



# HOKKAIDO UNIVERSITY

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|---------------------|---------------------------------------------------------------------------------------------------------------------------|
| Title               | Study on the clinical outcome and molecular changes of precursor-targeted immune-mediated anemia treated with splenectomy |
| Author(s)           | 須田, 芽伊                                                                                                                    |
| Degree Grantor      | 北海道大学                                                                                                                     |
| Degree Name         | 博士(獣医学)                                                                                                                   |
| Dissertation Number | 甲第15654号                                                                                                                  |
| Issue Date          | 2023-09-25                                                                                                                |
| DOI                 | <a href="https://doi.org/10.14943/doctoral.k15654">https://doi.org/10.14943/doctoral.k15654</a>                           |
| Doc URL             | <a href="https://hdl.handle.net/2115/97051">https://hdl.handle.net/2115/97051</a>                                         |
| Type                | doctoral thesis                                                                                                           |
| File Information    | Mei_Suda.pdf                                                                                                              |



**Study on the clinical outcome and molecular changes  
of precursor-targeted immune-mediated anemia  
treated with splenectomy**

(Precursor-targeted immune-mediated anemia (PIMA)に  
対する脾臓摘出術の臨床的有用性および  
分子生物学的変化に関する研究)

**Mei Sugawara-Suda**

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## Abbreviations

|        |                                                                        |
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| AUC    | areas under the curve                                                  |
| BM     | bone marrow                                                            |
| BPIFB4 | bactericidal/permeability-increasing fold-containing family B member 4 |
| CBC    | complete blood count                                                   |
| CR     | complete response                                                      |
| CR%    | corrected reticulocyte %                                               |
| CRP    | C-reactive protein                                                     |
| DEGs   | differentially expressed genes                                         |
| DIA    | data independent acquisition                                           |
| EMR    | erythroid-maturation ratio                                             |
| FC     | fold change                                                            |
| FCN1   | ficolin 1                                                              |
| FDRs   | false discovery rates                                                  |
| GMR    | granulocyte-maturation ratio                                           |
| IHC    | immunohistochemistry                                                   |
| IMHA   | immune-Mediated Hemolytic Anemia                                       |
| ITP    | immune thrombocytopenia                                                |
| IgG    | immunoglobulin G                                                       |
| LC     | liquid chromatography                                                  |
| MASP2  | mannan-binding lectin serine peptidase 2                               |
| MDs    | Miniature Dachshunds                                                   |
| MS     | mass spectrometry                                                      |
| MST    | median survival time                                                   |
| NRIMA  | non-regenerative immune-mediated anemia                                |
| PCA    | principal component analysis                                           |
| PCV    | packed cell volume                                                     |
| PIMA   | precursor-targeted immune-mediated anemia                              |
| PR     | partial response                                                       |
| PS     | phosphatidylserine                                                     |
| RI     | reference intervals                                                    |

## Notes

Contents of the present study were partially published in the following article list. The first article is corresponding to chapter 3. The second article is corresponding to chapter 1.

### **List of published articles**

- 1 . Sugawara-Suda M, Morishita K, Ichii O, Namba T, Aoshima K, Kagawa Y, Kim S, Hosoya K, Yokoyama N, Sasaki N, Nakamura K, Yamazaki J, Takiguchi M. Transcriptome and proteome analysis of dogs with precursor targeted immune-mediated anemia treated with splenectomy. PLoS One, DOI: 10.1371/journal.pone.0285415, 2023.
- 2 . Sugawara-Suda M, Morishita K, Iwanaga Y, Yamazaki J, Kagawa Y, Yokoyama N, Sasaki N, Ohta H, Nakamura K, Takiguchi M. Investigation of the therapeutic effects, predictors, and complications of long-term immunosuppressive therapy in dogs with precursor-targeted immune-mediated anemia. J Vet Med Sci, DOI: 10.1292/jvms.23-0010, 2023.

## General Introduction

Non-regenerative immune-mediated anemia (NRIMA) <sup>59,70)</sup> and precursor-targeted immune-mediated anemia (PIMA) <sup>1,2,5,37,38)</sup> have been described in dogs with non-regenerative anemia who display evidence of ineffective erythropoiesis. Both PIMA and NRIMA are currently understood to involve immune-mediated destruction of erythroid lineage cells. Recent studies indicate that PIMA is characterized by the destruction of more mature erythroid lineage cells due to the autoimmune system. Therefore, PIMA includes dogs with few signs of peripheral immune-mediated destruction, while NRIMA often includes dogs with symptoms of immune-mediated hemolytic anemia (IMHA), such as the existence of spherocytes and a positive saline agglutination test, which indicates peripheral blood destruction by the autoimmune system. Terminology and diagnostic criteria often vary between reports: in this study we use the term PIMA according to the inclusion criteria previously reported<sup>1,2,5,9,37,38)</sup>, since the dogs in this study did not have any concurrent IMHA symptoms.

In previous reports, it has been shown that in PIMA/NRIMA dogs, 50–88% of cases exhibited a regenerative response to immunosuppressive therapy within a median of 14–31 days<sup>5,38,59,70)</sup>. In addition, more than half of the dogs that showed a regenerative response achieved remission. However, it is important to note that 36% of dogs did not respond to immunosuppressive therapy and had to be euthanized within three months. Our facility has also experienced many cases of PIMA with resistance to immunosuppressive therapy, and these dogs are currently placing a significant burden on their owners due to the increased number of hospital visits and high treatment costs associated with multiple blood transfusions. Additionally, the administration of long-term immunosuppressive therapy, which often involves the use of corticosteroids, has the potential to worsen the prognosis due to its undesirable side effects. In light of these challenges, our facility has chosen to offer splenectomy as a therapeutic option for dogs diagnosed with PIMA who are unresponsive to immunosuppressive therapy. As a result, we found that several dogs that were refractory to immunosuppressive therapies indeed responded to splenectomy.

Splenectomy has been established as a therapeutic option for immune thrombocytopenia (ITP) and autoimmune hemolytic anemia in human patients<sup>7,48</sup>. While corticosteroids represent the primary course of treatment, splenectomy is typically reserved as a second-line option. In veterinary medicine, there have been reports of splenectomy being used as a treatment option for immunomediated hematological diseases<sup>9,20,31</sup>. This procedure is aimed at reducing autoantigen and eliminating a site for the destruction of thrombocytes and red blood cells. However, there are currently no reports on the use of splenectomy as a treatment for PIMA in veterinary medicine, and the mechanisms by which splenectomy may benefit dogs with PIMA have not been researched.

Thus, the purpose of this study was to investigate the clinical outcome and molecular changes of PIMA treated with splenectomy. In chapter 1, the author conducted a retrospective investigation to elucidate the limitations of the conventional treatment approach for PIMA, which involves immunosuppressive therapy. In addition, complications of immunosuppressive therapies were further investigated in all PIMA dogs. In chapter 2, the clinical outcomes of splenectomized dogs were retrospectively investigated. Finally in chapter 3, candidates for disease-related genes and serum protein were identified using RNA sequencing with spleen samples and proteomic analysis with serum before and after the splenectomy.

## **Chapter 1**

**Investigation of the therapeutic effects, predictors, and complications of long-term immunosuppressive therapy in dogs with precursor-targeted immune-mediated anemia**

# 1. Introduction

There are some reports that have described the clinicopathologic characteristics of dogs diagnosed with NRIMA/PIMA and their outcomes following immunosuppressive therapy. Several retrospective studies indicated that the median time to treatment response was about 14–31 days <sup>5,38,59,70</sup>), but there was significant variation among individuals, with some dogs requiring as long as 105 days <sup>38,67,70</sup>). Although some recent studies also examined predictors for survival, these studies were limited by the inclusion of cases in which the duration of immunosuppressive therapy was too short to assess the treatment response <sup>5,70</sup>). Therefore, it would be more appropriate to identify predictive factors influencing treatment responses only in dogs who have received immunosuppressive therapy for more than 105 days. Furthermore, in NRIMA/PIMA dogs, the prognosis is expected to be influenced by treatment response as well as complications associated with prolonged severe anemia or immunosuppressive therapy. However, the type, frequency, and timing of complications and adverse events are not known.

The purpose of this study was to investigate predictors of the therapeutic effect in PIMA dogs receiving immunosuppressive therapy for more than 105 days, which is considered a sufficient duration to determine treatment responses. In addition, complications or adverse events in PIMA dogs treated with immunosuppressive treatment, including those treated for less than 105 days, were summarized as evidence for informed consent to assist in the initiation of treatment.

## **2. Materials and methods**

### **2.1 Case selection**

First, client-owned dogs diagnosed with NRIMA or PIMA at Hokkaido University Veterinary Teaching Hospital between December 2012 and August 2020 were identified by electronic medical record review. Second, each dog was judged to ensure they met the diagnostic criteria of PIMA: (i) minimum 5-day history of severe non-regenerative anemia (hematocrit <20%) with an absolute reticulocyte count <  $60 \times 10^3/\mu\text{l}$ <sup>59,66</sup>; (ii) bone marrow (BM) cytology and/or histopathology results available and ineffective erythropoiesis identified as erythroid hypercellularity or an increase in early-stage erythroid precursors with maturation arrest that could not be attributed to a pre-regenerative response or other disease process (e.g., myelodysplastic disease or drug reaction); and (iii) complete blood count, serum biochemistry profile, and thoracic and abdominal imaging results revealed no underlying cause for severe non-regenerative anemia. Dogs were included if they fulfilled all the following data: signalments; clinical signs noticed by the owner; treatment before referral; physical examination findings; laboratory findings including complete blood count (CBC) conducted within 24 hours of bone marrow extraction or before their latest blood transfusion, corrected reticulocyte % (CR%)<sup>15</sup>, erythrocyte agglutination test; Coombs test; peripheral blood smear evaluation; treatment after referral; and treatment outcomes. Immunosuppressive therapy (e.g., more than 2 mg/kg of prednisolone or prednisolone with immunosuppressive drug/drugs) that was already started at the referral hospital was included in the treatment duration.

### **2.2 Assessment of response to immunosuppressive therapy**

To evaluate the response of long-term immunosuppressive therapy, only dogs that had been treated for more than 105 days were included in this assessment. Dogs were considered to have responded to immunosuppressive therapy if their absolute reticulocyte count increased to  $\geq 60,000/\mu\text{l}$  or they had a spontaneous increase in the packed cell volume (PCV) of >5%. A sole increase in reticulocyte without an increase

in PCV was not considered a treatment response. Dogs that failed to achieve the therapeutic response after a duration exceeding 105 days of immunosuppressive therapy were classified as non-responsive to immunosuppression. Relapse was defined as a worsening of non-regenerative anemia requiring the intensification of immunosuppressive therapy in responders.

BM cytology samples were evaluated by one clinician who was blinded to the case information, using BM smears obtained at the time of diagnosis. A total of 500 cells per slide were evaluated. The absence of polychromatophils and presence of rubriphagocytosis, and the macrophage percentage of all nucleated cells, were recorded. The myeloid-to-erythroid ratio, granulocyte-maturation ratio (GMR), and erythroid-maturation ratio (EMR) were investigated, as reported previously <sup>62,68</sup>. GMR was calculated using the following formula: (myeloblasts + promyelocytes + myelocytes)/(metamyelocytes + band neutrophils + segmented neutrophils). EMR was calculated using the following formula: (rubriblasts + prorubricytes)/(rubricytes + metarubricytes). The reference intervals (RI) of GMR and EMR were set at 0.04–0.15 and 0.02–0.12, respectively, according to a previous study <sup>68</sup>. A pathological examination of the BM was performed by a pathologist to assess fibrosis and nucleated cell and erythroblast fullness. The extent of fibrosis (mild, moderate, or severe) was graded subjectively, as described previously <sup>5</sup>.

### **2.3 Investigation of adverse events and complications**

Problems other than anemia that occurred during the implementation of immunosuppressive therapy were investigated. These included complications associated with PIMA and side effects from immunosuppressive drugs. Side effects that improved with the tapering of medications such as polyuria and polydipsia or did not require a new therapeutic intervention for the problem that arose were excluded. Because it was not always easy to distinguish between complications and side effects, the author used the term complications to describe both.

## **2.4 Statistical analysis**

All analyses were performed using commercial software (JMP Pro 16.0.0, SAS Institute Inc., Cary, NC, USA). Statistical significance was set as  $P < 0.05$ . Signalments, CBC, and BM cytology results were analyzed and compared between responders and non-responders to immunosuppressive treatment. Continuous variables (age, body weight, PCV, reticulocytes, platelets, CR%, EMR, GMR, and macrophage ratio) were analyzed by one-sided Wilcoxon's exact test. Sex, neutering status, existence or non-existence of polychromatophils and rubriphagocytosis in BM smears were analyzed by Fisher's exact test. Logistic regression analysis was performed using continuous variables that showed significant differences in the comparison between the two groups. From the results obtained, a receiver operating characteristic was drawn, and corresponding areas under the curve (AUC) were calculated to assess the predictive accuracies. The cutoff value of the continuous variable with the highest AUC was determined using the Youden Index.

## 3. Results

### **3.1 Population characteristics**

During the observation period, 50 cases presented to our clinic were diagnosed with PIMA based on bone marrow examination. The median age was 10 years (range: 4–14 years), and the dogs included 30 females (17 intact and 13 neutered) and 20 males (2 intact and 18 neutered). There were 10 different breeds represented, with the most frequent being Miniature Dachshunds (MDs) (n=35, 70%). Twenty-three dogs were on immunosuppressive therapy for less than 105 days, because of death during immunosuppressive therapy (n=8), loss to follow-up (n=8), or because they underwent splenectomy as an alternative therapy (n=7) (Figure 1).

### **3.2 Characteristics of responders and non-responders**

Twenty-seven dogs that could be followed for more than 105 days were evaluated for the effectiveness of the treatment. The median age was 10 years (range: 3–14 years) and there were 16 females (8 intact and 8 neutered) and 11 males (all neutered). The breeds were 17 MDs, 3 Toy Poodles, 2 mixed-breed dogs, and 1 each of Beagle, Labrador Retriever, Papillon, Shih Tzu, and Wire Fox Terrier. Seven dogs (26%) had been started on immunosuppressive therapy by the referring veterinarians; this comprised corticosteroids (n=7), azathioprine (n=3), cyclosporine (n=3), mycophenolate mofetil (n=4), and recombinant human erythropoietin (n=1). The median duration of immunosuppressive treatment at the referring hospitals was 21 days (range: 3–124 days) and 8 dogs received a median of 1.5 blood transfusions (range of the number of transfusions: 1–14).

On the first visit to our hospital, all dogs showed good consciousness levels and appeared tolerant of anemia. Thoracic radiography and abdominal ultrasonography showed the following findings: hepatomegaly (n=11), splenomegaly (n=8), gallstones (n=4), small amount of ascites (n=4), intra-gastric foreign bodies (n=2), mild microhepatia (n=2), and mild prostate calcification (n=1). No cases revealed any thrombus by ultrasonography.

