Only studies published in peer-reviewed journals were used.

Results

Aetiology

Loss of self tolerance in canine IMHA

IMHA is a type II antibody-mediated reaction characterized by the binding of anti-erythrocyte antibodies to the erythrocyte membrane leading to phagocytosis or complement-mediated lysis of antibody-coated erythrocytes (13). In dogs with IMHA different erythrocyte glycophorins with a molecular weight between 29 and 42 kD, and the anion-exchange molecule, band 3, were identified as major target antigens (14). In humans the dominant targets that have been identified are part of the Rhesus blood group antigen (15). Dog erythrocyte harbour a membrane molecule homologous to the human Rhesus antigen but it is not clear if this is a target in canine IMHA (16).

The loss of immunological tolerance to these epitopes may be the result of a change in autoantigen processing revealing cryptic epitopes or molecular mimicry to a cross-reactive environmental molecule (17). Molecular mimicry has also been proposed as a mechanism that prevents clonal deletion and induces activation of self-reactive B-cells (17). In dogs research has focused on assessing the activation status of autoreactive T cells in lymphocyte proliferation tests (18, 19). Secondary, or recall, proliferative responses peak earlier than primary proliferative responses (13). Clinically healthy dogs have autoreactive T-cells but showed a primary proliferation response only, in contrast to the presence of autoreactive T-cells with recall kinetics in dogs with IMHA suggesting the loss of tolerance (19). Interestingly, primed autoreactive T-cells were also present in siblings of dogs with IMHA which supports a genetic susceptibility to develop IMHA (13).

Ligand binding of the co-stimulatory molecule CTLA-4 on activated T-cells to the B7-2 counter receptor on antigen presenting cells results in down regulation of the T-cell response (20). Anti-CTLA-4 antibodies led to increased T-cell responses (21). Autoantibodies against CTLA-4 were demonstrated in several canine autoimmune diseases but not in dogs with IMHA, however (22).

Signalment and genetic factors

Breed predisposition and familial occurrence suggest that a genetic component contributes to the susceptibility for IMHA (Table 1) (23, 24). Moreover, some susceptible breeds may have a higher incidence of more than one immune-mediated disease (23-25). Canine IMHA is associated with both susceptible and protective DLA haplotypes which, interestingly, are associated with different effects in specific breeds (26) (Table 1). This suggests that the recognition of foreign proteins or self proteins by the major histocompatibility complex (MHC) proteins is one of the key events in the development of immune-mediated disease (27). The presence of autoreactive T-cells in dogs with IMHA supports the view that MHC molecules are candidate susceptibility genes for IMHA (19).

The distribution and frequency of both DLA alleles and haplotypes varies substantially between breeds as a result of selective breeding (28-31). Several other genes involved in the immune response cluster in the MHC region and show strong linkage disequilibrium with MHC genes (32). Therefore, susceptibility to IMHA might be linked to another gene within the MHC region. Odds ratio's or hazard rates attributed to breed are higher than those conferred by DLA haplotype which suggests that the risk for IMHA cannot be explained solely by the DLA haplotype (26, 33-35). An increased incidence of IMHA has been observed in female (11, 35-37) and neutered (11, 36) dogs and an association has been reported with oestrus and whelping (38).

Table 1. Breed predispositions, DLA-haplotypes, and sex in canine IMHA

Breed	OR	HR	95% CI	Reference
Airdale terrier	45.3	-	5.5-∞	(33)
Bichon frise	5.3	-	1.2-22.5	(35)
Cocker spaniel	-	-	-	(34, 36, 37, 39, 40)
	12.2	-	4.5	(35)
	-	3.3		(9)
	-	5.9		(40)
	-	5		(11)
	6.7	-		(10)
English Springer spaniel	10	-	1.3-74.7	(33)
	32.4	-	-	(10)
Finnish spitz	69.9	-	2.1-2287	(35)
Hungarian Viszla	10	-	1.3-74.7	(33)
Maltese	2.8	-	1.5-4.9	(33)
Miniature pincher	7.4	-	1.2-47.1	(35)
Miniature schnauzer	-	4	-	(11)
Old English sheepdog	-	-	-	(23)
Rough-coated Collie	22.2	-	-	(34, 35)
Shih Tzu	-	-	-	(39)
Welsh corgi	-	-	-	(39)
DLA-haplotype				
DRB1*00601/DQA1*005011/DQB1*00701	1.8	-	1.2-3	(26)
DRB1*015/DQA1*00601/DQB1*00301	2.6	-	1.1-5.8	(26)
DRB1*001/DQA1*00101/DQB1*00201	0.4	-	0.2-0.9	(26)
Sex				
Females	2.1		1.1-4.1	(35)
Females	-	-	-	(11, 36, 41)
Neutered females	-	-	-	(11, 36)
Neutered dogs	-	-	-	(11, 36)

Risk factors for IMHA were obtained in these studies by comparison of breed, haplotype, and sex incidence in a population with IMHA dogs versus the general hospital population. In case of Bichon frise, Finnish spitz, Miniature Pinscher, and the Rough-coated Collie the author acknowledged that the numbers of dogs with this breed with IMHA may have been too low in study to accurately assess risk on IMHA.
The study that evaluated the risk associated with DLA haplotype revealed that different breeds had different DLA associations with IMHA (26).
OR, Odds Ratio; HR, Hazard Ratio; -, not reported.

Clinical presentation

Clinical signs

IMHA can occur at any age. Though most reports describe an onset after the first year of age (3, 9, 10, 34, 42), idiopathic IMHA developed in 8 of 222 dogs before one year of age in another study (7). Mean age of onset of IMHA is 6 years (combined result of n= 340) (9, 10, 33, 34, 43).

Most dogs with IMHA develop anaemia rapidly, possibly over a period as short as 3 days (Table 2). Non-specific signs, such as lethargy and loss of appetite, occur in most dogs and are accompanied by vomiting and diarrhoea in 20-30% of cases (7, 8, 37). Signs more specific for haemolysis are yellow to orange discolouration of the faeces and red urine (7-10, 37). The physical examination reveals clinical signs caused by anaemia, such as, tachycardia, tachypnoea, steep pulse, pale mucous membranes, and systolic murmur (9, 10). Fever is a common clinical sign (44), occurring in 46% of dogs (7, 8). Petechiation as a result of concurrent severe thrombocytopenia is reported incidentally (2-5% of cases) (8, 9) and may be due to concurrent immune-mediated thrombocytopenia (ITP). Cranial organomegaly, due to splenomegaly and hepatomegaly, is found in up to 40% of cases (8-10, 34).

Table 2. Duration and incidence of clinical signs

	n=60 (9)[a]	n=42 (34)	n=18 (37)	n=70 (10)	n=149 (8)[b]	n=73 (7)[c,d]
Duration (days)	-	90% <3	83% <3	-	median 6	median 3
Lethargy (%)	93	86	94	99	98	-
Pallor (%)	77	76	-	97	98	99
Anorexia (%)	-	90	83	99	80	76
Vomiting (%)	-	-	30	-	30	37
Diarrhea (%)	-	-	17	-	15	23
Icterus (%)	45	50	67	51	37	37
Red urine (%)	32	-	44	13[e]	44	24
Heart murmur (%)	27	-	-	47	-	-
Organomegaly[f] (%)	38	25	-	43	34	-

In this table the results of six studies on canine idiopathic IMHA have been summarized. In three studies, however, 1-5% of dogs had petechiation that may have been due to concurrent ITP (7-9, 45). [a] In 1 dog (2%) petechiae were noted; [b] In 8 dogs (5%) petechiae were noted; [c] Fever in 46% of dogs; [d] Petechiae in 1% of dogs; [e] Haematuria; [f] Cranial abdominal organomegaly established during physical exam.

Complete blood count (CBC)

The CBC results are remarkably similar in different populations of dogs with idiopathic IMHA despite differences in study inclusion and exclusion criteria (Table 3). At the time of presentation, most dogs have severe anaemia with a haematocrit of 12–14%, but some dogs may show a more chronic disease course and have a higher haematocrit (13). Tissue oxygenation is severely impaired at an haematocrit below 12% (46) and can result in severe exercise intolerance, tachypnoea, and tachycardia, which may be the main reasons for referral since dogs with these symptoms require transfusion.

Pronounced leucocytosis with a left shift is a common laboratory feature at presentation, or alternatively, leucocytosis may develop in dogs with leucopenia or a leucocyte count that is within the reference range at presentation (47). Monocytosis is present in about 50% of cases (48).

Table 3. CBC in dogs with IMHA.

	Mean	Median	Range	References
Ht	14.8	12-14.5	4-32	(7-10, 33, 34, 36, 37, 42)
	n=353	n=412	n=444	
Corrected reticulocytes	1.3	0.9-2.7	0-19.2 %	(7-9, 34, 36)
	n=60	n=287	n=282	
WBC	34.4	21.7-38.7	2.1-130	(7-10, 33, 36, 37, 42)
	n=312	n=554	n=444	
Band neutrophils	2.1 ± 2.6	1-1.4	0-22.1	(7, 8, 36, 37, 45)
	n=72	n=294	n=294	

In this table the results of the CBC at presentation in a referral institution have been summarized for 614 dogs based on data from 9 different studies (7-10, 33, 34, 36, 37, 45). The means for Ht, reticulocytes, WBC and band neutrophils have been calculated from the mean and the number of dogs as reported in the studies referred to in this table. In case of the median the range of the medians that were reported are given. The range given in the table encompasses all the ranges reported in the respective studies. The inclusion criteria for these studies consisted of different combinations of the following criteria: a restriction on the Ht (< 30 – 35%), positive Coombs' test, autoagglutination, evidence of haemolysis (hyperbilirubinemia, bilirubinuria, haemoglobinemia, haemoglobinuria, and/or spherocytosis), and regenerative erythroid response. Exclusion criteria focused on the exclusion of possible underlying disorders such as vaccination, medication, neoplasia, and infectious disorders, pretreatment with cytostatics or longstanding immunosuppressive therapy.
WBC, White Blood Cell Count; Ht, haematocrit.

Coagulation

The prothrombin time is increased in up to 50% of dogs, and the activated partial thromboplastin time (APTT) is increased in 50–60% of dogs with idiopathic IMHA (8, 9, 36, 45). Thrombocyte counts are below 50 x 10^9/l in about 20% of dogs (8, 9, 34, 36, 45). These

changes suggest the presence of diffuse intravascular coagulation in many dogs, which is supported by the finding of a low fibrinogen concentration in 20% of dogs and increased concentrations of D-dimer and fibrin degradation products and a decreased antithrombin (AT) activity (8, 45) (Table 4). In a study of 149 dogs with miscellaneous diseases, low AT activity predicted mortality using a cut-off value of AT activity of 60% with a sensitivity of 58% and specificity of 85%: sixteen of 21 IMHA dogs in this study had low AT activity (49). However, another study found fibrinogen concentrations to be raised in 30–90% of dogs, which might be because fibrinogen is secreted during the acute-phase response (50).

Table 4. Coagulation test results and thrombocyte counts in canine idiopathic IMHA.

Test		Incidence %	n	Reference	Test		Incidence %	n	Reference
PT	↑	10	20	(45)	FDP	↑	57	28	(36)
		28	34	(9)			60	20	(45)
		28	32	(36)					
		46	98	(8)	AT	↓	45	20	(45)
APTT	↑	45	20	(45)	Thrombocytes		18	60	(9)
		47	32	(36)	< 50 x 10^9/l		22	72	(36)
		56	34	(9)			24	42	(34)
		67	98	(8)			25	20	(45)
							25	148	(8)
Fibrinogen	↑	34	96	(8)					
		62	24	(9)	< 100 x 10^9/l		38	42	(34)
		85	20	(45)			44	18	(37)
					< 200 x 10^9/l		65	20	(45)
	↓	18	96	(8)			68	60	(9)
							67	42	(34)
D-dimers	↑	80	20	(45)			70	72	(36)

This table summarizes the results of 6 studies that report on increases (↑) and decreases (↓) of coagulation test results and thrombocyte counts in at least 150 dogs with idiopathic IMHA (8, 9, 34, 36, 37, 45). All studies were retrospective except for one (45) and coagulation parameters were determined in a subset of the total number of dogs in the study only. The number of dogs in which the tests were performed is given per test in the table. It should be realized however that selection bias may have resulted in overestimation of the presence of abnormal coagulation test results.

Diagnosis

Diagnostic testing

The laboratory diagnosis of IMHA rests upon the demonstration of an immune-mediated mechanism for the haemolysis. Although the direct Coombs' test is still the main method used to demonstrate anti-erythrocyte antibodies, alternative methods include flow cytometry (51), ELISA (52), and a gel test (53), although these tests are not routinely available in veterinary practice. The diagnostic performance of the direct Coombs' test depends on many factors, such as use of polyspecific instead of monospecific Coombs reagents, a one-dilution only tube test instead of multititre plates, incubation at 37°C only versus additional incubation at 4°C (54). The differences in the set-up of the Coombs' test in different laboratories may explain the reported large range in sensitivity, 50–80% (55, 56).

