Diagnostic and Prognostic Significance of Rubricytosis in Dogs: A Retrospective Case-Control Study of 380 cases

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ABSTRACT

Peripheral nucleated red blood cells (pnRBC, rubricytosis) are observed in dogs in various disorders. In humans, pnRBC are associated with high morbidity and poor prognosis. This retrospective case-control study aimed to characterize the laboratory findings, diagnoses and prognoses of dogs with rubricytosis. Data from the medical records of 380 dogs with rubricytosis and 356 negative controls were compared using appropriate statistical methods. Dogs with pnRBC were older compared to controls (median age 7.3 years vs. 5. 7 years; $P<0.001$), had higher ($P<0.05$) counts of leukocytes, segmented and band neutrophils and lower hematocrit, hemoglobin concentration, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin concentration, and more serum chemistry abnormalities compared to controls. They had higher ($P<0.035$) occurrence of heatstroke, immune mediated hemolytic anemia (IMHA), lymphoma, hemangiosarcoma, anticoagulant poisoning, bite wounds and mast cell tumors compared to controls. Dogs with pnRBC had a higher mortality rate ($P=0.001$) compared to controls, and it significantly increased with the absolute pnRBC count. The mortality rate significantly ($P<0.0001$) increased with an increase in the absolute nRBC count quartile. However, the absolute pnRBC count was an inaccurate outcome predictor (area under the receiver operator characteristics curve, 0.62). Rubricytosis in dogs is associated with multiple hematological and serum chemistry abnormalities and higher mortality. Most rubricytosis-associated hematological abnormalities were due to regenerative anemia, likely because IMHA and blood loss were significantly more common in the rubricytosis group. In dogs, as in humans, presence of pnRBC was a negative prognostic indicator.

Key words: Anemia; Canine; Hematology; Metarubricyte; Normoblast; Nucleated Red Blood Cells.

INTRODUCTION

Erythropoiesis in healthy adult dogs occurs mostly in the bone marrow (1, 2). However, extramedullary hematopoiesis (EMH), especially splenic, is common in certain diseases. Erythropoiesis involves both proliferation and maturation. The proliferating erythroid precursors include the rubriblast, the earliest recognizable erythroid precursor using light microscopy, the prorubricyte and the basophilic and polychromatic rubricytes. The rubricyte differentiates further, yielding the metarubricyte, from which mitoses no longer occur. Further maturation and nuclear expulsion result in reticulocyte formation (1). Nuclei-containing erythroid precursors are often referred to as nucleated red blood cells (nRBCs) (2). In health, in both dogs and humans, reticulo-

cyte maturation occurs within the bone marrow, as well as in the peripheral blood. In the latter case, splenic macrophages play a role in the final removal of cytoplasmic organelles and nuclear remnants from maturing reticulocytes, resulting in mature erythrocytes (1). In healthy dogs, an erythropoietic stimulus will result in reticulocytosis within approximately five days (2).

Control of erythropoiesis is complex, involving various growth and transcription factors, including erythropoietin (Epo), granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin (IL)-11 and IL-6. Epo is the most important controlling factor of erythropoiesis, playing major roles in determining total erythrocyte mass, and promoting mitoses and differentiation of erythroid precursors, mostly within the bone marrow (2). In regenerative anemia, in the face of high RBC demands, the surface covered by bone marrow reticular barrier cells is decreased, and although the blood-bone marrow barrier is structurally intact, it allows release of nRBCs into the peripheral blood (2).

The presence of peripheral nRBCs (pnRBCs) in adult healthy dogs is more common compared to adult humans and farm animals (1). It is termed metarubricytosis or rubricytosis. It has been defined by the presence of at least 0.5 nRBC/100 leukocytes (WBC) (3). Rubricytosis is classified as appropriate (physiologic), or inappropriate (pathologic) (2). Appropriate rubricytosis is often characterized by presence of additional, concurrent peripheral evidence of erythroid regeneration, including reticulocytosis, polychromasia, macrocytosis, anisocytosis and hypochromia. It may be accompanied with increased mean corpuscular volume (MCV) and RBC distribution width (RDW), and decreased mean corpuscular hemoglobin concentration (MCHC) (2, 4). Appropriate rubricytosis occurs in response to increased Epo concentration, such as in hemorrhagic or hemolytic regenerative anemias, or in chronic hypoxemia (2). Inappropriate rubricytosis is characterized by a disproportionately high pnRBCs number, in relation to the extent of erythroid regeneration. Therefore, hematological evidence of regeneration is often absent, or is of a relatively low magnitude, potentially suggesting presence of bone marrow or splenic insults (1, 5). Inappropriate rubricytosis occurs due to impaired control of bone marrow (or other hematopoietic centers, such as the spleen, liver and lymph nodes) erythroid cell release (5). This results in release of immature, nucleated erythroid precursors into the peripheral blood, prior to completion of their maturation and elimination of their nuclei (1). It occurs in various bone marrow lesions, including inflammation, necrosis, hypoxia, thrombosis and infarction, hyperthermia, intoxication (e.g., lead), myelodysplasia, extramedullar neoplastic metastasis, primary neoplasia of non-hematopoietic bone marrow elements (e.g., hemangiosarcoma and fibrosarcoma) and lympho- and myelo-proliferative disorders (1, 2). Inappropriate rubricytosis may also occur upon EMH (mostly splenic), due to various causes, including trauma, neoplasia and inflammation (1, 6). Splenectomy has been shown to result in both rubricytosis and reticulocytosis in dogs (7). However, it might result in rubricytosis only, not necessarily correlating erythroid regeneration or bone marrow lesions. It is then due to loss of the splenic elimination of nuclei from peripheral blood nRBCs (2, 7-9).