After the first visit, all dogs were treated with prednisolone at a median dose of 2 mg/kg/day (range: 1.3–3 mg/kg/day). Twenty-two dogs also received immunosuppressive agents as follows: cyclosporine (n=14; median dose: 7.5 mg/kg/day, range: 5–10.5 mg/kg/day), mycophenolate mofetil (n=4; median dose: 27 mg/kg/day, range: 24–30 mg/kg/day), and azathioprine (n=2; median dose: 2.4 mg/kg/day, range: 2–2.7 mg/kg/day). Over 105 days of treatment, 18 dogs were classified as immunosuppressive responders and 9 as immunosuppressive non-responders. The median duration of immunosuppressive therapy was 224 days (range: 112–2,382 days) for all cases. The median time to observe a therapeutic response was 29 days in the immunosuppressive responders (range: 6–126 days), and most dogs responded to treatment within 60 days (16 of 18 dogs), but 2 required 93 and 126 days, respectively (Figure 2). Three of 18 immunosuppressive responders relapsed at 82, 212, and 233 days after the response date, respectively. All dogs relapsed when immunosuppressive therapy was being tapered. Three out of the non-responders continued immunosuppressive therapy until the last visit to our hospital, and 6 had their medication discontinued because of side effects or at the owner's request. The median duration of immunosuppressive therapy in the non-responders was 154 days (range: 112–268 days).

### **3.3 Clinicopathological data of responders and non-responders**

In 22 of the 27 dogs, CBC was evaluated within 24 hours before bone marrow examination. In the remaining 5 dogs, CBC was evaluated 3–4 days prior to the bone marrow examination. All dogs were severely anemic (median PCV 18%, range: 8–27), with low reticulocyte counts (median reticulocytes  $21.9 \times 10^3/\mu\text{l}$ , range: 0–50.5). Autoagglutination test results were negative in all 9 dogs tested, and 11 of 27 dogs that underwent Coombs testing were negative. Spherocytes were observed in 4 dogs, but none of these fulfilled the criteria of  $\geq 5$  spherocytes/ $\times 100$  oil immersion field<sup>22)</sup>. No dogs showed evident pancytopenia but 4 had bicytopenia: 1 showed mild neutropenia and 3 dogs had mild thrombocytopenia with anemia. Seven dogs had mild-to-moderate leukocytosis (range:  $17.1$ – $31.4 \times 10^3/\mu\text{l}$ ) without a left shift and 8 dogs showed thrombocytosis (range:  $494$ – $889 \times 10^3/\mu\text{l}$ ).

BM aspiration was performed in all dogs and core biopsies were obtained from 20 dogs. Aspiration was difficult in 1 dog (dry tap), which showed severe BM fibrosis on core biopsy. Fourteen dogs had erythroid hyperplasia, 8 had hypoplasia, and 4 had normoplasia. Regarding GMR and EMR, 15 dogs were within the RI and 5 were above the RI for GMR, whereas 7 dogs were within the RI and 19 were above the RI for EMR. The median macrophage ratio was 0.2% of all nucleated cells, but the ratio in 1 dog was 4.2%, which was above the RI <sup>27)</sup>. Rubriphagocytosis was detected in 8 dogs. In accordance with a previous report <sup>70)</sup>, BM polychromatophils were present in 19 dogs, despite the peripheral blood samples showing non-regenerative anemia. Among the 20 core biopsies evaluated, 13 dogs had fibrosis that was graded as mild, moderate, or severe in 6, 2, and 5 dogs, respectively.

### **3.4 Predictors of responses to immunosuppressive therapy**

The characteristics and clinicopathological data of the immunosuppressive responders and non-responders are shown in Table 1. Based on the characteristics at admission, immunosuppressive non-responders were prone to be older than responders ( $P = 0.049$ ). However, there were no significant differences in relation to sex, neutering status, body weight, and CBC results. EMR and GMR were significantly related to the response in BM, which was significantly lower in immunosuppressive responders compared with non-responders ( $P = 0.026$  and  $0.046$ ). A logistic regression analysis was conducted to determine whether age, EMR, and GMR were predictors of treatment response. Our results found that EMR and GMR were significantly associated with treatment response (age  $P = 0.100$  vs. EMR  $P = 0.015$  vs. GMR  $P = 0.024$ ). The AUC of EMR was higher than that of GMR (EMR AUC = 0.74 vs. GMR AUC = 0.71). Therefore, the author focused on EMR and a cutoff value of 0.17 was obtained based on the highest Youden Index (Figure 3). These cutoff values were associated with a sensitivity and specificity of 70.6% and 77.8%, respectively, to predict a treatment response.

### **3.5 Complications of immunosuppressive therapy**

Of the 50 PIMA-affected dogs included in this study, 8 died while on immunosuppressive therapy, and 2 died because of progressive anemia 14 and 33 days after starting immunosuppressive therapy. In these two cases, the medication was not tapered. The remaining 6 cases died of complications including pancreatitis (n=3), pneumonia (n=2), and neurological disorder (n=1). One of the 2 dogs that developed pneumonia also had megaesophagus. One dog that developed a neurological disorder presented with nystagmus and seizures and died 6 days later with a loss of consciousness. At the onset of complications, all dogs were receiving prednisolone. The details of treatments and outcomes are shown in Table 2.

Of the 27 dogs with continuous immunosuppressive treatment for more than 105 days, complications occurred in 6 dogs (22%). Of these, 4 were responders and 2 were non-responders. At the onset of the complications, 5 out of 6 dogs were treated with prednisolone and immunosuppressive drugs and 1 dog was treated with mycophenolate mofetil alone. Immunosuppressive therapy was discontinued on the day of complication onset in 4 dogs (hepatic abscess, pyothorax, prostate abscess, and pyometra). One dog developed aspiration pneumonia and died during the tapering of prednisolone. One dog developed pancreatitis 49 days after resuming immunosuppressive therapy following a relapse of PIMA. Therefore, this dog continued immunosuppressive therapy after the onset of pancreatitis. Regarding the outcomes, 2 dogs recovered from complications but the other 4 died within 30 days of diagnosis. The dog with prostate abscesses discontinued immunosuppressive therapy and received blood transfusions as necessary. The dog with pyometra resumed mycophenolate mofetil 18 days after hysterectomy.

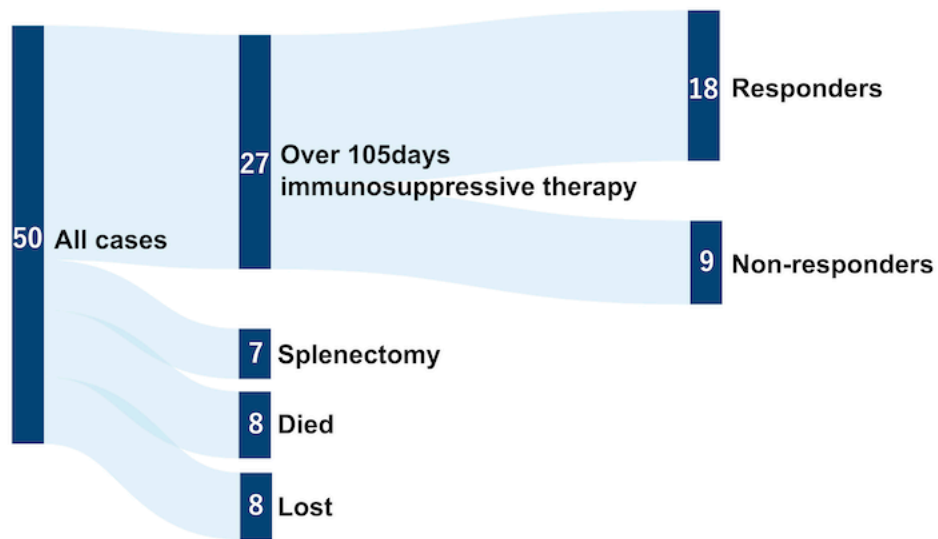


**Figure 1.** Sankey diagrams illustrating the study design of 50 dogs diagnosed with precursor-targeted immune-mediated anemia. Outcomes are written horizontally in each node with the height representing the number of dogs.

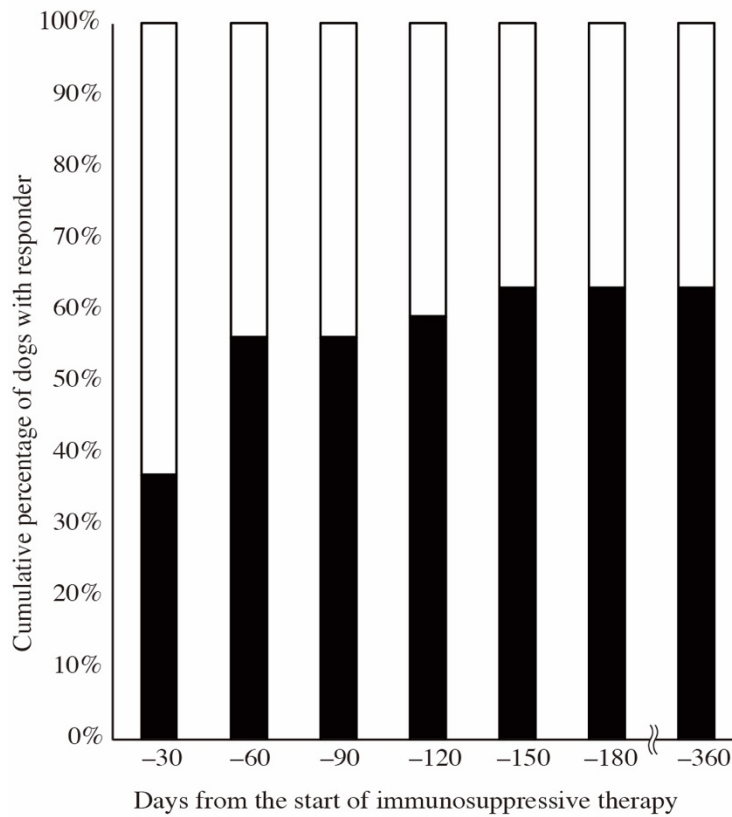


**Figure 2.** Stacked bar graph showing the cumulative percentage of responders to long-term immunosuppressive therapy per month in 28 dogs with precursor-targeted immune-mediated anemia. White = immunosuppressive non-responders; black = immunosuppressive responders.

**Table 1.** Characteristics and clinicopathological data for immunosuppressive

| Variable                                    | Reference interval | Responders <sup>a</sup>      | n  | Non-responders <sup>b</sup> | n | P value |
|---------------------------------------------|--------------------|------------------------------|----|-----------------------------|---|---------|
| Sex                                         | -                  | male 8 / female 10           | 18 | male 3 / female 6           | 9 | 0.692   |
| Neutering status (intact/neutered)          | -                  | intact 6 / neutered 12       | 18 | intact 2 / neutered 7       | 9 | 0.676   |
| Age (years)*                                | -                  | 9 (3–14)                     | 18 | 12 (5–14)                   | 9 | 0.049   |
| Body weight (kg)                            | -                  | 6.41 (4.1–30.5)              | 18 | 5.4 (3.48–7.66)             | 9 | 0.198   |
| PCV (%)                                     | 37–55              | 16.5 (8–27)                  | 18 | 18 (12–25)                  | 9 | 0.435   |
| Reticulocytes ( $\times 10^3/\mu\text{l}$ ) | 10–110             | 19.9 (0–50.5)                | 18 | 22.2 (5.5–38.9)             | 9 | 0.338   |
| Platelets ( $\times 10^3/\mu\text{l}$ )     | 148–484            | 366 (49–889)                 | 18 | 307 (136–843)               | 9 | 0.41    |
| CR%                                         | 0–1.0              | 0.26 (0–0.72)                | 18 | 0.34 (0.06–0.85)            | 9 | 0.202   |
| Result of bone marrow aspiration            |                    |                              |    |                             |   |         |
| Polychromatophils (+ / -)                   | -                  | observed 12 / not observrd 5 | 17 | observed 7 / not observrd 2 | 9 | 1       |
| Rubriphagocytosis (+ / -)                   | -                  | observed 5 / not observrd 12 | 17 | observed 3 / not observrd 6 | 9 | 1       |
| EMR*                                        | 0.02–0.12          | 0.14 (0.06–0.50)             | 17 | 0.42 (0.06–0.80)            | 9 | 0.026   |
| GMR *                                       | 0.04–0.15          | 0.05 (0.01–0.76)             | 17 | 0.05 (0–0.11)               | 9 | 0.046   |
| Macrophages (%)                             | <1                 | 0.2 (0–0.8)                  | 17 | 0.2 (0–4.2)                 | 9 | 0.235   |

**Abbreviations:** CR%, corrected reticulocyte %; EMR, erythroid-maturation ratio; GMR, granulocyte-maturation ratio

Data expressed as median (range) or number. \*Significant difference between immunosuppressive responders and non-responders ( $P < 0.05$ ).

