SUPPLEMENTARY MATERIAL

Details of dogs excluded from analysis because immune-mediated hemolysis status could not be reliably determined

The excluded DAT negative dog had been prescribed 27 days of immunosuppressive therapy before presentation (single dose of dexamethasone, 26 days of prednisolone at an immunosuppressive dose and 14 days of mycophenolate), but there was no evidence of hemolysis during hospitalization or on review of the referring veterinarian’s records. Anemia was not responsive to immunosuppression and direct antiglobulin testing performed at an external laboratory 22 days after initiation of immunosuppression was negative. Although IMHA was considered unlikely, an alternative diagnosis was not identified. SATs were positive at 1:1 dilution and negative at 1:4, 1:9 and 1:49 dilutions.

One excluded DAT positive dog had a weakly positive gel DAT test and positive SATs at all dilutions. There was no clinical evidence of hemolysis at the time of study sample collection, and anemia did not respond to 12 days of immunosuppressive-dose prednisolone. Clinical investigations did not identify an underlying cause of anemia. At necropsy, there was erythrophagia in the spleen and bone marrow, but the dog had received multiple packed red cell transfusions, and it was unclear if this was evidence of IMHA or phagocytosis of transfused erythrocytes. The other excluded DAT positive dog had suffered snake envenomation two days before presentation. In the interim, the dog had received crotalid antivenom, fresh frozen plasma, cryoprecipitate and dexamethasone. At presentation, hematocrit was 30 %, plasma was severely hemolyzed, urine was red, fibrinogen was < 30 mg/dL (110-275 mg/dL) and PT and aPTT were prolonged beyond the reportable range for the assays. The gel DAT test was weak positive, but the dog had received 4 units of crotalid antivenom, including a unit within an hour of collection of the study sample, and passive antibody adsorption could not be ruled out. This dog was excluded because it was unclear if hemolysis was a direct effect of venom components or an immune-mediated process. This dog had a positive SAT at a 1:1 dilution and SATs were negative at all other dilutions.
Clinical details of dogs with a failed DAT (i.e. positive auto-control) and evidence of a non-immune-mediated cause for anemia

One dog with a failed DAT test had a hematocrit of 27.3 %, no evidence of regeneration (absolute reticulocyte count below the linear range of the instrument), severe thrombocytopenia [15,000 /µL, reference interval (RI) 200,000-400,000], prolonged PT (11.6 s, RI 6.4-9.5) and aPTT (30.4 s, RI 11.6-25.1), decreased antithrombin (44 %, RI > 114 % normal human plasma), increased D-Dimers (792 ng/mL, RI < 371.5) and fibrinogen (292 ng/mL, RI 110-275) and excessive bleeding from an abscess. Clinically, anemia was considered to be due to anemia of inflammatory disease and blood loss secondary to disseminated intravascular coagulation. The other dog with a failed DAT test had a hematocrit of 23.1 % with a minimal increase in reticulocytes (102,400 /µL), severe thrombocytopenia (20,000 /µL), within reference interval PT, aPTT, fibrinogen and D-Dimers and a reduced antithrombin activity (63 %). Melena and petechiation were present, and anemia was attributed to blood loss resulting from thrombocytopenia induced by chemotherapy for lymphoma. A packed red cell transfusion was administered before sample collection in 1/2.

Clinical details of the non-immune-mediated hemolysis cases with positive DAT but no evidence of hemolysis

