Evaluation of responses to immunosuppressive therapy in dogs with suspected non-regenerative immune-mediated anaemia: 11 cases (2012–2018)

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Ethical statement

We declare that all relevant legal and ethical requirements have been met with regards to the humane treatment of animals described in the study. This study was approved by the Ethics screening committee in Hokkaido University Veterinary Teaching hospital (permission number 2022-003).

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Conflict of interest

No conflicts of interest have been declared.

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Summary

Objectives: We aimed to determine the response time to immunosuppressive therapy and time required to achieve a 5% increase in haematocrit among dogs with non-regenerative immune-mediated anaemia.

Methods: Eleven client-owned dogs with non-regenerative immune-mediated anaemia were evaluated. Inclusion criteria included a minimum 5-day history of anaemia, severe non-regenerative anaemia (haematocrit <20%) with an absolute reticulocyte count $<60\times10^3/\mu L$ and absence of any underlying disease. The first treatment regimen included prednisolone (2 mg/kg/day) and ciclosporin (up to 10 mg/kg/day) for 8 weeks. Dogs that did not respond to the first regimen proceeded to the second regimen comprising prednisolone and mycophenolate mofetil (15 mg/kg, twice a day). Reticulocyte count and haematocrit were monitored every 1–2 weeks. Treatment response was defined as an absolute reticulocyte count $>60\times10^3/\mu L$ or increasing haematocrit. With regard to haematocrit, the treatment response was defined only if the increase was unrelated to blood transfusion and a subsequent blood test showed an increasing trend.

Results: Treatment responses were observed in 8 of 11 dogs, and the median time to response was 39.5 days (range 8–92 days). The time required to achieve a 5% increase in haematocrit was assessed in six dogs, with a median of 55.5 days (range 8–135 days).

Clinical significance: Despite limitations related to differences in patient background, detailed information on response times, treatment response patterns, changes in treatment efficacy when changing immunosuppressive agents, side effects and complications when using predetermined immunosuppressive therapies can be used as preliminary knowledge for veterinary practitioners.

Introduction

Non-regenerative anaemia is a common form of anaemia in dogs (Chervier *et al*. 2012). Although its causes are extensive, many systemic illnesses such as inflammatory, renal, and endocrine disease result in non-regenerative anaemia (Hohenhaus & Winzelberg 2017). If systemic illness is absent, the possibility of bone marrow disease should be considered. Among multiple bone marrow disorders, non-regenerative anaemia with suspected immune-mediated pathogenesis was reported in dogs, some of whom showed evidence of an immune response to circulating erythrocytes and responded to immunosuppressive therapy (Stockham *et al.* 1980, Weiss *et al.* 1982, Kaplan *et al.* 1985, Dunn *et al.* 1986, Jonas *et al.* 1987, Holloway *et al.* 1990, Gilmour *et al.* 1991, Scott-Moncrieff *et al.* 1995, Stokol *et al.* 2000, Weiss 2008, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al.* 2021). For this reason, this type of anaemia has been referred to as a non-regenerative form of immune-mediated haemolytic anaemia (IMHA) (Jonas *et al.* 1987, Weiss 2008). Some researchers have also defined this as non-regenerative immune-mediated anaemia (NRIMA) (Stokol *et al.* 2000, Woolhead *et al.* 2021), noting the limited evidence of haemolysis in peripheral blood. Other researchers have defined the condition as precursor-targeted immune-mediated anaemia (PIMA) (Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Lucidi *et al.* 2021), focusing on the phagocytosis of erythroid cells by macrophages in the bone marrow. Although the terminology and diagnostic criteria vary, past reports are in agreement that the first choice of treatment is immunosuppressive therapy, the therapeutic outcomes of which have been reported in several papers (Stokol *et al.* 2000, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al.* 2021).

Owing to the rapid progression of anaemia in IMHA, if the haematocrit (Hct) stops decreasing or increases, the dog is considered to have responded to treatment (Swann *et al.* 2019). Additionally, several measures of disease activity (including spherocytosis, agglutination, serum bilirubin concentration, and reticulocyte count) were used to help assess the treatment response (Garden *et al.* 2019, Swann *et al.* 2019). However, in the case of non-regenerative anaemia with limited direct evidence of haemolysis, there are currently no indicators of disease activity other than Hct and reticulocyte count. If there is no change in Hct or reticulocyte count during treatment, it is necessary to determine whether the medication is not working or if more time is needed for a response. Therefore, knowing the appropriate duration of

immunosuppressive therapy to determine the therapeutic response is helpful when explaining to owners how long the initial treatment may be, especially as dogs can become transfusion-dependent during this period.