Anti-erythrocyte antibodies are characteristic of both secondary and idiopathic IMHA. The pattern of antibody reactivity in the direct Coombs' test may be of diagnostic significance. IgM reactivity is more common in dogs with underlying disorders, and in one study was associated with less severe anaemia (54, 57, 58). Low Coombs' titres may be seen in sick dogs without obvious signs of haemolysis (56, 59). These findings demonstrate that the choice for either polyspecific or monospecific reagents and the cut-off titre in the DAT protocol determine the diagnostic performance as has been demonstrated in a study that tested two different procedures for the DAT (56). The development of a commercially available gel test to detect antigen-antibody reactions suggests that it may be possible to standardize anti-erythrocyte antibody testing (53).

Currently, the use of multiple tests is advocated to overcome the problem of the low sensitivity of the direct Coombs' test for diagnosing IMHA. Spherocytosis can be the result of immune-mediated erythrophagocytosis, but may also be found in some hereditary spherocytic disorders, such as spectrin deficiency (60). These diseases are rare, and spherocytosis is generally accepted as pathognomonic for IMHA. Spherocytes are quantified microscopically, usually as counts per high-power field (61-63). Potential disadvantages of this method are a high interobserver variability and the fact that the number of spherocytes per high-power field depends on the haematocrit.

Autoagglutination is generally accepted as a diagnostic criterion for IMHA (4, 64, 65). To avoid a false-positive outcome, erythrocytes that aggregate due to the presence of anti-erythrocyte antibodies, the so-called true autoagglutination, must be distinguished from rouleaux, which are conglomerates of erythrocytes that develop for non-immunological reasons, especially at lower temperatures. The basis of the in-saline slide agglutination test used for this purpose is the fact that saline may break up rouleaux formation but not erythrocyte aggregates. The ratio of saline to erythrocytes used differs between protocols from a 1:1 to 10:1 ratio (4, 64, 65). Agglutination can be viewed macroscopically on a slide, but most investigators

prefer to confirm the absence of aggregates by microscopic examination. The disappearance of erythrocyte aggregates in the in-saline slide agglutination test does not exclude IMHA, because repeated washing of erythrocytes may break up erythrocyte aggregates (64).

Secondary IMHA

It is clinically important to distinguish between idiopathic IMHA and secondary IMHA because therapy and prognosis may be different. The incidence of secondary IMHA in dogs is 20-25% (8, 66).

Risk factors for secondary IMHA can be grouped into medications, vaccinations, neoplasia, infections, and systemic immune-mediated disorders (4) (Table 5). Most of these risk factors have been taken from the human literature (67, 68), and relatively few have been confirmed in dogs. Sulfonamides, cephalosporin, and carprofen have been linked with IMHA (69-73). The association of vaccination in one study was not confirmed in two other studies (36, 43, 74). In contrast with the situation in humans, where the occurrence of non-Hodgkin lymphoma is associated with IMHA (75), in dogs immune-mediated thrombocytopenia has been found to be associated with lymphoma, but no such association has been confirmed for IMHA (5). IMHA may be diagnosed as part of a systemic immune-mediated disease such as systemic lupus erythematosus (76), or as part of Evans' syndrome.

Many studies have reported on the seasonality of the onset of IMHA (9, 11, 33, 34, 43, 66), with increased exposure to infectious agents in certain seasons being suggested to trigger the disease. The occurrence of IMHA secondary to vector-borne diseases affects dogs that live or have visited the geographic area where the vector is endemic (77-79). Inconsistencies in disease incidence between countries might be explained by differences in exposure risk in different geographic locations. For example, despite cases that may suggest the opposite (80, 81), *Haemoplasma canis* is not an important trigger for IMHA in dogs in the UK (82).

Table 5. Reported environmental triggers for canine IMHA.

Medications	Reference	Vaccination	Reference
Sulfonamides	(69-71)	Increased risk on IMHA	(43)
Cephalosporin	(72)	No association IMHA	(36)
Carprofen	(73)	No association	(74)
Season		Infections	
No seasonal influence (Australia)	(33)	*Haemoplasma canis*	(80, 81)
No seasonal influence	(9)	*Haemoplasma canis,* no trigger	(82)
↑ May and June (Pennsylvania, VS)	(34)	*Ehrlichia canis*	(78)
↑ in the fall (Pennsylvania, VS)	(43)	*Babesia gibsoni*	(79)
↑ warm months (April-September)	(11)		
↑ cooler months (October – March)	(66)		
Malignancies		Miscellaneous	
Sarcoma	(83)	Bee sting	(84)

Increased incidence of IMHA (↑) has been associated with several diseases.

Differential diagnosis

The generally accepted diagnostic features of idiopathic IMHA include immune-mediated haemolysis and the exclusion of underlying triggering disorders (4). Knowledge of the known triggers for IMHA will optimize the identification of secondary IMHA. Many cases of secondary IMHA are relatively straightforward to diagnose because the dogs may display signs and symptoms due to the underlying disorder and different from those expected in idiopathic IMHA. Two clinical entities, Evans syndrome and pure red cell aplasia (PRCA), merit special attention since their presentation is similar to that of idiopathic IMHA.

IMHA and immune-mediated thrombocytopenia

Evans syndrome, defined as the combined or sequential occurrence of IMHA and ITP, was first reported by Evans et al. in 1951 and is a rare disease in humans (85). The incidence of Evans syndrome in dogs has been estimated at 0.01% (86). In addition to signs due to IMHA, dogs with Evans syndrome develop signs of haemorrhagic diathesis, such as petechiae or ecchymosis (in 13 of 21 dogs) (87), melena (in 4 of 12 dogs) (86), oral bleeding (in 4 of 21 dogs and 3 of 12 dogs) (86, 87), and haematemesis (in 1 of 12 dogs) (86). The diagnosis of ITP should be made based upon exclusion of other causes of thrombocytopenia, preferably in combination with the demonstration of antiplatelet antibodies (88-91); however, antiplatelet antibody testing is not routinely available in veterinary practice. The thrombocyte count cut-off to distinguish between ITP and disseminated intravascular coagulation in dogs has been reported as 15 x 10^9/l (92), although another study reported median thrombocyte counts of 32 x 10^9/l in dogs with ITP and 55 x 10^9/l in dogs with DIC (93).

The prognosis of Evans syndrome is reported to be worse than that of the individual constituent diseases (66, 87), although another study reported the prognosis to be similar to that of the individual constituent diseases (86). The thrombocyte counts in these studies varied between 10 – 66 x 10^9/l (n=10, IMHA + ITP; n=26, ITP), and 0 – 46 x 10^9/l, respectively, suggesting that cases with IMHA and DIC may not have been sufficiently excluded and that in fact the prognosis of dogs with IMHA and DIC has been reported (66). Contrastingly, a retrospective study in 12 dogs with IMHA and thrombocyte counts below 15 x 10^9/l revealed a mortality rate similar to mortalities reported in the literature for each disease alone (92).

Non-regenerative anaemia: non-regenerative IMHA or Pure Red Cell Aplasia (PRCA)

Up to 33% of dogs with idiopathic IMHA present with non-regenerative anaemia. As reticulocytosis takes about 4-5 days to develop and most dogs develop clinical signs about 3 days before presentation, regenerative anaemia should develop a few days after first presentation (4). If reticulocytosis fails to develop in this period, non-regenerative IMHA and PRCA should be considered in the differential diagnosis (94-97). PRCA is characterized by total erythroid aplasia, in contrast with non-regenerative IMHA, where there is bone marrow erythroid hyperplasia or erythroid maturation arrest (94, 96)

Dogs with non-regenerative IMHA and PRCA (n=43) had clinical signs for more than 7 days at presentation and were clinically stable seeming to tolerate the anaemia well suggesting physiological adaptation (96). In the non-regenerative IMHA cases, the median Ht at presentation varied between 11 - 15% and was even lower, around 8%, in dogs with PRCA (94). Reticulocyte counts < 60 x 10^9/l were used as inclusion criterion (94, 96)). Reticulocytopenia of this degree has been reported in positive in about 50% of cases with non-regenerative IMHA or PRCA (n=43) (96) but was negative in all 13 dogs diagnosed with PRCA in another study where one of the diagnostic criteria was selective erythroid aplasia in the bone marrow (97).

Dogs with non-regenerative IMHA characterized by erythroid hyperplasia were found to have a lower 60-day survival than dogs with non-regenerative IMHA characterized by erythroid maturation or PRCA (94). Besides erythroid hyperplasia, these dogs had multiple pathological events, such as dysmyelopoiesis, myelonecrosis and myelofibrosis, interstitial oedema, haemorrhage, acute inflammation, and haemophagocytic syndrome, that might have aggravated ineffective erythropoiesis (94). In contrast, in dogs with PRCA erythroid colony formation is suppressed by serum and immunoglobulin G (98).

Mortality

Mortality and thromboembolism

The death rate of canine IMHA may be as high as 80% (10), and most deaths occur in the first 2 weeks after diagnosis (7, 8). As many as half of these deaths are due to thromboembolism in the lung, liver, spleen, or in multiple organs, which was found in 50-80% of cases in three post-mortem studies (36, 40, 45). IMHA was diagnosed in many cases in studies that searched for the underlying disorders in splenic vein thrombosis (IMHA n=8/34; IMHA and ITP, n=2/34 (99), pulmonary thromboembolism (n=5/29) (100), and portal vein thrombosis (n=1/ 11) had IMHA. Risk factors inherent to IMHA are thrombocytopenia, increases in serum bilirubin concentrations and hypoalbuminaemia, but treatment-related factors, such as glucocorticoid therapy, blood transfusion, and intravenous catheterization, may contribute as well (36, 40, 101).

Table 6. Risk factors and incidence and location of thromboembolisms in canine IMHA.

Risk factors	Reference	Location of TE in postmortem examinations	Reference
Thrombocytopenia	(36)	n=25; Twenty dogs had TE located in lung (n=9), heart (n=6), liver (n=4), spleen (n=8), kidney (n=8), pituitary gland (n=2; n=10), or thromboembolisms in multiple organs	(36)
Increased serum bilirubin	(101)		
Serum bilirubin >5 mg/dl	(36)	n=6; One dogs had TE and 2 dogs had DIC	(45)
Serum bilirubin >10 mg/dl	(40)	n=34; Twenty-five dogs had macrothrombi, microtrombi, and widespread fibrin deposition, and haemorrhage; Ten dogs had pulmonary thromboembolism	(40)
Hypoalbuminemia	(36)		
Intravenous catheterization	(101)		
Blood transfusions	(101)		

In this table a summary of risk factors and incidence and location of thromboembolisms (TE) in 3 studies on idiopathic IMHA (36) and one study that also included cases of secondary IMHA (101) is given. In this last study, concurrent diseases, however, were not associated with increased risk of thromboembolism (101). The association with thromboembolism was not completely significant for the risk factor blood transfusions (35, 101). It seemed worth mentioning since it may be related to the risk that is conveyed by intravenous catheterization.

Virchow's triad states that thrombogenesis results from changes in blood constituents, blood flow, and the vessel wall (102). A retrospective study in 39 IMHA dogs identified 33 dogs as hypercoagulable by thromboelastography tracings (103), and a similar prospective study identified hypercoagulability in all 11 IMHA dogs in the study before glucocorticoid therapy had started (104). There is an inverse linear correlation between red blood cell mass and the diagnostic characteristics of thromboelastography tracings; according to some this may have influenced hypercoagulability measured in cases with IMHA (105).

One flow cytometry study demonstrated increased P-selectin expression by platelets in 15 of 20 dogs with IMHA and seven dogs in this study developed clinical signs consistent with thromboembolisms (106). In contrast, a similar study detected platelet activation only in cases with severe thrombocytopenia; this may have been because of different methodologies and the fact that in the latter study flow cytometry was performed in whole blood instead of in platelet rich plasma thus minimising *in vitro* platelet activation (107). Anti-endothelial antibodies have been shown to contribute to endothelial cell changes that contributed to thrombosis (108). A study in dogs with diseases that have a high incidence of TE showed no anti-endothelial antibodies in 21 IMHA dogs; low sensitivity of their assay and the small sample size in relation to the low incidence of TE may explain the low number of positive tests (2/91) in this study, however (109).

The results of the CBC revealed that an inflammatory response is present in most IMHA dogs (Table 3). As a matter of fact thrombogenesis and inflammation are interrelated. In inflammation, cell adhesion molecules called selectins are up regulated that facilitate leucocyte transmigration. Additionally, interactions of P-selectin with its receptor, PSGL-1, stimulate production of thrombogenic microparticles from leucocytes (mainly monocytes) along with platelets and endothelial cells and play an important role in thrombogenesis (102, 110). The presence of an inflammatory response in IMHA dogs is also evidence by the demonstration of activity of chemotaxins and peptides involved in the acute phase response (Table 7). One study that measured acute phase proteins in 27 dogs with IMHA reported that WBC was significantly increased in comparison to control dogs from day 1 till day 30. A decline in the WBC started on day 10 but a significant difference was measured in comparison to day 3, 5, and 7 on the next time point it was measured, day 30 (47). Monocytosis has been reported in IMHA dogs (48). Increases in monocyte chemoattractant protein-1 (MCP-1) were found in healthy (n=26), postoperative (n=35), and critically ill dogs (n=26) of which 7 had IMHA. MCP-1 was significantly increased in critically ill dogs, reaching the highest values in dogs with sepsis (111).

Table 7. Chemotaxins and peptides involved in the acute phase response in dogs with IMHA.