<sup>a</sup> Responders defined as absolute reticulocyte concentration increased to  $\geq 60,000/\mu\text{l}$  ; <sup>b</sup>Non-responders defined as absence of increased absolute reticulocyte concentration ( $< 60,000/\mu\text{l}$ ) up to 105 days after starting treatment

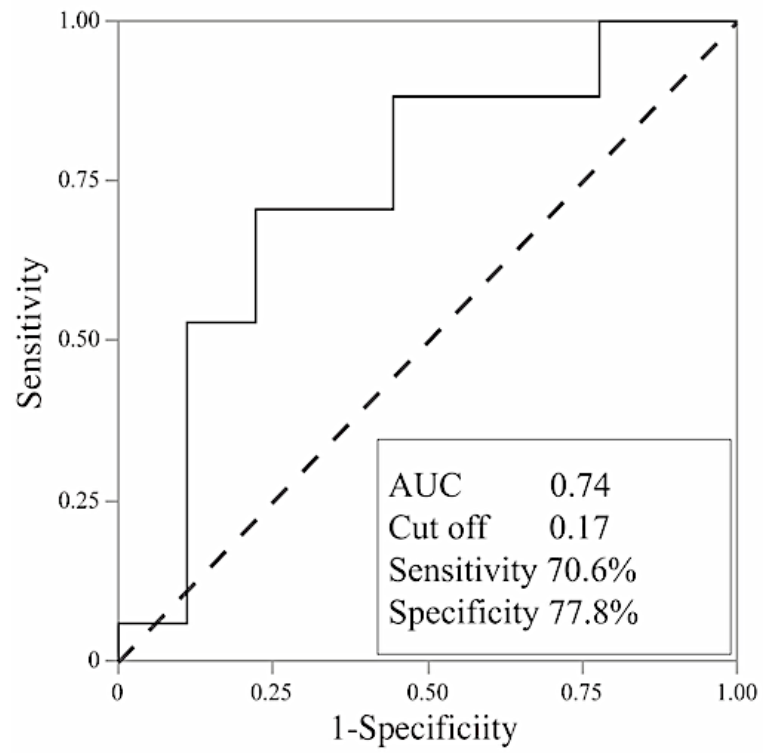


**Figure 3.** Receiver operating characteristic curves for the cutoff point of the erythroid-maturation ratio.

**Table 2.** Complications of immunosuppressive therapy with precursor-targeted immune-mediated anemia

| Complication          | Duration of immunosuppressive therapy at the time complication occurred (days) | Dosage at the time of complication (mg/kg/day) | Response for immunosuppressive therapy | Outcome              |
|-----------------------|--------------------------------------------------------------------------------|------------------------------------------------|----------------------------------------|----------------------|
| Aspiration pneumonia  | 7                                                                              | Pre (2) and MMF (20)                           | NE                                     | Died on the same day |
| Pancreatitis          | 15                                                                             | Pre (2) and CyA (8.7)                          | NE                                     | Died 13 days later   |
| Pancreatitis          | 22                                                                             | Pre (2.3) and CyA (11.5)                       | NE                                     | Died 4 days later    |
| Neurological disorder | 29                                                                             | Pre (1.9) and CyA (20)                         | NE                                     | Died 6 days later    |
| Pancreatitis          | 74                                                                             | Pre (1.6) and MMF (14.8)                       | NE                                     | Died the next day    |
| Orbital abscess       | 77                                                                             | Pre (1.4) and CyA (10)                         | NE                                     | Survived             |
| Pneumonia             | 102                                                                            | Pre (1.4) and Lef (4.3)                        | NE                                     | Died the next day    |
| Hepatic abscess       | 114                                                                            | Pre (1.4) and CyA (9.4)                        | Non-responder                          | Died the next day    |
| Aspiration pneumonia  | 116                                                                            | Pre (2) and MMF (26.8)                         | Responder                              | Died 10 days later   |
| Pyothorax             | 155                                                                            | Pre (0.2) and Lef (3.2)                        | Non-responder                          | Died the next day    |
| Prostate abscess      | 167                                                                            | Pre (1.2) and MMF (27)                         | Responder                              | Survived             |
| Pancreatitis          | 318                                                                            | Pre (2) and MMF (30.4)                         | Responder                              | Died 30 days later   |
| Pyometra              | 1,491                                                                          | MMF (12.5)                                     | Responder                              | Survived             |

**Abbreviations:** CyA, cyclosporine; MMF, mycophenolate mofetil; Lef, leflunomide; Pre, prednisolone; NE, not evaluated

## 4. Discussion

Thromboembolic events and CR% were not shown to indicate treatment response or to be prognostic factors in this retrospective study. Assenmacher et al. and Woolhead et al. previously showed that thromboembolic events and CR%, as a measure of the degree of hematopoiesis, were prognostic factors for survival in dogs with non-regenerative anemia that received immunosuppressive therapy as first-line treatment<sup>5,70</sup>). Therefore, these variables were investigated in our study population. In the present study, only 1 dog had portal vein thrombus and died. However, the cause of thrombus was suspected to be associated with pancreatitis. The author also compared the median CR% between immunosuppressive responders and non-responders. Woolhead et al. reported that a CR% < 0.2 was associated with decreased mortality at 3 months and 12 months in NRIMA dogs, and therefore, the author expected a lower CR% in the non-responders<sup>70</sup>). However, there were no differences in median CR% between responders (0.26) and non-responders (0.34). The median CR% reported by Woolhead et al. was 0.7 compared with 0.30 in the current study. This apparent discrepancy might be related to differences in the case populations between the studies. There were also differences between the studies in terms of tests used to examine immune-mediated erythrocyte destruction in the peripheral blood. Woolhead et al. reported positivity rates for the saline agglutination test, Coombs test, and spherocytosis of 54%, 8%, and 19%, respectively; however, these were all negative in our study<sup>70</sup>). These differences indicate that the cases in the study by Woolhead et al. had more clinical findings indicating an immune response to peripheral erythrocytes compared with our cases, which may have contributed to the differences in CR% between the two studies.

In contrast, the present results suggested that EMR might be a predictor of treatment response. The author observed a median EMR of 0.14 in the immunosuppressive responders and 0.42 in the non-responders. A higher EMR indicates a larger number of immature cells, which suggests that immature erythroblasts were more abundant in immunosuppressed non-responders, indicating more pronounced maturation arrest. To the best of our knowledge, no previous reports have examined EMR and treatment response in relation to non-regenerative anemia. Lucidi et al.

reported the presence of immunoglobulin G (IgG) and phosphatidylserine (PS) in erythroid lineage cells at each maturation stage of bone marrow cells in dogs with PIMA. Their study demonstrated that IgG and PS positive cells were more likely to be present in the mature rubricyte cells<sup>37)</sup>. This result suggested that mature nucleated red blood cells are susceptible to destruction by the immune system. Thus, the high EMR can be the result of active regeneration of the erythroid lineage to compensate for immune-mediated destruction of mature nucleated red blood cells. Although this study did not include cases with marked dysplastic features of bone marrow cells, high EMR is known to be a feature of primary myelodysplastic syndrome with refractory anemia<sup>68)</sup>. Tani et al. reported that they suspected the possibility of myelodysplastic syndrome in MDs that were resistant to immunosuppressive therapy because of higher platelet counts and higher frequencies of hyper-segmented neutrophil and spindle shape platelet in peripheral blood compared to responders<sup>62)</sup>. If non-regenerative anemia in MDs is difficult to distinguish between PIMA and myelodysplastic syndrome on bone marrow cytology, the higher EMR in the non-responders could be due to the inclusion of myelodysplastic syndrome cases. Further research is warranted to determine whether EMR can be a prognostic factor, including approaches to pathophysiology.

Overall, the treatment response in the current study was 29 days, which was similar to previous reports by Assenmacher et al. and Woolhead et al., reporting 29 and 31 days, respectively<sup>5,70)</sup>. The distribution of treatment response time showed that 9 of the 18 dogs responded within 30 days, 7 within 30–60 days, and the remaining 2 responded by 93 and 126 days, respectively. Therefore, to distinguish between responders and non-responders, treatment should be continued for at least 60 days, even if no response is detected by 30 days.

In this study, complications were observed over the entire study period from initial treatment to long-term immunosuppression. Pancreatitis (n=4), abscesses (n=3), and pneumonia (n=3) were observed in multiple cases. All three pneumonia cases in this study were diagnosed in MDs, with aspiration pneumonia secondary to megaesophagus in two of the three cases. In a retrospective study in Japan, megaesophagus was significantly overrepresented in MDs<sup>46)</sup>. Higher numbers of aspiration pneumonia cases associated with megaesophagus might have been observed in this study compared with

previous studies because of the high number of MDs. Likewise, pancreatitis was also reported in immune-mediated diseases treated with protocols similar to PIMA <sup>16)</sup>.

Although the relationship between pancreatitis and immune suppressive therapy or non-regenerative anemia is unknown, all dogs diagnosed with pancreatitis suffered from persistent anemia and were prescribed prednisolone at a dose of more than 2 mg/kg. Therefore, pancreatitis may be a complication requiring attention, especially during periods of uncontrolled anemia.

Additionally, pancreatitis and pneumonia were reported as complications in previous PIMA/NRIMA retrospective studies <sup>5,70)</sup>, our study is the first to report the presence of infection, including abscesses. These complications were observed in cases receiving immunosuppressive treatment from 77 to 167 days. In a report of opportunistic fungal infections of the skin, the median diagnosis of opportunistic infection was reported at a median of 43 days after the immunosuppressive treatment was initiated<sup>42)</sup>. Although at different sites of infection, the abscesses and pyothorax observed in this study were most likely opportunistic infections related to the underlying immunodeficiency caused by prolonged immunosuppressive therapy. Furthermore, 2 of the 4 dogs that developed a bacterial infection died because of complications, suggesting that opportunistic infections may have a significant impact on prognosis. Therefore, it is important to consider the following two points when planning a treatment course for PIMA: first, most dogs that responded to immunosuppressive treatment had an effective response within 60 days; and second, we should carefully consider treatment lasting more than 60 days, especially with regard to the onset of opportunistic infections associated with immunodeficiency.

This study had several limitations. First, the study population was small and had a breed bias. Although there were 65 suspected cases of PIMA, 15 dogs were excluded because BM examination was not performed. Thus, some comparisons may have lacked the required statistical power to show a significant difference. However, most previous reports focused on immunosuppressive responders, whereas the current study is the first to observe both responders and non-responders to immunosuppressive therapy for PIMA over a span of a certain period. Second, the study was limited by its retrospective nature. Because 26% of the dogs were referred to our hospital after the initiation of

treatment, we were unable to standardize the time interval between the onset of treatment and bone marrow testing, which could have affected the findings.

In conclusion, a novel finding of our study is that an  $EMR < 0.17$ , which can be calculated from the initial bone marrow examination findings, is a positive prognostic factor. EMR can be calculated from the initial bone marrow examination, and it may be useful for predicting treatment responses and initial treatment planning. Furthermore, few dogs improved from non-responders to responders after 60 days of treatment whereas more complications, including suspected opportunistic infection, were observed after 70 days. Therefore, the author concluded that treatment plans should be reconsidered if immunosuppressive therapy is ineffective, despite sustained administration for 60 days. The effectiveness of alternative treatments such as splenectomy <sup>9)</sup> should also be examined to reduce the risk of complications associated with the long-term use of immunosuppressive therapy.

## 5. Summary

Dogs with PIMA are commonly treated with immunosuppressive therapy, but information on predictors of treatment response and response time is limited. Therefore, the author retrospectively investigated predictive factors that influenced the treatment response and duration required to observe a response in dogs with PIMA receiving continuous immunosuppressive therapies for more than 105 days. Of 50 client-owned dogs that developed PIMA, 27 were included in this study, of which 18 were responders and 9 were non-responders to immunosuppressive therapies. Sixteen of the 18 responders responded to treatment within 60 days and the remaining 2 responded at 93 and 126 days, respectively. The author found that an erythroid-maturation ratio of  $< 0.17$  may be a useful predictor for treatment response. In addition, complications of immunosuppressive therapies were investigated further in 50 dogs. Pancreatitis and pneumonia occurred over the entire treatment period, and infections such as abscesses tended to be more common in dogs on an extended period of immunosuppressive therapy. These findings may be helpful when planning for the initial treatment and may provide evidence for informed consent about potential comorbidities throughout the treatment course.

## **Chapter 2**

### **Investigation into the therapeutic effects of splenectomy in dogs with precursor-targeted immune-mediated anemia**

# 1. Introduction

In chapter 1, it was indicated that adverse events should be considered possible when administering long-term immunosuppressive therapy and that if the dogs do not respond to treatment within 60 days, it is unlikely that they will respond to treatment thereafter. Hence, there is an imperative need for alternative therapies for PIMA, particularly for dogs that exhibit resistance to immunosuppressive treatment.

Splenectomy has long been known as a treatment for ITP in humans. Corticosteroids are the standard initial treatment, but splenectomy is chosen as a second-line treatment <sup>48)</sup>. In the case of ITP, the spleen performs a dual function as the primary site for both the production of autoantibodies and the destruction of antibody-coated platelets. Therefore, the removal of the spleen may reduce the levels of anti-platelet antibodies and antibody-coated platelets <sup>12)</sup>. Splenectomy is performed in cases of autoimmune hemolytic anemia with the expectation of a similar mechanism in humans <sup>7)</sup>. Recently, a consensus statement for the treatment of canine IMHA recommends splenectomy as a third-line therapeutic option <sup>60)</sup>.

To date, there have only been a few reports describing splenectomy as a potential treatment for immune-mediated hematological diseases in dogs, including IMHA and immune-mediated thrombocytopenia <sup>9,20,31)</sup>. Notably, the efficacy of splenectomy as a treatment option for PIMA in dogs has yet to be studied. Therefore, chapter 2 aims to investigate the therapeutic efficacy of splenectomy in dogs by describing the clinical outcomes of dogs diagnosed with PIMA who underwent splenectomy.

## **2. Materials and methods**

### **2.1 Case selection**

Client-owned dogs diagnosed with PIMA at the Hokkaido University Veterinary Teaching Hospital between April 2015 and June 2021 were recruited through reviewing an electronic medical record.

Each dog was assessed to decide if it met the diagnostic criteria of PIMA<sup>5)</sup>, which includes: (1) minimum 5-day history of severe non-regenerative anemia (hematocrit <20%) with an absolute reticulocyte count of  $<60 \times 10^3/\mu\text{l}$ <sup>19)</sup>. If a dog's reticulocyte count exceeded 60,000 / $\mu\text{l}$  at the time of their visit to our facility, they were only considered for inclusion if their count had been below this threshold at their initial visit to the referring veterinarian and treatment had commenced at that time; (2) The results of BM cytology and/or histopathology were obtainable. We included dogs that exhibited ineffective erythropoiesis, which was delineated as the existence of one of several erythropoietic response patterns (e.g., erythroid hypercellularity or an escalation in early-stage erythroid precursors with maturation arrest) that could not be associated with a pre-regenerative response or any other disease progression (e.g., myelodysplastic syndrome or drug reaction); and (3) Complete blood count, serum biochemistry profile, and thoracic and abdominal imaging results, which revealed no underlying cause of severe non-regenerative anemia.

Canines that fulfilled the aforementioned diagnostic criteria and underwent splenectomy were included in this chapter.

### **2.2 Medical record review**

The following data were retrieved for each dog: signalments; past medical history; laboratory findings, including CBC and biochemistry with first visit to our hospital; BM cytology results; the type and duration of immunosuppressive treatment; number of transfusions; the reasons for splenectomy; post-splenectomy progress, and the outcome of splenectomy.

### **2.3 Response to splenectomy**

The dogs were considered to have responded to splenectomy if their absolute reticulocyte count increased to  $> 60 \times 10^3/\mu\text{l}$ .

The criteria in determining the effectiveness of splenectomy were as follows:

- Complete response (CR): PCV reached the reference range and was maintained without any treatment.
- Partial response (PR): PCV did not reach the reference range but was maintained without blood transfusion and immunosuppressive therapy.
- Poor response: Splenectomy did not appear to result in clinical benefit and the dog's condition/treatment remained similar to, or was worse than, that before splenectomy.

### **2.4 Statistical analysis**

The statistical analyses were performed through the utilization of proprietary software (JMP Pro 16.0.0, SAS Institute Inc., SC, USA). The Kaplan-Meier approach was used to compute the aggregate survival duration.

### **3. Results**

#### **3.1 Signalment and clinical signs**

In total, 20 dogs met the inclusion criteria. The dogs ranged in age from 3 to 15 years (median, 11.5 years) and included six sexually intact females, six spayed females, and eight sexually intact males. The study population consisted of 15 MDs, 2 chihuahuas, and 1 each of the following: toy poodle, shih tzu, and mix breed.

