The diagnostic approach to anaemia in the dog and cat

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ABSTRACT
The term anaemia indicates a pathologic condition characterized by a decreased concentration of hemoglobin (Hb) in blood, usually associated with a decreased number of erythrocytes (RBC) and/or of hematocrit (Ht). Anaemia depends on two pathogenic mechanisms: 1) decreased RBC production due to toxic, infectious or idiopathic bone marrow diseases or to metabolic, neoplastic or infectious diseases that secondarily affect erythropoiesis, leading to the so-called “non regenerative anaemia”, on which no reticulocytes are released in blood from bone marrow; 2) decreased lifespan of mature RBC due to acute blood loss or hemolysis that leads to “regenerative anaemia” in which reticulocytes are released in blood as an attempt to restore the RBC mass. A stepwise diagnostic approach to anaemic dogs and cats may allow first to identify which of the two mechanisms is involved in the pathogenesis of anaemia, then to identify the possible cause of decreased RBC production or of decreased RBC lifespan. This approach must include clinical data, information regarding gross appearance of the sample, actual values of RBC counts, Ht and Hb concentration, RBC indexes (MCV, MCH, MCHC, RDW) and the magnitude of the reticulocyte response. Morphology of blood cells and additional laboratory tests may further address the diagnosis. With rare exceptions, non regenerative anaemia is normocytic normochromic, while regenerative anaemia is macrocytic hypochromic and characterized by anisocytosis and polychromasia, since reticulocytes are larger and have less Hb than mature RBC. However, blood loss or hemolytic anaemia are initially “pre-regenerative” (normocytic and normochromic), then they shift to the macrocytic hypochromic pattern in a few days, when reticulocytosis becomes relevant. Microcytic hypochromic anaemia is usually associated with iron deficiency. Once anaemia is classified into one of the categories listed above, morphology of RBC may suggest the possible cause, especially in regenerative anaemia, when the shape of RBC may be consistent with oxidative damage (eccentrocytes, Heinz bodies), immune-mediated mechanisms (agglutination, spherocytes, schistocytes, etc) or infectious diseases (e.g. mycoplasmosis, babesiosis). If needed, bone marrow cytology, Coomb’s test or flow cytometric detection of anti-RBC antibodies, coagulation profiles or additional biochemical or serological tests may be used to finalize the diagnostic approach.
**INTRODUCTION**

The term anaemia indicates a pathologic condition characterized by a decreased concentration of hemoglobin (Hb) in blood, usually associated with a decreased number of erythrocytes (RBC) and/or of hematocrit (Ht) (Stockham and Scott, 2008). This review describes a stepwise approach to anaemic dogs and cats, preceded by a short description of the pathophysiological aspects of RBC maturation and destruction that are essential to understand the rationale of this diagnostic approach.

**ERYTHROPOIESIS AND ERYTHRO-CATHESIS**

Erythrocytes (RBC) originate in the bone marrow from a stem cell common to all blood cells. This stem cell, under the influence of growth factors, cytokines, and finally erythropoietin (EPO, originating in the kidney), generates lymphoid and myeloid precursors. The myeloid precursor then divides in precursors of each myeloid cell type: granulocytes, monocytes, and platelets/erythrocytes. The latter develops into the so called burst-forming units-erythroid, the first precursor specifically committed to erythroid differentiation, that through a series of steps (erythroid colony-forming units, rubriblast, proerythrocyte, basophilic rubricyte, polychromatophilic rubricyte, metarubricyte reticulocyte), develops into mature RBC (Olver, 2010). These maturation stages differ also morphologically (Figure 1): mature RBC are biconcave discs that at the microscope appear as non-nucleated round cells (5-7 µm of diameter) with a central pale area surrounded by an eosinophilic rim. Conversely, precursors are large and nucleated cells, except reticulocytes, that appear as non-nucleated RBC larger and less eosinophilic than mature RBC. These features reflect the changes that occur during maturation, when the cell size progressively decreases, the nucleus becomes pyknotic and it is extruded at the metarubricyte stage, the number of intracytoplasmic organelles decreases (reticulocytes still contain mitochondria and endoplasmic reticulum) and they are finally extruded from the mature RBC.

Mature RBC perform only a few metabolic pathways. The most important is anaerobic glycolysis (Embden Meyerhof Pathway or EMP) that provides energy to sustain the viability of the cells. The EMP has 2 accessory pathways: the pentose phosphate pathway (PPP) generates NADH or NADPH, essential for antioxidant defenses, and the 2,3 diphosphoglycerate (2,3DPG) pathway produces 2,3DPG to facilitate the release of O₂ to hypoxic tissues (Harvey, 2010). These pathways may be activated when the need of O₂ increases (e.g. anaemia, hypoxia) (Paltrinieri et al., 2000). Conversely, the precursors synthesize Hb, the main constituent of mature RBC. Hb synthesis starts at the rubri-
cyte stage and continues until the reticulocyte stage. During Hb synthesis, globin chains and the heme group are synthesized and assembled. Then iron is incorporated and maintained in the reduced form (Fe^{2+}), able to bind O_2. Hb synthesis regulates also cell replication: precursors replicate until a given amount of Hb is formed (Olver et al., 2010).

Therefore, bone marrow includes a proliferative pool of replicating cells and a maturation pool composed by non-replicating cells (from rubricytes to mature RBC). Mature RBC are then released in blood. Aging of RBC is characterized by an increased cytoplasmic density and by oxidation of membranes. Cells other than RBC may replace damaged structures with newly synthesized molecules, but RBC cannot synthesize new proteins. Hence, the only defense against aging are the antioxidant defenses, that becomes progressively less efficient over time. Therefore, after about 2.5 months in cats and 4 months in dogs, senescent RBC are cleared from blood through an immune reaction against aged RBC proteins based on extravascular (phagocytosis by splenic macrophages, eventually after antibody binding) or intravascular hemolysis (complement activation). Then, the heme group is converted into bilirubin and transported to the liver, aminoacids of the globin chains are recycled and iron is bound to ferritin or hemosiderin (Christian, 2010).