Several studies have reported the time observed for a therapeutic response, with median values ranging from 14–31 days (Stokol *et al.* 2000, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al.* 2021). As all previous reports have been retrospective studies referring to medical records, the immunosuppressive agents used in treatment varied. We therefore designed a study to determine the time observed for therapeutic response to a predetermined immunosuppressive therapy in dogs with NRIMA. In addition to the treatment response, we evaluated complications, adverse effects of immunosuppressive therapy, and treatment outcomes at 6 months after the initiation of therapy. In this study, the inclusion criteria and evaluation methods were established following previous research (Stokol *et al.* 2000); therefore, anaemia was denoted as NRIMA.

Materials and Methods

Study design

This was a single-centre, case series to evaluate the response to immunosuppressive therapy in dogs with NRIMA.

Inclusion criteria

Client-owned dogs diagnosed with NRIMA in Hokkaido University Veterinary Teaching Hospital between December 2012 and May 2018 were enrolled. On the basis of previous research (Stokol *et al.* 2000), dogs were diagnosed with NRIMA when the following criteria were met: (1) a minimum 5-day history of anaemia, (2) severe non-regenerative anaemia (Hct <20%) with an absolute reticulocyte count $<60\times10^3/\mu L$, and (3) absence of any identifiable underlying disease that may induce severe anaemia. All dogs underwent thoracoabdominal radiography and abdominal ultrasonography to detect underlying diseases. Exposure to toxins and drugs that could cause anaemia were also ruled out through history taking with the dog's owners. All included dogs underwent bone marrow examination, and bone marrow cytology was evaluated by one

88 veterinarian (KM) in charge of haematology. We excluded dogs with marked dysplastic features
89 in any cell lineage on bone marrow examination, suggesting the possibility of myelodysplastic
90 syndrome.

91 The following data were collected for each dog: age, breed, sex, clinical signs, pre-existing
92 conditions, treatment administered by referring veterinarian, results of haematological analyses
93 performed on admission, and results of bone marrow examination. All haematological analyses
94 were performed in the hospital's clinical laboratory using ProCyte DX (IDEXX Laboratories,
95 Inc.). Complete blood count (CBC), including reticulocyte count, was performed using ProCyte
96 DX. Blood smears and bone marrow cytology were evaluated by one veterinarian. Coombs tests
97 were performed at an external laboratory (Fujifilm Vet Systems Co., Ltd.) if deemed necessary
98 by the physician in charge. Dogs that had been previously treated with immunosuppressive
99 dosages of prednisolone (≥ 2 mg/kg/day) or other immunosuppressive agents at referring
100 hospitals for more than 14 days were excluded. This study was approved by the Ethics screening
101 committee in Hokkaido University Veterinary Teaching hospital. Informed owner consent was
102 obtained in all cases.

103 **Treatment administered**

104 The first treatment regimen included prednisolone (5 mg tablets; Takeda) and ciclosporin
105 (Atopica; Kyoritsu Seiyaku) for 8 weeks. The planned initial dosage of prednisolone was targeted
106 to 2 mg/kg, PO, q24h and maintained for 4 weeks. The dose of prednisolone was then reduced by
107 25% and maintained for the following 4 weeks. The initial target dose of ciclosporin was 10
108 mg/kg, PO, q24h and adjusted according to the dosage forms. To reduce the incidence of
109 gastrointestinal side effects, starting at a low dose and increasing to the target within 2 weeks was
110 considered acceptable. Ciclosporin was given for 8 weeks without tapering. Dogs that responded
111 within 8 weeks continued treatment with gradual tapering of the dose.

112 Dogs that did not respond to the first treatment regimen within 8 weeks proceeded to the
113 second treatment regimen consisting of prednisolone and mycophenolate mofetil (CellCept;
114 Chugai Seiyaku). The starting dose of prednisolone was returned to that in the first treatment
115 regimen. Mycophenolate mofetil was administered at a dosage of 15 mg/kg, PO, q12h for 8

weeks without tapering. Dogs that did not respond to both regimens and could not maintain their Hct level without transfusion were defined as poor responders.

In addition to immunosuppressive agents, famotidine (0.5–1.0 mg/kg/day, Gaster Tablets 10 mg; LTL Pharma) was administered to all dogs. Supportive care, including fresh whole blood or stored packed red blood cell transfusion, was provided as needed at the discretion of the attending clinician (KM). Use of other medications to treat concurrent illnesses during treatment was allowed, except for immunomodulating agents.

Outcomes

CBC was performed every 1–2 weeks in all dogs and changes in Hct levels and reticulocyte counts were monitored. According to previous research (Stokol *et al.* 2000), response to therapy was defined as an absolute reticulocyte count $>60\times10^3/\mu L$ or an increase in Hct. Only three consecutive confirmed transfusion-independent increases in Hct were evaluated as an increase in Hct, and the day on which an increasing trend was observed was defined as the response day. No response was defined as no increase in reticulocyte count to $>60\times10^3/\mu L$ and no increase in Hct during the treatment period of up to 16 weeks. In addition to the time to response, the time to a transfusion-independent increase in Hct >5% from the previous evaluation was recorded in responders. Treatment outcomes at 6 months after the initiation of therapy were also evaluated.

