Anemia in canine chronic kidney disease is multifactorial and associated with decreased erythroid precursor cells, gastrointestinal bleeding, and systemic inflammation

Sofia Borin-Crivellenti, PhD1; Leandro Z. Crivellenti, PhD1; Chen Gilor, PhD2; Shir Gilor, MS2; Daniela G. Silva, PhD3; Suellen R. Maia, MS4; Paula B. Costa, MS1; Alef W. O. Alvarenga, DVM1; André L. S. Fernandes, MS3; Aureo E. Santana, PhD5

1Graduate Program in Veterinary Science (PPGCV)/College of Veterinary Medicine (FAMEV), Universidade Federal de Uberlândia (UFU), Uberlândia, Minas Gerais, Brazil
2Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL
3NUCLEON-Diagnósticos Veterinários, Ribeirão Preto, Brazil
4School of Veterinary Medicine and Animal Science (FMVZ), Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil
5Department of Veterinary Clinic and Surgery, Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil

*Corresponding author: Dr. Borin-Crivellenti (sofiabcrivellenti@ufu.br).

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OBJECTIVE
Compare erythropoiesis-related factors between different stages of canine chronic kidney disease (CKD).

ANIMALS
8 healthy adult dogs (controls), and 24 dogs with CKD, equally divided into 3 groups based on International Renal Interest Society-CKD Guidelines (stage 2, 3, and 4) were recruited between December 2012 and December 2014.

METHODS
The following were assessed in all dogs and then compared between groups: bone marrow cytology, CBC, reticulocyte count, urinalysis, serum biochemistry, blood pressure, occult gastrointestinal bleeding, and serum concentrations of parathyroid hormone (PTH), erythropoietin, interleukin-1β, interleukin-3, tumor necrosis factor-α (TNFα), and interferon-γ.

RESULTS
Erythropoiesis inducing and suppressing factors and the results of the bone marrow cytology of dogs in stage 2 CKD did not differ from the control group. The presence of reticulocytosis in CKD stage 2 suggests that blood loss or erythrocyte destruction might be contributing to developing anemia. Anemia in dogs with progressive CKD was associated with increasing PTH and TNFα and with elevation of the ratio of myeloid to erythroid precursor cells caused by hypoplasia of the erythroid series. The latter was represented mainly by a decrease in the population of polychromatophilic rubricytes and metarubricytes.

CLINICAL RELEVANCE
Increased PTH and TNFα seem to contribute to the reduced percentage of polychromatophilic rubricytes and erythroid population, thereby aggravating the anemia of dogs with advanced CKD. Gastrointestinal blood loss contributes to anemia in all canine CKD stages.

Keywords: bone marrow cytology, erythropoiesis, occult gastrointestinal bleeding, PTH, TNFα

Normocytic normochromic nonregenerative anemia often occurs in patients with chronic kidney disease (CKD)1 and is a universal finding in advanced stages (ie, International Renal Interest Society [IRIS]-CKD stage 3 and 4).2 compromising the quality of life of dogs with the disease.3 Anemia in people and dogs with CKD has multifactorial causes, but it is considered to be mainly the result of erythropoietic hypoproliferation, which in turn is caused by relative insufficiency of erythropoietin (EPO) production of failing kidneys,1,5–6 “functional” iron deficiency,6 nutritional imbalances caused by hypoxia/anorexia,3 and visible or occult gastrointestinal bleeding.6 In dogs, in contrast to people, there
is no decrease in red blood cell lifespan, probably because canine erythrocytes maintain antioxidant defenses in CKD.

A considerable proportion of people with CKD are resistant to erythropoiesis-stimulating agents. This resistance is probably the result of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and interferon-γ (IFNγ), as well as increased parathyroid hormone (PTH) levels. A recent study in a rodent CKD model with nonregenerative anemia showed the presence of increased EPO levels with reduced proliferation of erythroid precursor cells in the bone marrow. This study suggested that uremic toxins and natural organic inflammatory substances might be involved in different ways in the suppression of erythropoiesis, for example, by causing functional iron deficiency.