PATHOPHYSIOLOGY OF ANAEMIA

Anaemia may be caused by two pathogenic mechanisms: 1) decreased RBC production due to toxic, infectious or idiopathic bone marrow diseases or to metabolic, neoplastic or infectious diseases that secondarily affect erythropoiesis; 2) decreased lifespan of mature RBC (acute blood loss or hemolysis) (Tvedten, 2010). The causes and mechanisms inducing these types of anaemia will be described separately.

Decreased RBC production

Anaemia depends on a progressively reduced erythropoiesis, so that RBC cleared from blood after their normal lifespan are not replaced by new cells. Therefore, anaemia is classified as non regenerative, since no reticulocytes are released in blood. This group of anaemia includes:

- Nutritional deficiencies: proteins or aminoacids are not available for the synthesis of Hb and cell membranes, due to starvation or diseases that induce malabsorption or protein losses. In this case, however, anaemia is only part of the general symptoms such as emaciation and edema.
- Cobalamin (Vitamin B_{12}) deficiency: it may occur in Giant Schnauzers, due to an inherited deficiency of receptors for the intrinsic factor necessary for vitamin B_{12} absorption (Fyfe et al., 1991).
- Copper (Cu) deficiency: rare, resembles iron (Fe) deficiency (Cu^{2+} is a cofactor of Fe^{2+} metabolism) (Weiss, 2010c)
- Iron deficiency: common in dogs and cats due to chronic hemorrhages that induce iron losses that deplete body iron stores (ulcerated tumors, gastrointestinal ulcers, bloodsucking parasites) or, less frequently, to decreased iron intake due to primary dietary deficiencies, malabsorption or altered intestinal pH.
- Aplastic anaemia: rare condition on which the replication of early hemopoietic precursors is severely depressed, due to unknown causes. Therefore also leukopoiesis and thrombopoiesis are markedly reduced, and hematopoietic bone marrow is replaced by adipose tissue (Weiss, 2010a).
- Pure red cell aplasia (PRCA): is an immune-mediated reaction against erythroid precursors. It may be idiopathic or associated with infections such as parvovirus and feline leukemia virus (FeLV) or with the administration of human recombinant EPO (Weiss, 2010b).
- Non regenerative anaemia associated with myelo-suppressive drugs: lists of myelotoxic substances that interfere with erythropoiesis can be found in hematology or toxicology textbooks (Weiss, 2010b, Nabity and Ramaiah, 2012). Chemicals acting on early precursors induce pancytopenia, while chemicals that act only on the erythroid lineage induce only anaemia. Chronic lead poisoning may induce a peculiar form of anaemia due to the inhibition of enzymes involved in the synthesis of heme (Morgan, 1994).
- Anaemia of inflammatory disease (AID) or of...
chronic disease (ACD): it is probably the most common non regenerative anaemia in dogs and cats and has a multifactorial origin, in which iron sequestration, decreased iron absorption and transport in blood play an important role (Fry, 2010).

- Anaemia associated with infections: although some infectious agent may induce hemolytic anaemia (see below) many infectious diseases (e.g. chronic ehrlichiosis, leishmaniosis, feline immunodeficiency virus) induce non regenerative anaemia due to the presence of AID (see above) and to the infection of stromal cells, that normally sustain the hematopoietic activity (Wardrop, 2010).

- Anaemia associated with metabolic diseases: the bone marrow is fully efficient only if the supply of substrates and hormones needed for erythropoiesis is normal. Renal diseases may be associated with decreased EPO production and azotemia, that has a myelotoxic effect. Similarly, in liver failure the production and distribution of proteins and lipids and, as regards portosystemic shunt or microvascular dysplasia, iron, is abnormal (Meyer and Harvey, 1994; Allen et al., 1999, Fry, 2010).

- Anaemia associated with tumors: the availability of protein and energy substrates decreases during neoplastic cachexia. In hematopoietic tumors (leukemia, lymphoma) anaemia depends also on the replacement of erythropoietic bone marrow by leukemic cells or on the paraneoplastic production of cytokines that depress hemopoiesis (McDonough and Moore, 2000, Bokemeyer et al., 2005).

Decreased RBC lifespan

The lifespan of erythrocytes decreases in 2 cases: severe acute hemorrhage and hemolytic anaemia. In both cases the bone marrow is fully efficient and releases immature RBC as an attempt to restore the RBC mass, and anaemia is classified as regenerative. Details of the pathogenesis of these anaemias are the following:

- Acute hemorrhage: immediately after a severe blood loss the patient is hypovolemic but not anaemic, since both RBC and plasma are lost. In a few hours, water is resorbed from extracellular fluid to restore volemia, and Ht decreases but anaemia is still pre-regenerative (see below). Regeneration may be seen after 4-7 days, when the number of reticulocytes increases (Tvedten, 2010). In dogs with internal blood loss RBC may be resorbed through vascular walls, rapidly restoring the Ht (Clark and Woodley, 1959).