In addition to treatment response, all dogs were monitored for adverse effects of the administered drugs, which were recorded. The number of blood transfusions during treatment was also recorded for each dog. All monitoring in this study was performed at Hokkaido University Veterinary Teaching hospital.

Results

Study dogs

During the study period, 23 dogs fulfilled the inclusion criteria for NRIMA. Four dogs were excluded because they had received immunosuppressive agents for more than 14 days at the primary clinic. Three dogs were not enrolled in this clinical trial because their owners chose to treat them in primary clinics. One dog was not enrolled because the owner chose to treat it with

other medications for financial reasons. The remaining 15 dogs received the first treatment regimen. One of 15 dogs had severe non-regenerative anaemia (Hct 10.8%) and acute pancreatitis on admission, which required blood transfusion and hospitalisation for 6 days. Despite recovering from acute pancreatitis, anaemia remained non-regenerative in this dog, and its Hct continued to decrease. This dog underwent the first treatment regimen 40 days after the first admission. During the treatment regimen, two dogs were excluded because their owners failed to comply with the study protocol. Two dogs were excluded because they developed acute pancreatitis during the treatment period, making it difficult to adequately evaluate the treatment response. Finally, responses to therapy were evaluated in 11 dogs (Figure 1).

Dogs ranged from 6 to 13 years old (median age, 9 years) and included six castrated males, three intact females, and two spayed females. The study population included eight Miniature Dachshunds, one Shih Tzu, one Papillon, and one Wire Fox Terrier.

Clinical signs noted by the dog's owners included anorexia (n=9), lethargy (n=7), diarrhoea (n=4), exercise intolerance (n=3), weight loss (n=2), collapse (n=2), pica (n=1), polyuria/polydipsia (n=1), vomiting (n=1), and tachypnoea (n=1).

The median time between the discovery of anaemia at the referring clinic and the visit to our hospital was 16 days (range 5–107 days). Seven of the 11 dogs had been treated with prednisolone by the referring veterinarian. Two dogs received more than 2 mg/kg of prednisolone, one for only 1 day and the other for 3 days, tapering off on the second day. The dog with the longest treatment period (155 days) received a median prednisolone dose of 0.35 mg/kg (range 0.13–1.0 mg/kg/day) and was mostly (105/155 days) treated with <0.3 mg/kg/day. Blood transfusion was performed by the referring veterinarian in four dogs (once in two dogs, twice in two dogs). Three dogs had pre-existing conditions that included atopic dermatitis (n=2, no treatment), keratoconjunctivitis sicca (n=1, treated with ciclosporin eye drops), hypothyroidism (n=1, treated with levothyroxine), and mitral valve disease (n=1, stage B1, no treatment). The detailed medication history taken by the referring veterinarian for each dog is summarised in Supporting Information.

On the day of admission, all dogs were relatively alert and appeared tolerant of anaemia. Physical examination findings included mucous membrane pallor (n=7) and systolic cardiac murmur (n=5).

Laboratory test results

The results of haematologic testing on admission are summarised in Table 1. All dogs had moderate to severe anaemia (median Hct 15.1%) with absolute reticulocyte counts of $<60\times10^3/\mu L$. Four dogs previously received blood transfusion for non-regenerative anaemia at the referral clinics 2–87 days (median 10 days) before admission. The lowest Hct, including blood test results performed in referring clinics, was in the range 7.5%–20% (median 15.1%). Anaemia was characterised as normocytic normochromic (5/11), microcytic normochromic (3/11), normocytic hypochromic (2/11), and macrocytic hypochromic (1/11). Evaluation of blood smears revealed no spherocytosis in any dogs. Three dogs had mild thrombocytosis, and one dog had marked thrombocytopenia ($49\times10^3/\mu L$), which returned to normal soon after starting the first treatment regimen. Leucocytes were within the reference range in nine dogs. Neutrophils were increased in three dogs, all with a mild left shift. Two dogs had lymphocytopenia. All three dogs with neutrophilia and one of the two dogs with lymphocytopenia had been treated with prednisolone before admission. All Coombs tests performed in these four dogs were negative.

Regarding biochemical testing, the most common abnormalities were increases in liver enzyme activity and elevated C-reactive protein. The increases in liver enzyme activity and C-reactive protein were mild to moderate in most cases, but one dog with concurrent acute pancreatitis showed markedly increased alanine aminotransaminase (>1,000 IU/L, reference interval: 17–84 IU/L), aspartate transferase (>1,000 IU/L, reference interval: 17–44 IU/L), alkaline phosphatase (1,268 IU/L, reference interval: 47–254 IU/L), and C-reactive protein (8.0 mg/dL, reference interval: 0–1.0 mg/dL). These variables decreased (alanine transaminase 199 IU/L, aspartate transferase 39 IU/L, alkaline phosphatase 224 IU/L, C-reactive protein 0.2 mg/dL) and clinical symptoms related to pancreatitis were completely resolved when the first treatment regimen was introduced on day 40.