Acute phase proteins
↑ C reactive protein (47, 62, 112, 113)
↑ Alfa-1 –acid glycoprotein (47, 114)
↑ Ceruloplasmin (112)
↓ Albumin (47, 113)
Chemotactic peptides
↑ MCP-1 (111, 113)
↑ GM-CSF (113)

This table presents a summary of changes in both positive (↑) and negative (↓) acute phase proteins reported in dogs with IMHA.
MCP-1, Monocyte Chemoattractant Protein; GM-CSF, Granylocyte-Macrophage Colony Stimulating Factor

Risk factors for mortality

Several studies have identified risk factors for death, the most important being variables related to disseminated intravascular coagulation (DIC), inflammation, and liver and kidney failure (Table 8). The identification of similar risk factors in different study populations suggests that these factors may be truly informative and not coincidental findings. Similar risk factors were identified by multivariate modelling that aims to identify the relationship between variables (Table 9).

Tissue hypoxia due to the often severe anaemia that develops during IMHA has been suggested to play a central role in the development of pathology (8, 40), yet only a few investigators have identified anaemia as a risk factor (34, 39). It may be that a significant association with anaemia has been overlooked in other studies because the haematocrit was evaluated as a continuous rather than a dichotomous variable. Studies have shown that tissue oxygenation is severely impaired when the haematocrit is below 10% (46, 115), which suggests that tissue hypoxia only develops in the severest cases of anaemia. The duration of severe anaemia may be another factor to consider. A study of dogs with IMHA demonstrated that the duration of hyperlactaemia was associated with a poor survival, which provides indirect evidence for the impact of anaemia since lactate concentrations are inversely correlated with the haematocrit (116). The effect of hypoxia may be difficult to identify since blood transfusions are a standard component of palliative therapy in IMHA.

The cumulative effect of multiple independent risk factors can be assessed by multiplication of the independent risks. Multivariate models suggest that renal failure, DIC, and inflammation contribute independently to the risk of death (7, 8) (Table 9). Hypoxia may cause tissue damage (40), and chemotaxins released during the ensuing acute-phase response then orchestrate an inflammatory response. Local tissue necrosis, endothelial cell activation, and the production of thrombogenic particles by leucocytes may activate coagulation (102, 110). The occurrence of thromboembolism may disturb tissue perfusion and further aggravate hypoxic tissue necrosis. The observation that these risk factors may potentiate each other explains the severely increased mortality risk if they are simultaneously present.

Table 8. Variables influencing the mortality risk in canine idiopathic IMHA.

Risk factors	Hazard ratio	95% CI	Reference
Clinical characteristics			
Male	↑		(39)
Body weight	↑		(39)
Warm season, Japan	↑		(39)
Vaccination	↑		(43)
DEA 7 in Cocker Spaniel	↓		(35)
Icterus	2	1.07 – 3.77	(8, 43, 116)
Haematological laboratory characteristics			
Autoagglutination	↑		(33, 43)
Spherocytosis	0.48	0.25 – 0.92	(8)
Decreased PCV	↑		(34)
PCV < 20%	↑		(39)
Reticulocytopenia	↑		(34)
WBC > 60 x 10^9/l	↑		(9)
Bands > 3 x 10^9/l	↑		(11)
Thrombocytopenia	↑		(36)
< 150 x 10^9/l	↑		(11)
< 200 x 10^9/l	↑		(39)
per 50 x 10^9/l increase	0.902	0.821 – 0.991	(7)
PT > 10 s	↑		(9)
PT > 9 s	↑		(39)
PT per second increase	1.44	1.14 – 1.82	(8)
PT per second increase	1.46	1.05 – 2.02	(117)
APTT (s)	1.03	1.01 – 1.06	(8)
FDP ≥ 10 ug/ml	↑		(39)
Increase Fibrinogen (g/l)	0.77	0.65 - 0.9	(8)
Biochemistry laboratory characteristics			
Total protein < 60 g/l	↑		(39)
Hypoalbuminemia	↑		(36)
< 30 g/l	↑		(11)
< 26 g/l	↑		(39)
Increased bilirubin	↑		(43, 116)
	1.03	1 – 1.07	(117)
> 2 mg/dl	↑		(39)
> 5 mg/dl	↑		(36)
> 10 mg/dl	↑		(34)

Increased AP	↑		(116)
Sodium < 140 mmol/l	↑		(39)
Potassium < 3.5 mEq/l	↑		(11)
Serum CK > 250 U/l	↑		(11)
Increased urea	↑		(116)
> 25 mg/dl	↑		(39)
per 20 mmol/l increase	2.11	1.14 – 3.15	(8)
Creatinine per 50 umol/l increase	1.27	1.12 – 1.43	(8)
Decreased HCO3-	↑		(116)
Decreased BE	↑		(116)
Increased Lactime	↑		(116)

The factors that were associated with increased (↑) or decreased (↓) mortality risk are summarized in this table. These risk factors were identified either by univariate Cox's proportional hazards analysis or by contingency table analysis.
Lactime was defined as the duration of increased plasma lactate concentration. AP, Alkaline Phosphatase; BE, Base Excess.

Table 9. Multivariate Cox's proportional hazard models in canine IMHA

A: n=115 (8)	HR	95% CI
Urea (per 20 mmol/l increase)	2.85	1.69 - 4.81
Thrombocytes (per 50 x 10^9/l increase)	0.7	0.56 - 0.91
Bands (per 1 x 10^9/l increase)	1.12	1.02 - 1.10
Petechiae (present)	4.01	1.19 - 13.54
B: n=164 (7)		
Urea (per 20 mmol/l increase)	2.56	1.73 - 3.79
Icterus (present)	2.94	1.60 - 5.42
Spherocytes (present)	0.38	0.2 - 0.72

Multivariate models that resulted from Cox' proportional hazards analysis on survival in two studies are presented in this Table (7, 8). The presence of both thrombocytes and petechiae as independent predictors in model A is puzzling at first sight. Evans' syndrome was not an exclusion criterion, however, in this study and petechiae may have been the result of the combination of DIC and ITP which has been reported to have a worse prognosis than IMHA alone. The second model was calculated on a data base that included the dogs from study A.

Treatment

The level of evidence that can be obtained from study methods applicable to evaluating therapy follows a hierarchy with on top a systematic review of controlled randomized trial (CRT), followed by controlled randomized trials, systematic reviews of observational studies, single observational studies, and lastly, physiologic studies and unsystematic clinical observations (118). Observational studies share the limitations that they may inadequately assess treatment effects due to non random allocation of patients to treatment or control-

group, and they are confounded by concurrent therapy and changes in overall patient care especially in case of historical controls (119, 120). An observational study, however, may be advantageous to assess long-term treatment effects such as duration of therapy, the natural history of the disease including recurrences, and incidentally occurring side-effects.

Immunomodulation

Immunomodulation is the mainstay of treatment of IMHA, with the aim of decreasing erythrophagocytosis and suppressing immunoglobulin production, and can be combined with whole blood or packed red cell transfusions and anticoagulation (3). The effects of therapy in canine idiopathic IMHA identified in observational studies and controlled randomized trials have been summarized, respectively, in Table 10 and 11, and will be discussed below.

Although the efficacy of glucocorticoids in IMHA has never been investigated in a clinical trial, it is generally assumed that they are effective and their side-effects are taken for granted (121). In contrast with recommendations for lifelong immunosuppression, the results of two studies suggest that immunosuppression for approximately 3 months is sufficient (7, 8). However, at least 10% of dogs had a recurrence of IMHA up to 4 years after the first haemolytic crisis (7, 8). At least ten percent of dogs (n=222) have a recurrence of IMHA up to 4 years after the first haemolytic crisis (7, 8). The percentage of recurrences may have been higher since in these retrospective studies many dogs were lost to follow-up in time.

A cohort study compared prednisolon therapy to the combined treatment with prednisolon and azathioprine (7, 8). There was no difference in outcome between the groups, however, side-effects due to azathioprine occurred in 8% of the dogs (7, 8). Confounding of the outcome may have occurred due to use of a historical control group (8). Two studies (Table 10) suggest a beneficial effect for treatment with azathioprine (9, 10). However, based on the recommendation from a recent review that clinical effect of azathioprine may be expected after at least 11 days of treatment (121), the duration of azathioprine therapy in one study must be judged suboptimal (9). These studies suffered from inclusion bias and confounding by concurrent medication, however, and the difference in treatment effect that was found is not substantiated by the report of a confidence interval (9-11). Based on the available evidence there seems to be no evidence for a beneficial effect of azathioprine in canine IMHA.

There is also no evidence supporting the use of cyclophosphamide in canine IMHA. No difference between treatment groups was found in a controlled randomized trial that evaluated cyclophosphamide and prednisolon treatment versus prednisolone alone (117). And cyclophosphamide was associated with increased mortality in retrospective studies although cyclophosphamide may have been given in the most severe IMHA cases in these studies (9, 12). Both a controlled randomized trial (117) and retrospective studies on human intravenous immunoglobin (hIVIG) failed to demonstrate a beneficial effect (122, 123) (Table 11).

Splenectomy has been investigated in two small retrospective cohort studies of dogs with IMHA (n=3, n=10) (124, 125). Besides splenectomy all dogs received medical treatment and blood transfusions and neither study included a control group, therefore no definite conclusions can be drawn about the efficacy of splenectomy in IMHA. Liposomal clodronate improved survival in dogs with IMHA compared with that of a historical control group matched for disease severity (126).

Table 10. Retrospective studies on therapy results in canine IMHA.

Study	Mortality	Survival			Best protocol	Protocols
		Mean (days) (range)	Median (days) (range)	1-year		
A. n=60 (9)	45%	758 (1-2600)	21	30	3	1. Pr + cyclo dose A, n=41 2. Pr + cyclo dose B, n=9 3. Pr + cyclo + aza, n=13
B. n=70 (10)	41%	1. 57 2. 28 3. 974 4. 15 5. 1	1. 67 2. 215 3. 974 4. 15 5. 1	-	3	1. Pr, n=16 2. Pr + cyclo, n=28 3. Pr + aza, n=5 4. Pr + cyclo + aza, n= 16 5. died or euthanized ≤ 24 hrs, n=5
C. n=222 (7)	34%	-	-	1. 69% 2. 64%	ND	1. Pr, n=73 2. Pr + aza, n =149
D. n=88 (12)	51%	-	-	-	ND[a]	1. Pr, n=17 2. Pr + non immunosuppressive agents[b] 3. Pr + ≥ 2 immunosuppressive agents[c]
E. n=22 (122)	-	-	-	-	ND	1. Pr, n=13 2. Pr + hIVIG[d], n=9
F. n=37 (123)	38%	-	460 (0-1075)	1. 77% 2. 87%	ND	1. pr + hIVIG[e] 2. pr (n=24) All dogs: cyclo, antibiotics, and heparin

Significant differences between the outcomes of dogs with IMHA treated with different immunomodulators in 6 observational studies are summarized in this table. In all studies dogs received whole blood transfusion, packed red cells, intravenous fluid therapy, and supportive medication such as gastric mucosal protectants at the discretion of the treating clinician. Most reports indicate that if patients could not tolerate oral prednisolone, it was exchanged for intravenous or subcutaneous dexamethasone.
Several aspects that decrease the value of the outcome of the studies are identified. In study A dose and duration of azathioprine therapy were less than is recommended as effective: in 7 dogs less than < 7 doses, only 6 dogs treated > 15 days (9, 40). Treatment arms were not randomized in these studies, and disease duration and severity may have influenced treatment choice in these dogs confounding the trial outcome. Improvements in general patient care may have confounded the outcome of study C that had a historical control group.

[a] Treatment with cyclophosphamide and bovine haemoglobin solution was associated with an increased RR, respectively, 1.59 and 2.13; [b] Danazol, bovine Hb solution, n=8; [c] Additional immunosuppressive drugs: azathioprine, n=27; cyclosporine, n=24; hIVIG, n=7; cyclophosphamide, n=32; [d] 0.35 g/kg [e] Clinical status dictated hIVIG therapy; hIVIG dose: 0.5 mg/kg, single dose, n=11; in 1 dogs 0,5 mg/kg 2dd; in 1 dog 0.25 g/kg 2dd.

Pr, prednisone or prednisolone; cyclo, cyclophosphamide; aza, azathioprine; hIVIG, human intravenous immunoglobin; ND, no difference.

Table 11. Prospective studies evaluating survival in canine IMHA.

Study type	Selection criteria	Best protocol	Protocols
A. Blinded CRT n=28 (117)	PCV < 28% and autoagglutination, spherocytosis, or positive DAT. Excluded were dogs that had glucocorticoids for > 48 hrs.	ND	1. pr + hIVIG (0.5 mg/kg for 3 days) 2. pr + placebo All dogs: Low molecular weight heparin, sq, 2dd.
B. CRT N=18 (37)	PCV < 20% Onset < 1wk Haemolytic anaemia, autoagglutination or positive DAT	ND	1. Pr (n=10) 2. Pr +cyclo (n =8)
C. Prospective study with historical control group, matched for disease severity (126)	Regenerative anaemia, anti erythrocyte antibodies (flow cytometry), plus at least 3 of the following: spherocytosis, polychromasia, hyperbilirubinemia, autoagglutination, DAT.	1	1. liposomal clodronate (n=7) 2. – (n=31) All dogs: pr + aza + heparin

In this table the results of 2 controlled randomized trials (CRT) (117) and one prospective study using a historical control group (126) are summarized. Autoagglutination in the inclusion criteria means agglutination of erythrocytes that cannot be dispersed by saline. In all reports exclusion criteria were similar; cases with specified underlying disorders causing haemolytic anaemia such as neoplasia, infections (ehrlichiosis, babesiosis, bacterial infections, among others) were excluded. In study C dogs were excluded that had prior treatment with human or equine immunoglobulins, bovine oxyglobin, serious concurrent renal or hepatic disease. Most reports indicate that if patients could not tolerate oral prednisolone, it was exchanged for intravenous or subcutaneous dexamethasone. Most reports indicate that if patients could not tolerate oral prednisolone, intravenous or subcutaneous dexamethasone was given instead.