- Hemolytic anaemia: intra- or extravascular hemolysis may occur earlier than the normal lifespan if RBC are damaged by toxins or oxidants or if intrinsic or extrinsic factors accelerate RBC aging. The most common causes of hemolysis in dogs and cats are the following:
  o Inherited enzyme deficiency: a reduced function of EMP, PPP or antioxidant enzymes does not allow the RBC to produce energy or to be protected from oxidation. The two most important inherited enzyme deficiencies in dogs and cats include phosphofructokinase (PFK) in spaniel dogs, pyruvate kinase (PK) in the Basenji, Beagle, terrier dog breeds, Abyssinian and Somali cats. PFK deficiency induces hemolysis after exercise since RBC lyse when the respiratory alkalosis induced by hyperventilation occurs; PK deficiency progressively induces myelofibrosis (Harvey, 2006).
  o Hemolysis induced by chemicals that directly lyse the RBC membranes (e.g. bacterial hemolysins, toxins contained in snake or insect venom) (Dell’Orco et al., 2005; Goddard et al., 2011).
  o Hemolysis induced by oxidants (e.g. acetaminophen, especially in cats, onions, Vitamin K₃, propofol, zinc, endogenous oxidants produced during diabetes, lymphoma, hyperthyroidism). Oxidants may induce methemoglobinemia (oxidation of iron in the heme group, that in turn induces hypoxia and cyanosis), Heinz bodies (due to the oxidation of globin chains of Hb) or eccentrocytes (due to oxidation of membranes that binds to each other), described below (Desnoyers, 2010).
  o Anaemia associated with infectious agents: many bacteria, viruses or parasites may induce anaemia, mostly through immune-mediated mechanisms (see below). Additionally, some infectious agents induce anaemia by releasing toxins (see above) or by inducing mechanical damage to RBC during replication (e.g. Babesia spp.) (Allison and Meinkoth, 2010, Riegel and Stockham, 2010).
Different systems are used to classify anaemia (e.g. based on cell size, cell morphology, severity of anaemia, etc.). However, in routine practice it is important to classify anaemia according to the two main pathogenic mechanisms (decreased RBC production or reduced RBC lifespan), since the identification of the pathogenic mechanism may allow to select the most appropriate therapeutic approach (Tvedten, 2010). Therefore, the diagnostic approach must be focused on the differentiation of clinical and laboratory features associated with one or the other type of pathogenesis, followed by additional investigation to discriminate, within each type of anaemia, the most likely causative agent. This approach is easier if data are evaluated step by step as follows:

**Clinical findings**

The rapid onset of signs associated with anaemia (depression, dyspnea, tachycardia, pale mucous membranes) is consistent with a hyperacute/acute decrease of the RBC mass (hemolysis or acute hemorrhage) that induces a rapid and severe hypoxia. Hemorrhages may be directly detected during physical examination, while in the case of hemolysis additional signs such as hemoglobinuria and/or icterus, and splenomegaly may be found. Conversely, anaemia due to decreased RBC production develops gradually over time and affected animals do not suffer from severe hypoxia since they have time to develop adaptive mechanisms to redistribute blood in peripheral tissue and to facilitate the release of O₂ to tissues. Therefore, symptoms associated with anaemia may appear only when the oxygen demand increases (e.g. intolerance to exercise) and mucous membranes may be mildly pale, even in the presence of very low Ht, Hb or RBC counts. The clinical signs due to the primary disease (e.g. renal or hepatic failure) may be more severe than those associated with anaemia.

**Macroscopic appearance of the blood sample**

The observation of the sample or of centrifuged microhematocrit tubes may provide useful information. For example, it may rapidly allow to estimate

- Immune-mediated hemolytic anaemia (IMHA), in which IgM or IgG antibodies bind antigens on the RBC membrane and activate the complement cascade, whose end product (membrane attack complex or perforin) lyse the RBC. Alternatively, phagocytes within hemocatheretic organs uptake and destroy RBC bearing antibodies on the cell surface. IMHA can occur when drugs (e.g. penicillin) or infectious agents (e.g. viruses, antigens from bacteria or parasites) are exposed on the RBC membranes and attacked by the immune system that ultimately also destroys the cell. A similar mechanism may be triggered against endogenous antigens in the case of autoimmune hemolytic anaemia (AIHA), frequently seen in female large dog breeds, often in association with other auto-immune disorders (e.g. autoimmune thrombocytopenia, lupus erythematosus), in which the immune reaction is directed against erythrocyte antigens (McCullough, 2003; Pick, 2011). Moreover, IMHA may occur after transfusions with incompatible blood types or in neonatal isoerythrolysis. In both cases, the reaction develops after contact of antibodies and RBC of two different animals. Neonatal isoerythrolysis occurs in type A or AB kittens receiving the colostrum of a type B queen (Tocci, 2010). Acute hemolytic transfusion reactions may occur if the donor and the recipients have a different blood type. Details of the main group types that may be found in cats (A, B, AB and Mik) and in dogs (Dog erythrocyte antigens or DEA 1-1, 1-2, 3 to 7 and Dal) may be found in hematology textbooks (Andrews and Penedo, 2010). However, it must be stressed here that transfusion reactions frequently occur in group B cats receiving type A or AB blood (since all type B cats have natural anti-A antibodies) and, less frequently, in type A or AB cats receiving type B blood (since no natural anti-B antibodies are present). Similarly DEA 1-1 is highly immunogenic but no natural antibodies exists: therefore, transfusion reactions may occur only in dogs previously sensitized by contact with DEA 1-1. Conversely, natural antibodies may exist against other DEA antigen (e.g. DEA 3, 5, 7), but these antibodies rarely induce severe transfusion reactions.
the severity of anaemia (very low hematocrit) or abnormalities of other cell populations: a severe thickness or a pinkish coloration of the buffy coat may indicate respectively leukocytosis (e.g. severe inflammation or leukemia) or presence of erythroblasts, to be verified by microscopic analysis. In addition, the color of plasma may indicate icterus (yellow), hemolysis (pink to red) or lipemia (with and milky), that may be associated with diseases responsible for anaemia.