Bone marrow aspirates and core biopsies were performed on 11 dogs and nine dogs, respectively. In one dog, only core biopsy was evaluated because adequate aspirate smears could

not be obtained owing to hypocellularity of the bone marrow. All core biopsy specimens were considered adequate for evaluation, and the results agreed with those of aspirate smears. Bone marrow cellularity was hypercellular in nine dogs, normocellular in one dog, and hypocellular in one dog. Myelofibrosis was present in two of nine dogs, which was described as mild to moderate in one dog with hypercellular bone marrow and moderate to marked in one dog with hypocellular bone marrow. Myeloid-to-erythroid ratios were determined in 10 dogs (excluding one dog with hypocellular bone marrow) and ranged from 0.16:1 to 12.26:1. Eight of 10 dogs had a myeloid-to-erythroid ratio less than 1.0. The erythroid status was judged as hyperplasia in eight dogs, normoplasia in one dog, and hypoplasia in two dogs. The erythroid maturation sequence was evaluated in 10 dogs. There were increased proportions of rubriblasts, prorubricytes, and basophilic rubricytes relative to polychromatophilic rubricytes, metarubricytes, and polychromatophilic erythrocytes in all dogs, indicating maturation arrest. Erythrophagocytosis and rubriphagocytosis were identified in four of 10 dogs. Myeloid and megakaryocyte numbers were relatively low in two dogs with myelofibrosis, but the others were judged as normal, even the dog with thrombocytopenia. Maturations of myeloid cells and megakaryocytes were balanced in all dogs.

Therapeutic response to immunosuppressive therapy

Eleven dogs were treated with the first regimen. The median dosages of prednisolone and ciclosporin were 2.0 mg/kg, PO, q24h (range 1.9–2.3 mg/kg) and 9.0 mg/kg, PO, q24h (6.0–11.0 mg/kg), respectively. Three dogs (cases 7, 8, and 10) were started on a low dose of ciclosporin. In cases 7 and 8, the ciclosporin dose was increased after 10 and 7 days, respectively. Case 10 that was given an initial ciclosporin dosage of 6 mg/kg, PO, q24h responded to treatment before the planned dose increase; therefore, the dose was maintained. In the first regimen, seven of the 11 dogs responded to treatment, with the remaining four dogs proceeding to the second regimen. The median dosage of prednisolone and mycophenolate mofetil were 2.0 mg/kg, PO, q24h (range 1.8–2.3 mg/kg) and 14.5 mg/kg, PO, q12h (13.2–15.0 mg/kg), respectively. Of the four dogs, a treatment response was observed in only one dog; the remaining three dogs were non-responsive after 16 weeks of immunosuppressive therapy.

Therapeutic responses to immunosuppressive therapy are summarised in Table 2. A therapeutic response to immunosuppressive therapy was observed in eight of 11 dogs, and the

median time to response was 39 days (range 8–92 days). Three (Cases 1, 5, and 11) in eight responders had already been treated with >0.5 mg/kg/day of prednisolone at the referring clinics 1–3 days before admission. Adding the treatment history at referring clinics, the median time to a response was 39.5 days (range 8–92 days).

Two responders (Cases 3 and 5) were unable to continue the first treatment regimen and were changed to the second regimen owing to anorexia and nausea, possibly induced by ciclosporin; withdrawal of ciclosporin improved their symptoms. Thus, the time to achieve a 5% increase in Hct was evaluated in the remaining six dogs, which was a median of 55.5 days (range 8–135 days). Adding the treatment history at referring clinics in two dogs (Cases 1 and 11), the median time to achieve a 5% increase in Hct was 55.5 days (range 8–135 days).

Case 4 was changed to the second treatment regimen because of a decreasing trend in Hct on day 116 during the gradual decrease of prednisolone. Thus, three of the eight responders (Cases 3, 4, and 5) were switched to the second regimen and followed up. In two dogs (Cases 3 and 4), Hct showed a temporary downward trend after changing to the second regimen, followed by an upward trend. In Case 5, Hct showed an upward trend even after changing to the second regimen.