Pr, prednisone or prednisolone; cyclo, cyclophosphamide; aza, azathioprine; hIVIG, human intravenous immunoglobin; CRT, Controlled Randomized Trial; ND, no difference.

Anticoagulation

A retrospective cohort study reported superior survival in IMHA dogs treated with ultra low dose aspirin (Table 12) (11). No difference was found in a CRT between the effectiveness of ultra-low dose aspirin versus clopidogrel, also an antiplatelet drug (44). However, the fact that no control group without antiplatelet treatment was included may have obscured the clopidogrel or aspirin effect.

Although used as an additional therapy in many canine IMHA patients (Table 10, 11), the effectiveness of heparin therapy has not been proven. Heparin dosing can be can be problematic due to differences in the pharmacokinetic profile between healthy and diseased dogs (127). Pharmacokinetic studies suggest that therapeutic levels both in unfractionated heparin and low molecular weight heparin therapy can only be safely reached with individual dose adjustments based on anti-Xa activity and that coagulation tests such as APTT are not

helpful (128-130). With this in mind it is promising that a recent controlled randomized trial, in which heparin dose was adjusted based on plasma anti-Xa activity, demonstrated superior survival in the heparin treatment arm (131).

Table 12. Studies on anticoagulant therapy in canine IMHA

Study type	Inclusion criteria and analysis	Best Protocol	Protocols[bcd]
A. Retrospective cohort study n = 151 (11)	PCV≤ 35%, autoagglutination, or positive DAT, or evidence of hemolysis[e] Comparison survival of treatment arms by log-rank test.	2	1. Pr + aza, n=27 2. Pr + aza + asp, n=76 3. Pr + aza + hep, n=13 4. Pr + aza + asp + hep, n=27 Additionally: Heparin, and other immunosuppressives[f]
B. Prospective trial with historical control group. n = 26 (48)	PCV ≤ 25%, spherocytosis, autoagglutination. Comparison of antithrombin activity at 30 minutes and 4h hrs by ANOVA. Comparison of incidence of TE to historical control group.	-	1. pr + heparin[g] + single plasma transfusion 2. pr + heparin (historical controls) Azathioprine was added if no increase in Ht after 10-14 days or if more than two transfusion of packed RBC were necessary.
C. RCT[h] n = 24 (44)	PCV < 30% Reticulocytes > 60.000/µl, Hemolysis[i] and at least one of the following: spherocytes[j], positive DAT[J.] Comparison of survival rate at discharge and 90 days	-	1. Clopidogrel[k] 2. ultra-low dose aspirin 3. Clopidogrel + ultra-low dose aspirin All dogs: docycycline, prednisone, azathioprine, famotidine, sucralfate.
D. Prospective controlled RCT n = 16 (131)	PCV < 30% with reticulocytes > 60000/µl, evidence of hemolysis[l], autoagglutination, spherocytosis[m], or a positive DAT. All cause death was compared between treatment arms by log-rank test.	2	1. pr + heparin, constant dosing[n] 2. pr + individual adjusted dosing

[a] In all reports exclusion criteria were similar; cases with specified underlying disorders causing hemolytic anemia such as neoplasia, infections (ehrlichiosis, babesiosis, bacterial infections among others) were excluded. In some reports also prior treatment with glucocorticoids or other immunosuppressives was an exclusion criterion. In study D, additional exclusion criteria were anticoagulation therapy before enrollment, severe thrombocytopenia (<50) and preexisting hypocoagulability (PT or APTT > 2 x the upper reference limit).

[b] Pr = prednisone or prednisolone. Most reports indicate that if patients could not tolerate oral prednisolone, it was exchanged for intravenous or subcutaneous dexamethasone.

[c] Cyclo = cyclophosphamide, aza = azathioprine, hIVIG = human intravenous immunoglobin, asp = aspirin.

[d] In all studies dogs received additional support such as whole blood transfusion, packed red cells, intravenous fluid therapy, and supportive medication such as gastric mucosal protectants at the discretion of the treating clinician.
[e] hyperbilirubinemia, hemoglobinemia, or hemoglobinuria, or spherocytosis
[f] Mixed molecular weight heparin, 75-125 U/kg, q 6 to 8 hours.Cyclosporine (7/151), Cyclophosphamide (7/151), Vincristine (3/151), Danazol (2/151), Leflunomide (1/151), HIVGI (1/151)
[g] Heparin dose was adjusted to obtain APTT of 1,5 – 2 times reference value. Despite this heparin was dosed below therapeutic range as extrapolated from human medicine.
[h] RCT = Randomized Clinical Trial
[i] Hemolysis as indicated by hyperbilirubinemia, hemoglobinemia, bilirubinuria, hemoglobinuria.
[j] At least 11-50 spherocytes per HPF
[k] DAT = Direct Agglutination Test
[l] Antiplatelet drug that inhibit ADP receptors.
[m] Hyperbilirubinemia, hemoglobinemia, bilirubinuria, hemoglobinuria.
[n] Present or absent.

Blood transfusion

It is not possible to evaluate the true effect of a blood transfusion on survival since they are a part of the supportive therapy in both retrospective studies and controlled randomized trials. Physiological studies suggest that tissue oxygen delivery is severely impaired at an haematocrit below 10% (46, 115). Several retrospective studies failed to find transfusion to be associated with survival (9, 11, 33, 36), and one found transfusion to adversely affect survival (8), possibly because transfusions were given to the most severely ill patients. However, there seems to be no evidence that transfusions are contraindicated. Bovine oxyglobin transfusion may be an alternative to blood transfusions, but no randomized controlled trials have been reported. One retrospective study reported an increased relative mortality risk in dogs with IMHA that had been treated with bovine haemoglobin solution (12).

Discussion

Despite numerous preclinical and clinical studies, the prognosis of canine IMHA remains poor. There is no evidence that immunomodulators in addition to glucocorticoids have a convincing positive effect on the outcome of IMHA. This may be due to a true lack of effect of this additional therapy or may be the reflection of a suboptimal study design. To optimize the ability of a trial to detect treatment related differences in outcome, it is essential that factors that have an impact on outcome, such as leucocytosis, left shift, bilirubinaemia, low thrombocyte counts, and increased coagulation times, are equally distributed between treatment arms. This is especially true because risk factors for death appear to be multiplicative such that dogs with icterus (HR 2), increased creatinine by 20 µmol/L (HR 1.7), increased PT by 5 seconds (HR 1.4), increased APTT by 10 seconds (HR 1.05), and thrombocyte counts of 100 x 10^9/l (HR 1.1) (see Table 8) have a 30-fold higher risk of death

than dogs without these risk factors. Retrospective studies may suffer from inclusion bias, but in a prospective randomized controlled trial the randomization procedure should equally distribute the variation in patient characteristics that could bear an impact on the outcome. A recent trial (44) used the estimate of 50% difference in survival in the setup of a trial that evaluated the therapy of clopidogrel, ultra low dose aspirin, or the combination of both. The power calculations in this study suggested that 3 groups of 8 dogs would be sufficient to find this difference in survival with a power of 80% (α=0.05). The range of clinicopathological data that were obtained at the time of presentation demonstrates that risk factors such as leucocytosis, left shift, bilirubinemia, low thrombocyte counts and increased coagulation times were present possibly leading to large differences in mortality risk between the treatment arms. The knowledge of these mortality risk factors may be used as part of the inclusion criteria in addition to the randomisation procedure to improve equalisation of mortality risk between treatment arms.

A recent study showed that dogs with IMHA can be stratified by risk of death according to the haematocrit (<20%), thrombocyte count < 200 x 10^9/l, total protein concentration (< 60 g/l), sex, and season (39); however, other investigators have not identified sex and season as mortality risk factors (Table 8). A more solid scoring system that is universally valid should be developed using mortality risk factors identified in different populations, such as those given in Table 8. The scoring system that evolves from this may be validated in existing data sets, adapted if necessary, and then evaluation should be performed in a prospective study.

The risk of death is highest during the first 2 weeks after diagnosis (7, 8), and the main factors that contribute to this risk are coagulation disturbances, and liver and kidney failure, which are probably due to anaemia-induced tissue hypoxia (7, 8, 40, 116). It may be unrealistic to expect a single therapy to be effective in these virtually moribund patients. Dogs at intermediate risk of dying may be a better population in which to study therapies to ameliorate the major risk factors within this 2-week time frame, such as anticoagulant therapy, immunomodulators to stop antibody-mediated erythrophagocytosis, or therapies that reverse anaemia and hypoxia. The subset of IMHA dogs with a low mortality risk score is perfectly suited to the study of immunomodulating therapies that have an expected onset of longer than 2 weeks, such as azathioprine and cyclosporine. The duration, dosing, and efficacy of immunosuppression should be evaluated in this subset of IMHA dogs.

A power analysis is an important tool to address the question how many dogs should be entered in a trial. The calculations necessitate an estimate of the expected effect size due to the therapy. Let us assume an, very optimistic, effect of a new medication of 50% decrease in mortality. If we test this new medication in a group of dogs with IMHA with 50% mortality we expect the mortality after treatment with the new medication to be 25%. According to the power analysis (Figure 1) for this situation 66 dogs per treatment arm are needed to have a power of 80% to detect this difference. The 95% CI intervals in previous studies are

consistent with lower mortality proportions, in which case many more study subjects will be needed (7, 8). It will be difficult at least or impossible for one institute only to perform a trial of this magnitude. The case load for IMHA cases of five referral institutes varied between 9-25 cases per hospital per year (8-11, 33). Our hypothetic trial above would last at least five years even with an optimistic estimate for the yearly caseload. Trial periods for five years or longer are unwanted for several reasons: funding may easier obtained for shorter projects, work contracts of researchers are often shorter than the estimated trial duration, and supportive care may change during the trial. It may be possible to increase the number of available IMHA cases for example by offering a reduction on the treatment price if a dog enters a trial or more patients may be attracted if new medications are used. It should be realized, however, that the incidence of IMHA is low and due to the severity of clinical signs many dogs would have been referred to these university hospitals anyway. The conclusion is that multicenter trials are the only solution to assemble study groups large enough to study treatment effect in canine IMHA.

We are planning a study of independent cases and controls with 1 control(s) per case. Prior data indicate that the failure rate among controls is 0.5. If the true failure rate for experimental subjects is 0.25, we will need to study 66 experimental subjects and 66 control subjects to be able to reject the null hypothesis that the failure rates for experimental and control subjects are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. We will use a continuity-corrected chi-squared statistic or Fisher's exact test to evaluate this null hypothesis.

Figure 1. The output of PS Power and Sample Size Calculations.
PS is an interactive program for performing power and sample size calculations by Dupont, W.D., and Plummer, W.D. (http://biostat.mc.vanderbilt.edu/PowerSampleSize; accessed 27/3/2011) that is freely available on the web. PS was used to calculate sample size for a hypothetical randomized controlled trial of a new treatment that would lead to 50% less mortality in dogs with IMHA with a mortality risk of 50%.

The diagnostic criteria for IMHA will certainly be a subject for debate at the start of a multicenter trial. The inclusion and exclusion criteria for idiopathic IMHA of both observational and prospective studies differ (Table 4, 5, 10-12), the diagnostic performance of the DAT differs, autoagglutination and spherocytosis are not always part of the inclusion criteria and are poorly standardized. A major drawback in comparing results of DAT and autoagglutination testing between laboratories is that duration and circumstances of transport may influence test results. Therefore the question arises if it is reasonable to expect that similar populations of dogs with idiopathic IMHA will be included in different centres that would contribute to a multicenter trial. The summary of clinical and laboratory characteristics in the first part of this review shows that similar clinical presentations arise from these observational studies on canine idiopathic IMHA: the results of the mean and median haematocrit, WBC, and bands are strikingly identical, as are the proportions of dogs having reticulocytopenia, thrombocytopenia, and abnormal coagulation tests. This maybe unexpected uniformity merits some discussion.

Idiopathic IMHA is a diagnosis made largely by hypothetico-deductive reasoning. Each step forward in the deductive process increases the prior probability on IMHA. Evidence for immune-mediated erythrocyte destruction is in most cases obtained through the DAT (Coombs' test). It is mostly the low sensitivity of the DAT and the lack of standardized methodology that has worried researchers. Though this is certainly true, it should be realized that not only sensitivity but also disease prevalence determines the positive predictive value of a diagnostic test. The largest change from pre-test probability to post test probability for any diagnostic test may be expected around a prior probability of around 50% (132). The influence of pre-test probability on the predictive value of the DAT is illustrated in Table 13 for five possible situations in which a DAT may be used as part of the diagnostic process in an IMHA patient.