If IMHA is suspected, a drop of blood on a glass slide may be examined to assess the possible presence of macro- or microagglutinations (see below), that are highly consistent with this condition. Agglutination must be differentiated from pseudoagglutination (due to the high concentration of proteins typical of inflammatory conditions) by mixing a drop of blood with a drop of saline solution: if RBC clumping disappear pseudoagglutination is more likely. Finally, the macroscopic analysis of the tube may allow to verify the presence of hyper- or hypoviscosity and, in case of methemoglobinemia induced by oxidants, a brownish coloration of blood. This latter change is easily detectable placing a drop of blood on a white paper towel.

**Evaluation of RBC indexes**

RBC indexes are directly generated by automated counters using calculation procedures that are different from those reported in textbooks regarding manual methods (Stockham and Scott, 2008; Moritz and Becker, 2010). Mathematical formulae are mentioned below to allow comprehension of what each index indicates. RBC indexes include:

- Mean corpuscular volume (MCV = (Ht x 10) / RBC/ml): indicates the mean volume of each RBC. Based on this index, RBC may be defined as normo, micro- or macrocytic.

- Mean corpuscular hemoglobin concentration (MCHC = (Hb x 100) / Ht): indicates the percentage of Hb within each RBC. Based on this index, RBC may be classified as normo- or hypochromic. Hyperchromic RBC usually suggest an analytical artifact such as overestimation of Hb concentration.

- Mean corpuscular hemoglobin (MCH = (Hb x 10) / RBC/ml): indicates the content of Hb in each RBC. The MCH may also be used to classify RBC as normo- or hypochromic.

- Red cell distribution width (RDW): calculated by the instrument based on the width of the RBC histogram and on the MCV. It indicates how much the RBC population is dispersed around the MCV and therefore indicates anisocytosis since it increases when macro- or microcytes are present. Therefore it may detect regeneration earlier than MCV since the few reticulocytes released in blood in early regeneration are not able to influence the MCV but are detectable as larger cells that increase the RDW.

- Some laser-based counters provide additional
indexes that may be useful in certain types of anemia: the corpuscular hemoglobin concentration mean (CHCM) and the cellular hemoglobin (CH) are directly read by the laser beam, that estimates the intracellular hemoglobin more accurately than MCHC and MCH, that may be artifactualy altered if free Hb is present in plasma (e.g. during hemolytic anemia) (March et al., 2005). Moreover, laser counters calculate the volume and Hb content of reticulocytes (MCVr and CHr, respectively), providing an additional tool to better characterize the reticulocyte response. This is useful in iron deficiency anemia (Steinberg and Olver, 2005; Fry and Kirk, 2006).

MCV, MCH, and MCHC are essential to classify the type of anemia, as follows (Tvedten, 2010):

- Normocytic normochromic (normal MCV and MCH or MCHC): the size and the Hb content of RBC are normal, as it occurs in anemia due to decreased RBC production, on which Ht, Hb or RBC number decrease because normal RBC at the end of their lifespan are not replaced by new cells. Therefore, the normocytic normochromic pattern is consistent with a diagnosis of non regenerative anemia. The only exception is the so-called “pre-regenerative anemia”, i.e. the condition occurring just after acute blood loss or hemolysis: although bone marrow releases reticulocytes early (macrocytic and hypochromic), reticulocytosis becomes apparent some days after the onset of anemia and peaks in about one week. Therefore, in the first days anemia is normocytic normochromic because the number of reticulocytes in blood is too low to modify the MCV or to induce anisocytosis and polychromasia (see below). Nevertheless, reticulocytosis associated with early regeneration may increase the RDW, as stated above.

- Macrocytic hypochromic: it is typical of regenerative anemia, when reticulocytes are released in blood in sufficient number to determine an increase of MCV and a decrease of MCH or MCHC. The only exception is anemia associated with cobalamin deficiency which is macrocytic and hypochromic in the absence of reticulocytosis since cobalamin is essential for cell replication. Therefore, when cobalamin is lacking division of precursors is slower than normal and daughter cells are larger than normal.

- Microcytic hypochromic: it is typical of iron deficiency anemia, since iron is essential for Hb synthesis, that in turn modulates the replication of RBC precursors. In the absence of iron, precursors continue to replicate generating smaller mature RBC (Weiss, 2010c). However, microcytosis is not a sensitive indicator of iron deficiency anemia, unless particularly severe (Paltrinieri et al., 2010a). Therefore, when microcytic hypochromic anemia associated with inappropriate reticulocytosis and poikilocytosis is found, a sideremic profile (see below) is mandatory to confirm the diagnosis.

- Macrocytic normochromic: it may occasionally occur in cats infected by the feline leukemia virus (FeLV). Type C FeLV alters the maturation of erythroid precursors, inducing macrocytosis in the absence of hypochromasia. However, macrocytosis of normochromic RBC may also be an artifact due to in vitro swelling of RBC after sampling. Alternatively it may occur if storage is prolonged, so that sources of energy are consumed and RBC in the tubes cannot extrude the anticoagulant that tends to enter the cytoplasm, or in hypernatremic patients whose RBC have an higher cytoplasmic concentration of Na⁺ that induces an osmotic influx of buffers in the counter.

- Hyperchromasia is usually an artifact that may be found in hemolytic anemia (MCHC and MCH are overestimated by the instruments that include in the formula both intracellular and extracellular hemoglobin) or in Heinz body anemia, since Heinz bodies lead to an artifactual increases of Hb, and therefore to an erroneous calculation of MCHC and MCH (Tvedten and Moritz, 2010).