The course of anaemia at 6 months after the start of treatment was evaluated in eight responders. Four dogs (Cases 1, 3, 5, and 10) did not require blood transfusion after the treatment response was observed; at 6 months, Hcts levels in these dogs were within the reference range. Case 2 was also never transfused after the treatment response was observed, but its Hct rose slowly, with a level of 23.3% at 6 months. Case 4, which showed a recurring trend of anaemia at day 114, had a low Hct of 14.6% at 6 months. Case 9 developed a hepatic abscess on day 114 of the initial treatment regimen while continuing prednisolone and ciclosporin and died on day 119. Case 11 showed worsening anaemia at 90 days. The anaemia was regenerative (reticulocyte count 339,500 /μL) with extravascular haemolysis (total bilirubin 3.3 mg/dL). The diagnosis of IMHA was made based on the increase in spherocytes and erythrocyte autoagglutination, and treatment was resumed with the second regimen. However, the dog developed pneumonia and died on day 125.

Other complications observed during the treatment period were prostate abscess (improved with percutaneous drainage and antibiotics) and calcinosis cutis in Case 5. Of the eight

responders, only two dogs required a single blood transfusion during the treatment period. The three non-responders received one, two, and three transfusions during the 4-month treatment period.

Discussion

In this study, we evaluated the time observed for a therapeutic response to immunosuppressive therapy in dogs with NRIMA. We found that the median time required to observe a therapeutic response was 39.5 days. Additionally, to confirm an upward trend in Hct, we evaluated the time required to achieve a 5% increase in Hct; the median value was 55.5 days. Together with the findings of previous retrospective studies (Stokol *et al.* 2000, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al.* 2021), the information obtained in this study might help the explanation to owners of what duration of initial treatment is needed to achieve a therapeutic response.

Stokol *et al.* (2000) retrospectively summarised the clinicopathological features of 41 dogs with NRIMA and two dogs with pure red cell aplasia, and they evaluated the outcome of immunosuppressive therapy. The authors reported a median treatment response of 2 weeks (range: 1–10 weeks), which was short compared with our clinical experience. Therefore, we conducted a study using the same diagnostic and evaluation criteria as Stokol *et al.* (2000) to provide objective data. Factors that prolonged the time to therapeutic response in the present research compared with that previous study cannot be easily identified because of differences in study design, drugs used for treatment, and characteristics of the enrolled dogs. Among these factors, we focused on differences in the characteristics of enrolled cases. In comparison to the present study, there was considerable variability in bone marrow findings in the study by stokol *et al*. (2000) and even included dogs that were unable to aspirate bone marrow or to obtain enough marrow particles. It cannot be ruled out that incomplete bone marrow assessment may have led to the inclusion of diseases other than NRIMA, resulting in discrepancies with the present study. Furthermore, we noted that this study had a higher proportion of dogs receiving immunomodulating agents at referring clinics than those in the report by Stokol *et al.* (2000) (64% vs 42%, respectively). Most dogs that had previously received immunosuppressive agents

had very short medication histories or received low doses, so we considered that the treatment on admission had a minimal effect on the treatment response among enrolled dogs. However, if private clinics in our area tend to perform trial immunosuppressive therapy, patients with early responses, such as Case 10, are less likely to be referred to our institute. Given the small number of dogs in this study, bias in the referral cases likely affected the results.

During our research period, a few retrospective studies on canine non-regenerative anaemia were published (Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Tani *et al.* 2020, Woolhead *et al*. 2021). Assenmacher *et al.* (2019) defined dogs with persistent non-regenerative anaemia and ineffective erythropoiesis in the bone marrow as PIMA and reported their clinicopathological features, therapeutic response, and prognosis. Interestingly, a regenerative response (defined as a reticulocyte count >76,000 /μL) in 51 dogs diagnosed with PIMA was observed within a median of 29 days (range 2–111 days), which is similar to the results of our study. Although the inclusion criteria differ from those of our research, most of our cases showed maturation arrest in the erythroid lineage, which may be consistent with the diagnostic criteria for PIMA. Furthermore, the number of dogs with spherocytosis (0/11) and positive Coombs test results (0/4) in our study was similar to that of dogs diagnosed with PIMA (spherocytosis, 2/66, 3%; positive Coombs test results, 3/19, 16%) (Assenmacher *et al.* 2019). Given the similar patient backgrounds, the time observed for a therapeutic response may be similar in both studies. In another recent retrospective study, Woolhead *et al.* (2021) reported that the median time to treatment response was 34 days (interquartile range: 16–38 days) in 33 NRIMA-affected dogs that survived 3 months after the start of immunosuppressive treatment. Although there were differences in the treatments and the evaluation methods in each study, our study, with its predetermined immunosuppressive therapy, confirmed the results of recent retrospective studies (Assenmacher *et al.* 2019, Woolhead *et al*. 2021) that reported a median time to treatment response of approximately 1 month.