In adult humans, presence of pnRBC (termed normoblastemia or erythroblastemia) is extremely uncommon, and is mostly abnormal (10). Studies in ill human patients (fetuses and neonates excluded) under various clinical settings, have shown that normoblastemic patients have a significantly higher mortality rate (range 42%-52%) compared to negative controls (4.2%) (10-14).

In dogs, rubricytosis was reported in various clinical conditions, including splenomegaly (due to benign or malignant tumors, splenic injury, torsion or infarction) (1, 7, 15, 16), bone marrow disorders (e.g., dyserthyropoiesis, myelodysplastic syndrome, dyscrasia and myelofibrosis) (17-21) , hematopoietic (e.g., polycythemia vera) (22) and non-hematopoietic neoplasia (19, 20, 23, 24), lead poisoning (25, 26), mycotoxicosis, immune-mediated hemolytic anemia (IMHA) and thrombocytopenia (IMT), systemic lupus erythematous, organophosphate toxicity, muscle disease (2), heatstroke (27, 28), and babesiosis (29, 30).

The morphology of nRBC, and their presence in the peripheral blood, in certain specific conditions, is relatively well documented (2). However, although the diagnostic and prognostic usefulness of rubricytosis in specific conditions, such as heatstroke, acute trauma and systemic inflammatory response syndrome (SIRS), and in critically ill dogs in general have been described (28, 31-33), the overall clinical, diagnostic and prognostic significance of rubricytosis in ill dogs, in general, have never been assessed in a large-scale study.

The objective of this study was therefore, to characterize the laboratory findings, diagnoses, morbidity and mortality of

a relatively large population of ill dogs presenting rubricytosis, compared to ill, negative controls, in a university teaching hospital setting.

MATERIALS AND METHODS

Selection of dogs and data collection

The clinical records of ill dogs presented to the Hebrew University Veterinary Teaching Hospital (HUVTH) between 1999 and 2000 were reviewed retrospectively. This particular period was selected as during this period all the blood smears of dogs presented to the HUVTH were examined by a single clinician (IA). Dogs presenting pnRBCs (>0.5/100 leukocytes) upon examination of blood smears stained with modified-Wright's staining solution were consecutively included in the study (nRBC) group. These dogs were time-matched with ill, negative controls presenting no evidence of rubricytosis upon blood smear examination. Each control was selected if presented within two weeks before or after a corresponding study dog, and if the CBC and the blood smear evaluation of the time of its presentation to the hospital were available. In both groups, when several consecutive CBCs were performed in a dog during hospitalization, only the first CBC, done at presentation to the hospital, was selected for this study. Examination of all blood smears was performed, as part of a routine CBC, by a single clinician (IA) before these dogs were selected for the present study.

Data collected included the signalment, history, clinical signs, laboratory tests results, specific diagnoses, the hospitalization time-period and the survival at 30 days post discharge from the hospital. The non-survivors included both dogs that died and those that were euthanized.

The diagnoses were divided into non-hemic and hemic disorders (including hemorrhage, anticoagulant intoxication, disseminated histiocytic sarcoma, lymphoma, mast cell tumor [MCT], hemangiosarcoma, IMHA, immune mediated thrombocytopenia (IMT), monocytic ehrlichiosis, babesiosis, *Hepatozoon canis* infection and disseminated intravascular coagulation [DIC]). The number of diagnoses exceeded the number of dogs, because in some dogs, several diseases coexisted.

Laboratory Tests

Blood samples for CBC were collected in potassium-ethylenediaminetetraacetic acid tubes at presentation, and analyzed within 30 minutes using automated impendence cell analyzers (Abacus or Arcus, Diatron, Wien, Austria). Differential leukocyte (WBC) counts were done manually, by counting 100 leukocytes in stained blood smears, while pnRBCs were counted as nRBC/100 WBC, and when present, the WBC count (WBCC) was corrected accordingly, and the absolute pnRBC count (as cells/μL) was then calculated (32) and used for all analyses involving pnRBC.