Although the classification above quite reliably classify the anemic process, it may be kept in mind that anemia is a dynamic process and therefore the hematologic pattern may vary over time. Apart of the example of pre-regenerative anemia mentioned above, non regenerative or iron deficiency anemia may become macrocytic and hypochromic if the causative condition is removed (e.g. supplementation of iron, treatment of inflammation) and bone marrow activity starts again, releasing reticulocytes in blood.
regenerative (i.e. to restore mature RBC that are lost due to a pathological condition), the magnitude of reticulocytosis should be inversely proportional to the severity of anaemia. Therefore, in human medicine the following additional parameters able to correct the magnitude of reticulocytosis by the severity of anaemia have been defined.

- The corrected reticulocyte percentage is calculated using the formula: Corrected Ret% = Ret% x (measured Ht/mean Ht of the species), where the mean Ht of cats is 37 and of dogs is 45.

- The reticulocyte production index (RPI) is calculated by dividing the Corrected Ret% by the maturation time of reticulocytes in blood, that is longer in severe anaemia, since the reticulocytes are released at an earlier maturation stage and take a longer time to become mature RBC. In humans this time is 1 day for an Ht of 45, 1.5 days for an Ht of 35, 2 days for an Ht of 25 and so on. However, no information on the actual maturation times in dogs and cats are available.

Presence and magnitude of bone marrow regeneration

This step is essential to classify the anaemia as regenerative or not. The most relevant sign of an increased bone marrow activity is the release of reticulocytes in blood. Therefore, the detection and quantification of reticulocytosis is the most reliable sign of regeneration. Reticulocytes may be counted using laser-based instruments that provide a relatively accurate enumeration of reticulocyte counts, or manually in smears stained with new methylene blue or brilliant cresyl blue (Figure 1). In cats, it is important to differentiate punctate reticulocytes, that are normally present in blood, from aggregate reticulocytes, that are less mature and that are the only cells suggestive of regeneration in cats (Tvedten and Moritz, 2010).

Manual or instrumental counts calculate the percentage of reticulocytes on RBC. However, this information alone is not enough to classify the anaemia as regenerative, since in order to be effectively regenerative (i.e. to restore mature RBC that are lost due to a pathological condition), the magnitude of reticulocytosis should be inversely proportional to the severity of anaemia. Therefore, in human medicine the following additional parameters able to correct the magnitude of reticulocytosis by the severity of anaemia have been defined.

- The corrected reticulocyte percentage is calculated using the formula: Corrected Ret% = Ret% x (measured Ht/mean Ht of the species), where the mean Ht of cats is 37 and of dogs is 45.

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Based on the limitations above, veterinary textbooks do not recommend the use of RPI or the Corrected Ret% and to evaluate regeneration using the absolute number of reticulocytes, that may be calculated based on the percentage of reticulocytes and on the actual number of RBC (for example if reticulocytes are 10% of 2 millions of RBC/µL, the absolute number would be 200.000 reticulocytes/µL) (Tvedten and Moritz, 2010). However, there is no consensus on the actual threshold to be used for reticulocyte numbers, and ultimately all the reticulocyte parameters, including Ret% and RPI may be suggestive of regeneration pending that factors such as the severity of anaemia and the time span from the initial blood loss are considered (Cowgill et al., 2003). When in doubt, these parameters must be evaluated over time: for example, if the percentage or the number of reticulocytes or the RPI are below the reference limit of the laboratory but the sequential samplings reveal an increase of these values over time, active regeneration is very likely.

In the absence of reticulocyte counts, regeneration may be estimated based on morphological features such as anisocytosis and especially polychromasia, that is the most reliable morphological sign of reticulocytosis in blood smears (Hodges and Christopher, 2011). Conversely, the presence of nucleated RBC alone is not consistent with regeneration except when anisocytosis, polychromasia and reticulocytosis are present. In the absence of these

Figure 2: morphological changes of feline and canine RBC detectable at low magnification (May Grünwald-Giemsa stain, 400X magnification). A) microagglutination: clumps of RBC of different size are detectable; B) rouleaux: erythrocytes are lined up and resemble stacks of coins; C) anisocytosis and polychromasia: cells of different size and staining properties are detectable in the smears. In this image cells that are larger and have a more basophilic rim of cytoplasm are likely interpretable as reticulocytes (see also figure 1E for the appearance of reticulocytes in blood stained with May Grünwald-Giemsa); D) poikilocytosis: the smear contains cells of different shape. In this image several abnormal cell shapes can be found (see figure 3 for details for each abnormal cell type): polychromatophils (grey arrowhead) acanthocytes (black arrowhead) stomatocytes (large black arrow), spherocytes (large grey arrow), schistocytes (black thin arrow), knizocytes (grey thin arrow).
findings, nucleated RBC in blood indicate the possible presence of an altered blood-marrow barrier, dysplastic changes in the bone marrow or chronic lead poisoning (Mandell et al., 1989).

**Morphology of RBC**

The morphology of RBC may provide useful diagnostic information. Therefore, it is always mandatory to prepare, stain and observe under a microscope a blood smear, even when automated cell counters already provide complete information about cell indices and differential leukocyte counts. Important aspects such as size, shape and color of RBC, presence of nucleated RBC, or intra-extraerythocytic parasites are not provided by cell counters and their presence must be verified on blood smears (Barger, 2010). Guidelines on how to prepare blood smears and on the most common staining solutions are available on many textbooks (Villiers and Blackwood, 2005). It is recommended for clinicians to practice in order to obtain good quality smears, with a wide monolayer area, were the morphological features of red cells are clearly interpretable.