R1
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R39

Table 13. Effects of change in pre-test probability for two different Direct Agglutination Test (DAT) sensitivities on the posterior probability of IMHA.

		Sensitivity Coombs' test 60%					Sensitivity Coombs' test 90%			
Pre-test Probability (%)	DAT	IMHA			Post test probability (%)	DAT	IMHA			Post test probability (%)
University hospital population										
		+	-				+	-		
4	+	24	48	72	33	+	36	48	84	43
	-	16	912	928	2	-	4	912	916	0
		40	960	1000			40	960	1000	
Anaemic dogs										
		+	-				+	-		
25	+	150	36	186	80	+	225	36	261	86
	-	100	713	813	12	-	25	713	738	3
		250	750	1000			250	750	1000	
Dogs with haemolytic anaemia, estimate I										
		+	-				+	-		
60	+	360	120	470	77	+	450	120	570	97
	-	240	380	620	49	-	150	380	530	28
		600	400	1000			600	400	1000	
Dogs with haemolytic anaemia, estimate II										
		+	-				+	-		
80	+	480	10	490	98	+	720	10	730	99
	-	320	190	510	63	-	80	190	270	30
		800	200	1000			800	200	1000	

Estimates of prevalence, or pre-test probability, of IMHA within different subsets of dogs were searched in the literature in order to calculate the effect of DAT sensitivity on the posterior test probabilities for IMHA. Pre-test probability in a population of nonanaemic dogs was estimated by Bayesian analysis as 4.1% (95% CI: 0.5 – 11.3%) (51). Another estimate for the pre-test probability of IMHA was taken from a report on a general university hospital (5) in which IMHA accounted for 2197 of the total of 1036428 that were seen between 1964 – 1991. Assuming that the more recent publication would be closer to the current prevalence of IMHA in a university hospital population composed of referral patients a point estimate of 4% was used.
In a study by Slappendel (58) IMHA dogs represented 10% of 327 dogs with anaemia. Morley (51) reported 29.7% (95CI% 7.6 – 59.1) as a Bayesian estimate of the true IMHA prevalence in a population of anaemic dogs. In the calculation above an intermediate estimate of 25% was chosen.
Reported specificities for the DAT vary between 95-100% (56). For these calculations estimates for sensitivity of 60% and 90% (55) and for specificity of 95% were used.

The calculations in Table 13 demonstrate that increases in pre-test probability on IMHA have profound impact on the posterior test probability. The calculations reveal that, assuming a sensitivity of the DAT of 90%, the post test probability of IMHA is 97% at a pre-test probability of 60%. In other words, a diagnosis of IMHA is almost certain if a DAT with a sensitivity of 90% is positive. Raising the pre-test probability for IMHA to 60% asks from the attending veterinarian to consider other causes of anaemia than haemolysis and exclude them if applicable by additional diagnostic tests (4). Non immune mediated causes of haemolysis such as acquired and genetic erythrocyte membrane defects, enzyme deficiencies, as well as infectious causes such as *Babesia canis* are part of the differential diagnosis for haemolysis (4). The estimate of the pre-test probability of IMHA at this point depends on the incidence of other components of the differential diagnosis of haemolysis. In endemic countries the pre-test probability of a *Babesia canis* infection will be high and pre-test probability of IMHA may be relatively low. The pre-test probability on IMHA in dogs with haemolysis examined in a first line veterinary practice may be relatively low in comparison to a university hospital situation that receives referrals only. The prevalence of IMHA, either primary or secondary within a population of dogs with haemolysis was not found in the literature, and therefore two estimates were used, starting with a conservative 60%, and ending with a higher estimate of 80%. An essential step in the work-up of an idiopathic IMHA patient is the use of exclusion criteria in order to differentiate primary from secondary IMHA. The results of the diagnostic tests done to exclude underlying disorders will also help to further increase the pre-test probability of having IMHA and create the situation that tests with lower sensitivities are efficient in confirming IMHA.

We can conclude from these calculations that the diagnosis of IMHA is a multistep procedure of which the DAT is an essential but not the only part determining the post test probability and the current diagnostic procedure may suffice as a starting point for multicenter trials. This does not mean that researchers should not strive to agree on objective standardized criteria by which IMHA is diagnosed and ensure that these criteria are validated in different study populations. Not only studies on treatment efficacy will benefit from collaborative efforts but also research on pathophysiological and etiological aspects of IMHA such as the involvement of erythrocyte membrane antigens or genetic causes may benefit from the increased availability of samples from well defined canine IMHA cases.

The evaluation of both traditional and alternative diagnostic tests, such as the gel test (53), may be simultaneously validated in multiple different study populations. Such an evaluation is traditionally performed by contingency table analysis with a gold standard test as a reference. This procedure assumes perfect sensitivity and specificity of the gold standard. In the case of canine IMHA the imperfect sensitivity and specificity of the DAT as currently used will introduce reference bias and result in wrong estimates of the diagnostic characteristics of the new test. With the DAT unfit as a gold standard, a Bayesian approach may be suited

to estimate true IHA prevalence with the use of one or more diagnostic tests (133, 134). At least one of the assumptions in Bayesian methodology, the independency of diagnostic tests, is not true in IMHA, since they all rely on, the consequences of, anti-erythrocyte antibody detection. Recently, a modification of the Bayesian approach was published that offers a possible solution (133).

The analysis of studies on prognosis and treatment in dogs with idiopathic IMHA suggests that collaborative efforts may be the only way to ameliorate the outcome in canine idiopathic IMHA. Multicenter trials will be the ultimate goal but a direct spin off will be the necessity to agree on diagnostic criteria for canine idiopathic IMHA with emphasis on the laboratory testing. A prognostic scoring system should be developed to enable stratification of dogs according to their mortality risk. Multicenter trials will allow for the possibility to standardize and validate current tests using a Bayesian probability approach and support the development of alternative diagnostic tests. Studies on etiological and pathophysiological aspects of canine idiopathic IMHA will benefit from the improved access to samples from well defined canine IMHA cases.

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R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

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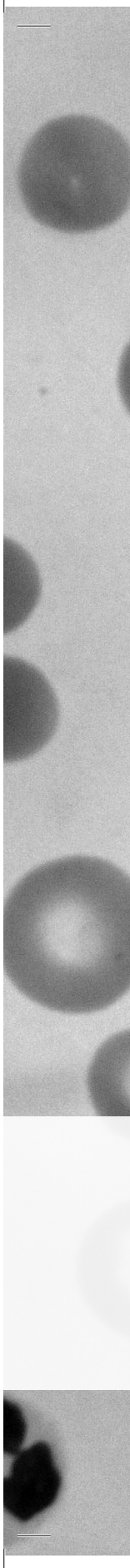

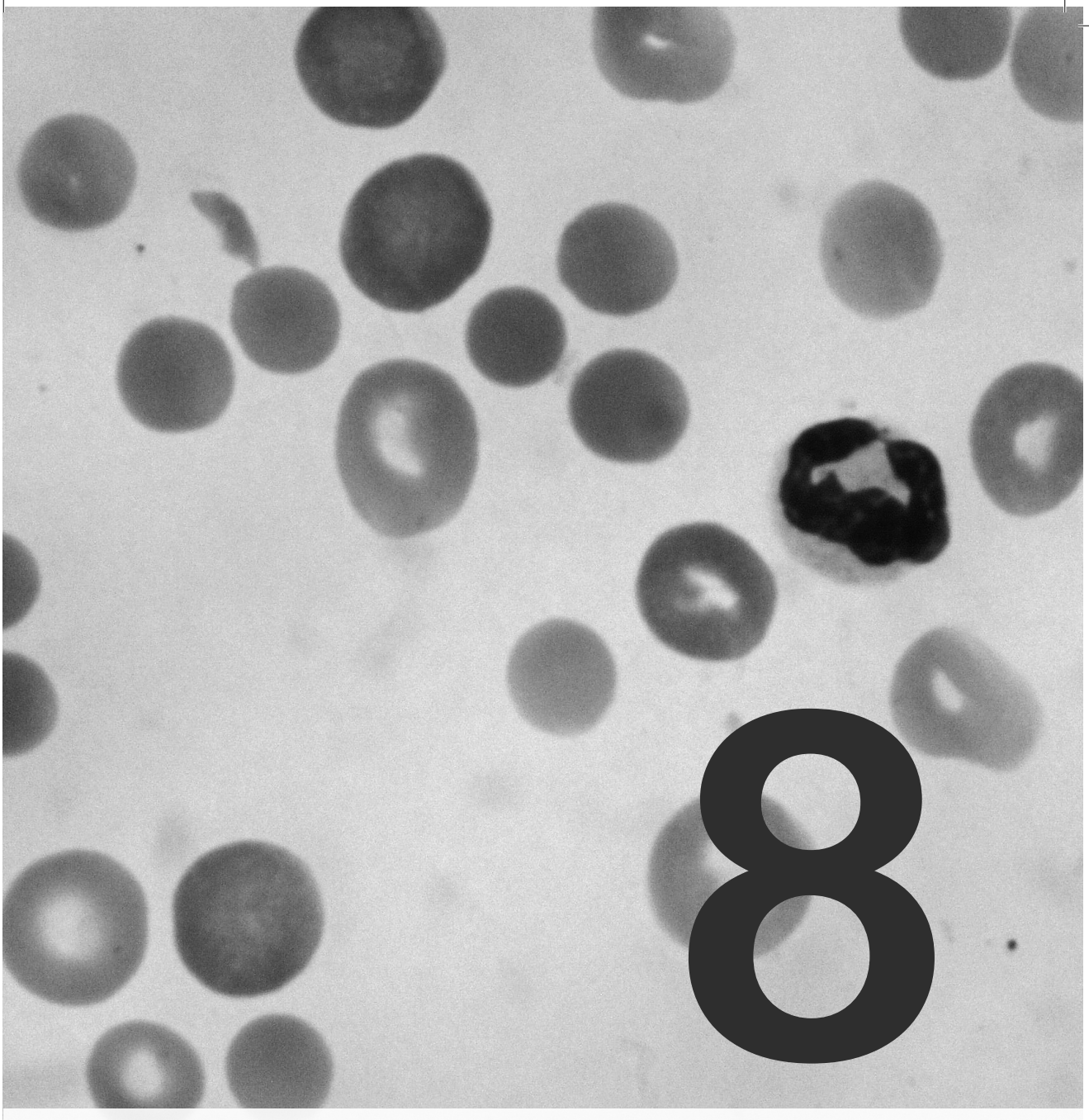

8

General Discussion

Canine idiopathic immune-mediated haemolytic anaemia (IMHA) is a disease with a high mortality especially during the initial hospitalisation period (1-3). As outlined in **Chapter 1** the central theme of this thesis was the analysis of clinical, therapeutic, and pathophysiological factors that contribute to the outcome of canine idiopathic IMHA.

The research questions that were formulated were as follows:

a) What is the estimated survival time in dogs with idiopathic IMHA and which clinical and laboratory characteristics determine this outcome? In addition, is a protocol of 3 months immunosuppression sufficient to maintain remission of IMHA?
b) Is there an additional beneficial therapeutic effect in dogs with idiopathic IMHA of a protocol that includes azathioprine and prednisolone versus prednisolone alone?
c) How does a fast and simple to perform gel-based direct agglutination test (DAT) perform in comparison to a traditional DAT and is such a test useful as a diagnostic tool in the diagnosis of IMHA?
d) Which reference genes are suitable for future quantitative RT-PCR studies into idiopathic IMHA in canine whole blood?
e) What is the contribution of whole blood gene expressions of tissue factor and interleukin-8 (IL-8) to the inflammatory response and the coagulation abnormalities in dogs with idiopathic IMHA?
f) What is the current state of evidence in canine IMHA and why has research as yet failed to improve outcome of canine IMHA?

These questions were explored in **Chapters 1 – 7**. The findings and their relevance are discussed below.

Mortality percentages in canine idiopathic IMHA have been reported between 20 % - 70 % with most deaths in the first two weeks after diagnosis (1, 4-6). These findings are supported by the estimated Kaplan Meier survival times in **Chapters 2, 3, and 6** (2, 3). Calculations of survival times were performed in three different cohorts of dogs with idiopathic IMHA and had very similar results. Respectively, in the cohort of 149 dogs treated with prednisolone and azathioprine (AP protocol) the half year survival was 72.6% (95% CI: 64.9 – 81.3%) (**Chapter 2**), in the cohort of 73 dogs treated with prednisolone (P protocol) the 1-year survival was 64% (95% CI: 54 – 77%) (**Chapter 3**), and in the cohort of 24 dogs treated with prednisolone the half year survival was 75% (95% CI: 59.5 – 94.5%) (**Chapter 6**) (2, 3).

Eighty percent of dogs with idiopathic IMHA in the cohort treated with azathioprine and prednisolone (n=149) had a leucocytosis and a left shift at presentation (**Chapter 2**) which is similar to the findings in the cohort treated with prednisolone alone (n=73) (**Chapter 3**). The 24 dogs in the group with idiopathic IMHA in **Chapter 6** had significantly increased leucocytes, band neutrophils, and monocytes. The summary of CBC data from other studies on canine idiopathic IMHA (**Chapter 7**) supports the conclusion that an inflammatory response consisting of a pronounced leucocytosis (1-3, 5, 7-10) and a left shift (2, 3, 7-9) are common laboratory features at presentation.