Blood for serum chemistry was collected in plain tubes with gel separators, allowed to clot for 30 minutes, centrifuged, and serum was either analyzed immediately, or stored at 4˚C pending analysis, which was performed within 24 hours of collection, using a wet chemistry analyzer (Cobas-Mira, Roche, Mannheim, Germany, at 37˚C).

Statistical Analysis

The distribution pattern of continuous parameters was examined using the Shapiro-Wilk's test. Normally and non-normally distributed continuous parameters were compared between groups using the Student's *t*- or Mann-Whitney *U*-tests based on their distribution. The Fisher's exact test was used to compare categorical variables and proportions of diagnoses between groups. Logistic regression analysis was performed to assess the relationship of pnRBC with the outcome. For the statistical analysis, the nRBC group was divided into four subgroups, based on the nRBC quartiles. Each quartile was then treated as a categorical variable, and was compared, using logistic regression analysis, to the negative control group, used as a reference category. Additionally, based on the absolute pnRBC count, the whole study population was divided into the following four groups: group 0, negative controls; group 1, $0<\text{pnRBC}\leq 0.16\text{x}10^9$/L; group 2, $0.16<\text{pnRBC}\leq 0.33\text{x}10^9$/L; group 3, $0.33<\text{pnRBC}\leq 0.96\text{x}10^9$/L and group 4, $\text{pnRBC}>0.96\text{x}10^9$/L.

The association of the absolute pnRBC count with the outcome was also assessed by the receiver operator characteristics (ROC) procedure, with its area under the curve (AUC), and cut-off points, with their corresponding sensitivity and specificity for prediction of the outcome were selected. The optimal cut-off point was defined as the one that was associated with the least number of misclassifications. All tests were two-tailed, and in all, a $P\leq 0.05$ was considered statistically significant. All calculations were performed using a statistical software package (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL).

Table 1: Complete blood count results and assessment of polychromasia of 380 dogs with rubricytosis and 356 time-matched negative controls

Parameter	nRBC[a]					Controls					Reference interval	*P* value
	n [b]	Median	IQR[c]	%<RI[d]	%>RI[d]	n [b]	Median	IQR[c]	%<RI[d]	%>RI[d]		
Corrected leukocytes[3] ($x10^9$/L) †	380	15.1	13.32	4.47	40.5*	355	12.4	11.5	5.6	32.9*	5.0-16.0	0.01
Red blood cells ($x10^{12}$/L) †	380	5.1	2.46	58.9*	4.2	355	5.94	1.77	36.9*	6.2	5.50-8.00	<0.001
Hemoglobin (g/L) †	380	119	6.8	50.3*	10.0	355	138	4.6	30.4*	13.5	120-175	<0.001
Hematocrit (L/L) †	380	0.344	16.9	51.8*	9.5	355	0.396	11.6	28.5*	9.9	0.35-0.50	<0.001
Mean corpuscular volume (fL) †	377	68.0	5.0	2.7	5.6*	355	67.0	4.0	4.5	1.1*	60-77	<0.001
Mean corpuscular hemoglobin (ρg)	380	23.3	2.6	5.6	26.6	355	23.3	2.1	4.8	20.6	19.5-24.5	0.138
MCHC[4] (g/L) †	380	341	3.34	22.9*	20.5	355	346	2.6	10.7*	23.9	320-360	0.012
Segmented neutrophils ($x10^9$/L) †	379	11.11	11.95	5.0	49.0	344	9.41	10.75	6.1	41.3	3.0-11.5	0.025
Band neutrophils ($x10^9$/L) †	380	0.0	0.28	NA	71.0	344	0.00	0.18	NA	76.6	0.00-0.30	0.007
Monocytes ($x10^9$/L) †	380	0.83	1.36	4.35	33.4	344	0.81	1.29	6.5	30.8	0.10-1.35	0.560
Lymphocytes ($x10^9$/L)	380	1.29	1.48	42.8	4.9	344	1.22	1.34	46.5	3.9	1.00-4.00	0.219
Eosinophils ($x10^9$/L)	379	0.14	0.47	NA	14.9	344	0.12	0.44	NA	16.1	0.00-1.00	0.50
Basophils ($x10^9$/L)	380	0.00	0.00	NA		344	0.00	0.00	NA		rare	0.001
Polychromasia ^	n [b] (%)					n [b] (%)						
Absent	62 (16.4%)					205 (59.2%)						
Mild	133 (35.3%)					134 (38.7%)						
Moderate	69 (18.3%)					6 (1.7%)						<0.001
Marked	113 (30.0%)					1 (0.3%)						
Any**	315 (83.6%)					141 (40.8%)						
Total	377 (100%)					346 (100%)						