Erythropoiesis in people is complex and influenced by multiple hormones and interleukins, with cytokines having a proapoptotic action in bone marrow cells. However, these characteristics vary significantly in terms of the type of erythroid progenitor and its response to circulating toxins. Therefore, bone marrow evaluation is an important predictor of mortality in people.

In view of the absence of data on bone marrow responses in dogs with CKD and the hormonal and inflammatory influence on erythroid precursors, this study aimed to compare bone marrow cytological features as well as hormones and interleukins involved in erythropoiesis between dogs in different stages of CKD.

Methods

The study was approved by the Ethics Committee for Animal Use of the University Estadual Paulista (UNESP), Jabiobacal, SP, Brazil (protocol No. 006137-09). Dogs were recruited between December 2012 and December 2014 after obtaining owners’ written consent. The study groups consisted of 8 healthy adult dogs (controls) and 24 dogs with confirmed CKD, equally distributed into 3 groups of 8: CKD stage 2, CKD stage 3, and CKD stage 4.

The inclusion criteria for CKD subjects followed the IRIS guidelines and included a documented clinical course of more than 90 days and stable serum creatinine concentrations above 1.4 mg/dL in hydrated patients, assessed at 3 different time points over the course of 2 to 3 weeks.

Patien exclusion criteria for all groups included blood transfusion or treatment with drugs that stimulate hematopoiesis (recombinant human granulocyte colony-stimulating factor, nandrolone, thymomodulin, and EPO), as well as comorbidities such as neoplasms or chronic inflammatory conditions.

Dogs in the control group were fed a variety of commercial dog foods, and the CKD dogs were fed a variety of prescription renal diets. They also were routinely treated with antiparasitic drugs (at least every 6 months) and had no evidence of gastrointestinal parasites upon Willis-Mollay test and direct fecal exam. Baseline parameters including CBC (performed on an automated hematology analyzer; ABC Vet; Horiba ABX), manual reticulocyte count, manual urinalysis (obtained by cystocentesis), quantitative urinary total protein and creatinine (to determine urinary protein creatinine [UPC] ratio), as well as serum creatinine, urea, phosphorus, total calcium, total protein, and albumin performed on a semiautomated analyzer (LABQUEST® Labtest Diagnostica), serum and urinary sodium concentrations (to determine urinary sodium fraction excretion) performed using ion-specific method (ISLAB; Labtest Diagnostica) and blood pressure levels measured by Doppler ultrasound (Microem; Microem) were used to verify the health of the control group, and to categorize CKD dogs into stages according to IRIS guidelines (2023).

Occult gastrointestinal bleeding (OGIB) in healthy and CKD dogs was evaluated by examining fresh fecal samples using the Benzidine-type indicator method described by Crivellenti et al and Narita et al. A qualitative scoring system was used to verify the absence (grade 0; zero) or presence and severity of gastrointestinal bleeding as follows: grade 1, weakly positive (the smeared surface turns slightly blue green); grade 2, moderately positive (the smeared surface turns blue green), and grade 3, strongly positive (the smeared surface becomes dark blue).

Serum hormone and cytokine concentrations were quantified using ELISA for the quantitative measurement of canine parathyroid hormone, EPO, IL-1β, IL-3, TNFα, and IFNγ, according to the manufacturer’s instructions (intra-assay CV < 10%; interassay CV < 12%; lower limit of detection, 3.11 pg/mL, 11.6 pg/mL, 6.2 pg/mL, 6.5 pg/mL, 6.0 pg/mL, and 5.5 pg/mL, respectively; USCN Life Science Inc).