The microscopic analysis of blood smears must always start at low magnification, to observe the eventual presence of the following changes (Figure 2):

- Microagglutination: clumps of RBC due to the presence of anti-RBC antibodies.
- Rouleaux: “columns” of RBC lined up to resemble stacks of coins, consistent with high blood viscosity, as it can occur during hyperproteinemia or inflammation. It is a relatively normal finding in cats.
- Anisocytosis: RBC of different size, usually due to reticulocytosis. A mild to moderate degree of anisocytosis may be normal when detected in cats.
- Polychromasia; RBC have different staining properties, mostly due to the presence of reticulocytes that are polychromatophilic due to their RNA content.
- Poikilocytosis: RBC of different shape. If a given shape is prevalent (see below) it should be described separately. Poikilocytosis is consistent with iron deficiency in dogs and with liver failure in cats.
- Presence of extracellular parasites (e.g. larvae of *Filaria* spp.)

At higher magnification, the following morphological changes can be seen (Figure 3):

- Echinocytes: RBC with small and numerous cytoplasmic spikes. Usually, echinocytosis indicates an artifact due to improper slide handling and staining, unless they are diffused throughout the smear. In this case they may be consistent with abnormal composition of membrane lipids (e.g. liver failure).
- Acanthocytes: RBC with evident and thick spikes, due to altered membrane deformability, suggestive of liver failure or of microangiopathic diseases (disseminated intravascular coagulation, tumors).
- Leptocytes: RBC with a larger central pale area due to a poor Hb content. They can be found in many types of non regenerative anaemia, and particularly in iron deficiency anaemia
- Codocytes or target cells, due to an abnormal distribution of Hb within the cytoplasm. They may be an artifact or depend on an altered membrane deformability potentially associated with liver diseases.
- Keratocytes: RBC with two cytoplasmic projections resembling “horns”, due to the uptake of part of the RBC cytoplasm by macrophages. Therefore they are suggestive of hemolytic anaemia.
- Schistocytes: cytoplasmic fragments of RBC, irregularly shaped, suggestive of RBC destruction.
- Spherocytes: RBC that appear smaller and more intensely stained than normal and lack the central pale area, since Hb is redistributed in a smaller cell after removal of part of the cytoplasm by phagocytes.
- Eccentrocytes: RBC with the pale area shifted to a pole of the cell due to oxidation of Hb and of membranes that binds to each other.
- Stomatocytes: the pale area forms a line at the center of the cytoplasm, something resembling a “smile”, due an altered osmotic equilibrium induced by abnormal membrane pumps (Bonfanti et al., 2004)
- Knizocytes: RBC irregularly folded that appears with irregular pale or densely stained area. It is a common change in reticulocytes or leucocytes.
- Basophilic stippling (small and numerous
Figure 3: morphological changes of feline and canine RBC detectable at high magnification (May Grünwald-Giemsa stain unless otherwise indicated in the figure legend, 1000X magnification): A) Echinocytes in a feline blood smear; B) Acanthocytes (arrows) in the blood of a dogs with a splenic tumor; C) Leptocytes, characterized by a large central pale area, from a dog with severe iron deficiency; D) codocytes or target cells in the blood smear of a dog with severe iron deficiency; E) Keratocyte (arrow) from a dog with immune-mediated hemolytic anaemia; F) Schistocyte or RBC fragment (arrow) from a dog with immune-mediated hemolytic anaemia; G) Spherocytes, that appears as smaller and intensely stained (eosinophilic) cells (arrow) from a dog with immune-mediated hemolytic anaemia; H) Eccentrocytes, characterized by the lack of the pale central area and by a rim of pale cytoplasm on a pole of the cell, from a dog with anaemia due to oxidants; I) Stomatocytes (arrows) from a dog with iron deficiency anaemia; cells are characterized by a linear or curvilinear pale area in the center of the cell instead of the typical round pale area; J) Knizocyte (arrow) from a dog with regenerative anaemia: the cell at the arrow is a RBC (in this case more likely a reticulocyte due to its size and basophilic cytoplasm) with a central folding that appears more intensely stained; K) Heinz bodies (arrows) representing aggregates of oxidized hemoglobin on a pole of the cells in the blood of a cat with anaemia induced by oxidants; L) Same cat of section I: the smear has been stained with brilliant cresyl blue, that allows a better identification of Heinz bodies (arrows); M) Howell-Jolly body (arrow) that is a nuclear remnant in a mature RBC; N) Babesia canis in the RBC of a dog with pre-regenerative anaemia the arrow indicates the RBC that contains 4 oval-pyriform parasites; O) Blood smear from a cat infected with Mycoplasma hemofelis: bacteria are seen as single blue dots on the center of the cell (black arrow) or as multiple blue dots, often arranged in chains or located at the periphery of the cell (grey arrows); P) Amastigotes within a macrophage from the bone marrow of a dog with canine leishmaniosis.
basophilic dots), consistent with lead poisoning.
- Heinz bodies: round protrusions on the cell membrane, better visualized with new methylene blue stain, due to oxidized Hb precipitated on the cell membrane. Heinz bodies are frequent in cats, in which Hb is rich of sulphhydryl groups and the spleen is less efficient in removing affected RBC. Therefore, Heinz bodies are considered pathologic only if affect more than 40% of feline RBC (Tvedten and Moritz, 2010).
- Howell Jolly bodies: round deeply basophilic inclusions (approximately 2 µm of diameter) that are residuals of the pyknotic nuclei. They are suggestive of intense regeneration, if associated with reticulocytosis, or of erythrodysplasia if reticulocytosis is absent.
- Infectious agents such as Mycoplasma spp., that appear as small round basophilic dots on the RBC or at the periphery of the cell, often arranged in small chains, or Babesia spp., round or pyriform structures, of 1-2 mm (B. gibsoni) or 3-5 mm (B. canis or B. vogeli), with a clear center surrounded by a thin basophilic rim. Since Babesia species replicate within the RBC usually 2, 4 or more parasites are seen in a single cell. Mycoplasmas that bind the cell membrane through a Ca²⁺ dependent mechanism, may detach from the RBC if blood is stored in EDTA, that chelates Ca²⁺. Therefore, observation of smears prepared before placing the blood in the anticoagulant is recommended. Moreover, bacteraemia may be cyclic. Therefore, if the clinical suspicion is high, blood sampling and microscopic observation of smears should be repeated (Tasker, 2010). The detection of Babesia is facilitated if capillary blood or the feathered edge of blood smears are observed (Comazzi et al., 1999). Conversely, infectious agents such as Leishmania or Ehrlichia may be found in bone marrow rather than in blood. When bone marrow is not available, the observation ofuffy coat smears increases the likelihood of microscopical identification of these agents (Mylonakis et al., 2003; Allison and Little, 2013).