A total of 17 dogs visited the referral hospital presenting with a medical history that included anorexia (n=9), lethargy (6), hematuria (3), polyuria/polydipsia (2), vomiting (1), pica (1), weakness (1), and collapse (1). There were two dogs whose symptoms remained undocumented in the medical record. One dog presented without any clinical signs, and anemia was discovered during a routine physical examination. On admission, 16 dogs were already receiving medication. Treatment administered included corticosteroids (n=16), mycophenolate mofetil (5), cyclosporine (4), and azathioprine (1). The median duration of treatment was 30 days (range: 8–242 days). Four dogs had received blood transfusions (range: 1–14 times).

#### **3.2 Laboratory test results**

All of the dogs had severe non-regenerative anemia (median 15.4%, range: 7.5–25.0%) and a low reticulocyte count (median 21,600/ $\mu$ l, range: 55,000–201,500/ $\mu$ l) at the time of admission to the veterinary teaching hospital. Regarding blood biochemistry, the elevation of C-reactive protein (CRP) was observed in seven dogs. Selected results from the CBC and serum biochemical tests from dogs with PIMA are shown in Table 3. The direct Coombs test was performed on 13 dogs, the results of which were all negative.

All 20 dogs underwent bone marrow aspiration, while core biopsy was performed on 16 of them. Bone marrow cellularity was judged to be normal in 1, increased in 11, and decreased in 3 dogs. There were 18 dogs with erythroid hyperplasia and 7, with erythroid hypoplasia. The evaluation of the core biopsies revealed myelofibrosis in nine dogs, which was graded as mild, moderate, or severe in five, one, and three dogs, respectively.

### **3.3 Treatment before splenectomy**

Of the canines, 19 continued with immunosuppressive therapy following their referral to our hospital. The median duration of immunosuppressive therapy prior to splenectomy was 113 days (range: 0–295 days). Treatment modalities included corticosteroids for all 19 dogs, cyclosporine for 20, mycophenolate mofetil for 9, and leflunomide for 2. Blood transfusions were administered to the dogs before their splenectomy based on their respective PCV levels. Specifically, 12 dogs received transfusions one day prior to their surgeries, while 2 other dogs received transfusions two days prior. One of the dogs did not require a transfusion, while the other five dogs were transfused more than a week prior to their splenectomy and therefore did not need a transfusion immediately prior to their procedures. These five dogs had received transfusions 7, 9, 14, 23, and 35 days before their respective surgeries.

### **3.4 Splenectomy and subsequent treatment course**

The background of splenectomy of the 20 dogs were as follows: 13 dogs exhibited no response to immunosuppressive drugs, 5 experienced a relapse despite an initial positive response, 1 suffered from an infection, and 1 underwent splenectomy as a first-line treatment. One of the dogs had to discontinue immunosuppressive therapy due to the development of a prostate abscess, despite its effectiveness in treating anemia. The other dog, which was diagnosed with otitis externa at the time of PIMA diagnosis, initially did not receive immunosuppressive therapy due to the risk of worsening the infection. Instead, splenectomy was chosen as the primary treatment for PIMA.

All of the spleens were examined via the histopathology and were diagnosed with extramedullary hematopoiesis. No dogs had neoplastic disease.

There were 2 dogs who died within 7 days after splenectomy; hence, the response was only evaluated in 18 dogs. The suspected cause of death was the progression of anemia and aspiration pneumonia associated with megaesophagus, respectively. In one of the two dogs, delayed transfusion-related anemia might have been induced by the blood transfusion before the splenectomy. Since the onset of the reaction occurred after the patient's discharge from the hospital, we were unable to intervene in their treatment.

The development of aspiration pneumonia in the other dog, associated with megaesophagus prior to splenectomy, made it inadvisable to continue with immunosuppressive therapy; thus, we opted for splenectomy. Unfortunately, the aspiration pneumonia worsened, and the dog eventually died.

For the remaining 18 dogs, regular checkups were performed every two to three weeks post-surgery and once a month after three months post-surgery. Immunosuppressive therapy was discontinued immediately after splenectomy in 12 dogs. For the remaining seven dogs, immunosuppressive therapies were gradually reduced before being discontinued. Among these dogs, six discontinued prednisolone within one month following splenectomy, and one discontinued the drug within two months. All 18 dogs had increased reticulocytes up to 60,000/ $\mu$ l, and the median time required was 16 days (range: 0–35 days). The median maximum value of reticulocytes during the whole observation period was 130,000/ $\mu$ l (range: 71,700–368,200/ $\mu$ l), and the median number of days required to reach this value was 28 days (range: 2–207 days).

At the end of the three-month observation period, the 18 dogs were classified as follows; 8 dogs as CR, 7 dogs as PR, and 3 dogs as poor. Most of the cases of CR and PR showed a clear decrease in transfusion frequency after splenectomy. Two dogs included in the PR category required temporary transfusion because of decreased activity and hemolysis, respectively. Except for this period, an increase in reticulocytes was confirmed and did not require the administration of immunosuppressants. Three dogs were classified as having a poor response. One dog determined to be poor responded to the resumption of immunosuppressive therapy. The remaining two patients were only treated symptomatically with repeated blood transfusions. The median of the transfusion duration was 35 days (range: 8–35 days) before and 37.5 days (range: 6–99 days) after splenectomy, with no significant difference. A comparison of PCV, reticulocyte count, and number of transfusions before and after splenectomy and the treatment effect are summarized in Table 4.

### **3.5 Long-term Outcome**

The median observation period after splenectomy was 681 days (range: 85–2299 days) in 18 dogs. During the observation period, no adverse event, such as thrombus formation, a known adverse event of splenectomy in humans and dogs, was identified, and no cases developed opportunistic infections such as those reported in chapter 1. The median survival time (MST) was 1,150 days in the total study population of 20 dogs (Figure 4).

Among these, 11 dogs were alive and 9 dogs were dead by the end of the observation period. The suspected causes of death were delayed acute hemolysis (n=2), neoplastic disease (n=2), aspiration pneumonia (n=1), uremia due to ureteral stones (n=1), and unknown (n=3).

**Table 3.** Selected results from CBC and serum biochemical tests from dogs with PIMA at the time of admission to our hospital

| Variable        | Units                | Median | range         | Number of Dogs with Values |     |      | RI             | n  |
|-----------------|----------------------|--------|---------------|----------------------------|-----|------|----------------|----|
|                 |                      |        |               | <LRL                       | WRL | >URL |                |    |
| PCV             | %                    | 15.4   | 7.5–25.0      | 20                         | 0   | 0    | 37–55          | 20 |
| MCV             | fl                   | 64.95  | 52.6–82.0     | 8                          | 10  | 2    | 61.6–73.5      | 20 |
| MCHC            | g/dl                 | 33.2   | 28.8–36.8     | 5                          | 15  | 0    | 32.0–37.9      | 20 |
| Reticulocyte    | /μl                  | 21,600 | 5,500–201,500 | 2                          | 17  | 1    | 10,000–110,000 | 20 |
| Platelet        | ×10 <sup>3</sup> /μl | 384.5  | 172–1,375     | 0                          | 11  | 9    | 14.8–48.4      | 20 |
| Leukocytes      | /μl                  | 13,970 | 6,230–106,120 | 0                          | 12  | 8    | 5,050–16,760   | 20 |
| Total protein   | g/dl                 | 6.6    | 5.3–7.5       | 0                          | 14  | 2    | 5.0–7.2        | 16 |
| Albumin         | g/dl                 | 3.25   | 2.3–3.7       | 1                          | 13  | 0    | 2.6–4.0        | 16 |
| Total bilirubin | mg/dl                | 0.2    | 0.1–0.5       | 0                          | 15  | 0    | 0.1–0.5        | 15 |
| CRP             | mg/dl                | 0.6    | 0–7.0         | 0                          | 11  | 7    | 0–1            | 18 |

**Abbreviations:** PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; CRP, C-reactive protein

**Table 4.** Packed red blood cell, reticulocyte count, and transfusion requirements for the individual dogs

| Dog | Effects of splenectomy | Hct (%)<br>(S) | Hct (%)<br>(PS) | Reticulocyte count (/μl)<br>(S) | Reticulocyte count (/μl)<br>(PS) | Transfusion # (BS) | Transfusion # (AS) |
|-----|------------------------|----------------|-----------------|---------------------------------|----------------------------------|--------------------|--------------------|
| 1   | CR                     | 11.6           | 34.5            | 19,900                          | 102,400                          | 5                  | 0                  |
| 2   | CR                     | 10.4           | 29.9            | 22,500                          | 46,700                           | 2                  | 0                  |
| 3   | CR                     | 15.4           | 37.5            | 28,300                          | 75,000                           | 3                  | 0                  |
| 4   | Poor                   | 9.9            | 11.6*           | 10,000                          | 42,600                           | 5                  | 20                 |
| 5   | Poor                   | 18.1           | 12.9*           | 11,200                          | 12,300                           | 20                 | 25                 |
| 6   | CR                     | 15.4           | 37.6            | 59,800                          | 64,300                           | 2                  | 0                  |
| 7   | NE                     | 12.5           | —               | 15,300                          | NE                               | 5                  | NE                 |
| 8   | CR                     | 22.3           | 34.1            | 13,800                          | 83,200                           | 4                  | 0                  |
| 9   | PR                     | 8.6            | 19.4            | 33,600                          | 59,900                           | 3                  | 2                  |
| 10  | NE                     | 14.3           | —               | 8,100                           | NE                               | 2                  | NE                 |
| 11  | CR                     | 12.5           | 43.3            | 15,100                          | 83,700                           | 1                  | 0                  |
| 12  | Poor                   | 16.7           | 20.4**          | 56,400                          | 10,100                           | 3                  | 1                  |
| 13  | PR                     | 14.6           | 25.4            | 26,300                          | 96,100                           | 1                  | 2                  |
| 14  | PR                     | 12.2           | 28.1            | 12,800                          | 44,500                           | 5                  | 0                  |
| 15  | PR                     | 14.2           | 25.8            | 42,700                          | 102,000                          | 3                  | 0                  |
| 16  | PR                     | 17.1           | 25.6            | 55,800                          | 122,300                          | 2                  | 0                  |
| 17  | PR                     | 10.6           | 23.2            | 11,400                          | 74,900                           | 9                  | 1                  |
| 18  | PR                     | 14.1           | 28.5            | 48,700                          | 225,200                          | 1                  | 0                  |
| 19  | PR                     | 15.6           | 26.8            | 38,700                          | 48,400                           | 2                  | 0                  |
| 20  | CR                     | 16.4           | 35.1            | 19,800                          | 55,600                           | 4                  | 0                  |

Abbreviations: CR, complete response; PR, partial response; Poor, poor response; S, at splenectomy; PS, three months post splenectomy; #BS, number before splenectomy; #AS, number after splenectomy

\*: Blood transfusions were performed approximately once a month due to transfusion dependence.

\*\* : Immunosuppressive therapy was also continued after splenectomy.

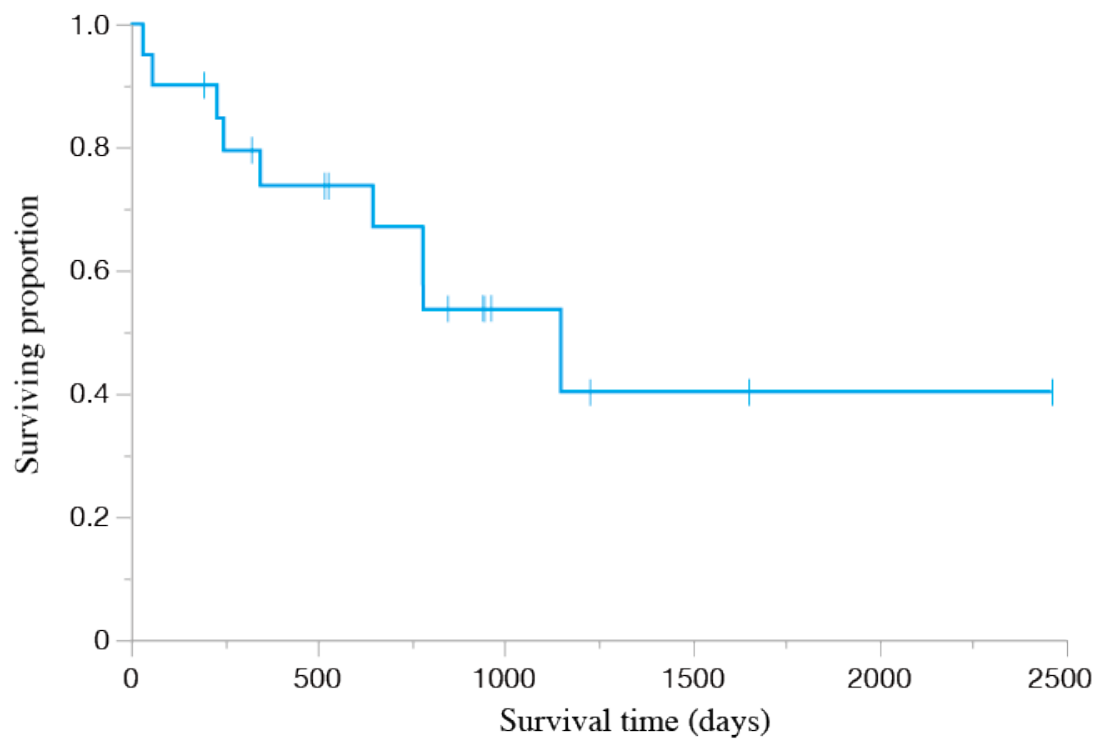


**Figure 4.** A Kaplan-Meier survival curve was generated to analyze the survival rate of 20 dogs who underwent splenectomy, resulting in a median survival of 1,150 days.

## 4. Discussion

This study found that 75% of dogs with PIMA who were treated via splenectomy had a favorable prognosis. They achieved a discontinuation of immunosuppressive therapy and showed improvement in terms of blood transfusion dependence. In total, 18 dogs were observed in the post-splenectomy course, and an improvement of non-regenerative anemia was obtained in 15 of them (8 dogs with CR and 7 dogs with PR). Moreover, the MST of the present study (1,150 days) was considerably enhanced compared to a previous study of PIMA (913 days). These results indicate that splenectomy is an effective treatment for PIMA dogs that are resistant to immunosuppressive therapy.

The literature on splenectomy for IMHA, ITP, and Evan's syndrome indicates that approximately 44–80% of patients achieved the successful cessation of immunosuppressive therapy following the procedure <sup>9,20,31,32</sup>. Although the diseases are different and so cannot be directly compared, the study finding that immunosuppressive therapy could be discontinued in 75% of cases indicates that splenectomy may be a more efficacious or equivalent treatment option for PIMA based on previously reported success rates in immunomediated hematological diseases.

From the standpoint of complications during the treatment period, no apparent adverse event that related splenectomy were reported in this study. A study examining the outcome of dogs undergoing splenectomy for non-splenic neoplastic disease revealed that 15% (31 out of 203 dogs) died in the immediate postoperative phase. Most of the fatalities during this period were attributed to shock and respiratory failure due to abdominal hemorrhage in the early postoperative recovery stage <sup>57</sup>. In the present study, two dogs died due to suspected delayed transfusion reaction and aspiration pneumonia secondary to megaesophagus. Therefore, the authors do not consider splenectomy to be the direct cause of death in these cases.