A small volume of bone marrow (0.5 to 1.0 mL) was harvested from the sternal manubrium by applying negative pressure using an 18- to 21-gauge sterile hypodermic needle attached to a 10 mL syringe containing 1% sterile EDTA in 0.6 mL saline as anticoagulant (0.4 mL of EDTA to rinse the needle and 0.2 mL retained in the syringe). Slides were prepared immediately after sample collection. A 500-cell differential count was performed on each of two bone marrow aspiration smears by a veterinary clinical pathologist (AES) who was blinded to group assignment. The cellular composition of aspirated bone marrow was calculated as a percentage by a differential count of 500 nucleated cells under X1,000 magnification. Only fragments containing areas of the marrow in which the cells were present as a monolayer were counted. The cell types of the differential count, immature to mature erythroid precursor cells ratio (I:Me), and erythroid maturation index were defined as described by Mischke and Busse. Briefly, I:Me was calculated by dividing the number of immature erythroid precursors, defined as rubriblasts and proerythroblasts, by the number of mature erythroid precursors, defined as polychromatophilic rubricytes and metarubricytes.

Commercially available statistical software JMP (SAS Institute Inc) and GraphPad Prism 4 (GraphPad Software, Inc) were used to compare data across the 3 stages of CKD and to verify the absence (grade 0; zero) or presence and severity of gastrointestinal bleeding as follows: grade 1, weakly positive (the smeared surface turns slightly blue green); grade 2, moderately positive (the smeared surface turns blue green), and grade 3, strongly positive (the smeared surface becomes dark blue).
Software) were used for statistical analysis. Normal distribution of data was assessed by the Kolmogorov-Smirnov test. The majority of the data were normally distributed and subjected to a one-way ANOVA, followed by Tukey’s post hoc test. Occult blood score, EPO, and PTH concentrations were the only data sets that were not normally distributed. For these, groups were compared using Kruskal-Wallis as a nonparametric test, followed by Dunn’s post hoc test. Pearson’s correlation coefficient was used to analyze the relationship between hormones, cytokines, and erythroid precursor cells. Spearman’s rank correlation was used to analyze the relationship between the grade of occult fecal blood and other variables. The significance level was set at $P < .05$.

### Results

The control group (CG) was comprised of healthy mixed breed dogs including 2 females (1 intact and 1 spayed) and 6 males (3 intact and 3 neutered) ranging in age from 6 to 10 years (8 ± 1.3 years) and weighing 10.5 to 28.5 kg (18.4 ± 7.2 kg). The CKD groups included 10 females (2 intact and 8 spayed) and 6 males (3 intact and 3 neutered) ranging from 2 to 16 years of age (9.5 ± 4.4 years) and weighing 2.75 to 52 kg (16.7 ± 14.5 kg). In the CKD group, mixed breed dogs were most commonly represented (n = 10), followed by Toy Poodle (5). Other small breeds such as Maltese, Fox Terrier, and Shih Tzu, as well as medium breeds such as Cocker Spaniel, Chow Chow, and Boxer, large breeds such as Weimaraner and Labrador Retriever, and also a giant breed such as Saint Bernard were represented by 1 dog each.

Concentrations of serum creatinine, urea, and phosphorus increased with CKD progression, as did urinary sodium fraction excretion, UPC, and systolic blood pressure, when compared to the control group (Table 1). Urine specific gravity was within the physiological range in controls but was decreased in CKD in all the stages.

Anemia (including low hematocrit, low hemoglobin, and low RBC) was more severe in advanced CKD stages (Table 2). Reticulocyte concentrations were consistent with a regenerative response in CKD stage 2 but with a nonregenerative response in more advanced CKD stages.

In CKD stage 4, there was a significantly lower percentage of polychromatophilic rubricytes, as well as an overall reduction in the erythroid population as expressed by an increase in the myeloid-to-erythroid ratio (Table 3), with a normal WBC count.

### Table 1—Results of serum biochemical, urine, and blood pressure parameters and occult bleeding scores of 8 healthy adult dogs (controls) and 24 dogs with CKD, equally divided into 3 groups based on IRIS-CKD Guidelines (IRIS-CKD stage 2, 3, and 4).