In summary, the absence of morphological changes is consistent with a non regenerative anaemia, while blood loss or hemolytic anaemia is usually characterized by typical morphological changes (anisocytosis, polychromasia, agglutination, spherocytes, schistocytes). However, in some non regenerative anaemia bone marrow activity may be secondarily depressed by systemic disease. In this case, morphological changes consistent with the primary disease may be found (e.g. rouleaux, codocytes, acanthocytes, leucoctyes).

**Additional tests**

Once the approach above identified a potential pathogenetic mechanism of anaemia, additional tests may be used to establish the final diagnosis. Some of these are serum biochemistry or serological tests: for example, renal and hepatic functions should be investigated as well as the serum concentration of markers of inflammation. In cats, serology for FIV and FeLV is always recommended, as well serology or PCR for Leishmania or Ehrlichia infections in dogs (Levy et al., 2008; Paltrinieri et al., 2010b; Allison and Little, 2013). Others are specialized hematological tests to be selected based on the clinical presentation and on the results of the basic hematological approach mentioned above. Among these, some are run only in specialized laboratories (e.g. genetic testing, osmotic fragility, activity of intracellular enzymes), while others are routinely run in many veterinary laboratories.

The most important of the aforementioned tests are:
- **Cytology/histology of bone marrow:** it is recommended in the presence of non regenerative anaemia, pancytopenia, leukemia, suspected leishmaniosis that is not confirmed by serology or PCR or in any other case on which laboratory data are potentially consistent with bone marrow involvement (e.g. hypercalcemia, monoclonal gammopathy). Bone marrow biopsies must be collected according to standard procedures, described in textbooks of internal medicine (Ettinger and Feldman, 2010). Ideally, both cytology and histology should be performed and a blood sample should be collected and analyzed at the same time (also if blood has been already analyzed). Cytology may be performed on bloody bone marrow or on smeared core biopsies. After staining, the following aspects must be evaluated:
  - **quality:** the sample is considered adequate if
contains fat, bone spicules and megakaryocytes
- cellularity: the sample is classified as hypo-normo- or hypercellular if adipose tissue accounts for more than 75%, 25-75% or less than 25%, respectively. Low cellularity may depend on sampling errors or physiological processes (e.g. aging) on myelofibrosis or on depressed hematopoiesis. Histology may allow to differentiate sampling artifacts from true hypocellularity or myelofibrosis.
- Myeloid:erythroid (M:E) ratio: it is calculated by counting an 200 to 500 cells of both the lineages. The normal M:E ratio is close to 1. Different values may be due to hyperplasia of one lineage, hypoplasia of the other or both. Results must be evaluated in comparison with the peripheral blood: for example erythroid hyperplasia is appropriate if regeneration is seen in blood, and pathological in patients with suspected non regenerative anaemia
- Evaluation of cell lines: this aims to assess whether a pyramidal distribution is present (e.g. high number of mature cells and progressively lower number of early stages) or if blasts are prevalent (blasts exceed 20% in leukemia or myelodysplastic syndromes), or if a maturation arrest is present (only early precursors are detectable), suggesting myelodysplasia or immune-mediated disorders.
- Abnormal findings: abnormal cells, asynchronous maturation (i.e. mitotic nuclei in mature cells), parasites (e.g. Leishmania) or cells not normally present in bone marrow (e.g. abundant lymphocytes, plasma cells or mast cells, tumor cells) must be recorded. The amount of iron stores (hemosiderin deposits) should be also assessed eventually after Prussian blue staining.

- Coomb’s test or flow cytometric detection of anti-RBC antibodies. It is recommended when immune-mediated anaemia is suspected. Blood of the patient is added with anti-canine IgG or IgM antibodies that, in the flow cytometric test, are labeled with a fluorochrome. These antibodies bind the IgG or IgM exposed on the RBC membranes of patients with IMHA inducing RBC agglutination (Coomb’s test) or a fluorescent signal detectable by a flow cytometer (Kucinskiene et al., 2005). Occasionally, a few dogs without IMHA may be positive with the very sensitive flow cytometric technique (Morley et al., 2008).

- Blood typing: Although in dogs acute hemolytic reaction may not occur at first transfusion, it is better to type at least the more immunogenic blood antigens of donors and recipients before to transfuse a dogs. Transfusion reactions are common in cats. Therefore blood typing must always be performed in cats. In both species, standardized methods must be used (Giger et al., 2005; Seth et al., 2011).