[a], Nucleated red blood cells; [b], Number of dogs in which the test was available; [c], Interquartile range; [d], Reference interval; NA, not applicable; MCHC, mean corpuscular hemoglobin concentration; *, Significant (P<0.05) difference between groups in the proportion of abnormality; †, Significant (P<0.05) difference between groups; ^, absent, polychromatophils 0-1%; mild, polychromatophils 1-4%; moderate, polychromatophils 5-20%; marked, polychromatophils >20%; **, polychromatophils >1%

RESULTS

For 24 control dogs, the blood smears were unavailable, and these were therefore excluded. The study and control groups included 380 and 356 dogs, respectively, with no sex and neutering-status group differences. The age of the rubricytosis group was significantly (P<0.001) higher compared to the controls (median, 7.25 years; range 0.08-17.00 vs. median, 5.67 years; range 0.17-16.00, respectively).

Dogs with rubricytosis had a significantly (P<0.05) lower RBC count, hemoglobin concentration and hematocrit, and higher corrected WBCC, segmented, and band neutrophil counts, MCV and frequency of macrocytosis compared to the controls (Table 1). When the nRBC group was divided into quartiles, an increase in the nRBC quartile was significantly (P<0.0001) and inversely associated with the hematocrit, and positively associated with the proportion of polychromasia.

Dogs with rubricytosis had significantly higher (P<0.05) serum activities of alanine transferase (ALT), amylase, aspartate transferase (AST), creatine kinase (CK), γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH), as well as higher concentrations of globulins, sodium, bilirubin, triglycerides, creatinine and urea, and a higher frequency of hyperbilirubinemia, compared to controls (Table 2).

Dogs with rubricytosis had significantly (P<0.02) higher occurrence of anticoagulant intoxication, bite wounds, hemorrhage, DIC, canine monocytic ehrlichiosis (CME), IMHA, Evans' syndrome, heatstroke, hemangiosarcoma, intoxication (in general), lymphoma, MCT and other sarcomas (combined) compared to the negative controls (Table 3). Hemic diseases were significantly (P<0.001) more frequent in the rubricytosis group compared to the controls. Dogs with hemic diseases had a

Table 2: Serum chemistry results of dogs presenting with rubricytosis and time-matched negative controls

Parameter	nRBC[a]					Controls					Reference interval	*P* value
	n[b]	Median	IQR[c]	%<RI[d]	%>RI[d]	n[b]	Median	IQR[c]	%<RI[d]	%>RI[d]		
Alanine transferase (U/L) †	155	55	96	3.2	41.9*	173	40.9	47.5	3.5	24.8*	10-70	0.001
Albumin (g/L)	89	30	0.70	17.9	1.1	98	31	0.862	21.4	4.1	26-40	0.58
Alkaline phosphatase (U/L) †	92	254	524.25	0.0	56.5	95	150	360	0.0	44.2	13-190	0.12
Amylase (U/L) †	87	625	490	21.8	11.5*	96	456	352	29.2	2.1*	340-1110	0.02
Aspartate transferase (U/L) †	88	54.5	61	0.0	31.8*	96	32	25	0.0	15.6*	12-80	<0.001
Calcium (mmol/L)	82	2.47	1.6	13.4	3.7	96	2.5	1.6	14.6	1.0	2.175-2.95	0.513
Chloride (mmol/L)	81	108.8	6.6	14.8	4.94	91	110.6	5.9	12.1	6.6	102.0-117.0	0.073
Cholesterol (mmol/L)	88	6.26	136.5	6.8*	43.1	97	6.02	71.5	1.0*	29.9*	3.1-6.72	0.123
Creatine kinase (U/L) †	86	257	451	0.0	69.8*	94	158	224	0	48.9	20-160	0.004
Creatinine (µmol/L) †	164	74.26	0.55	8.4	14.5	179	64.53	0.51	13.4	17.3	44.2-141.44	0.085
γ-glutamyl-transferase (U/L) †	81	4.5	4.3	3.7	11.1	92	3.45	3.35	8.7	10.9	1.0-13.0	0.023
Globulin (g/L) †	83	39	0.0	0.0*	60.2*	96	33	2.42	5.2*	38.5*	19-35	<0.001
Glucose (mmol/L)	93	5.48	40.1	14.1	33.7	83	5.53	33	13.25	3	3.88-6.10	0.75
Lactate dehydrogenase (U/L) †	87	573	677	1.2	77.0	97	461	492.5	6.2	62.9	34-360	0.004
Phosphorus (mmol/L) †	79	1.35	2.51	15.2	16.5	96	1.15	2.25	20.8	9.4	0.81-2.00	0.125
Potassium (mmol/L)	151	4.1	1.2	30.7	5.3	173	4.2	1.0	26.3	3.5	3.8-5.6	0.54
Sodium (mmol/L) †	143	148.0	6.95	11.0	13.8*	160	146.7	6.0	16.5	5.7*	140-154	0.003
Total bilirubin (µmol/L) †	87	7.69	0.76	0.0	35.6*	97	6.15	0.26	1.0	15.3*	1.71-10.26	0.013
Total protein (g/L)	83	68	1.4	12.1	24.1	96	65.5	1.57	15.6	16.7	55-75	0.16
Triglycerides (mmol/L) †	81	1.00	76	4.9*	38.3	90	0.84	64.75	15.6*	31.1	0.45-1.13	0.043
Urea (mmol/L) †	168	15.24	44.35	6.1*	53.8*	187	13.17	26.7	13.9*	43.9*	7.49-14.28	0.008