Other adverse effects following splenectomy in both dogs and humans have been reported. An example is infectious diseases, especially sepsis and thrombus formation <sup>69</sup>. However, in the present study, there was no adverse event reported that raised

concern for these complications. On the other hand, reduced vascular reservoir function and reduced capacity for extramedullary hematopoiesis are concerns after splenectomy<sup>28</sup>). During our study, splenic pathology revealed extramedullary hematopoiesis in all dogs with PIMA. This raised concerns about the potential for a shortened transfusion interval in patients who were dependent on hematopoiesis in the spleen. Nevertheless, a comparison of transfusion intervals before and after splenectomy in the two dogs who were unresponsive to splenectomy demonstrated no significant changes. This finding had a favorable impact on cases where splenectomy was ineffective. However, the underlying reasons for this outcome were not elucidated in the present study. Further investigation is warranted due to the limited number of cases and the brief observation period.

Previous research documented those patients with PIMA treated exclusively with immunosuppressive therapy had an MST of 913 days with a positive treatment response. This study reveals that splenectomy demonstrated longer MSTs of 1,150 days. However, an accurate interpretation of this result requires careful consideration of the study limitations. Because splenectomy is not yet a firmly established treatment for PIMA, it was selected for use in circumstances where immunosuppressive therapy proved to be ineffective in the present study group. This means that all of the dogs in this study survived for two to three months, despite being unresponsive to immunosuppressive therapy. This may have induced a potential bias in favor of an extended MST by naturally selecting dogs that could survive for a minimum of two to three months. On the other hand, if splenectomy had been ineffective or if complications had arisen, their survival period would have been significantly shortened. Therefore, the author concluded that the splenectomy could be an effective therapy for PIMA. Since this study aimed to evaluate the therapeutic efficacy of splenectomy as a second-line therapy for PIMA, further prospective studies are warranted to investigate the therapeutic effect of splenectomy as a first-line treatment.

## **5. Summary**

This chapter presents a retrospective analysis aimed at evaluating the therapeutic potential of splenectomy in dogs with PIMA. A total of 20 dogs underwent splenectomy, and 18 of them were followed up for the assessment of outcomes. Of these, 15 dogs were successfully treated post-splenectomy, with immunosuppressive therapy discontinued and transfusion dependence withdrawn. Furthermore, the MST was observed to be 1,150 days, which is longer than in previous studies that utilized immunosuppressive therapy alone. The data obtained from this chapter suggests that splenectomy may serve as an effective therapeutic for PIMA in cases where the dogs fail to exhibit a satisfactory response to immunosuppressive therapy.

## **Chapter 3**

**Transcriptome and proteome analysis of dogs with  
precursor targeted immune-mediated anemia treated  
with splenectomy**

# 1. Introduction

According to chapter 2, seventy-five percent of dogs refractory to immunosuppressive therapy responded to splenectomy which suggested effective treatment for PIMA dogs.

Intriguingly, splenectomy has been recommended as an alternative treatment for refractory immune-mediated hematopoietic diseases in both humans and dogs <sup>3,9,11,20,31</sup>). In humans, spleen-specific plasma cells that produce anti-platelet antibodies were reported to be responsible for the improvement in immune-mediated thrombocytopenia following a splenectomy; however, there have been no similar veterinary studies to determine the mechanism of splenectomy in animals <sup>41</sup>).

Therefore, the objective of chapter 3 is to investigate the factors that might improve non-regenerative anemia after splenectomy with two approaches: transcriptome analysis of the spleen with PIMA dogs and healthy dogs and proteomic analysis with pre- and post-splenectomy sera of the same individual dogs of PIMA.

## **2. Materials and methods**

### **2.1 Ethics statement**

Spleen and serum samples were obtained from client-owned dogs for veterinary diagnostic and treatment purposes with informed consent and the permission of the Ethics Screening Committee, Veterinary Teaching Hospital, Hokkaido University (permission number 2022-003). As controls, we used spleen samples that had been collected and preserved from healthy dogs that were euthanized in another study approved by the Hokkaido University Faculty of Veterinary Medicine Institutional Animal Care and Use Committee (#20-0081).

### **2.2 Study population**

This study included 21 client-owned dogs diagnosed with PIMA that were presented to the Department of Internal Medicine at the Veterinary Teaching Hospital of Hokkaido University. The inclusion criteria were as follows: (1) minimum 5-day history of severe non-regenerative anemia (hematocrit < 30%) with an absolute reticulocyte count <  $60 \times 10^3/\mu\text{l}$  <sup>59,66</sup>; (2) bone marrow cytology and/or histopathology results available with a diagnosis of ineffective erythropoiesis, defined as the presence of an erythropoietic response pattern (such as erythroid hypercellularity or increase in early-stage erythroid precursors with maturation arrest) not attributable to a pre-regenerative response or other disease process (such as myelodysplastic disease or drug reaction); (3) complete blood count, serum biochemistry profile, and thoracic and abdominal imaging results revealing no underlying cause for severe non-regenerative anemia; and (4) splenectomy performed because the animal had responded poorly to immunosuppressive therapy for > 2 months, or because the animal was unable to receive immunosuppressive therapy due to suspected infection. Details of all the dogs are summarized in Table 5.

### **2.3 Spleen samples**

The histopathology of the spleen samples was evaluated by a member of the American College of Veterinary Pathologists. Spleens from healthy control dogs

showed no significant changes, but spleen samples from three non-PIMA and 21 PIMA dogs showed extramedullary hematopoiesis. Splenic masses were diagnosed as hematomas and adipose nodules. Spleen samples were collected and stored in 10% neutral buffered formalin or RNAlater (Qiagen, Hilden, Germany) buffer at  $-80^{\circ}\text{C}$  for further histopathological and molecular analyses.

#### **2.4 Serum samples**

Serum samples were only collected from dogs with PIMA. Blood samples were collected before and after the splenectomy by jugular venipuncture into tubes containing a coagulation activator and a gel separator. The tubes were then kept at room temperature until visible clotting had occurred. The samples were then centrifuged at  $1,000 \times g$  for 10 min and the serum was separated and stored at  $-80^{\circ}\text{C}$  until analysis. Pre-splenectomy serum samples were collected before blood transfusion. Post-splenectomy serum samples were collected approximately 2 months after surgery to eliminate the invasive effects of surgery, which may have affected the results.

#### **2.5 Transcriptome analysis**

Total RNA was extracted from PIMA and non-PIMA spleen tissues using an RNeasy Mini Kit (Qiagen). RNA integrity was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies Japan, Tokyo, Japan), and the RNA integrity number of the total RNA isolated from each spleen was  $> 8.5$ . Sequencing libraries were prepared using a QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) (Lexogen GmbH, Wien, Austria). RNA sequencing (75-bp single-end) was conducted with NextSeq 500 (Illumina, San Diego, CA, USA) using a NextSeq 500/550 High Output Kit v2.5 Kit (Illumina), and a minimum of 2.1 million reads were generated for each sample. Quantified read counts and differentially expressed genes (DEGs) were determined using RaNa-Seq (a bioinformatics tool for the analysis of RNA-seq data; [https:// ranaseq.eu/index.php](https://ranaseq.eu/index.php)). FASTQ files were pre-processed with the Fastp tool <sup>13)</sup>, and expression was quantified using Salmon <sup>47)</sup> with the reference genome Can\_Fam\_3.1. RNA sequences have been deposited in the repository of the DNA Data Bank of Japan with the accession number DRA015678 (mdpima-0002\_Submission).

Differential expression was analyzed using DESeq2, and DEGs were determined based on an adjusted *P*-value of  $< 0.05$  and  $\log_2$  fold change (FC)  $> |2.0|$ . The distinct gene expression patterns were visualized using principal component analysis (PCA) and volcano plots using the program R <sup>49)</sup> and RJSplot <sup>8)</sup>.

## **2.6 Immunohistochemistry (IHC) of the spleen**

IHC was performed using deparaffinized histological sections with a thickness of 3  $\mu\text{m}$ . The sections were incubated in 10 mM citrate buffer (pH 6.0) for 15 min at 110 °C, treated with 0.3% H<sub>2</sub>O<sub>2</sub>/methanol solution for 20 min, and blocked using 10% normal goat serum (SABPO kit; Nichirei Bioscience, Tokyo, Japan). The sections were then incubated overnight with primary antibodies to S100A8/A9 (1:5000; NBP2-45295; Novus Biologicals, Centennial, Co, USA.) at 4 °C, followed sequentially by incubation with biotinylated goat anti-mouse IgG(H+L) (1:100; 1031-08, Southern Biotech, Birmingham, AL, USA) for 30 min at room temperature, incubation with streptavidin-horseradish peroxidase using the SABPO kit (Nichirei Bioscience) for 30 min, and then incubation with 3,3-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with hematoxylin and examined under a microscope (Olympus, Tokyo, Japan), and were then converted to virtual slides using a Nano-Zoomer 2.0-RS (Hamamatsu Photonics, Shizuoka, Japan) at 40 $\times$  magnification.

## **2.7 Quantitative analysis for IHC samples**

S100A8/A9 stained slides were analyzed with QuPath ver 3.2.0 <sup>6)</sup>. Three areas of 1 mm<sup>2</sup>, each containing one lymph nodule, were selected in each slide for S100A8/A9 signal counting. Hematoma and other lesions disrupting normal splenic structures were excluded from the analysis. To avoid the edge effects, areas within 500  $\mu\text{m}$  from the tissue edge were not used. Cell segmentation was performed using the StarDist extension for QuPath based on optical sum density values <sup>51)</sup>. S100A8/A9 positive signals were distinguished from hemosiderin and formalin pigments during annotation. Annotation was performed for every slide. The average number of positive cells per 1 mm<sup>2</sup> area was used for further analysis.

## **2.8 Serum proteome analysis**

Serum samples from 4 different dogs with 2 different conditions (pre-splenectomy and post-splenectomy) were procured to conduct serum proteomic analysis individually. Albumin was removed from eight serum samples using a ProMax albumin removal kit (Bangs Laboratories Inc., Fishers, IN, USA). 10  $\mu$ l of serum was added to 35  $\mu$ l of ProMax Binding/Wash Buffer and 50  $\mu$ l of ProMax particles, followed by gentle mixing for 20 min at room temperature. The particles were then collected by magnetic separation and the albumin-containing supernatant was removed. The particles were washed three times with 500  $\mu$ l ProMax Binding/Wash Buffer and then mixed for 10 min at room temperature in 80  $\mu$ l of 100 mM Tris-HCl pH 8.5 and 0.5% sodium dodecanoate. The supernatant was transferred to a fresh 1.5 mL tube after magnetic separation of the particles. 20  $\mu$ l of the sample was treated with 10 mM dithiothreitol at 50 °C for 30 min, followed by alkylation with 30 mM iodoacetamide in the dark at room temperature for 30 min. The reaction was stopped with 60 mM cysteine and incubated for 10 min. The mixture was then diluted with 150  $\mu$ l of 50 mM Tris-HCl pH 8.0 and digested by the addition of 400 ng of Trypsin/Lys-C mix (Promega, Madison, WI, USA) and incubation overnight at 37 °C. The digested sample was acidified with 30  $\mu$ L of 5% trifluoroacetic acid and then sonicated (Bioruptor UCD-200, CosmoBio, Tokyo, Japan) for 5 min. The mixture was then shaken for 5 min and centrifuged at 15,000  $\times$  g for 5 min. The sample was desalted using C18-StageTips<sup>50</sup>), then dried using a centrifugal evaporator. The dried peptides were dissolved in 3% acetonitrile and 0.1% formic acid, and the concentration was measured using a colorimetric peptide assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The samples were finally transferred to a hydrophilic-coated low-adsorption vial (ProteoSave vial; AMR Inc., Tokyo, Japan).

For liquid chromatography (LC) separation, the mobile phases consisted of 0.1% (v/v) formic acid as solvent A and 0.1% (v/v) formic acid/80% (v/v) acetonitrile as solvent B. Each peptide sample (200 ng) was injected directly onto a 75  $\mu$ m  $\times$  12 cm nanoLC nano-capillary column (Nikkyo Technos Co., Ltd., Tokyo, Japan) at 40 °C and then separated by a 40 min gradient at a flow rate of 150 nL/min using an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). Peptides eluted from the

column were analyzed using a Q Exactive HF-X (Thermo Fisher Scientific) for overlapping window data-independent acquisition-mass spectrometry <sup>4,35</sup>. Mass spectrometry (MS) 1 spectra were collected in the range of 495–785 m/z at 30,000 resolution to set an automatic gain control target of 3e6 and maximum injection time of 55. MS2 spectra were collected for ions > 200 m/z at 15,000 resolution to set an automatic gain control target of 3e6, maximum injection time of “auto”, and a normalized collision energy of 28%. The isolation width for MS2 was set to 4 m/z and overlapping window patterns in 500–780 m/z were window placements optimized by Skyline v4.1 <sup>40</sup>.

MS files were searched against a dog spectral library using Scaffold data independent acquisition (Proteome Software, Inc., Portland, OR, USA). The dog spectral library was generated from a dog protein sequence database (Proteome ID: UP000002254) by Prosit <sup>23,55</sup>. The Scaffold DIA (data independent acquisition) search parameters were as follows: experimental data search enzyme, trypsin; maximum missed cleavage sites, 1; precursor mass tolerance, 8 ppm; fragment mass tolerance, 8 ppm; static modification, cysteine carbamidomethylation. The protein identification threshold was set for both peptide and protein false discovery rates (FDRs) of < 1%. Peptide quantification was calculated using the EncyclopeDIA algorithm <sup>54</sup> in Scaffold DIA. For each peptide, the four highest quality fragment ions were selected for quantification. Protein quantification was estimated from the summed peptide quantification. We only used proteins that met the following criteria: peptide FDR < 1%; protein FDR < 1%; number of peptide fragments  $\geq 2$ . The proteomic datasets have been deposited into the repository of ProteomeXchange and Japan ProteOme STandard Repository with the accession numbers PXD039913 and JPST002024 respectively. Ultimately, the average value of protein quantification for all four samples, both pre- and post- splenectomy, were computed and subjected to statistical analysis. The thresholds for altered proteins were  $P < 0.05$  (t-test) and fold change > 2 or < 0.5 between the pre- and post-splenectomy groups.

## **2.9 Pathway analysis**

DEG- and differentially expressed protein-related pathways were analyzed with PANTHER 17.0 (<http://www.pantherdb.org/>), using the annotation dataset “Reactome pathway” to identify the activated pathways. We only included significantly ( $P < 0.05$ ) upregulated or downregulated pathways in our results.

### **2.10 Statistical analysis**

Statistical analysis of the IHC results was performed using JMP Pro 16.0.0 (SAS Institute, Cary, NC, USA). The Steel–Dwass test was used to compare positive cells in PIMA spleens, non-PIMA spleens, and healthy spleens in IHC slides. Statistical significance was defined as  $P < 0.05$ . Statistical analysis of the proteome results was performed using Microsoft Excel for Mac 16.64 (Microsoft Corporation, Redmond, WA, USA).