<table>
<thead>
<tr>
<th>Analyte (unit)</th>
<th>Controls (n = 8)</th>
<th>IRIS-CKD2 (n = 8)</th>
<th>IRIS-CKD3 (n = 8)</th>
<th>IRIS-CKD4 (n = 8)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.04 ± 0.04$^a$</td>
<td>1.79 ± 0.06$^a$</td>
<td>3.46 ± 0.13$^a$</td>
<td>7.33 ± 0.97$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>29.93 ± 3.61$^a$</td>
<td>93.61 ± 9.47$^a$</td>
<td>154.3 ± 11.94$^b$</td>
<td>233.7 ± 23.66$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>4.40 ± 0.20$^a$</td>
<td>4.52 ± 0.36$^a$</td>
<td>6.39 ± 0.74$^a$</td>
<td>9.64 ± 2.43$^b$</td>
<td>.0166</td>
</tr>
<tr>
<td>Total calcium (mg/dL)</td>
<td>10.01 ± 0.23</td>
<td>9.80 ± 0.42</td>
<td>10.84 ± 0.33</td>
<td>10.83 ± 0.33</td>
<td>.0792</td>
</tr>
<tr>
<td>Total protein (mg/dL)</td>
<td>7.13 ± 0.18</td>
<td>6.67 ± 0.40</td>
<td>6.18 ± 0.16</td>
<td>6.47 ± 0.22</td>
<td>.0719</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>3.01 ± 0.26</td>
<td>2.50 ± 0.35</td>
<td>2.52 ± 0.52</td>
<td>2.56 ± 0.43</td>
<td>.0667</td>
</tr>
<tr>
<td>Urine specific gravity</td>
<td>1.041 ± 0.08$^a$</td>
<td>1.018 ± 0.02$^b$</td>
<td>1.013 ± 0.03$^c$</td>
<td>1.011 ± 0.02$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>133.8 ± 7.44$^a$</td>
<td>172.5 ± 13.09$^a$</td>
<td>184.3 ± 30.41$^b$</td>
<td>193.6 ± 34.73$^c$</td>
<td>.0002</td>
</tr>
<tr>
<td>UPCR</td>
<td>0.07 ± 0.01$^a$</td>
<td>0.91 ± 0.29$^b$</td>
<td>1.39 ± 0.47$^c$</td>
<td>1.39 ± 0.28$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>FENa (%)</td>
<td>0.43 ± 0.06$^a$</td>
<td>0.94 ± 0.11$^a$</td>
<td>3.16 ± 0.74$^b$</td>
<td>6.85 ± 1.18$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Fecal occult blood (score 0 to 3)</td>
<td>0 (0–0)$^a$</td>
<td>2 (0–3)$^{a,b}$</td>
<td>2 (0–2)$^c$</td>
<td>3 (2–3)$^b$</td>
<td>.0002</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD (normally distributed) except for the fecal occult blood, which were not normally distributed and are reported as median and range.

CKD = Chronic kidney disease. FENa = Urinary sodium fraction excretion. IRIS = International Renal Interest Society. UPC = Urinary protein creatinine ratio. 

$^a$cValues with different superscripts in the same row are significantly ($P < 0.05$) different.

### Table 2—Results of a CBC parameters of control dogs and dogs in different stages of chronic kidney disease as described in Table 1.