[a] Nucleated red blood cells; [b] Number of dogs in which the test was available; [c] Interquartile range; [d] Reference interval;
* Significant (P<0.05) difference between groups in proportions of abnormality; † Significant (P<0.05) difference between groups.

significantly (P<0.001) higher pnRBC count compared to those with non-hemic disorders (median, 0.31x10^9/L, range, 0.00-17.30x10^9/L vs. median, 0.00x10^9/L, range, 0.00-35.50x10^9/L, respectively).

The mortality rate was significantly (P=0.001) higher in the nRBC group, compared to the controls (24.4% vs.14.6%, respectively; odds ratio [OR], 1.88; 95% confidence interval [CI$_{95\%}$], 1.28-2.78). The mortality rate significantly P<0.0001) increased with an increase in the absolute nRBC count quartile (Fig. 1). Conversely, ROC analysis showed that the absolute nRBC count, as a sole variable, was an inaccurate outcome predictor in the general study population (AUC, 0.62, CI$_{95\%}$, 0.57-0.68). It also did not discriminate between the outcome groups within immune-mediated or neoplastic disorders, or within specific diseases, including hemangiosarcoma, lymphoma, IMHA and CME.

DISCUSSION

Several studies of human patients have demonstrated that the presence of pnRBC is clinically significant (10-14). However, there is a dearth of studies investigating the associations of rubricytosis with hematological and other laboratory variables, specific diagnoses and its prognostic significance in dogs in general. The present relatively large-scale, case-control study attempted to perform a comprehensive analysis of such associations in dogs.

In this study, dogs presenting rubricytosis were older compared to controls, similarly to findings in normoblastemic human patients, where normoblastemia occurs in 10% of patients older than 60 years (11, 35). This association, of rubricytosis with older age in dogs likely results from higher proportions in the rubricytosis group, of certain diseases that occur more commonly in aged dogs, when compared

Table 3: Proportions of selected* diagnoses in 380 dogs with rubricytosis and 356 time-matched negative controls**

Diagnosis	nRBC [a]	Control	*P*
	n [b] (%)	n [b] (%)	value
Monocytic ehrlichiosis	39 (10.3)	15 (4.2)	0.001
Hemangiosarcoma	25 (6.6)	1 (0.3)	<0.001
Immune mediated hemolytic anemia	24 (6.7)	2 (0.6)	<0.001
Lymphoma	17 (4.8)	3 (0.8)	0.002
Hit by car	15 (3.9)	10 (2.8)	0.316
Bite Wounds	14 (3.7)	4 (0.8)	0.018
Evans' syndrome	14 (3.7)	0 (0.0)	<0.001
Heatstroke	10 (2.6)	0 (0.0)	0.001
Acute kidney injury	9 (2.4)	15 (4.2)	0.208
Bacterial cystitis	9 (2.4)	10 (2.8)	0.806
Disseminated intravascular coagulation	9 (2.4)	1 (0.3)	0.011
Pneumonia	9 (2.4)	8 (2.2)	0.820
Post-surgery	8 (2.1)	14 (3.9)	0.189
Hemorrhage	8 (2.1)	0 (0.0)	0.005
Congestive heart failure	8 (2.1)	7 (2.0)	0.803
Hyperadrenocorticism	8 (2.1)	5 (1.4)	0.407
Hepatopathy	8 (2.1)	4 (1.1)	0.248
Pyometra	8 (2.1)	20 (5.6)	0.020
Bone fracture	7 (2.0)	7 (1.8)	0.992
Mast cell tumor	7 (2.0)	0 (0.0)	0.008
Sepsis	7 (2.0)	6 (1.7)	1.0
Anticoagulant intoxication	6 (1.7)	0 (0.0)	0.014
Lung contusion	6 (1.7)	4 (1.1)	0.530
Other sarcomas (combined) [c]	6 (1.7)	0 (0.0)	0.014
Septic peritonitis	6 (1.7)	13 (3.7)	0.101
Daboia (Vipera) palaestinae snakebite	6 (1.7)	4 (1.1)	0.530
Diabetic ketoacidosis	5 (1.3)	2 (0.6)	0.258
Nonspecific bacterial enteritis	5 (1.3)	16 (4.5)	0.014
Other carcinomas (combined) [d]	4 (1.1)	0 (0.0)	0.124
Canine distemper virus infection	4 (1.1)	8 (2.3)	0.240
Acute gastritis	4 (1.1)	22 (6.2)	<0.001
Gastric dilatation and volvulus	4 (1.1)	5 (1.4)	0.731
Intervertebral disk disease	4 (1.1)	10 (2.8)	0.102
Mammary gland tumor	4 (1.1)	13 (3.7)	0.026
Acute metritis	4 (1.1)	1 (0.3)	0.176
Myositis	4 (1.1)	0 (0.0)	0.045
Canine parvovirus infection	4 (1.1)	22 (6.2)	<0.001
Other trauma [e]	4 (1.1)	4 (1.1)	0.994
Uveitis	4 (1.1)	0 (0.0)	0.045