Median hematocrit was 25.3 % (range 20-30), and reticulocytes were > 100,000 /µL in 13/27. There was no evidence of hemolysis in 25/27. For the remaining 2 dogs, 1 had a single hemolyzed plasma sample, but other samples collected during hospitalization were not visibly hemolyzed. Hemolysis was therefore considered likely to be an artifact introduced during or after venipuncture. Clinically, mild non-regenerative anemia (hematocrit 29.4 %, reticulocyte concentration 70,300 /µL) was assessed as due to anemia of inflammatory disease, as the dog had a congenital portosystemic shunt and urinary tract infection. Anemia improved without immunosuppression after initiation of antibiotic therapy. For the other dog, there was a moderate positive heme reaction on urine dipstick. This was attributed to color interference from bilirubin because the bilirubin dipstick result was strongly positive. Plasma bilirubin
was 2.5 mg/dL (RI < 0.9) with elevated ALP and ALT and histopathologic evidence of lymphoma involving the liver. This dog did not receive immunosuppressive therapy, and the mild, regenerative anemia (hematocrit 30 %, reticulocytes 140,100 /µL) was attributed to surgical blood loss during exploratory laparotomy and hepatic biopsy. In total, 6/27 dogs had elevated bilirubin, which was attributed to hepatobiliary disease or functional icterus due to sepsis based on clinical, laboratory and imaging findings (Supplementary Table 1). 2/27 dogs had immune thrombocytopenia (ITP), but anemia was considered likely secondary to blood loss rather than evidence of Evans syndrome, based on relatively mild anemias (24 % and 30 %) and lack of evidence of hemolysis. Packed red cells had been transfused before sample collection in 4/27, with clinical diagnoses of ITP (1), disseminated Histoplasmosis (1) and trauma (2).

Clinical details of non-immune-mediated hemolysis cases with a negative DAT

One dog (hematocrit 26.1 %, reticulocytes 186,500 /µL) did receive doxycycline and an immunosuppressive dose of prednisolone for possible IMHA, but this was tapered after 26 days because anemia did not improve. This dog was being treated with doxorubicin for splenic stromal sarcoma, and at necropsy, there was a low volume hemorrhagic abdominal effusion and evidence of acute and chronic hemorrhage in abdominal lymph nodes. The remaining 108 dogs were not treated for IMHA at any point. For the 109 DAT negative dogs, median hematocrit was 27 % (range 12-30 %) and 23/107 dogs had > 100,000 reticulocytes /µL. Reticulocyte counts were not available for the remaining 2 dogs. Plasma was hemolyzed for 5/109, but for 4/109 this was considered likely an artifact introduced during or after collection as there were no other findings consistent with hemolysis. The remaining dog had > 5 spherocytes/HPF, hemoglobinuria and increased plasma bilirubin. This dog had developed clinical evidence of hemolysis within hours of envenomation by large numbers of bees, and based on the short interval between envenomation and hemolysis and the negative DAT, hemolysis was considered to be a direct effect of venom components rather than an immune-mediated process. Urine dipstick was positive for heme in 5 other dogs, but urine sediment contained erythrocytes for 4/5 and for the remaining dog,
myoglobin release was suspected as severe muscle damage had been sustained during a dog attack.

Plasma bilirubin was increased in 15/109, and for 13/15 this was accompanied by an increase in at least one of ALP, ALT or GGT. In the remaining 2/15, functional icterus induced by sepsis was suspected, as one dog had multiple infected dog bite wounds and the other an intra-abdominal abscess. For the 12/109 dogs with ITP, Evans syndrome was considered unlikely based on negative DAT, lack of evidence of hemolysis and bleeding recorded in 11/12 (melena 4/12; hematochezia 4/12, hematemesis 2/12, petechiae 6/12, ecchymoses 3/12, vulval bleeding 1/12, gingival bleeding 1/12, injection site bleeding 1/12, hematoma 1/12). Hematocrits in dogs diagnosed with ITP ranged from 15.1\% to 29.3\% and platelet counts from 500 to 58,000 /µL, with the 2 dogs with platelet concentrations > 30,000 /µL both receiving immunosuppression at the time of sample collection. 14/109 dogs had received at least one packed red cell or whole blood transfusion before sample collection (bacterial hepatitis 1/14, bee envenomation 1/14, bile peritonitis 1/14, bleeding gastric ulcer 3/14, hypoadrenocorticism with gastrointestinal bleeding 1/14, ITP 2/14, precursor targeting immune mediated anemia 1/14, surgical blood loss 3/14, trauma 1/14).