Several studies have reported that Miniature Dachshunds are predisposed to non-regenerative anaemia (Assenmacher *et al.* 2019, Tani *et al.* 2020, Woolhead *et al*. 2021). The Miniature Dachshund is a popular breed in Japan, and most dogs enrolled in this study (8/11 dogs) were Miniature Dachshunds. Focusing on their disease predisposition, Tani *et al.* (2020) investigated the clinicopathological features of non-regenerative anaemia in this breed and reported that the sensitivity to immunosuppressive treatments could be predicted based on thrombocytosis and

dysplastic features in the peripheral blood. We did not enrol Miniature Dachshunds that had marked thrombocytosis or dysplastic features in peripheral blood; therefore, we cannot discuss the relationship between these findings and treatment responses. The overall treatment response rate (8/11, 72.7%) in this study was not significantly different from those of previous reports (73% in Stokol *et al.* 2000; 83% in Assenmacher *et al.* 2019), but given that all three non-responders (cases 6, 7, and 8) were Miniature Dachshunds and all three non-Miniature Dachshunds (cases 1, 5, and 10) were responders, Miniature Dachshunds appeared to experience a poorer response to treatment in this small cohort of dogs. Further studies are warranted to determine whether Miniature Dachshunds have a different response to treatment and prognosis than other breeds.

Four dogs that did not respond to the first treatment regimen proceeded to the second treatment regimen, and one responded after the change in regimens. Reportedly, some dogs have shown therapeutic response after changing the immunosuppressant used in combination with prednisolone (Weiss *et al.* 1984, Kaplan *et al.* 1985, Scott-Moncrieff *et al.* 1995, Stokol *et al.* 2000, Weiss 2002). However, the therapeutic response in NRIMA varies considerably from individual to individual, and dogs showing a therapeutic response within a maximum of 15 weeks have also been reported (Stokol *et al.* 2000, Weiss 2002, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al.* 2021). Case 2, which showed a therapeutic response during the second treatment regimen, responded 92 days after the start of the first treatment regimen, but it is impossible to determine whether this response was the result of long-term immunosuppressive therapy or the change to the second treatment regimen. However, because immunosuppressive treatment was performed for at least 16 weeks in all cases, the number of dogs in which the observation period ended before showing a therapeutic response was minimal and is not considered to be a limitation in assessing treatment responses.

Although two dogs responded to the first treatment regimen, ciclosporin continued to cause anorexia and nausea, forcing withdrawal of ciclosporin. Case 5, which showed a therapeutic response on day 38, was changed to the second treatment regimen on day 45. Its Hct levels showed an upward trend even after changing treatment, with continued improvements in anaemia. Case 3, which showed a therapeutic response on day 29, was changed to the second treatment regimen on day 66. In contrast to Case 5, reticulocytes in Case 3, which had been

increasing, decreased once and required 89 days to increase again. Although it is difficult to clarify the relationship between temporary weakening of the regenerative response and the change in medication, the regenerative response may have been diminished because of ciclosporin withdrawal.

After starting immunosuppressive therapy, several complications were observed. Of particular concern is the fact that Case 9 developed a hepatic abscess while on immunosuppressive therapy and died on day 114. This dog had pancreatitis at the time of initial diagnosis, and immunosuppressive therapy was started after recovery from pancreatitis. It has been reported that some dogs that develop hepatic abscesses have a history of pancreatitis (Farrar *et al.* 1996, Schwarz *et al.* 1998), and this association is suspected in our case. In humans, the incidence of hepatic abscess increases with age and comorbidities (diabetes, malnutrition, immunodeficiency) (Lardière-Deguelte *et al.* 2015), and the administration of immunosuppressive therapy may be the cause. Time is needed to achieve a therapeutic response in NRIMA, but immunosuppressive therapy over a long period may increase the risk of opportunistic infections (Peterson *et al.* 2012, Dowling *et al.* 2016, McAtee *et al.* 2017). Although the treatment regimen in this study was mainly designed in terms of providing sufficient immunosuppressive therapy to properly evaluate treatment responses, further studies are needed to determine whether prednisolone alone or a combination of prednisolone and other immunosuppressive agents should be used.

The cases in this study received predetermined immunosuppressive therapy; however, there were some limitations, especially regarding differences in case backgrounds. First, because our veterinary hospital mainly deals with referral cases, seven of the 11 dogs were receiving some form of medication at their first visit. To minimise the effect of medication at the referring hospital, we excluded dogs that had received immunosuppressive agents for more than 14 days. Therefore, we believe that the impact of medication in these seven dogs was minimal. In future studies, including dogs that are not receiving any immunosuppressive drugs, including glucocorticoids, is ideal. The second limitation is the large number of excluded cases, which prevented us from evaluating predictors of a treatment response. Third, the protocol consisted of an initial treatment regimen with prednisolone and ciclosporin followed by a second treatment regimen with prednisolone and mycophenolate mofetil. We were therefore unable to compare the clinical efficacy of ciclosporin and mycophenolate mofetil. In particular, the second regimen

seemed to be strongly influenced by the first regimen, although the dose of prednisolone was returned to the initial dose. Fourth, for ciclosporin, pharmacokinetic monitoring or pharmacodynamic monitoring should have been performed to confirm that the dose was appropriate. Lastly, whether the combination of ciclosporin or mycophenolate mofetil provides any advantage over prednisolone alone remains unclear because we did not have a prednisolone monotherapy group.