The literature survey in **Chapter 7** suggests that as many as half of the deaths in idiopathic IMHA are related to thromboembolisms (7, 8, 11-14). From the summary of clinical observational studies of dogs with idiopathic IMHA in this chapter it can be concluded that up to 50% of dogs with idiopathic IMHA present with abnormalities in coagulation parameters suggesting the presence of disseminated intravascular coagulation (DIC). This is confirmed by the results for the coagulation times, fibrinogen concentration, and thrombocyte counts in **Chapters 2 and 3**. The prothrombin time and activated partial thromboplastin time were increased in respectively 46% and 67% (n=98), and a decreased fibrinogen concentration and a thrombocyte count below 50 x 10^9/l were found in respectively 18% (n=96) and 25% (n=148) of the cohort of dogs with idiopathic IMHA treated with azathioprine and prednisolone (**Chapter 2).** These coagulation tests en thrombocyte count results were not significantly different from those found in the cohort of dogs with idiopathic IMHA treated with prednisolone **(Chapter 3).** Decreases in individual coagulation factor activities fitting DIC were found in the cohort of 24 dogs with idiopathic IMHA **Chapter 6** as well. A low mean platelet content (MPC), indicative of platelet activation, was found in the group of dogs diagnosed with DIC and in the group with idiopathic IMHA (**Chapter 6**) (15-17). In addition, large platelets, characterized by an increases in mean platelet volume (MPV) and mean platelet mass (MPM) were found in the dogs with idiopathic IMHA, which most likely results from an increase in platelet production rate (15, 17, 18). It has been reported that large platelets are associated with an increased haemostatic capacity (15). In conclusion, the dogs with idiopathic IMHA in **Chapter 6** have decreased MPC and increased MPV and MPM reflecting a high platelet turnover due to the continuous platelet activation that occurs in DIC.

Uni- and multivariate analysis with the aim to identify prognostic variables and their relationship has been performed in 3 different cohorts of dogs with idiopathic IMHA in this thesis (**Chapters 2, 3, and 6**). The finding of increased plasma urea or creatinine concentration, the presence of icterus, the presence of increased band neutrophils, increased monocyte counts, thrombocytopenia, and prolonged APTT as prognostic variables in the respective multivariate models indicates that parameters that signal the presence of renal failure, liver failure, inflammation, and DIC, alone, or in combination are robust independent

predictors of mortality. The finding of the same or related prognostic factors by other authors (Table 8, **Chapter 7**) supports this conclusion.

Hypoxia is a risk factor found by some (19, 20), but could not be confirmed in the univariate analyses in **Chapters 2, 3, and 6** in this thesis. Nevertheless, a pathology study that established a positive relation between the presence of hypoxic necrosis, especially in the liver, and increased leucocyte counts (11) and a study that related increased duration of hyperlactemia to mortality (21) both support that anaemia may be central in the development of a high mortality risk. Oxygen delivery was impaired in a canine isovolemic anaemia model below a haematocrit of 10% (22, 23). The fact that icterus and failing liver functions form an important risk factor is in agreement with hypoxia as risk factor, since the failing liver functions can be entirely attributed to centrolobular hypoxic hepatocyte necrosis resulting from severe anaemia.

The establishment of both the inflammatory response and the thrombotic tendency as major independent factors that determine outcome in dogs with idiopathic IMHA was the immediate cause to investigate the possible underlying shared pathophysiological mechanisms. Tissue factor (TF) expression by inflammatory cells, especially monocytes has been reported as a link between inflammation and coagulation (24, 25). This occurs mainly through the NF-kB signaling pathway and leads to increases in cytokines such as IL-8 which is a major chemotaxin for leucocytes (26, 27). It was hypothesized that blood levels of TF and IL-8 in dogs with idiopathic IMHA are increased due to increased expression by inflammatory cells (**Chapter 6**). Total leucocyte counts and band neutrophils are increased in 80% of dogs with idiopathic IMHA at presentation (**Chapters 2 and 3**) and further increase during the hospitalisation period (21). The high leucocyte turnover suggests a continuing production of IL-8. Similarly, to explain a thrombotic tendency a continuous intravascular source for TF must be present. Therefore we choose to measure TF and IL-8 gene expressions during the hospitalisation period by quantitative RT-PCR. Gene expressions were measured in whole blood since isolation procedures of leucocytes may cause up regulation of cytokine expressions (28).

One of the solutions to control for the internal variation that affects the outcome of the quantitative RT-PCR reaction is the use of reference genes as an internal standard (29, 30). Reference genes are selected based on the supposition that their expression is stable in all cells regardless of the tissue or individual used in the study (31-34). In **Chapter 5** the suitability of nine frequently used canine genes as reference genes for quantitative RT-PCR in whole blood was investigated. The analysis revealed that white blood cell count and disease category had a statistically significant effect on the expression of the potential reference genes in canine whole blood. Two software applications were used to select the potential reference genes that had the most stable expression and the number that was needed to provide an optimal normaliser within the experimental setting in **Chapter 6**. Normfinder

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selects those reference genes that have the most stable expression between groups and GeNorm selects the genes with the least variation in individual samples (30, 35). It was concluded that multiple reference genes are necessary to provide a stable normaliser for quantitative RT-PCR in canine whole blood samples and, since expression may be influenced by the experimental conditions, that it is necessary to assess the stability of expression for each experimental situation anew (36).

In **Chapter 6**, the hypothesis was rejected that the presence of hypercoagulability and an inflammatory response in dogs with idiopathic IMHA is due to increased expression of IL-8 and TF by monocytes. Our study demonstrated that whole blood TF expression was increased but IL-8 expression was not significantly different from the IL-8 expression in healthy dogs and significantly lower than in the groups with systemic disease, neoplasia and DIC, and sepsis. The decreases in coagulation factors FII, FV, FVII, FIX, FXI, FXII confirmed the presence of DIC in many of the dogs with idiopathic IMHA. The high FVIII and fibrinogen activity in the dogs with idiopathic IMHA suggested an acute phase response. Much evidence has been reported in the literature for the presence of an acute phase response in dogs with idiopathic IMHA (37-41). Increased leucocyte counts and turnover such as documented in **Chapters 2, 3 and 7** have been related to severity of centrolobular hepatocyte necrosis (11). In the first part of the acute phase response macrophages activated by liver hypoxia release mediators such as interleukin-1 and tumor necrosis factor which is followed by the release of IL-8 and monocyte chemoattractant protein by local fibroblasts and endothelial cells (42). We identified an increased monocyte count as independent negative prognostic parameter in the multivariate model predicting death in dogs with idiopathic IMHA in **Chapter 6**. In a recent study monocyte count in dogs with idiopathic IMHA was not identified as a prognostic factor. But serum cytokine concentrations related to monocyte recruitment (monocyte chemoattractant protein-1, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 15 and interleukin-18) were increased, and two of them, monocyte chemoattractant protein -1 and interleukin-18, were independently associated with higher mortality (40). And similar to our findings, the results for the serum IL-8 concentration (median 2.6 µg/l, range (1.2–32.0), n=20) in this study were not significantly different from the healthy controls (median 1.6 µg/l, range (0.6–5.4), n=6) (40).

Whole blood TF expression was increased in dogs with idiopathic IMHA (**Chapter 6**) and thus contributes to the consumptive coagulopathy documented in dogs with idiopathic IMHA (**Chapter 2, 3, and 6**). TF expression in monocytes is regulated via the NF-kB signaling pathway and activation of this pathway is expected to result in increased expression of IL-8 as well (24). Since IL-8 expression in dogs with idiopathic IMHA was not increased, our results suggest that another source than blood monocytes is responsible for the increased whole blood TF expression. Platelets have been reported to express TF but not IL-8 and may be the alternative source of TF expression in our study (43). This is supported by the fact that

p-selectin, a platelet activation marker was elevated in dogs with idiopathic IMHA (44-46).
The summarized evidence that has been gathered from the literature on canine idiopathic IMHA in **Chapter 7** demonstrates that research results supporting the use of immunomodulators in addition to glucocorticoids are lacking, despite the efforts that have been made in observational studies and randomized clinical trials (1, 2, 5, 9, 47-50). The analysis of the prerequisites of such trials with regard to inclusion criteria in **Chapter 7** suggests that it may be advantageous to categorize dogs with idiopathic IMHA based on their probability of survival in addition to the randomisation procedure. The estimates of the effect size in power analyses to estimate sample sizes necessary for clinical trials in canine idiopathic IMHA may have been too optimistic and led to false negative outcomes (51). An example of a power analysis shows that larger sample sizes are needed than hitherto used in the literature. In fact, our retrospective cohort study in **Chapter 3** had respectively 149 dogs in the AP protocol group and 73 dogs in the P protocol group and is one of the largest studies comparing treatment in canine idiopathic IMHA. This study is an observational study using an historical control group, however, and not a randomized controlled trial.
As discussed in **Chapter 7,** observational studies may be more suited than controlled randomized trials to establish the required duration of immunosuppression, the natural history of the disease studied, and to detect occasionally occurring side effects. We were able to demonstrate in the retrospective cohorts of dogs with idiopathic IMHA in **Chapters 2 and 3** that in contrast with general recommendations that include lifelong immunosuppression, an immunosuppression with prednisolone alone or in combination with azathioprine for 3 months is sufficient to obtain remission of idiopathic IMHA. Side-effects due to azathioprine were observed in 8% of dogs (n=222) (2). It was established that recurrences of a haemolytic crisis may occur in at least 10% of the dogs with idiopathic IMHA (n=222) (**Chapters 2 and 3**). In **Chapter 3** we reported a lack of additional therapeutic effect of azathioprine in the cohort of dogs with idiopathic IMHA treated with azathioprine and prednisolone in comparison to the cohort treated with prednisolone only. As discussed in **Chapters 3 and 7**, the use of historical controls may have resulted in confounding due to improvement of supportive care within the time span of both cohorts.
In **Chapter 7** it was concluded that a mortality risk based classification of dogs with idiopathic IMHA is needed to ensure that dogs that enter a trial have similar mortality risks. Indeed, the survival probabilities between the treatment arms may have differed in the study in **Chapter 3** since the dogs in the trial arm treated with azathioprine and prednisolone had lower thrombocyte counts and longer duration of clinical signs which may have obscured an additional treatment effect of the azathioprine. Therefore a blinded randomized clinical trial is still needed to establish the true effect of azathioprine. In view of the expected slow onset of azathioprine it is unlikely that a benefit of azathioprine can be discerned in the first 1-2 weeks after the start of treatment. Therefore such a trial should be conducted in the subset

of dogs with idiopathic IMHA that are likely to survive the initial hazardous hospitalization period.

In **Chapter 7** it was concluded that progress in treatment improvements in canine idiopathic IMHA is slow and that multicenter trials may be the only solution to obtain study groups with enough power to find differences in outcome due to treatment. The datasets of the cohorts of dogs with idiopathic IMHA in **Chapters 2, 3, and 6**, and other previously reported data sets, may be utilized to provide the basics for a mortality risk based scoring system. Such a system, however, should be validated in a prospective study that preferably incorporates data from dogs from different research groups working on canine idiopathic IMHA to ensure that the resulting scoring system is properly validated for general application.

Universal agreement on the diagnostic criteria for canine idiopathic IMHA will be a necessary prerequisite for the initiation of such multicenter trials. The use of similar inclusion and exclusion criteria in the studies on canine idiopathic IMHA summarized in **Chapter 7** predicts that criteria that researchers agree upon will consist of the presence of moderate to marked anaemia, and diagnostic procedures that ensure exclusion of pathophysiological routes of development of anaemia other than haemolysis (1, 2, 5, 9, 47-50). However, ultimately, the diagnosis of immune-mediated haemolysis depends upon demonstration of anti-erythrocyte antibodies which is most commonly done by the conventional direct agglutination test (52). The execution of the DAT is poorly standardized and source for much debate (52). In **Chapter 4** it was shown that the results of a fast polyvalent gel-based DAT agreed very well with the results of the conventional DAT as it is performed in two veterinary university clinic laboratories specialized in haematology. Since a gel-based DAT may be commercially produced these results are encouraging with regard to future standardisation of diagnostic testing in canine idiopathic IMHA. A potential additional advantage may be that the gel-based DAT was less often positive in secondary IMHA (**Chapter 4**).