* Diseases were selected if recorded in ≥ 4 dogs; ** The total number of diseases exceeds the total number of dogs within each study group because some dogs had more than a single disease;
[a] Nucleated red blood cells; [b] Number of dogs in the group; [c] Including osteosarcoma (3 cases), esophageal sarcoma, splenic liposarcoma and intestinal sarcoma (1 each); [d] Including hepatic, squamous cell and adrenal carcinoma, thyroid adenocarcinoma; [e] Other trauma, excluding dogs hit by car.

to the controls, including neoplasia (i.e., hemangiosarcoma, MCT, lymphoma and other sarcomas) (36-39) and IMHA (including Evans' syndrome), which occurs more commonly in middle-aged to older dogs (40, 41). These diagnoses comprised 21% of the nRBC group, compared to only 1.3% of the controls.

Anemia and erythroid regeneration were more commonly observed in the rubricytosis group. This probably accounts for the significantly lower hemoglobin concentration, RBC count and hematocrit, and higher MCV in this group, compared to the controls. Therefore, as expected, rubricytosis in dogs is associated with regenerative anemia, whether, hemorrhagic or hemolytic, and is likely, mostly physiologic and appropriate. Moreover, based on the significant association of both the hematocrit (negative association) and the occurrence of polychromasia (positive association) with the absolute nRBC count, it can be concluded that in most cases of appropriate rubricytosis, the more severe and regenerative the anemia, the more pronounced the rubricytosis. To the best of our knowledge, this is the first study to demonstrate this universally accepted interpretation of appropriate rubricytosis in dogs.

Presently, dogs with rubricytosis had higher WBCC and higher proportions of neutrophilia and left shift compared to the controls. Several possible mechanisms might have played a role in promoting leukocytosis and neutrophilia in the rubricytosis group. First, in various conditions characterized by rubricytosis, a concurrent inflammation has been proven to occur. IMHA was significantly more frequent in the rubricytosis group. Increased serum concentrations of IL-2, IL-4, and tumor necrosis factor-α and an imbalance of IL-10 and IL-12 concentrations have been identified in human IMHA patients (42). In another study, concentrations of C-reactive protein, IL-2, IL-10, keratinocyte chemoattractant protein, and monocyte chemoattractant protein-1 were significantly increased in dogs with primary IMHA compared to healthy controls (43). Secondly, human studies have demonstrated an association between presence of pnRBCs and increased Epo, IL-6 and IL-3 concentrations (35, 44, 45). Both IL-6, IL-11 and GM-CSF are involved in regulation of erythropoiesis, but are also increased in certain inflammatory conditions (35). In septic dogs, IL-6 levels were significantly and positively with disease severity and mortality (46). Therefore, inflammatory processes or hypoxic injuries (e.g., IMHA), which are associated with