**Clinical details of immune-mediated hemolysis cases**

Plasma was hemolyzed for 7/9; 8/9 had ≥ 5 spherocytes /HPF; 2/9 had large numbers of ghost cells on a freshly prepared blood smear; 1/9 had hemoglobinuria and 8/9 had increased plasma bilirubin, although this was accompanied by an increase in at least one of ALP, ALT or GGT for 3/8. Secondary IMHA was considered likely in 2/9 dogs, as a cephalosporin antibiotic was prescribed 2 weeks before onset of anemia for one dog, and the other was 42 days pregnant. For 6/9 IMHA cases, IMHA was likely primary as an underlying cause was not identified by diagnostic investigations including abdominal ultrasound (6/6), thoracic radiographs (6/6) and vector borne disease testing (6/6 point of care testing for *Dirofilaria immitis* antigen and antibodies against *Ehrlichia* spp., *Anaplasma* spp. and *Borrelia burgdorferi* (C6); 1/6 *Babesia* spp. PCR and 1/6 PCR for *Anaplasma, Babesia, Bartonella, Ehrlichia,*
Rickettsia, and hemotropic Mycoplasma spp. with immunofluorescence antibody assays for Ehrlichia canis, Rickettsia rickettsia, Babesia gibsoni and Bartonella spp.).

Median hematocrit was 15 % (range 6-30 %). Of the two dogs with hematocrits over 20 %, one had received immunosuppressive therapy for IMHA for at least 2 months before sample collection and the other was affected by an immunological hemolytic transfusion reaction. For the 7/9 with hematocrit < 20%, 3 were receiving immunosuppressive doses of dexamethasone or prednisolone at the time of study sample collection, but the duration of immunosuppression was < 24 hours in all cases. For 6/9 dogs, an automated reticulocyte count was > 100,000 /µL or a corrected manual reticulocyte count was > 1 %. Packed red cells were transfused to 4/9 dogs before study sample collection.
Supplementary Table 1. Clinical findings in dogs with a positive DAT and elevated bilirubin attributed to hepatobiliary disease and/or functional icterus, rather than hemolysis. Of the 27 dogs with a positive DAT that were classified as anemic due to a non-immune-mediated process, 6 had above reference interval (RI) bilirubin. Clinical details which contributed to classification of these cases as non-hemolytic are presented below.

| Case | Bilirubin (0.0-0.8 mg/dL) | ALT (10-130 U/L) | ALP (24-147 U/L) | GGT (0-25 U/L) | Other biochemistry findings potentially consistent with hepatobiliary disease | Imaging findings consistent with hepatobiliary disease | Clinical history consistent with hepatobiliary disease and/or functional icterus of sepsis |
|------|---------------------------|------------------|------------------|----------------|-----------------------------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------------------------------|
| 1    | 0.9                       | 233              | 354              | 11             | Elevated ammonia (64 ug/dL, RI: 0-50)                                       | Intrahepatic portosystemic shunt confirmed on CT    | Elevated ammonia and marginal increase in bilirubin resolved after shunt attenuation |
| 2    | 1.0                       | 84               | 2899             | <10            | Hyperechoic liver and gall bladder mucocele on abdominal ultrasound        |                                                     | Cytology consistent with vacuolar hepatopathy                                      |
| 3    | 1.1                       | 83               | 1588             | <10            | Enlarged liver with hyperechoic nodules on ultrasound                      |                                                     | Long term history of phenobarbital treatment for idiopathic epilepsy               |
| 4    | 1.2                       | 134              | 401              | <10            | Hypoalbuminemia (1.6 g/dL, RI: 2.4-3.6)                                     |                                                     | Septic abdomen after dehiscence of jejunal resection and anastamosis due to a foreign body |
| 5    | 2.5                       | 319              | 1892             | 14             | Elevated cholesterol (353 mg/dL, 120-247)                                   |                                                     | Cytologic diagnosis of intermediate cell lymphoma involving the liver, with concurrent vacuolar hepatopathy |
| 6    | 2.6                       | 265              | 446              | 44             | Mildly hyperechoic liver and findings consistent with mild acute pancreatitis, pancreatic edema or inflammation of peri-pancreatic fat on ultrasound |                                                     | Cytologic findings consistent with vacuolar hepatopathy.                           |
Supplementary Table 2. Diagnoses in dogs with false positive and false negative results for saline agglutination tests performed at saline to blood ratios of 1:1, 4:1, 9:1 and 49:1. Results were considered false negatives if the dog met our criteria for immune-mediated hemolysis but erythrocyte aggregates were not seen in five x 40 objective fields. Results were considered false positives if the dog did not meet our criteria for immune-mediated hemolysis but erythrocyte aggregates were identified in at least one of five x 40 objective fields. AID: Anemia of inflammatory disease, DAT: Direct antiglobulin test; FUO: Fever of unknown origin; IMH: Immune-mediated hemolysis; IMHA: Immune-mediated hemolytic anemia; IMPA: Immune-mediated polyarthritis; PIMA: Precursor-targeting immune-mediated anemia.