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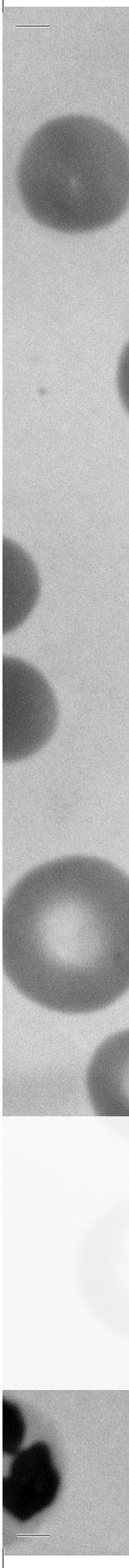

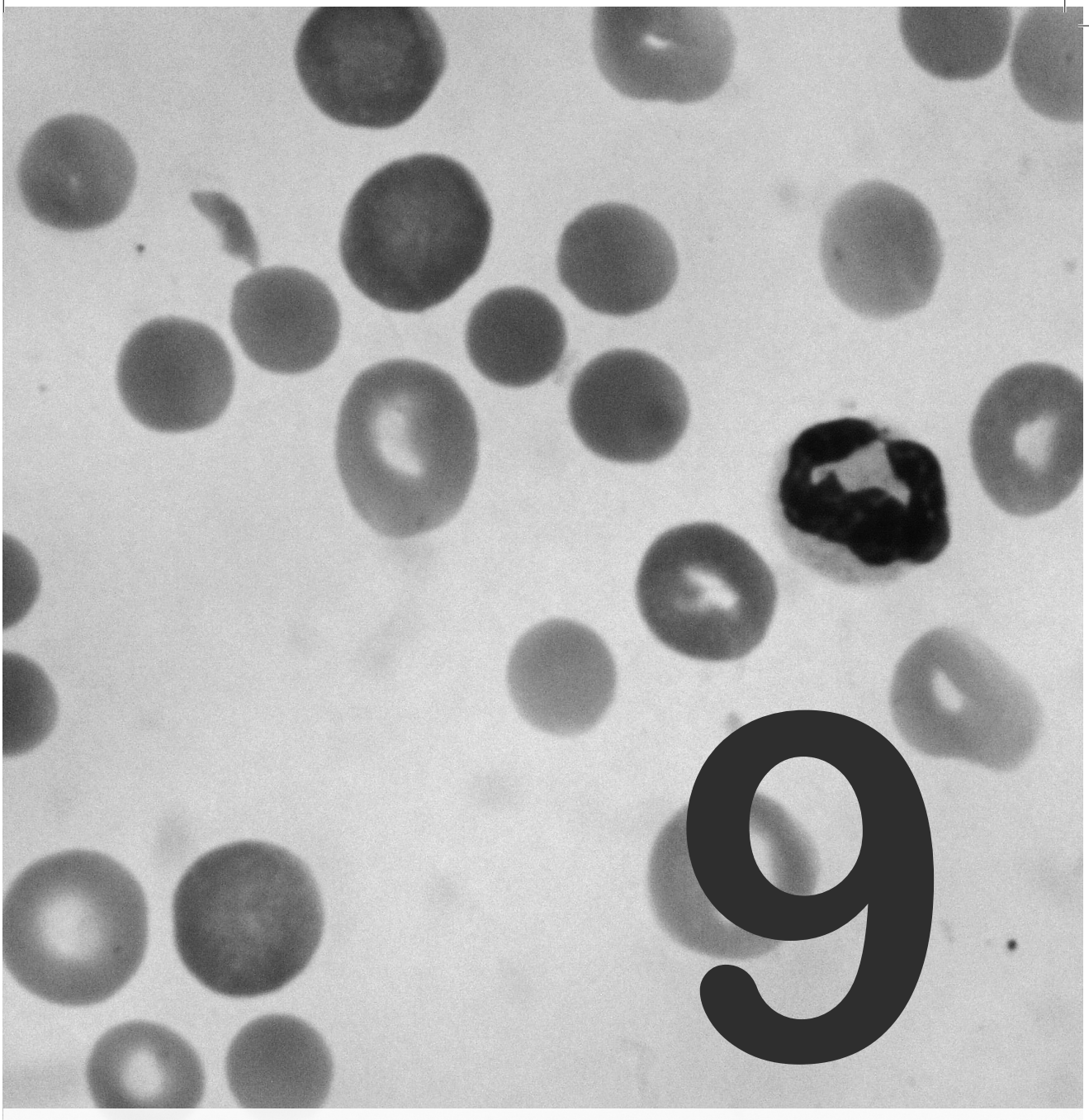

9

Nederlandse samenvatting

Idiopatische immuungemedieerde hemolytische anemie is een van de meest voorkomende immuungemedieerde ziektes bij de hond (1, 2). De meerderheid van de honden met idiopatische immuungemedieerde hemolytische anemie (IHA) heeft op het moment van aanbieden een ernstige anemie die binnen enkele dagen ontstaan is (3-11). De oorzaak van deze anemie is het binden van antilichamen aan de erytrocyt met als gevolg afbraak door complement binding, fagocytose, of een combinatie van beide (12). De diagnose immuungemedieerde hemolytische anemie wordt gesteld op basis van de aanwezigheid van een hemolytische anemie in combinatie met een positieve directe agglutinatie test die de aanwezigheid van anti-erytrocytaire antilichamen bevestigt (13). De directe agglutinatie test is ontwikkeld door de dierenarts dr. Robert Coombs en is ook bekend onder de naam Coombs test (14). Het vinden van microsferocyten in een bloeduitstrijkje is een andere goede manier om de diagnose IHA te bevestigen (12). Microsferocyten zijn kleine bolvormige erytrocyten die ontstaan wanneer met antilichamen beladen erytrocyten herkend worden door macrofagen en vervolgens stapsgewijs gefagocyteerd worden. Als kan worden uitgesloten dat de IHA een reactie is op onderliggende neoplastische en infectieuze aandoeningen, of vaccinaties en medicaties is er per definitie sprake van een idiopatische IHA (2, 15, 16).

Idiopatische IHA bij de hond is een aandoening met een hoge mortaliteit. De overleving in drie onafhankelijke cohorten in dit proefschrift varieerde van een halfjaarsoverleving van 72.6% (95% CI: 64.9 – 81.3%) (**Hoofdstuk 2**), 75% (95% CI: 59.5 – 94.5%) (**Hoofdstuk 6**) en een 1-jaars overleving van 64% (95% CI: 54 – 77%) (**Hoofdstuk 3**) (8, 9). Dit past binnen de schattingen van mortaliteit bij honden met idiopatische IHA door andere auteurs (**Hoofdstuk 7**) (3, 9, 10, 17-19). Het centrale thema van dit proefschrift is het analyseren van de klinische therapeutische en pathofysiologische factoren die bijdragen aan de slechte prognose van idiopatische immuungemedieerde hemolytische anemie bij de hond.

Uit de klinische gegevens van de 3 bovenstaande cohorten en de literatuur komt een consistent klinisch beeld van een hond met IHA naar voren. De mediane hematocriet ten tijde van de diagnose idiopatische IHA ligt rond de 13% (3-11). De anemie ontstaat snel, meestal binnen een periode van een ongeveer 3 dagen (3, 5, 8-10). Het blijkt dat ongeveer 80% van de honden met idiopatische IHA een ontstekingsbloedbeeld op het moment van diagnose heeft die bestaat uit een leukocytose (3, 4, 6-11). Dit beeld wordt verklaard door een neutrofilie in combinatie met een linksverschuiving en een monocytose (4, 6, 8, 9, 20). Meer dan 50% van de sterfte in honden met idiopatische IHA is gerelateerd is aan het voorkomen van trombose (4, 8, 9, 20-24). Met behulp van pathologisch onderzoek worden in organen zoals longen, lever en nieren, aanwijzingen voor trombose gevonden (4, 20, 21). Bij ongeveer 50% van de honden met idiopatische IHA worden op het moment van de diagnose afwijkingen gevonden die passend zijn binnen het beeld van diffuse intravasale stolling (DIS) (**Hoofdstuk 2, 3, 6, 7**) (3-6, 8, 20). Ook wijzen de trombocytenparameters erop dat zowel het volume als de massa van de bloedplaatjes bij honden met idiopatische IHA is

toegenomen (**Hoofdstuk 6**). Uit de literatuur is bekend dat grote bloedplaatjes een grotere bijdrage kunnen leveren aan de stolling. Thromboelastografisch onderzoek wijst uit dat bij vrijwel alle honden met IHA sprake is van hypercoagulabiliteit. (25, 26). Samenvattend kan geconcludeerd worden dat er sprake is van hypercoagulabiliteit bij honden met idiopatische IHA.

Een belangrijke vraag is of, en hoe, de klinische bevindingen gerelateerd zijn aan de prognose van een hond met idiopatische IHA. Met behulp van multivariaat analyse werd vastgesteld dat gestegen plasma ureum of creatinine concentraties, de aanwezigheid van icterus, een linksverschuiving, neutrofilie, monocytose, trombocytopenie, en een verlengde APTT op het moment van diagnose gerelateerd zijn aan de sterfte als gevolg van IHA (**Hoofdstuk 2, 3, 6**) (8, 9). Deze bevinding wordt ondersteund door het feit dat meerdere auteurs identieke of verwante prognostische factoren hebben gevonden in univariaatmodellen (Tabel 8, **Hoofdstuk 7**) (3, 27, 28). Deze combinatie van variabelen geeft aan dat bij honden met idiopatische IHA de aanwezigheid van verstoringen in de nier- en leverfunctie, diffuse intravasale stolling, alleen optredend of in combinatie, een hoge kans op sterfte voorspelt. De postmortale veranderingen bij honden met idiopatische IHA als gevolg van de door anemie veroorzaakte hypoxie bestaan onder andere uit voornamelijk centrolobulair gelocaliseerde levernecrose (21). Het is gebleken uit postmortaal onderzoek dat de leukocytose in ernst toeneemt naarmate de schade door hypoxie van de weefsel ernstiger is (21). Een sterk gedaalde Ht (< 0,20 l/l) is beschreven als een risicofactor die leidt tot hoge mortaliteit (27). Tevens blijkt bij honden met IHA die langdurig verhoogde serum lactaat condentratie hadden als gevolg van hypoxie een verhoogd sterfte risico te bestaan (28). Hieruit kan geconcludeerd worden dat het lang bestaan van ernstige anemie de bepalende factor is voor een hoog sterfte risico bij een individuele patiënt.

Een belangrijke vraag is of er een pathofysiologische relatie is tussen de anemie en de hypercoagulabiliteit. Zoals al eerder besproken heeft het overgrote deel van de honden met idiopatische IHA op het moment van de diagnose een ontstekingsbloedbeeld dat gekenmerkt wordt door een neutrofilie met een linksverschuiving (3, 4, 6-11). Bij honden met idiopatische IHA zijn veranderingen in de concentraties van acute fase eiwitten gevonden die passen bij de aanwezigheid van een zogenaamde acute fase respons in deze patiënten (29-34). Interleukine-8 is een van de cytokines die stijgt gedurende deze acute fase respons en is betrokken bij de transendotheliale migratie van de leukocyten doordat de oppervlakte-expressie van specifieke celadhesiemoleculen op de leukocyten toeneemt. Leukocyten spelen daarnaast een belangrijke rol bij het ontstaan van trombose door het produceren van weefseltromboplastine dat het extrinsieke stollingspad activeert, met name door monocyten (35, 36).

De hypothese dat honden met idiopatische IHA een gestegen expressie hebben van interleukine-8 en weefseltromboplastine werd onderzocht met behulp van kwantitatieve RT-PCR. De hoeveelheid eiwit die door een cel geproduceerd wordt is gerelateerd aan de hoeveelheid RNA dat in de celkern wordt afgelezen. De stappen die in een dergelijke RT-PCR doorlopen worden bestaan uit het isoleren, vermeerderen en meten van het voor interleukine-8 en weefseltromboplastine specifieke mRNA. De expressie van eiwitten in een cel is onderhevig is aan invloeden van buiten af en om betrouwbaar veranderingen in die expressie vast te kunnen stellen worden deze gemeten in relatie tot de expressie van zogenaamde referentiegenen. Een ideaal referentiegen voldoet aan de voorwaarde dat zijn expressie stabiel is onder alle omstandigheden. Als voorbereiding op het bepalen van de expressies van interleukine-8 en weefseltromboplastine in honden met idiopatische IHA zijn negen potentiële referentiegenen onderzocht op hun geschiktheid voor een kwantitatieve RT-PCR in volbloed van de hond (**Hoofdstuk 5**). Met behulp van twee software applicaties, GeNorm (37) and Normfinder (38), werd vastgesteld dat een combinatie van vijf tot zes referentie genen nodig is als interne standaard in kwantitatieve RT-PCR experimenten bij honden met idiopatische IHA (39). Uit de RT-PCR experimenten bleek dat de expressie van weefseltromboplastine in volbloed hoger was in honden met idiopatische IHA, maar de interleukine-8 expressie was niet significant verschillend van de expressie in gezonde honden en significant lager dan in de groepen met systemische interne ziekten, de groep met tumoren en diffuse intravasale stolling, en de groep met sepsis (**Hoofdstuk 6**).

De verhoogde expressie van weefseltromboplastine verklaart de hypercoagulabiliteit in honden met idiopatische IHA. Weefseltromboplastine expressie wordt in monocyten gereguleerd via het NF-kB signaalpad. Activatie van dit signaal pad leidt ook tot verhoogde expressie van interleukine-8 (40). Dit suggereert dat, aangezien de interleukine-8 expressie in de bloedbaan niet gestegen was honden met idiopatische IHA, een ander celtype dan de monocyten zorg draagt voor de expressie van weefseltromboplastine. Trombocyten zijn een andere mogelijke bron voor weefseltromboplastine (41). Daarnaast kunnen trombocyten endotheel cellen stimuleren tot weefsel tromboplastine expressie via CD40L-CD40 interactions (42, 43), interacties met monocyten (43), en neutrofielen (43). Dit wordt ondersteund door het feit dat p-selectine, een marker die aangeeft dat trombocyten geactiveerd zijn, verhoogd was in honden met idiopatische IHA (26, 44, 45). Verder onderzoek is nodig, maar een voorzichtige conclusie is dat anti-trombotische middelen gericht tegen trombocyten mogelijkheden kunnen bieden voor een effectievere therapeutische interventie. Retrospectief onderzoek suggereerde eerder al dat acetylsalicylzuur mogelijk een toegevoegde therapeutische waarde zou hebben bij honden met idiopatische IHA.