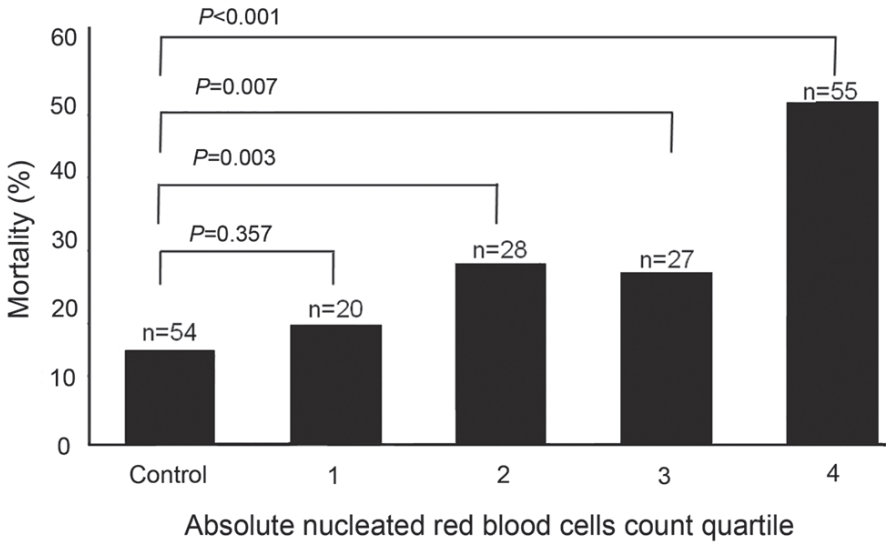

Figure 1: mortality rate of dogs with rubricytosis divided by their absolute peripheral nucleated red blood cell (pnRBC) count quartiles as follows: quartile 1, 0<nRBC≤0.16 x10^9/L; quartile 2, 0.16<nRBC ≤0.33x10^9/L; quartile 3, 0.33<nRBC≤0.96x10^9/L, and quartile 4, nRBC>0.96x10^9/L. The mortality rate of each quartile was compared to the negative control group, used as a reference category. Note the significantly increasing mortality rate with an increase in pnRBC count quartile, compared to the negative controls, reaching approximately 50% mortality rate of the dogs with rubricytosis in the fourth quartile.

leukocytosis, might play a role in promotion of rubricytosis (35, 47). Certain diseases, more frequent in the nRBC group compared to the controls, are indeed characterized by inflammation, including IMHA and neoplasia, and are often manifested by leukocytosis and neutrophilia (48-50). Rubricytosis was also described in dogs with SIRS, where it was associated with death, similarly to the present study (30). However, inflammation cannot be the only underlying mechanism responsible for rubricytosis, because certain neoplastic diseases (e.g., mammary gland neoplasia), as well as bacterial diseases in general, which often lead to inflammation, were actually significantly more frequent among the controls. Secondly, severe regenerative anemia in dogs and cats is often associated with a leukoerythroblastic response, characterized by concurrent release of bone marrow nRBC and immature neutrophils into the peripheral blood (6, 8). Thirdly, bone marrow lesions, and injuries to the blood-bone marrow barrier, induced by certain diseases, might have led to concurrent release of nRBC and mature as well as immature neutrophils (33). For example, heatstroke, significantly more frequent in the nRBC group, was previously associated both with pathologic rubricytosis and bone marrow lesions (27, 28, 51, 52). Hemic diseases, including IMHA, IMT, and neoplasia involving blood vessels and the hemolymphatic system, such as hemangiosarcoma, lymphoma and MCT, which were also more frequent in the rubricytosis group, might have affected the bone marrow or spleen, leading to the concurrent release of nRBCs, as well as segmented and band neutrophils. In contrast, mammary gland neoplasia, more frequently recorded among the controls, usually spares the spleen and bone marrow, and was therefore likely not associated with rubricytosis.

The association of rubricytosis with CME is unclear. In most dogs with CME, acute or pancytopenic (i.e., 'myelosuppressive'), a normocytic normochromic non-regenerative anemia is present (53). This anemia, during the acute disease stage, is likely secondary to inflammation (53), and possibly secondary to bleeding due to thrombocytopenia. In the pacytopenic stage, it is consequent to bone marrow destruction and hypocellularity (53). During the acute stage of CME, when the bone marrow is hypercellular, (53) and potentially responsive, severe bleeding (e.g., profound epistaxis) possibly results in regenerative anemia, and hence, consequently in physiologic rubricytosis. In the chronic pancytopenic

stage, rubricytosis might be secondary to bone marrow lesions, splenic EMH, or both. It has been shown in mice infected with *Ehrlichia muris*, a pathogen closely related to *E. chaffeensis* (the agent of human monocytic ehrlichiosis) and *E. canis*, resulted in anemia, thrombocytopenia, and marked reduction in bone marrow cellularity, while striking changes were observed in spleen cellularity and architecture, and infected mice exhibited extensive splenic EMH. The authors hypothesized that inflammation associated with *Ehrlichia* infection suppresses bone marrow function, induces the emigration of B cells, and establishes hematopoietic activity in the spleen (54). Such splenic EMH might lead to pathological rubricytosis.

In human normoblastemic patients monitored during hospitalization, no significant changes in serum biochemistry analytes related to liver damage and renal function were observed within the following three days after normoblastemia has been diagnosed, suggesting that normoblastemia is unassociated with failure of these organs or occurrence of organ lesions (47). In contrast, presently, concurrent multiple serum chemistry abnormalities were significantly more frequent in the rubricytosis group compared to the controls, likely reflecting the underlying rubricytosis-associated diseases. Such diseases, especially hemolysis, likely account for the higher bilirubin concentration and higher frequency of hyperbilirubinemia in the rubricytosis group compared to the controls (41, 55).