**Table 5.** Clinical and histological characteristics of dogs with precursor-targeted immune-mediated anemia and healthy control dogs

| Dog        | Age | Sex | Breed            | PCV (%) | Clinical diagnosis | Histopathological diagnosis of spleen | Outcome of splenectomy for non-regenerative anemia |
|------------|-----|-----|------------------|---------|--------------------|---------------------------------------|----------------------------------------------------|
| healthy 1  | 3   | F   | beagle           | -       | Healthy            | no significant change                 | —                                                  |
| healthy 2  | 3   | F   | beagle           | 43      | Healthy            | no significant change                 | —                                                  |
| healthy 3  | 3   | F   | beagle           | 43      | Healthy            | no significant change                 | —                                                  |
| PIMA 1     | 4   | CM  | MD               | 17      | PIMA               | EH                                    | Improved                                           |
| PIMA 2     | 9   | SF  | MD               | 19      | PIMA               | EH                                    | Improved                                           |
| PIMA 3     | 10  | CM  | Mix              | 13      | PIMA               | EH                                    | Improved                                           |
| PIMA 4     | 12  | SF  | MD               | 14      | PIMA               | EH                                    | Improved                                           |
| PIMA 5     | 11  | F   | MD               | 24      | PIMA               | EH                                    | Improved                                           |
| PIMA 6     | 9   | CM  | MD               | 14      | PIMA               | EH                                    | Unable to evaluate                                 |
| PIMA 7     | 4   | F   | MD               | 12      | PIMA               | EH                                    | Improved                                           |
| PIMA 8     | 14  | F   | MD               | 11      | PIMA               | EH                                    | Improved                                           |
| PIMA 9     | 15  | SF  | MD               | 21      | PIMA               | EH                                    | Improved                                           |
| PIMA 10    | 12  | CM  | MD               | 18      | PIMA               | EH                                    | Improved                                           |
| PIMA 11    | 13  | F   | MD               | 27      | PIMA               | EH                                    | Improved                                           |
| PIMA 12    | 13  | SF  | MD               | 20      | PIMA               | EH                                    | not improved                                       |
| PIMA 13    | 13  | SF  | MD               | 11      | PIMA               | EH                                    | not improved                                       |
| PIMA 14    | 5   | M   | Chihuahua        | 13      | PIMA               | EH                                    | not improved                                       |
| PIMA 15    | 13  | F   | MD               | 17      | PIMA               | EH                                    | Unable to evaluate                                 |
| PIMA 16    | 10  | CM  | MD               | 15      | PIMA               | EH                                    | Improved                                           |
| PIMA 17    | 12  | SF  | Mix              | 16      | PIMA               | EH                                    | Improved                                           |
| PIMA 18    | 5   | CM  | MD               | 17      | PIMA               | EH                                    | Improved                                           |
| PIMA 19    | 13  | CM  | Shih Tzu         | 18      | PIMA               | EH                                    | Improved                                           |
| PIMA 20    | 13  | F   | MD               | 16      | PIMA               | EH                                    | Improved                                           |
| PIMA 21    | 14  | SF  | Toy poodle       | 13      | PIMA               | EH                                    | Improved                                           |
| non-PIMA 1 | 14  | CM  | MD               | 45      | Spleen mass        | EH, adipose nodule                    | —                                                  |
| non-PIMA 2 | 11  | CM  | Golden retriever | 38      | Spleen mass        | EH, hematoma                          | —                                                  |
| non-PIMA 3 | 13  | SF  | MD               | 38      | Spleen mass        | EH, hematoma                          | —                                                  |

CM, castrated male; EH, extramedullary hematopoiesis; F, female; M, male; MD, Miniature Dachshund; PIMA, precursor-targeted immune-mediated anemia; SF, spayed female.

### 3. Results

#### **3.1 Transcriptome analysis and pathway analysis**

The author analyzed spleen samples from 15 dogs with PIMA and three healthy dogs to identify PIMA-related DEGs in the spleen. A total of 15,903 genes were detected by transcriptome analysis. The gene expression profiles were analyzed by PCA (Figure 5), which demonstrated the clear separation between normal and PIMA spleen samples.

Total of 1,385 DEGs were identified between spleen samples from healthy dogs and dogs with PIMA (8.7% of all identified genes), with a  $P < 0.05$ . Of these, 707 genes were upregulated and 678 were downregulated in dogs with PIMA compared with healthy dogs (Figure 6). The top ten over- and under-expressed genes are shown in Table 6. We then performed pathway analysis of the 300 most strongly upregulated and downregulated DEGs. The top ten pathways that were enriched according to the upregulated genes included the cell cycle and DNA replication, possibly because extramedullary hematopoiesis was detected by histopathologic examination in all dogs with PIMA, but not in healthy dogs. The top ten over- and under-expressed pathways are shown in Table 7.

#### **3.2 IHC for S100A8/A9**

Transcriptome analysis revealed that *S100A12*, *S100A8*, and *S100A9* were the most highly overexpressed genes in the spleens of dogs with PIMA ( $p < 9.6\text{E-}11$ ,  $2.9\text{E-}10$ , and  $6.8\text{E-}10$  respectively; Table 6). We focused on the heterodimeric protein S100A8/A9, which plays a role in erythrocyte hematopoiesis and complement activation<sup>52,53</sup>). We verified the protein expression levels and localization of S100A8/A9 in spleen samples from 21 dogs with PIMA, three healthy dogs, and three non-PIMA dogs, to investigate the relationship between S100A8/A9 and extramedullary hematopoiesis. Few S100A8/A9-positive cells were observed at the white pulp margin in healthy dogs and non-PIMA dogs, while numerous S100A8/A9-positive cells were observed in the medullary red pulp as well as the white pulp in dogs with PIMA (Figure 7). The number of S100A8/A9-positive cells was significantly higher in PIMA spleen

samples compared with healthy and non-PIMA spleen samples ( $P= 0.0412$  and  $P = 0.0412$ , respectively; Figure 8).

### **3.3 Differentially expressed proteins and pathways in pre- and post-splenectomy serum samples in dogs with PIMA**

We performed proteomic profiling of serum samples using LC tandem mass spectrometry to examine the physiological changes that occur before and after splenectomy. We collected pre- and post-splenectomy samples from four dogs with PIMA (totaling eight samples). From these samples, we identified 432 proteins, of which 342 met the analysis criteria. A total of 22 proteins were differentially expressed between the pre- and post-splenectomy serum samples ( $P < 0.05$ , fold change  $\geq 2$ ; Table 8). 12 proteins were downregulated and 11 were upregulated in the post-splenectomy serum samples compared with the pre-splenectomy serum samples.

Finally, we focused on the pathways that appeared to be enriched, according to analysis of the 22 serum proteins that we found to be significantly altered. The Reactome pathway “ficolins bind to repetitive carbohydrate structures on the target cell surface” was significantly up-regulated in pre-splenectomy samples ( $P= 4.91\text{E-}06$ ). This pathway included ficolin 1 (FCN1) and mannan-binding lectin serine peptidase 2 (MASP2), which were up-regulated 4- and 2.8-fold, respectively, in pre- compared with post-splenectomy samples.

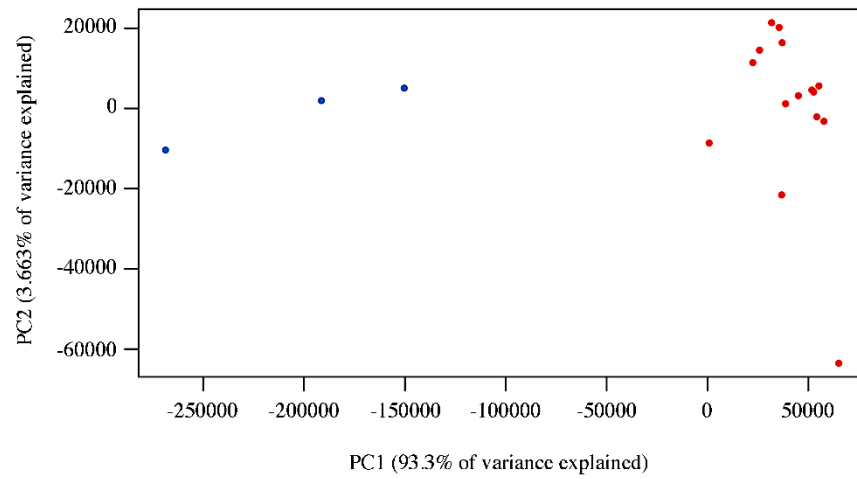


**Figure 5.** PCA of spleen samples from dogs with PIMA and healthy dogs. Red: dogs with PIMA; blue: healthy dogs.

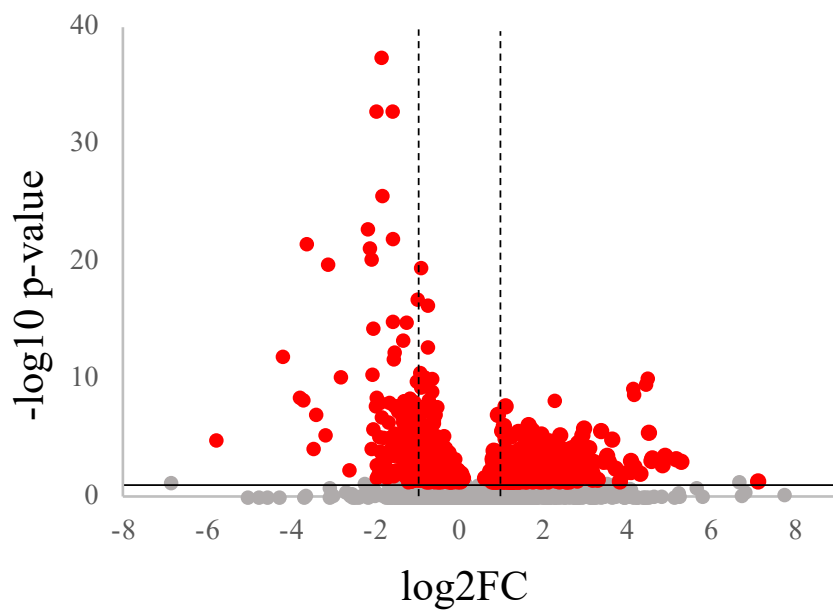


**Figure 6.** Volcano plot of DEGs detected by transcriptome analysis in dogs with PIMA compared with healthy dogs. Red points indicate genes that were significantly increased or decreased in dogs with PIMA compared with healthy dogs ( $P < 0.05$ ). The x-axis shows  $\log_2$  fold-changes in expression and the y-axis shows the  $-\log_{10} P$ -value of the DEG. The two dotted lines show the size cut-offs for  $\log_2$  fold-changes while the horizontal line shows the  $P$ -value cutoff.

**Table 6.** Top ten over- and under-expressed genes in dogs with PIMA vs healthy dogs

| Ensembl ID           | Gene Symbol          | Mean Expression | log2 Fold Change | adjusted p value |
|----------------------|----------------------|-----------------|------------------|------------------|
| ENSCAFG00000023324.2 | S100A12              | 8755.88         | 4.49             | 9.60E-11         |
| ENSCAFG00000017557.2 | S100A8               | 8888.91         | 4.44             | 2.90E-10         |
| ENSCAFG00000029470.1 | S100A9               | 839.68          | 4.15             | 6.80E-10         |
| ENSCAFG00000013763.3 | LTF                  | 192.64          | 4.16             | 2.30E-09         |
| ENSCAFG00000007851.3 | HMGB2                | 327.60          | 2.28             | 7.20E-09         |
| ENSCAFG00000009488.5 | MTDH                 | 546.70          | 1.09             | 1.80E-08         |
| ENSCAFG00000012057.4 | EIF3A                | 126.76          | 0.91             | 1.00E-07         |
| ENSCAFG00000006593.3 | CBX5                 | 227.34          | 1.64             | 7.30E-07         |
| ENSCAFG00000012528.3 | PSMA7                | 609.24          | 1.05             | 8.70E-07         |
| ENSCAFG00000001211.2 | HMGA1                | 47.22           | 2.97             | 1.50E-06         |
| ENSCAFG00000011677.4 | EPB41                | 86.04           | -1.84            | 4.00E-38         |
| ENSCAFG00000007478.3 | CHMP4B               | 75.71           | -1.59            | 1.60E-33         |
| ENSCAFG00000006164.4 | CYP1B1               | 315.00          | -1.96            | 1.60E-33         |
| ENSCAFG00000002513.4 | TMEM196              | 24.76           | -1.82            | 2.30E-26         |
| ENSCAFG00000016920.4 | ABI3                 | 5.02            | -2.17            | 1.70E-23         |
| ENSCAFG00000010377.4 | ENSCAFT00000048356.2 | 39.12           | -1.60            | 9.10E-23         |
| ENSCAFG00000000364.3 | WIF1                 | 4.33            | -3.65            | 2.70E-22         |
| ENSCAFG00000008756.3 | ACTG2                | 19.44           | -2.15            | 6.70E-22         |
| ENSCAFG00000017326.3 | CNN1                 | 41.78           | -2.10            | 5.10E-21         |
| ENSCAFG00000018078.4 | ENSCAFT00000028730.4 | 16.39           | -3.13            | 1.40E-20         |

**Table 7.** Top ten upregulated or downregulated pathways from DEGs in dogs with PIMA

| Pathway name                                       | Fold enrichment | Raw P-value | regulate | No. of DEG |
|----------------------------------------------------|-----------------|-------------|----------|------------|
| Unclassified                                       | 0.57            | 8.81E-45    | up       | 245        |
| Cell Cycle                                         | 6.05            | 1.86E-43    | up       | 100        |
| Cell Cycle, Mitotic                                | 6.31            | 1.14E-41    | up       | 93         |
| Cell Cycle Checkpoints                             | 8.35            | 4.65E-31    | up       | 57         |
| M Phase                                            | 5.94            | 2.56E-28    | up       | 66         |
| Mitotic Anaphase                                   | 7.73            | 1.43E-26    | up       | 51         |
| Mitotic Metaphase and Anaphase                     | 7.69            | 1.74E-26    | up       | 51         |
| Separation of Sister Chromatids                    | 8.24            | 1.44E-25    | up       | 47         |
| Metabolism of RNA                                  | 4.17            | 1.60E-23    | up       | 75         |
| S Phase                                            | 8.85            | 6.73E-23    | up       | 40         |
| Signal Transduction                                | 1.92            | 4.41E-11    | down     | 114        |
| RHO GTPase cycle                                   | 2.96            | 1.34E-08    | down     | 38         |
| Smooth Muscle Contraction                          | 11.8            | 1.05E-07    | down     | 10         |
| RAC1 GTPase cycle                                  | 3.78            | 7.33E-07    | down     | 21         |
| Signaling by Rho GTPases                           | 2.35            | 7.35E-07    | down     | 43         |
| Signaling by Rho GTPases, Miro GTPases and RHOBTB3 | 2.3             | 1.57E-06    | down     | 43         |
| Muscle contraction                                 | 3.9             | 3.01E-06    | down     | 18         |
| CDC42 GTPase cycle                                 | 3.8             | 4.23E-06    | down     | 18         |
| Cell Cycle                                         | 0.06            | 4.61E-06    | down     | 1          |
| Cell Cycle, Mitotic                                | 0.07            | 2.09E-05    | down     | 1          |