Immuunsuppressie staat centraal in de behandeling van idiopatische IHA met als doel het remmen van de erytrofagocytose en het onderdrukken van de antilichaamproductie en wordt indien nodig gecombineerd met erytrocyten transfusies en antistolling (1, 46).

Corticosteroïden vormen de belangrijkste component van de behandeling, maar kunnen als de klinische toestand van de patiënt onvoldoende verbetert of de bijwerkingen van corticosteroiden onacceptabel zijn in combinatie met andere andere immuunsuppressieve stoffen of cytostatica toegepast worden. Azathioprine, een thiopurine analoog, is een cytotoxisch farmaceuticum dat interfereert met de DNA synthese door competitie met adenosine en wordt als onderdeel van een standaardprotocol in de behandeling van IHA veel toegepast ondanks het feit dat bewijs voor de effectiviteit van een dergelijke combinatie ontbreekt (3, 9, 10, 17-19). Een vervelend neveneffect van cytostatica is de carcinogeniteit voor contactpersonen (47).

Er bleek geen verschil te zijn tussen de overleving van de groep honden met idiopatische IHA die behandeld werden met prednisolon en azathioprine en de groep behandeld met alleen prednisolon (8, 9) (**Hoofdstuk 3**). Tevens bleek dat ondanks de huidige aanbeveling om levenslang immuunpressie toe te passen in honden met idiopatische IHA een immuunsuppressieve therapie van drie maanden volstaat (8, 9) (**Hoofdstuk 2, 3**). Beenmergsuppressie, een bijwerking van azathioprine, werd in 8% van de honden gezien (8). Een recidive van de hemolytische crisis trad op bij minstens 10% van de honden in beide studies (8, 9).

Het kan zijn dat door het gebruik van een historische controlegroep een verschil in het effect van de twee behandelingen verborgen is gebleven omdat aannemelijk is dat in de loop der jaren de ondersteunende behandeling op de intensieve zorg afdeling verbeterd is. Een tweede verklaring kan zijn dat er een verschil in sterfterisico bestond tussen beide groepen. De honden die behandeld werden met azathioprine en prednisolon hadden een lager trombocyten aantal en de klinische verschijnselen duurde langer voorafgaand aan de diagnose. Dit kan geleid hebben tot het verborgen blijven van een additioneel behandelingseffect van de azathioprine. Echter het feit dat in een grote trial met 222 honden geen positief effect kon worden vastgesteld van azathioprine suggereert dat het potentiële gunstige effect klein is of slechts in enkele honden optreedt. Geconcludeerd kan worden dat azathioprine geen onderdeel hoort uit te maken van een standaard behandelingsprotocol voor idiopatische IHA bij de hond.

Op basis van de bevindingen in dit proefschrift kunnen een aantal aanbevelingen gedaan worden voor toekomstige klinische trials. De incidentie van idiopatische IHA bij de hond is, met ongeveer 0.2% in universitaire centra, laag waardoor het lastig is binnen een werkbaar tijdsbestek voldoende patiënten te includeren in een onderzoek (48). Het uitvoeren van zogenaamde klinische multicenter trials waarbij honden uit verschillende behandelingscentra deelnemen aan de trial kan dit voor een deel ondervangen. Een tweede probleem is het gevolg van het grote verschil in sterfterisico tussen honden met idiopatische IHA. Deels kan dit worden genivelleerd door randomisatie met als doel het verschil in sterfterisico zo gelijk mogelijk over de groepen te verdelen zodat het effect van de interventie het best

meetbaar wordt. De ontwikkeling van een prognostisch score systeem gebaseerd op de in dit proefschrift vastgestelde prognostische kenmerken kan, na validatie in onafhankelijke proefgroepen, bijdragen aan het samenstellen van een studiepopulatie met een gelijk sterfterisico in de verschillende interventiegroepen.

Om multicenter trials te kunnen opstarten is het noodzakelijk algemene overeenstemming te bereiken over de diagnostische criteria voor idiopatische IHA. Een belangrijke stap in het stellen van de diagnose idiopatische IHA is de directe agglutinatie test (DAT) (14). Het blijkt lastig DAT uitslagen van verschillende laboratoria te vergelijken omdat er weinig uniformiteit bestaat wat betreft de uitvoering (49-51). Recent is er een DAT (Diamed Benelux BV) ontwikkeld die de agglutinatie van de erytrocyten door antilichamen zichtbaar maakt door ze door een gel te filtreren. Het bleek dat in twee universitaire laboratoria de resultaten van de gel test goed correleerde met de traditionele DAT (**Hoofdstuk 4**). In vergelijking met de traditionele DAT is de DAT op gelbasis snel en gemakkelijk uitvoerbaar is, en heeft een hogere specificiteit heeft voor idiopatische IHA. Het feit dat deze test commercieel geproduceerd kan worden en daarmee veel van de stappen uit het traditionele DAT protocol standardiseert kan leiden tot de vereiste uniformering van de diagnose idiopatische IHA.

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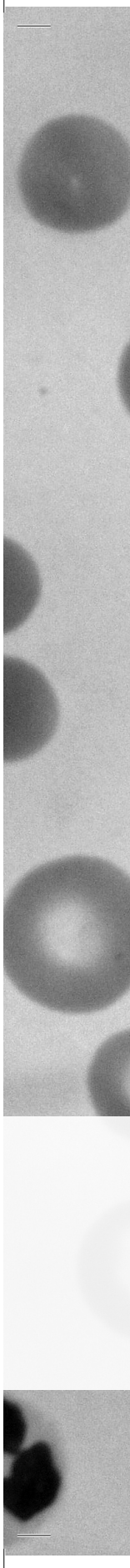

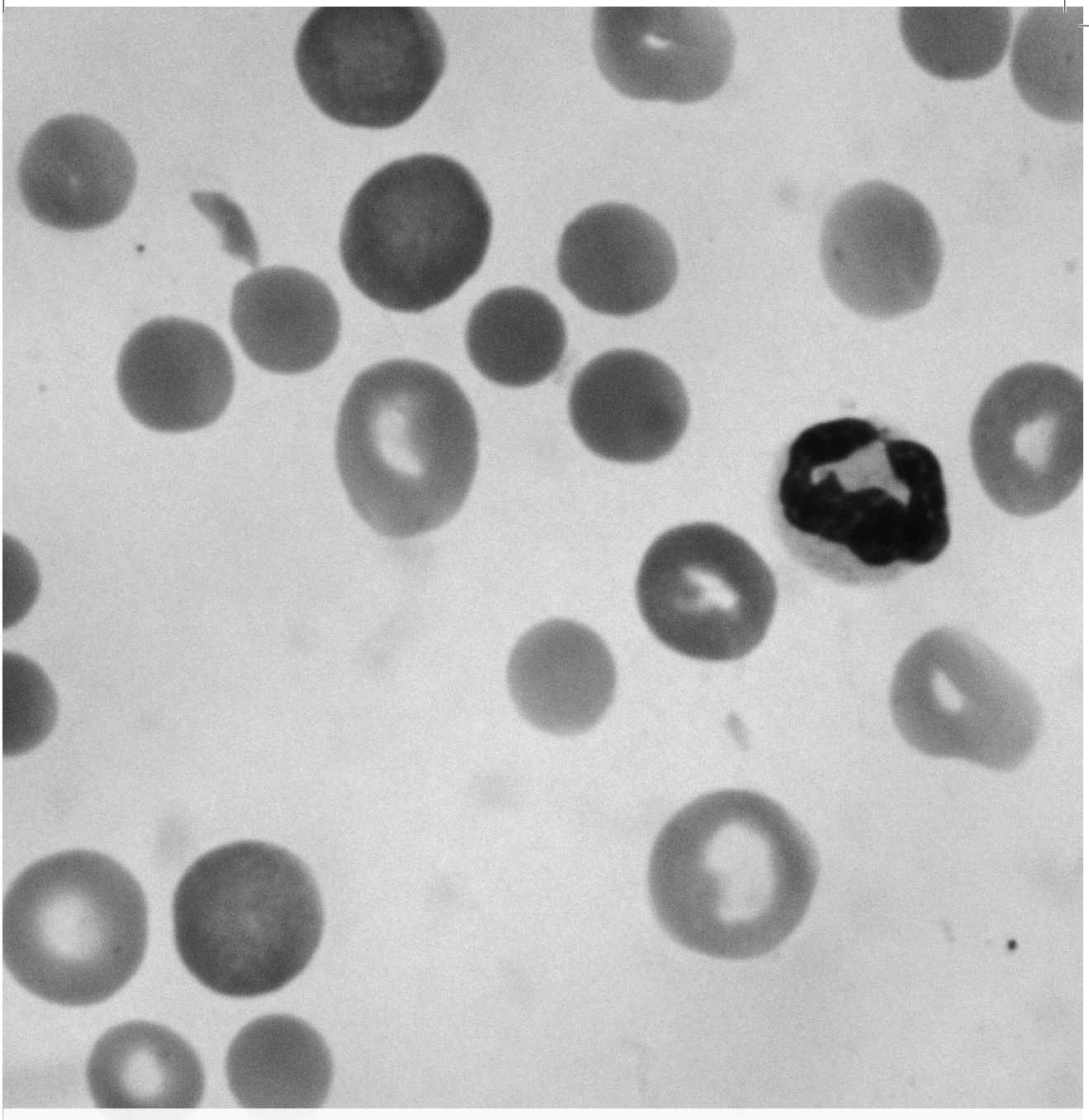

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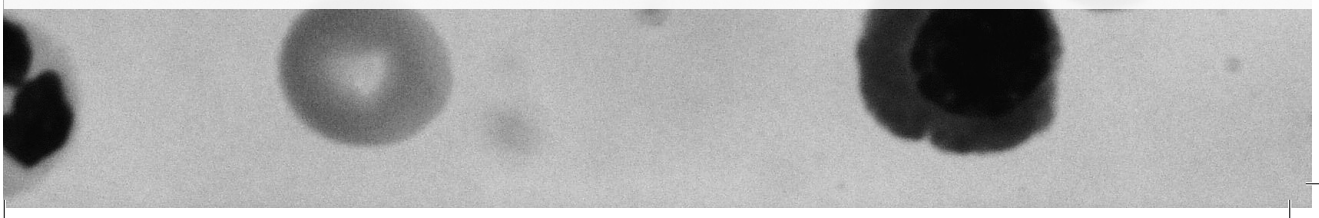
Dankwoord
Curriculum vitae
Publications



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Curriculum vitae

Born on June 9th, 1966, in Hazerswoude, The Netherlands, Dr. Christine Piek graduated from the Gymnasium Camphusianum in Gorinchem. She obtained her Doctor of Veterinary Medicine degree from the Faculty of Veterinary Medicine at the University of Utrecht in 1991. After graduation Dr. Piek worked in several primary care companion animal clinics before joining the first Dutch emergency clinic, (Spoedkliniek voor Dieren in Amsterdam), where she worked as emergency veterinarian. In 1994 she was successful in obtaining an internship in companion animal medicine, followed by a veterinary internal medicine residency in Companion Animals at the Department of Clinical Sciences at the Veterinary Faculty at the University of Utrecht. Dr. Piek successfully completed her veterinary internal medicine residency, passed her board examination and became a Diplomate of the European College of Veterinary Internal Medicine – Companion Animals (Dipl. ECVIM-CA) in 1999.

Dr. Piek was hired as contract faculty clinician and clinical instructor in the Department of Clinical Sciences at the Veterinary Faculty at the University of Utrecht. While providing support for the general companion animal internal medicine service, especially in emergency medicine, nephrology, and neurology, she obtained her Basic Teaching Qualification of the Utrecht University. Dr. R. J. Slappendel became her mentor during her advanced training in haematology and she succeeded him as haematologist in 2001 on his retirement. Her current responsibilities are overseeing the veterinary haematology service of the Department of Clinical Sciences of Companion Animals at the University of Utrecht, providing and overseeing the haematology lectures and labs in the veterinary curriculum, and training of internal medicine residents in veterinary haematology. Dr. Piek received advanced training in cytology by Dr. E. Teske and participates in the cytology caseload of the Veterinary Diagnostic Laboratory of the Department of Clinical Sciences of Companion Animals.

Dr. Piek has been Resident Adviser for the ECVIM-CA training programme for several years. She co-chaired the committee that developed the course "Blood and haematopoietic organs" at the start of the Bachelor School in 2007, and is currently the chair of this committee.

She is active in national and international post academic education in general veterinary internal medicine with emphasis on the problem oriented approach, laboratory medicine, haematology, and vector-borne diseases. She has spent time at the University of Dublin as a locum clinical pathologist and worked as locum companion animal internist at the Atlantic Veterinary College, University of Prince Edward Island. She is a member of the scientific board of the Dutch Veterinary Journal (Tijdschrift voor Diergeneeskunde).

As veterinary haematologist working with dogs with idiopathic immune-mediated haemolytic anaemia and being confronted with the adverse outcome in these patients the idea for this PhD thesis was born.

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Und jetzt Atempause…