| Saline to blood ratio | 1:1 | 4:1 | 9:1 | 49:1 |
|-----------------------|-----|-----|-----|------|
| **False negatives**   |     |     |     |      |
| Transfusion reaction 1|     |     |     | Transfusion reaction 1; Primary IMHA 2 |
| AID/non-hemolytic paraneoplastic anemia 7 | AID/non-hemolytic paraneoplastic anemia 9 | AID/non-hemolytic paraneoplastic anemia 5 |
| Congestive heart failure 1; Infectious 3 (Distemper 1, Pythiosis 1, septic abdomen, 1); Inflammatory 1 (pancreatitis, 1); Neoplastic 2 (lymphoma 2) | Congestive heart failure 1; Congenital 1 (portosystemic shunt 1); Infectious 3 (Distemper 1, Pythiosis 1, septic abdomen 1); Inflammatory 1 (pancreatitis 1); Neoplastic 3 (lymphoma 3) | Congestive heart failure 1; Infectious 3 (Distemper 1, Pythiosis 1, septic abdomen, 1); Neoplastic 1 (lymphoma 1) |
| Blood loss 1 | Blood loss 1 | Blood loss 1 |
| [Hemangiosarcoma 1] | [Immune thrombocytopenia 1] | [Immune thrombocytopenia 1] |
| Chemotherapy 1 | Chemotherapy 2 |
| [Lymphoma/lymphoid leukemia 1] | [Lymphoma/lymphoid leukemia 2] |
| Other 2 | Other 2 |
| [Phenobarbital toxicity 1, splenic torsion 1] | [Phenobarbital toxicity 1, splenic torsion 1] |
| **False positives**   |     |     |     |      |
| Non-IMH/DAT positive |     |     |     |      |
| Non-IMH/DAT negative |     |     |     |      |
| AID/non-hemolytic paraneoplastic anemia 24 | AID/non-hemolytic paraneoplastic anemia 22 | AID/non-hemolytic paraneoplastic anemia 11 |
| [Immune-mediated 3 (myositis 1, IMPA 1, polyneuritis 1); Infectious 7 (abdominal abscess 1, Dirofilaria immitis 1; otitis media 1; Spirocerca lupi 1; wound infection 3); | [Endocrine 1 (hypoadrenocorticism 1); Immune-mediated 2 (IMPA 1, polyneuritis 1); Infectious 7 (abdominal abscess 1, bacterial hepatitis 1, Dirofilaria immitis 1, otitis Spirocerca lupi 1); | [Endocrine 2 (diabetes mellitus 1, hypoadrenocorticism 1); Infectious 4 (abdominal abscess 1, bacterial hepatitis 1, Dirofilaria immitis 1, Spirocerca lupi 1); |
| AID/non-hemolytic paraneoplastic anemia 26 | AID/non-hemolytic paraneoplastic anemia 11 |
| [Endocrine 1; hypoadrenocorticism 1; Infectious 7 (abdominal abscess 1, bacterial hepatitis 1, Dirofilaria immitis 1, otitis Spirocerca lupi 1); | [Multiple chronic systemic disorders 1] |
| Blood loss 2 | [Coagulopathy secondary to hepatic Histoplasmosis 1; Trauma 1] |