In conclusion, we evaluated the response to treatment in 11 dogs with NRIMA and found that the median time to response was 39.5 days (range 8–92 days, n=8), and the time to obtaining a 5% increase in Hct was 55.5 days (range 8–135 days, n=6). Although there have been several studies on treatment responses (Stokol *et al.* 2000, Lucidi *et al.* 2017, Assenmacher *et al.* 2019, Woolhead *et al*. 2021), this was the first study using a defined treatment protocol. Further studies to determine the best therapeutic approach for the long-term management of affected dogs are warranted.

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Conflict of interest

No conflicts of interest have been declared.

Fugure 1. Sankey diagrams illustrating the study design of 23 dogs diagnosed with non-regenerative immune-mediated anaemia in terms of treatment response. Outcomes are written horizontally in each node with the height representing the number of dogs.

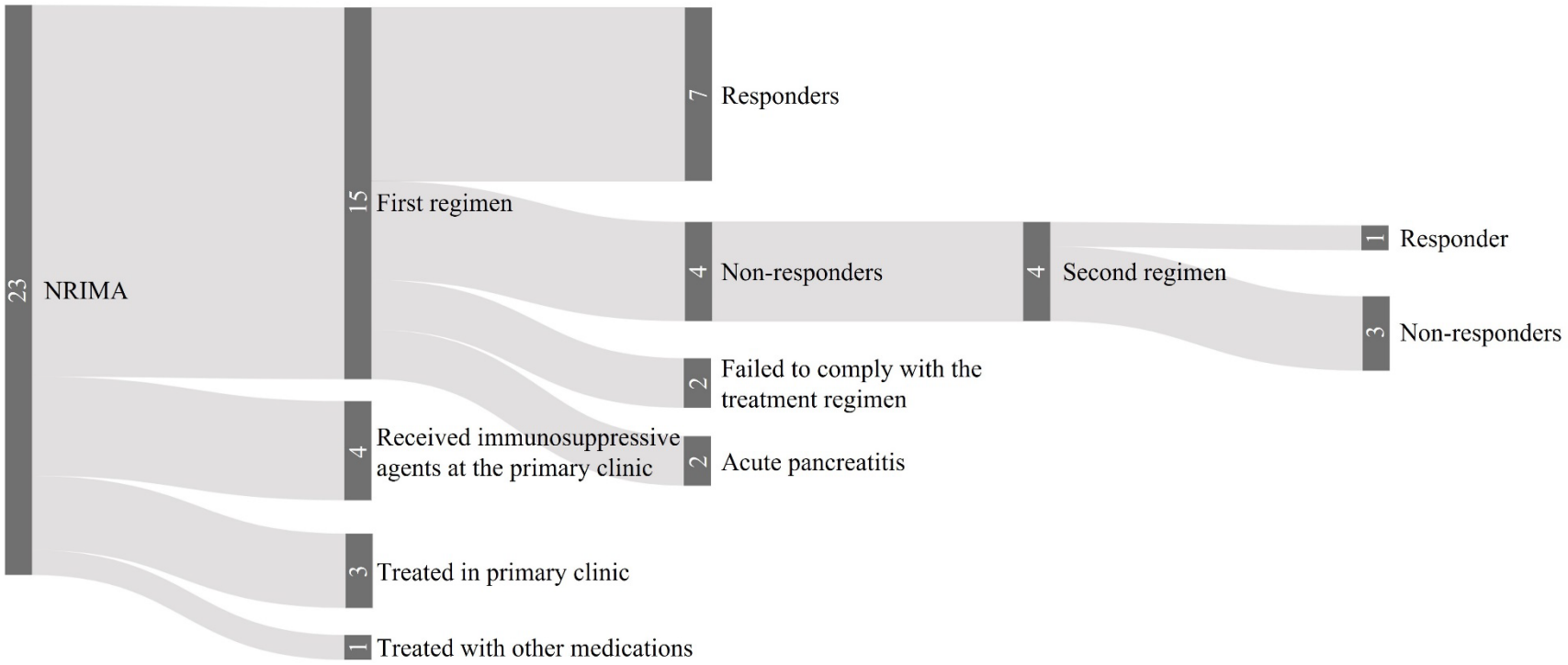

Table 1. Selected haematologic and biochemical test results in 11 dogs with NRIMA at first admission.