- Sideremic profile: It may be useful to confirm iron deficiency or to differentiate iron deficiency by other anaemia potentially associated with a trend to microcytosis. The profile includes the measurement of serum iron and of total iron binding capacity (TIBC, an indirect estimate of transferrin concentration) followed by the calculation of the percentage of transferrin saturation (Fe/TIBC) and of unsaturated iron binding capacity (UIBC=TIBC – Fe). Ferritin may also be measured using immunological method, although the serum concentration of ferritin may increase in dogs with tumors or inflammation independently on the actual amount of iron stores (Weiss, 2010c). In iron deficiency anaemia serum iron, ferritin and transferrin saturation are low, TIBC is not decreased and UIBC increases. Conversely, low iron, decreased TIBC and UIBC and increased ferritin may be found in inflammation.

- Coagulation profile: it may be recommended, independently on the actual platelet counts, when blood loss anaemia is diagnosed, especially when no evident traumatic events are reported by the owners. The panel should include coagulation times and possibly fibrin/fibrinogen degradation products and d-dimer assessment.

SUMMARY OF THE MAIN DIAGNOSTIC FINDINGS IN ANAEMIC DOGS AND CATS
Hematological findings in common forms of canine and feline anaemia may be interpreted as follows:
- Normocytic normochromic anaemia is consistent with a non regenerative origin, to be confirmed by the lack of anisocytosis or polychromasia on blood smears and by low reticulocyte counts. Theoretically, this profile is also consistent with pre-regenerative anaemia, that however may be supported by an increased RDW, by clinical signs (signs of blood loss or hemolysis) or assessed through repeated samplings. Once non regenerative anaemia is confirmed, additional signs that may identify the cause or the pathogenic mechanism should be investigated clinically or through laboratory tests such as serology for infectious diseases, serum biochemistry or more importantly, bone marrow cytology. Information on numbers of other blood cell types is also important: leukemia may be detected by peripheral blood analysis, a peripheral bi- or tri-cytopenia associated with a severe bone marrow hypoplasia of all the lineages is consistent with a disease of early hematopoietic precursors (myelotoxicosis or aplastic anaemia). Conversely, the lack of changes in the leukogram is consistent with a toxic damage restricted to RBC precursors or with immune-mediated destruction of precursors (PRCA): in both cases bone marrow may reveal erythroid hypoplasia, although in PRCA hyperplasia of early precursors with a maturation arrest may be present and Combs’s test or anti-RBC antibody testing may be positive. Chronic lead poisoning may also be associated with inappropriate erythroblastosis (circulating nucleated RBC in the absence of reticulocytosis) and basophilic stippling within mature RBC (Morgan, 1994). However, inflammation, infections or metabolic diseases are the most frequent causes of non regenerative anaemia. The diagnosis may be supported by additional hematological findings (e.g. poikilocytosis in liver diseases, a trend to microcytosis in inflammation), by biochemical tests for liver and kidney function or for inflammatory markers, or by serology for infectious diseases.

- Microcytic hypochromic anaemia is usually associated with iron deficiency, to be confirmed with a sideremic profile. Leptocytes and often poikilocytosis with frequent knizoschistocytes may be found on blood smears. Reticulocytosis may be present, although not proportional to the severity of anaemia, and bone marrow cytology reveals an hyperplastic erythroid lineage with decreased iron stores. A similar profile may be found in blood of dogs with portosystemic shunts or microvascular dysplasia, that, however, may be differentiated clinically and/or by diagnostic imaging or testing liver functions. Once iron deficiency has been confirmed, the condition responsible for this change (e.g. chronic hemorrhage, ulcers) should be investigated through a thorough clinical investigation.

- Macrocytosis may be associated with storage artifacts or with FeLV infection, especially if hypochromasia and reticulocytosis are not present. If these conditions are ruled out (e.g. through FeLV serology), and if hypochromic RBC are present, anaemia is most likely regenerative since cobalamin deficiency, the other possible cause of macrocytic hypochromic anaemia, is rare. Regenerative anaemia is also characterized by anisocytosis and polychromasia on blood smears and by an increased RDW. In this case, the enumeration of reticulocytes is essential to quantify the magnitude of regeneration. Once regeneration is confirmed, the cause of the decreased RBC lifespan must be investigated clinically (e.g. signs of hemorrhage or hemolysis) or by looking at additional hematological findings consistent with the different causes of hemolysis (e.g. agglutination, spherocytes, schistocytes, consistent with IMHA, eventually supported by positive Coombs’s test or anti-RBC antibodies; eccentrocytes, Heinz bodies or methemoglobinemia consistent with oxidation, presence of hemoparasites). The diagnostic approach may also include additional tests (e.g. coagulation profiles in the case of blood loss, genetic testing in the case of suspected inherited enzyme deficiency, tests for infectious diseases) or sequential samplings to assess the evolution of the regenerative response over time.

CONCLUDING REMARKS

Anaemia is a frequent condition in dogs and
cats. It may be due to a reduced production of RBC for primary or secondary bone marrow diseases or to a reduced RBC lifespan (blood loss or hemolysis). These two main pathological findings may be differentiated to each other by assessing the presence and the magnitude of reticulocytosis, that reflects bone marrow activity. This may be achieved with a stepwise diagnostic approach that combines clinical and laboratory data, eventually associated with additional hematological investigations aimed to identify morphological findings consistent with the possible pathogenic mechanism and/or by additional tests to identify the cause of abnormal bone marrow activity or of erythrocyte loss or destruction.

CONFLICT OF INTEREST STATEMENT

The author of this paper does not have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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