| Inflammatory 4 (aspiration pneumonia 1, bile peritonitis 1, pancreatitis 2); Multiple chronic systemic disorders 1; Neoplasia 6 (disseminated adenocarcinoma 1, lymphoma 2, osteosarcoma 1, soft-tissue sarcoma 1, suspected but not histologically confirmed 1); Open/other 3 (FUO 1, chronic diarrhea 1, cerebrovascular event 1) | Blood loss 20 [Coagulopathy secondary to hepatic Histoplasmosis 1; Gastrointestinal lesions 8; Idiopathic hematuria 1; Immune thrombocytopenia 4; Surgical blood loss 3; Hemorrhage secondary to thrombocytopenia associated with a large splenic sarcoma 1, Trauma 2] Chemotherapy 9 [Lymphoma/lymphoid leukemia 5, Mast cell neoplasia 4] Renal failure 6 [Acute on chronic 2, Chronic 4] Other 3 [Ehrlichiosis associated pancytopenia 1, PIMA 1, Venom induced hemolysis 1] | media 1, Spirocerca lupi 1, wound infection 2); Inflammatory 3 (aspiration pneumonia 1, bile peritonitis 1, pancreatitis, 1); Multiple chronic systemic disorders 1; Neoplasia 5 (disseminated adenocarcinoma 1, lymphoma 3, soft-tissue sarcoma 1); Open/other 3 [FUO 1, chronic diarrhea 1 pulmonary hypertension 1] Blood loss 21 [Coagulopathy secondary to hepatic Histoplasmosis 1; Gastrointestinal lesions 5; Idiopathic hematuria 1, Bleeding associated with non-gastrointestinal neoplasm 3 (Disseminated neuroendocrine carcinoma 1, Hemangiosarcoma 2); Surgical blood loss 5; Hemorrhage secondary to thrombocytopenia associated with a large splenic sarcoma 1; Trauma 1] Chemotherapy 6 [Lymphoma/lymphoid leukemia 2, Mast cell neoplasia 3, Multiple myeloma 1] Renal failure 3 [Chronic, 3] Other 3 [Hemodilution due to large volume resuscitation after anesthesia adverse event 1, Parvo infection 1, PIMA 1] | Inflammatory 2 (aspiration pneumonia 1, pancreatitis 1); Multiple chronic systemic disorders 1; Neoplasia 1 (disseminated adenocarcinoma 1); Open/other 1 [pulmonary hypertension 1] Blood loss 13 [Coagulopathy secondary to hepatic Histoplasmosis 1; Gastrointestinal lesions 5; Idiopathic hematuria 1, Bleeding associated with non-gastrointestinal neoplasm 2 (disseminated neuroendocrine carcinoma, 1, hemangiosarcoma, 1); Surgical blood loss 3; Hemorrhage secondary to thrombocytopenia associated with a large splenic sarcoma 1] Chemotherapy 3 [Lymphoma/lymphoid leukemia 1, Mast cell neoplasia 2] Renal failure 2 [Chronic 2] Other 2 [Hemodilution due to large volume resuscitation after anesthesia adverse event 1, PIMA 1] |
| Diagnosis                              | Count | Count | Count |
|---------------------------------------|-------|-------|-------|
| Non-IMH/DAT failed (n = 2)            | 0/2   | 0/2   | 0/2   |
| Sepsis 1, Lymphoma, 1                 | 0/2   | 0/2   | 0/2   |