<table>
<thead>
<tr>
<th>Analyte (unit)</th>
<th>Controls (n = 8)</th>
<th>IRIS-CKD2 (n = 8)</th>
<th>IRIS-CKD3 (n = 8)</th>
<th>IRIS-CKD4 (n = 8)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>50.5 ± 4.4$^a$</td>
<td>41.0 ± 7.8$^b$</td>
<td>33.7 ± 5.9$^c$</td>
<td>28.9 ± 5.8$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Erythrocytes (X10$^6$/µL)</td>
<td>7.3 ± 0.7$^a$</td>
<td>6.0 ± 1.1$^b$</td>
<td>4.9 ± 1.0$^c$</td>
<td>4.2 ± 1.0$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>16.3 ± 1.2$^a$</td>
<td>13.0 ± 2.5$^b$</td>
<td>10.9 ± 1.6$^c$</td>
<td>9.1 ± 1.8$^c$</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Reticulocytes (cells/µL)</td>
<td>40,943 ± 7,887$^b$</td>
<td>94,606 ± 22,973$^b$</td>
<td>39,824 ± 7,992$^a$</td>
<td>20,138 ± 8,102$^a$</td>
<td>.0033</td>
</tr>
<tr>
<td>MCV (FL)</td>
<td>69.5 ± 3.0</td>
<td>68.4 ± 3.7</td>
<td>69.5 ± 3.9</td>
<td>69.9 ± 4.0</td>
<td>.88</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.1 ± 1.4</td>
<td>31.8 ± 1.2</td>
<td>32.4 ± 1.4</td>
<td>31.6 ± 1.1</td>
<td>.64</td>
</tr>
<tr>
<td>Total leucocytes (cells/µL)</td>
<td>9,425 ± 2,801</td>
<td>9,358 ± 2,524</td>
<td>7,700 ± 2,584</td>
<td>7,000 ± 1,971</td>
<td>.17</td>
</tr>
<tr>
<td>Monocytes (cells/µL)</td>
<td>375.5 ± 91.3</td>
<td>265.1 ± 52.0</td>
<td>168.4 ± 30.8</td>
<td>211.6 ± 70.4</td>
<td>.14</td>
</tr>
<tr>
<td>Neutrophils (cells/µL)</td>
<td>7,653 ± 2,944</td>
<td>7,135 ± 2,322</td>
<td>6,136 ± 2,109</td>
<td>6,084 ± 2,265</td>
<td>.52</td>
</tr>
<tr>
<td>Lymphocytes (cells/µL)</td>
<td>1,615 ± 199.1$^a$</td>
<td>1,374 ± 141.7$^{a,b}$</td>
<td>1,180 ± 119.8$^{a,b}$</td>
<td>811.4 ± 115.3$^b$</td>
<td>.013</td>
</tr>
</tbody>
</table>

MCHC = Mean corpuscular hemoglobin concentration. MCV = Mean corpuscular volume.

See Table 1 for the remainder of the key.
in peripheral blood (Table 2). Erythropoiesis was also assessed by the erythroid maturation index and the I:Me, which did not differ between groups.

None of the control dogs tested positive for OGIB. Dogs with CKD had significantly higher OGIB scores at all stages of disease. There was a moderate positive correlation between OGIB scores and serum creatinine concentrations ($r = 0.70$). There was also a moderate negative correlation between OGIB scores and serum phosphorus levels ($r = 0.58$, $95\%$ CI = 0.38 to 0.78), myeloid precursor cells ($P = .0026$, $r = 0.52$, 95\% CI = 0.19 to 0.74), and TNFα ($P = .025$, $r = -0.40$, 95\% CI = -0.66 to 0.05).

**Discussion**

Based on our data and previous studies, we hypothesize that late erythroid precursor cells (polychromatophilic rubricytes and metarubricytes) could possibly be inhibited by humoral factors such as PTH and TNFα. The serum concentrations of IL-3 and IFNγ did not differ between controls and CKD groups. Likewise, there were no differences in the relative counts of rubriblasts and prorubricytes. Among cytokines, IFNγ has been previously identified as one of the most potent inhibitors of the proliferation of CFU-erythroid and burst-forming unit-erythroid in human beings.20,21 IL-3 has also been previously identified as a multilineage hematopoietic growth factor, acting mainly in the early stages of hematopoiesis in synergy with other cytokines.22–24 Hence, it appears that these 2 factors are not involved in the pathogenesis of canine CKD anemia and that the cause of erythroid hypoplasia is not directly related to the imbalance of immature erythroid precursor cells.
Conversely, a moderate negative correlation was found between erythrocyte counts and PTH, erythrocyte counts, and TNFa, as well as TNF and metarubricytes. Considering these correlations and the observation that metarubricytes initially declined in CKD3, we also hypothesize that progression of the disease not only contributes peripherally but can also interfere in the process of proliferation and maturation of erythroid precursors, perhaps by reducing the population of erythroid precursors by virtue of a hypoproliferative activity.