Variable (units)	RI	Median (range)	Number of dogs with values below RI (%)	Number of dogs with values above RI (%)
Haematocrit (%)	37–55	15.1 (7.5–25.4)	11 (100)	0 (0)
MCV (fL)	61.6–73.5	68.5 (54.1–75.5)	3 (27)	1 (9)
MCHC (g/dL)	32.0–37.9	33.1 (28.0–37.2)	3 (27)	0 (0)
Reticulocytes (/μL)		14,200 (0–41,200)		
Platelets ($\times 10^3$/μL)	148–484	373 (49–832)	1 (9)	3 (27)
Leukocytes (/μL)	5,050–16,760	14,980 (6,000–40,170)	0 (0)	3 (27)
Neutrophils (/μL)	2,950–11,640	10,580 (4,320–31,700)	0 (0)	3 (27)
Lymphocytes (/μL)	1,050–5,100	1,830 (468–7,400)	2 (18)	1 (9)
ALT (IU/L)	17–78	51.0 (27–>1,000)	0 (0)	5 (45)
AST (IU/L)	17–44	30 (17–>1,000)	1 (9)	2 (18)
ALP (IU/L)	47–254	286 (138–>3,500)	0 (0)	6 (55)
T-Bil (mg/dL)	0.1–0.5	0.2 (0.1–0.7)	0 (0)	1 (9)
CRP (mg/dL)	0–1.0	1.3 (0.2–12.0)		7 (64)

[†]Values are expressed as median (range). MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; T-Bil, total bilirubin; CRP, C-reactive protein; RI, reference interval.

Table 2. Summary of treatment results in each case.

Case (Breed)	Time to response	Rationale for response	Time to achieve a 5% increase in Hct	Outcome at 6 months
1 (Papillon)	52 days	Reticulocyte count increased from 0 (on admission) to 66,360 /μL (response day)	66 days	Hct 37.5%
2 (MD)	92 days	Reticulocyte count Increased from 49,000 (on admission) to 118,590 /μL (response day)	135 days	Hct 23.3%
3 (MD)	28 days	Reticulocyte count increased from 11,790 (on admission) to 73,320 /μL (response day)	NE	Hct 38.4%
4 (MD)	42 days	Reticulocyte count increased from 9,600 (on admission) to 65,200 /μL (response day)	42 days	Hct 14.6%
5 (Shih Tzu)	36 days	Reticulocyte count increased from 13,000 (on admission) to 68,700 /μL (response day)	NE	Hct 37.8%
6 (MD)	No response	-	-	-
7 (MD)	No response	-	-	-
8 (MD)	No response	-	-	-
9 (MD)	42 days	Hct increased from 16% (Day 29) to18.4% (Day 43)	70 days	NE
10 (WFT)	8 days	Reticulocyte count increased from 22,700 (on admission) to 100,100 /μL (response day)	8 days	Hct 42.0%
11 (MD)	18 days	Reticulocyte count increased from 25,600 (on admission) to 75,000 /μL (response day)	32 days	NE

Time to response was defined as an absolute reticulocyte count > 60×10^3/μL or an increase in Hct. Hct, haematocrit; NE, not evaluated; MD, Miniature Dachshund; WFT, Wire Fox Terrier.

Supporting Information. Medication history taken by the referring veterinarian for each dog

Case	Prednisolone administration in referring clinics	Duration of prednisolone preadministration in referring clinics	Medications at referring clinics other than prednisolone	Transfusion history prior to first visit
1	2.0 mg/kg/day for 1 day, then 1.0 mg/kg/day for 1 day, then 0.5 mg/kg/day for 1 day	3 days	-	2 days prior to visit (once)
2	-	-	ampicillin	-
3	-	-	enrofloxacin	-
4	0.35 mg/kg/day for 3 days, then 0.45 mg/kg/day for 5 days, then 0.23 mg/kg/day for 11 days, then 0.45 mg/kg/day for 5 days, then 0.23 mg/kg/day for 4 days, then 0.125 mg/kg/day for 5 days, then 1.0 mg/kg/day for 7 days, then 0.5 mg/kg/day for 7 days, then 0.23 mg/kg/day for 21 days, then 1.0 mg/kg/day for 6 days, then 0.63 mg/kg/day for 3 days, then 0.43 mg/kg/day for 14 days, then 0.3 mg/kg/day for 64 days	155 days	-	116 days and 87 days prior to visit (twice)
5	2.0 mg/kg/day for 1 day	1 day	cyclosporine eye drops	-
6	1.0 mg/kg/day for 11 days	11 days	-	-
7	1.0 mg/kg/day for 5 days	5 days	-	-
8	0.8 mg/kg/day for 14 days	14 days	-	14 days prior to visit (once)
9	-	-	-	-
10	-	-	-	-
11	0.9 mg/kg/day for 2 days	2 days	levothyroxine	33 days and 6 days prior to visit (twice)