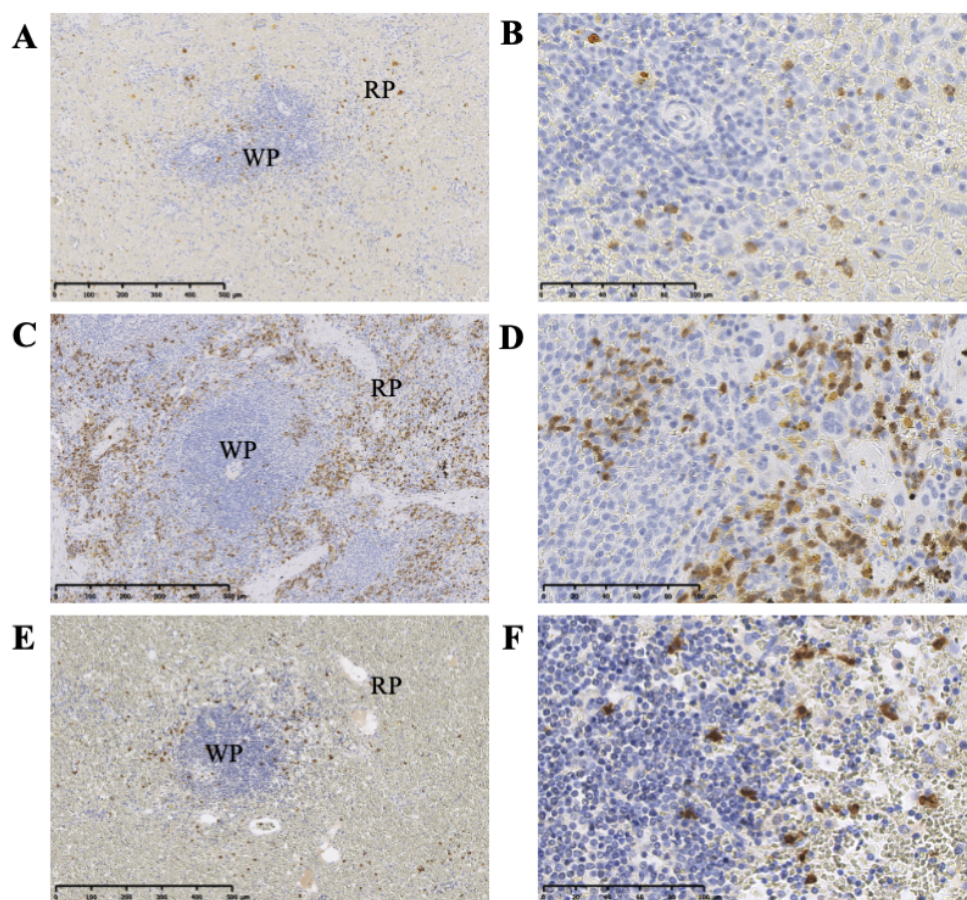


**Figure 7.** IHC analysis of S100A8/A9 in spleen samples from dogs with and without PIMA. Representative positive staining in a healthy dog (A, B), a dog with PIMA (C, D), and a dog with non-PIMA (E, F). RP = red pulp (in red), WP = white pulp (in blue). S100A8/A9 stained-cell (shown in brown). Scale bars = 500  $\mu$ m (A, C, E) and 100  $\mu$ m (B, D, F).

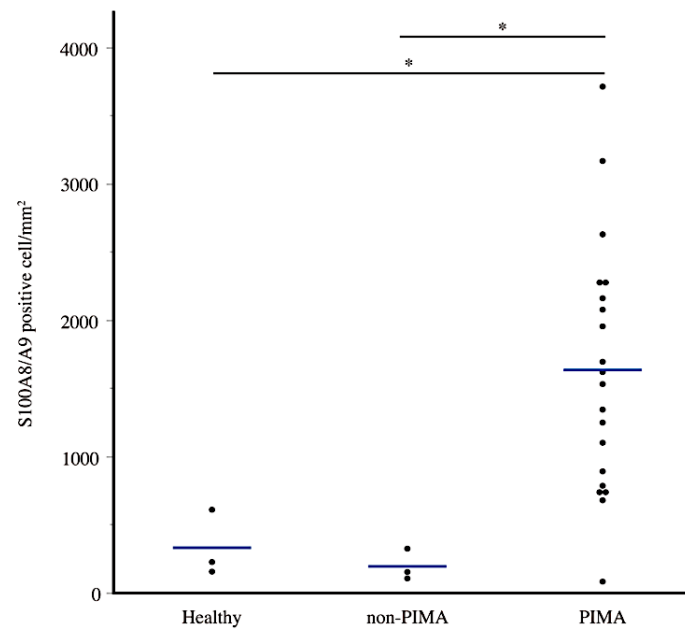


**Figure 8.** Numbers of S100A8/A9-positive cells in the spleen assessed by IHC. Dot plots represent the mean number of S100A8/A9-positive cells per mm<sup>2</sup> for each spleen sample. Statistical analysis was performed using the Steel–Dwass test. \* $P < 0.05$ .

**Table 8.** Over- and under-expressed proteins in pre- and post-splenectomy serum samples from dogs with PIMA

| Uniprot Ac. | Protein name                                           | Gene name | p value  | ratio >2 or ratio <0.5 |
|-------------|--------------------------------------------------------|-----------|----------|------------------------|
| F1PPL0      | BPI fold containing family B member 4                  | BPIFB4    | 3.76E-02 | 26.99                  |
| P19006      | Haptoglobin                                            | HP        | 5.07E-03 | 6.54                   |
| J9P7F7      | Ficolin 1                                              | FCN1      | 3.16E-02 | 4.00                   |
| F1PH87      | Serpin family A member 3                               | SERPINA3  | 2.97E-02 | 3.60                   |
| F6USM4      | Mannan binding lectin serine peptidase 2               | MASP2     | 4.48E-02 | 2.80                   |
| Q28260      | Vascular cell adhesion protein 1                       | VCAM1     | 6.49E-03 | 2.59                   |
| E2RNL2      | Insulin like growth factor binding protein 7           | IGFBP7    | 1.68E-04 | 2.58                   |
| F1PCE5      | Serpin family A member 1                               | SERPINA1  | 4.62E-02 | 2.21                   |
| J9P9J6      | Uncharacterized protein                                | -         | 1.64E-02 | 2.21                   |
| A0A5F4DC46  | Endostatin domain-containing protein                   | -         | 3.02E-03 | 2.14                   |
| E2RLS3      | Heat shock protein 90 alpha family class B member 1    | HSP90AB1  | 4.64E-02 | 2.10                   |
| F1PGM9      | Complement component 4 binding protein alpha           | C4BPA     | 2.34E-02 | 0.48                   |
| A0A5F4C3Y4  | Sex hormone binding globulin                           | SHBG      | 4.48E-02 | 0.45                   |
| A0A5F4D8N3  | Lymphocyte cytosolic protein 1                         | LCP1      | 4.18E-02 | 0.41                   |
| A0A5F4BYK6  | Tenascin C                                             | TNC       | 2.37E-02 | 0.37                   |
| E2RJY0      | Potassium channel tetramerization domain containing 12 | KCTD12    | 1.44E-03 | 0.31                   |
| A0A5F4BVB4  | ANTXR cell adhesion molecule 1                         | ANTXR1    | 1.20E-02 | 0.29                   |
| A0A5F4BTK2  | Anthrax toxin receptor                                 | ANTXR2    | 2.66E-02 | 0.28                   |
| A0A5F4CN27  | Periostin                                              | POSTN     | 1.11E-02 | 0.26                   |
| A0A5F4CTD0  | Alpha-1-B glycoprotein                                 | A1BG      | 1.14E-02 | 0.25                   |
| A0A5F4D9Z3  | Serpin family A member 7                               | SERPINA7  | 1.38E-02 | 0.23                   |
| E2RNR9      | Osteomodulin                                           | OMD       | 3.93E-02 | 0.21                   |

## 4. Discussion

In this study, the author investigated the mechanisms responsible for the effects of splenectomy in dogs with PIMA by using transcriptomic analysis of spleen samples and proteomics analysis of pre- and post-splenectomy serum samples. Based on the results of the transcriptome analysis, it was determined that S100A8 and S100A9 were differentially expressed in canines with PIMA, whereupon the protein expression of S100A8/A9 was confirmed in spleen samples via IHC analysis.

PCA of the transcriptomic data from spleen samples showed that 15,903 genes showed a clear differential between dogs with PIMA and healthy dogs, suggesting that PIMA may significantly affect gene expression in the spleen. Among these, 1,385 genes were identified as DEGs, and the genes *S100A12*, *S100A8*, and *S100A9* were found to be the three most highly overexpressed genes in PIMA spleens. Consistently, IHC analysis showed that the heterodimeric protein complex S100A8/A9 was highly expressed, especially in PIMA samples. The S100 protein family comprises the largest subgroup within the Ca<sup>2+</sup>-binding EF-hand protein superfamily<sup>29)</sup>. S100A8/A9 and S100A12 are released from activated monocytes and granulocytes and act as proinflammatory endogenous Toll-like receptor 4-ligands<sup>21,39,64)</sup>. Serum levels of S100A12 and S100A8/A9 have been shown to be increased in various inflammatory and autoimmune diseases, and complement activation was reported to occur at sites expressing S100A8/A9<sup>29,33,45,53,65)</sup>. Previous studies have suggested that PIMA may be an autoimmune disease<sup>37)</sup>, which may partially explain the elevated S100A8/A9 levels.

One of the significant clinicopathological findings is the presence of ineffective erythropoiesis in dogs with PIMA. S100A9 has been shown to suppress erythroid differentiation through inactivation of Rps14 in both the experimentally-induced deletion 5q subtype of myelodysplastic syndrome and in a wild-type mouse model, specifically the hematopoietic stem cells and progenitor cells<sup>52)</sup>. Moreover, serum levels of S100A8 have been shown to be significantly elevated in patients with myelodysplastic syndrome compared with healthy controls<sup>24)</sup>. Thus, the inhibitory

effect of S100A9 on erythroblast differentiation may be responsible for the ineffective erythropoiesis in PIMA dogs.

The author also carried out proteomic analysis of pre- and post-splenectomy serum samples and found that 22 proteins were differentially expressed. Bactericidal/permeability-increasing fold-containing family B member 4 (BPIFB4), haptoglobin, and FCN1 were specifically downregulated in post-splenectomy serum samples compared with pre-splenectomy serum samples. BPIFB4 serum levels were 26-fold higher before the splenectomy and the RNA transcript levels were significantly higher in the spleens of dogs with PIMA compared with healthy spleens ( $P=0.003$ ). Given these results, BPIFB4 may be produced in the spleen, and may be expressed at higher levels in dogs with PIMA. The concentration of BPIFB4 in serum appears to then decrease after a splenectomy. However, these data are inconsistent with the fact that BPIFB4 is typically more abundant in the serum of healthy, long-lived individuals compared with frail individuals<sup>58</sup>). This has been postulated to be due to advantageous anti-inflammatory effects through macrophage polarization<sup>14</sup>) and the correction of hypertension in humans<sup>63</sup>). Unfortunately, there is insufficient information to understand the precise role of BPIFB4 in non-regenerative anemia, and therefore its association with the pathogenesis of PIMA remains unclear.

A study that investigated the clinical progression of horses after splenectomy found that two months after the procedure, the concentration of haptoglobin was significantly reduced compared with those that had not been splenectomized<sup>25</sup>). Our findings are consistent with these data, and the collection timelines were similar because the author collected post-splenectomy serum samples from the dogs more than 2 months after the procedure. However, haptoglobin levels can be influenced by the administration of corticosteroids to dogs, and increased haptoglobin levels are often observed after corticosteroid therapy<sup>26,44</sup>) and in spontaneous hyperadrenocorticism<sup>43</sup>). Together, these data suggest that changes in serum haptoglobin could be affected by the splenectomy; however, we cannot exclude the possibility of elevated haptoglobin due to corticosteroid therapy.

Factors involved in the lectin pathway of complement activation were over-represented in our list of proteins that were upregulated before the splenectomy. The

complement system can be activated through three major pathways: the classical, lectin, and alternative lectin pathways. Complement activation, regardless of the pathway, ultimately triggers three effector pathways (membrane attack complex assembly, anaphylatoxins, and opsonization) that enable the complement to fulfill its physiological role in host defense. The lectin pathway is initiated by binding of pattern-recognition molecules, including FCN1, and the pathway is accelerated by MASP2, which was upregulated in pre-splenectomy serum <sup>17)</sup>. FCN1 is a multimeric protein consisting of an N-terminal collagen-like domain and a C-terminal fibrinogen-like domain, is present in secretory granules, and is primarily expressed in granulocytes and monocytes. Serum levels of FCN1 are elevated in vasculitis syndrome and arthritis, and FCN1 has thus gained attention as a potential therapeutic target for the treatment of autoimmune diseases <sup>34)</sup>. FCN1 RNA levels were also shown to be increased in the peripheral blood in dogs with IMHA <sup>10)</sup>. In the present study, FCN1 and MASP2 were upregulated 4- and 2.8-fold, respectively, in serum collected pre- compared with post-splenectomy, suggesting that uncontrolled non-regenerative anemia with complement activation may be relevant to the immune-mediated destruction of erythroid progenitor cells, which has been considered as a pathophysiology of PIMA <sup>37)</sup>. However, FCN1 expression data could not be determined from our transcriptome analysis, and MASP2 expression levels were similar between samples from dogs with PIMA and healthy dogs, despite their significant upregulation in serum before the splenectomy. MASP2 is reported to be produced mainly in the liver <sup>56)</sup>, and FCN1 is located primarily in peripheral blood <sup>30,36)</sup>, suggesting that these genes are not highly expressed in the spleen, which may explain this discrepancy between the transcriptomic and proteomic data. Nevertheless, since FCN1 and MASP2 are involved in the lectin pathway that induces the activation of complement pathway <sup>18)</sup>, the author speculate that the changes in levels of these proteins in the serum may be affected by the release of a specific factor, such as S100A8/A9 (Figure 9).