There was no difference in EPO concentrations between controls and CKD at all stages. This might be related to technical issues such as nonspecific ELISA or lack of statistical power. However, this might also represent a balance between opposing pathologies, i.e., increased stimulus to secrete EPO and decreased capacity to produce it. As has already been observed in people, dogs with CKD might also have decreased response to EPO (i.e., EPO resistance), caused by the chronic inflammatory process and the excess of circulating PTH. Relative EPO deficiency (and not resistance) might also play a role, considering that dogs that suffer from severe anemia associated with chronic inflammation, respond, at least temporarily, to supplementation of EPO. If the inflammatory process was the only cause of EPO resistance, a compensatory response would have been expected, resulting in increased EPO concentrations, as seen in other endocrine systems (for example, peripheral insulin resistance leads to a compensatory response in beta cells, which then secrete more insulin).

The suppressive effects of TNFa on EPO-producing cells have been described in vitro as well as the relative decline in EPO production capacity resulting from decreased functional renal mass. Furthermore, it should be noted that the response of erythroid cells to stimuli for proliferation and differentiation is highly dependent on an adequate supply of nutrients, especially iron and vitamins, and this supply is usually impaired in patients with anemia caused by chronic disease and in dogs with CKD.

EPO-based therapies have been indicated for patients in stages 3 and 4 of CKD, who already have a significant decrease in erythrometric measurements and are exhibiting clinical signs attributable to anemia, such as hyporexia, lethargy, and weakness. EPO abnormalities are identified in the literature as a main trigger of CKD anemia, our data suggest that the suppressive effects of PTH and TNFa are important as well, since their concentration changes even in the early stage of the disease. Moreover, contrary to the opinion that anti-EPO antibodies are the main concern in the treatment of CKD in both people and animals, we believe that treatment strategies using adjuvant anti-inflammatory cytokine therapies could be more important. Anti-TNFa treatments in particular might be useful in reducing resistance to EPO as well as other deleterious effects of proinflammatory cytokines during the treatment of CKD anemia in dogs.

Although RBC counts and hemoglobin concentrations were within the reference range, they were both decreased in CKD2. It is possible that hematopoiesis was already negatively affected by increased PTH and TNFa in this early stage, considering the trend for an increase in both together with a trend toward decreased erythrocyte precursors. However, this is not supported by the increase in the absolute reticulocyte count in peripheral blood. We believe that an important contributor to this “relative anemia” at CKD2 could be a chronic gastrointestinal blood loss, as evidenced by increased scores of fecal occult blood test.

Small sample size might be a study limitation and may explain some of the negative findings in our experiment, for example, no difference in EPO between groups and contradiction between reticulocyte count and bone marrow findings (increased reticulocytes with no corresponding change in the marrow). In this context, we suggest that future studies, comparing data from a larger population of dogs with CKD in different stages of the disease, be carried out to complement or even reaffirm the preliminary results observed here. Moreover, as well as for dogs, further investigation of the subject would be extremely significant for cats.

Anemia in dogs with CKD is associated with increased PTH and TNFa concentrations. It is also related to erythroid hypoplasia, represented mainly by a declining population of polychromatophilic rubricytes and metarubricytes, which constitute, in part, the basis of the anemic state in advanced stages of CKD in dogs (IRIS-CKD stage 3 and 4).

Acknowledgments

None reported.

Disclosures

Part of this study was presented in oral abstract form at the World Small Animal Veterinary Association/Federation of Asian Small Animal Veterinary Associations (WSAVA/FASAVA) 2013 Annual Conference in Auckland, New Zealand. All the procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice where the studies were conducted. The authors declare that there were no conflicts of interest. No AI-assisted technologies were used in the generation of this manuscript.

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