Considering the pathogenesis of the disease, the author predicted that S100A8/9 would be present at high levels in the spleen of dogs with PIMA, resulting in activation of the lectin pathway (Figure 9). However, the reason behind the upregulation of S100A8/A9 in the spleen remains unknown. Expression levels of S100A8 and S100A9

in various organs have been reported to increase with aging <sup>61)</sup>. PIMA is most commonly reported in middle-aged and older dogs; however, a previous study reported a median onset age of 6.5 years <sup>5)</sup>. In contrast, the median age in the current group was 12 years, which is similar to a retrospective study of Miniature Dachshunds with suspected PIMA in Japan (which reported a median age of 10.9 years) <sup>62)</sup>. Despite the differences in median age between these studies <sup>5)</sup>, the author speculated that there might be an association between the characteristic clinical course of PIMA and the high expression of S100A8/A9 present in dogs older than 6.5 years.

There are several limitations to this study. The study uses a low number of control samples from both non-PIMA and healthy dog groups, because of the difficulty in obtaining samples. Furthermore, most of the samples were Miniature Dachshunds, which is consistent with previous reports, but does not allow a more diverse understanding of PIMA in dogs more generally <sup>5)</sup>. Lastly, the disease pathophysiology may differ depending on the responsiveness to the splenectomy. Further large-scale studies in different breeds and organs, as well as in different treatment settings are required to identify the precise pathology of PIMA in dogs.

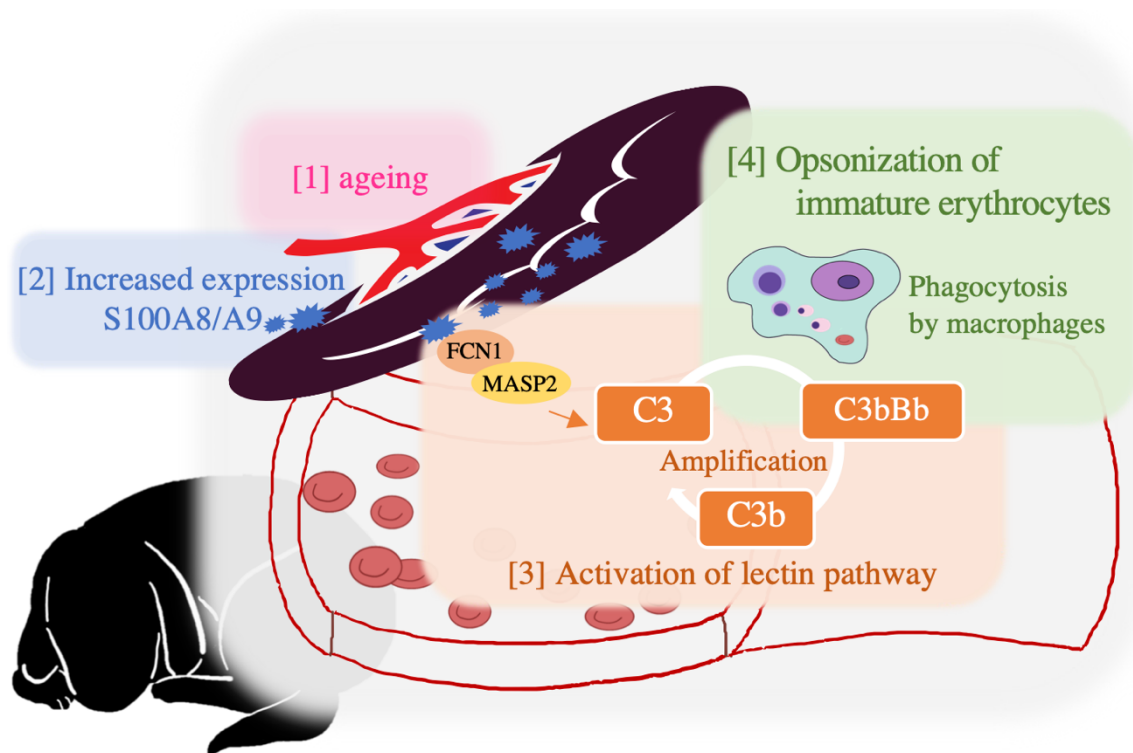


**Figure 9.** Schematic diagrams illustrating possible mechanisms underlying PIMA in dogs. [A], [B]: Age-related changes in the spleen lead to increased expression of S100A8/A9. [C] S100A8/A9 promotes FCN1 recognition and MASP2 regulation, which together activate the lectin complement pathway. [D] The lectin complement pathway triggers opsonization of immature erythrocytes and phagocytosis by macrophages.

## 5. Summary

In this chapter, gene expression levels in the spleen of dogs with or without PIMA and in serum before and after splenectomy were analyzed. A total of 1,385 genes were found to express differentially in the spleens from dogs with PIMA compared with healthy dogs by transcriptome analysis, of which 707 genes, including *S100A12*, *S100A8*, and *S100A9* were up-regulated that are linked directly to the innate immune system and have been characterized as endogenous damage-associated molecular patterns. Furthermore, immunohistochemistry confirmed that S100A8/A9 protein expression levels were significantly higher in dogs with PIMA compared with those in healthy dogs. A total of 22 proteins were found to express differentially between the serum samples collected before and after splenectomy by proteome analysis, of which 12 proteins were up-regulated in the samples before. The lectin pathway of complement activation was identified by pathway analysis in pre-splenectomy samples. The author speculated that S100A8/9 expression may be increased in the spleen of dogs with PIMA, resulting in activation of the lectin pathway before splenectomy. These findings enhance our comprehension of the pathology and mechanisms of splenectomy for PIMA.

## General Conclusion

This study focused on splenectomy as a treatment for PIMA and aimed to (1) examine the clinical outcomes of conventional immunosuppressive therapy and splenectomy, and (2) investigate the molecular changes after splenectomy. PIMA is considered as an immune mediated hematological disease in dogs, though immunosuppressive therapy has not been successful in some cases where its pathogenesis of disease remains unclear. Moreover, pertinent information pertaining to the factors that predict treatment response and response time is limited.

In chapter 1, the author focused on the limitations of conventional treatment, specifically immunosuppressive therapy. The predictive factors influencing treatment response and the duration of time required to respond in dogs with PIMA were retrospectively investigate, where the subjects were limited to dogs that remained on treatment for at least 3 months. The author found that erythroid-maturation ratio of  $< 0.17$  could be utilized as a predictor for treatment response. Moreover, a small number of cases transition from non-responders to the responders only after being treated for 60 days or more. In addition, the study explored the potential complications associated with immunosuppressive therapies in dogs with PIMA and identified the prevalence of pancreatitis and pneumonia during the entire treatment period. Dogs undergoing extended periods of immunosuppressive therapy were also found to be more susceptible to infections, such as abscesses.

Chapter 2 retrospectively investigated the efficacy of splenectomy as a treatment for dogs with PIMA. In this study, twenty dogs underwent splenectomy, and progress was observed in eighteen of them. After splenectomy, treatment was deemed successful in 15 out of the 18 dogs, with the cessation of immunosuppressive therapy and transfusion dependence. The median observation period of 301 days yielded no complications that could be attributed to splenectomy. These findings suggest that splenectomy may be a highly effective treatment strategy for PIMA in cases where immunosuppressive therapy has failed to produce results.

In chapter 3, the author delves into the molecular changes associated with PIMA in dogs. Through gene expression analysis of the spleens and serum samples, the study

identified 1,385 differentially expressed genes in the spleens of dogs with PIMA compared to healthy dogs, with 707 of those genes up-regulated. Among the up-regulated genes were S100A12, S100A8, and S100A9, which are known to be endogenous damage-associated molecular patterns. This finding was confirmed by immunohistochemistry, which demonstrated significantly higher S100A8/A9 protein expression levels in dogs with PIMA compared to healthy dogs. The study also analyzed 22 differentially expressed proteins in serum samples collected before and after splenectomy, with 12 proteins up-regulated before splenectomy. Pathway analysis identified the lectin pathway of complement activation in pre-splenectomy samples, suggesting that S100A8/9 expression may be increased in the spleen of dogs with PIMA, resulting in activation of the lectin pathway before splenectomy.

In conclusion, the present study elucidates that in the management of PIMA, the prolonged use of immunosuppressive therapy beyond the duration of 60 days appears to have a questionable efficacy and is associated with a high probability of adverse effects. Instead, the study recommends splenectomy as a potential alternative therapy with a high response rate and good long-term prognosis, especially for cases where immunosuppressive therapy has been unsuccessful. Additionally, genetic and protein analysis of spleen and serum samples revealed overexpression of S100A8/A9 and activation of the lectin pathway before splenectomy, suggesting their potential involvement in the pathogenesis of PIMA. Further studies which clarify the details of these targets are necessary to elucidate the pathogenesis of PIMA.

## Acknowledgement

This PhD study is not one that I could have achieved alone. I would like to express my gratitude to all those who have supported and encouraged me.

First and foremost, I would like to express my gratitude towards my supervisor, Dr. Mitsuyoshi Takiguchi for his constant encouragement, support, advice, and guidance throughout the process of this thesis.

I am also grateful to Drs. Mutsumi Inaba, Kensuke Nakamura, and Hiroshi Ohta (Rakuno Gakuen University) for taking the time and effort to provide advice and critical comments, which helped make this dissertation better.

I am also greatly indebted to my mentors, Dr. Keitaro Morishita and Dr. Jumpei Yamazaki, who had taken their time and effort to critically evaluate my research, papers, presentations and this dissertation. Without their insightful guidance, my work would not have reached its current state of improvement.

I also thank Drs. Noboru Sasaki, Nozomu Yokoyama, Osamu Ichii, Keisuke Aoshima, Kenji Hosoya, Kim Sangho, Yumiko Kagawa (North Lab), Noriyuki Nagata, Kazuyoshi Sasaoka, Tatsuyuki Osuga (Miyazaki University) and Masahiro Tamura (Rakuno Gakuen University) for their constant support and advice. My gratitude also extends to my wonderful comrades; Drs. Yong Bin Teoh, Takashi Namba, Kazuki Kojima, Thandar Oo and all members of the Laboratory of Veterinary Internal Medicine for their priceless advice and support. In addition, I also wish to thank all the staff of Hokkaido University Teaching Hospital for their support.

Last but not least, my utmost gratitude goes to my family, husband and friends for their encouragement and support.

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## Summary in Japanese (和文要旨)

### **Study on the clinical outcome and molecular changes of precursor-targeted immune-mediated anemia treated with splenectomy**

(Precursor-targeted immune-mediated anemia (PIMA) に対する脾臓摘出術の臨床的有用性および分子生物学的変化に関する研究)

慢性的に非再生性貧血を呈する犬の骨髓像の一つとして、赤芽球過形成や成熟停止を特徴とする無効造血が報告されている。このような症例に対する詳細な病態解明はなされていないものの、免疫抑制療法が一定の効果を示すことから免疫異常がその原因であると推測されている。従来、本疾患は非再生性貧血に加え免疫介在性溶血性貧血(Immune-mediated hemolytic anemia, IMHA)に特徴的な臨床検査所見を有する症例が一定数存在したことから、非再生性免疫介在性貧血(Non-regenerative immune-mediated anemia, NRIMA)と定義されていた。一方、IMHA の所見に乏しい非再生性貧血のみを呈した犬ではマクロファージによる赤芽球貪食像が高頻度に観察されるという知見に基づき、赤血球前駆細胞に対する免疫異常に限定した precursor-targeted immune-mediated anemia (PIMA) という疾患概念が近年提唱された。現時点で PIMA は主に赤芽球に対する免疫反応、NRIMA は赤芽球から赤血球まで幅広い分化段階に対する免疫反応と理解されている。本研究では近年提唱された診断基準に従い PIMA と診断された症例を対象に研究を行った。

過去の報告では、PIMA/NRIMA 罹患犬は免疫抑制療法により 50–88% が中央値 14–31 日までに赤血球再生像が認められ、このうち半数以上が寛解を達成したとされている。しかしながら、これらの報告には免疫抑制療法が奏功せず、3 ヶ月以内に安楽死された犬も多く含まれており、長期間治療を継続した場合の予後については情報が不足している。一方、長期間の免疫抑制療法の実施は、合併症や副作用の問題、さらには通院回数の増加や複数回の輸血に伴うオーナーの経済的な負担の増大など多くの問題があることは否定できない。このような観点から、北海道大学動物医療センターでは、免疫抑制療法に反応を示さない PIMA 症例に対して、代替療法として脾臓摘出術(脾摘)を実施し、一定の効果を経験してきた。本研究は PIMA の治療法として脾摘に焦点を当て、①従来の免疫抑制療法と脾摘の臨床成績を検討すること、②脾摘前後の血清蛋白質の変化および脾臓における遺伝子発現について網羅的に検討することを目的とした。

第1章では、従来の治療法である免疫抑制療法への反応性や有害事象に着目し、PIMA 罹患犬において3ヶ月以上治療を継続した症例に限定して治療効果に影響を与える因子と治療反応を示すまでに要する期間を回顧的に調査した。その結果、Erythroid-maturation ratio(EMR)が0.17未満であることは、治療反応に対する正の予後予測因子である可能性が示され、60日以上治療を継続した場合に非反応群から反応群へと移行する症例はわずかであることが明らかとなった。さらに、PIMA 罹患犬全症例で免疫抑制療法の有害事象を調査したところ、膵炎および肺炎は全治療期間にわたり発生し、膿瘍などの感染症は免疫抑制療法を長期間行っている犬でより多く発生する傾向が示された。

第2章ではPIMA 罹患犬に対する脾摘の有効性を回顧的に調査した。脾摘を実施した20頭中、18頭で長期的な経過観察が可能であった。18頭中15頭は術後、免疫抑制療法の中止および輸血依存の離脱が出来、治療が奏功したと考えられた。また、術後観察期間(中央値301日)で脾臓摘出術の合併症は認められなかった。これらの結果から、犬が免疫抑制療法に反応しない場合、脾摘がPIMA に対する有効な治療戦略である可能性が示された。

第3章では、PIMA 罹患犬と健常犬の脾臓におけるトランスクリプトーム解析およびPIMA 罹患犬の脾摘前後の血清中蛋白質を網羅的に解析した。PIMA 罹患犬の脾臓では1,385の発現変動遺伝子が検出され、炎症関連タンパク質である*S100A12*、*S100A8*、*S100A9*などの707遺伝子の発現が対象と比べて高い値であった。さらに、免疫組織化学的手法により、PIMA 発症犬では*S100A8/A9*蛋白質の発現量が健常犬に比べ有意に高いことが確認された。血清プロテオーム解析では、脾摘前後の同一個体のサンプル間で22種の蛋白質発現に有意差が認められ、パスウェイ解析により脾摘前のレクチン経路を主とした補体活性化が示唆された。

本研究により、PIMA の治療に対する知見として、60日以上にわたる免疫抑制療法に反応を示さない場合は有害事象発生の観点から積極的な代替療法の考慮が望まれることが明らかとなった。その代替療法として、脾摘は奏効率が高く、長期的な予後が期待できる有効な治療法となり得ることが回顧的研究により示された。さらに脾臓を用いた網羅的な遺伝子解析や脾摘前・後の血清を用いた血清中蛋白質の網羅的解析により*S100A8/A9*の過剰発現や脾摘前におけるレクチン経路の活性化がPIMA の病態に関与している可能性が示された。