A variety of adhesion molecules play a part in the interactions between erythroid cells, central macrophages and the extracellular matrix within the bone marrow, and during differentiation they undergo dynamic changes (54). These include matrix laminins and fibronectin, erythroblast-macrophage protein, VCAM-1 and $\alpha_4\beta_1$ integrin (56-58). Pathologic rubricytosis might occur due disruption and damage to one or several of these adhesive molecules, some of which have been shown to be crucial for erythroid cell enucleation, with subsequent release of pnRBCs (56). Damage to these might occur during hypoxia, hyperthermia, intoxication or neoplasia. Although the exact pathophysiology of normoblastemia in adult humans is mostly unknown, studies have suggested that both inflammation and decreased tissue oxygenation play important roles in its induction (45). This is supported by the higher occurrence of acute and chronic anemia, leukemia, neoplasia, particularly myeloproliferative disorders, and severe inflammation and infection in normoblastemic humans, compared to negative controls (11). Dogs have been previously reported to present rubricytosis, often pathologic, in cases of splenomegaly of several causes, and with neoplasia in general, immune diseases, trauma, intoxication, SIRS, acute trauma, heatstroke and critical conditions in general (2, 9, 15, 23, 25, 26, 28, 50, 51), which were all more frequent in the rubricytosis group herein.

Nevertheless, presently, most cases of rubricytosis are probably physiologic, because of the significant association of rubricytosis with laboratory findings suggestive of erythroid regeneration (i.e., polychromasia, anisocytosis and macrocytosis). Studies have shown that erythropoiesis occurs in erythroid islands within the bone marrow, composed of erythroid progenitor cells, surrounding a central macrophage. Extruded nRBC nuclei are phagocytized by the latter, in a process of nuclear engulfment which is mediated by phosphatidylserine engulfment, upon conclusion of terminal erythroid differentiation (56, 59). Erythroid progenitors have been hypothesized to move within the bone marrow towards its sinusoids, by attaching and reattaching to central macrophages. With increased erythropoiesis and an increase in nRBC numbers exceeding the reattachments sites on macrophages, nRBC can escape the bone marrow and appear in the peripheral circulation (56). This process, along with the decreased surface covered by bone marrow reticular barrier cells (2), might account for the association of regenerative anemia with presence of pnRBCs in the present study group in cases of physiologic rubricytosis.

This study demonstrates that dogs with rubricytosis have poorer prognosis compared to negative, ill controls, in agreement with studies in human patients, hospitalized in both medical and surgical intensive care wards (11, 12, 14, 47, 60), as well as in critically ill dogs, and those with acute trauma and SIRS (31-33). The occurrence of normoblastemia in human intensive care patients is 17.5 to 19.2%, and their mortality rate is five to seven-fold higher compared to negative controls. Furthermore, the mortality rate increases with an increase in the number of pnRBCs, and an absolute nRBC count >500/μL was associated with a lethal outcome (10, 12). The present findings show a similar trend, as the mortality rate was not only associated with the presence of nRBC, but also increased significantly with an increase in the nRBC count quartile. It was concluded in human patients, that normoblastemia should alert clinicians to institute appropriate,

more intensive care in such patients, to increase their survival chances (10-13). In the present study, parallel findings in dogs, similarly suggest that intensive monitoring and therapy are warranted in dogs with rubricytosis, especially when the pnRBC count is high.

Several possible explanations account for the higher mortality in the nRBC group. Firstly, neoplastic diseases (e.g., lymphoma, MCT and hemangiosarcoma), IMHA (and Evans' syndrome), DIC and heatstroke, all characterized by high mortality (28, 38-40, 41, 51), were significantly more frequent in this group. Conversely, bacterial and viral infections, which mostly have better prognoses, were significantly more frequent in the control group. Secondly, dogs with rubricytosis were significantly more anemic compared to the controls, and this likely contributed to their higher mortality.

This study has three major limitations. First, over 100 comparisons were performed, and it is thus possible that some of the associations recorded were statistically significant due to pure chance (type-I error). However, as this study is a 'generate-hypothesis' study, we elected to perform multiple comparisons in order to retain its high sensitivity, at a cost of identifying some spurious associations. Thus, interpretation of the results must be made with caution and with understanding the pathophysiology of the currently included diseases. Clearly, further, preferably prospective, research is warranted, to strengthen the presently described associations, to shed light on the mechanisms that induce rubricytosis, and to assess the sensitivity and specificity of such changes for the diagnosis and prognosis of various diseases and conditions. Furthermore, this constituted a retrospective study, and some data were missing, thereby limiting the power of statistical analyses (type-II error). Nevertheless, we have attempted to overcome this limitation by including a rather large study cohort. Lastly, this study is limited by the nature of the diagnoses made herein, which obviously did not include other commonly observed canine diseases, and was influenced by the clinical setting in which it was performed, namely a referral academic hospital. Thus, the results should be applied with caution to other clinical settings.

In conclusion, rubricytosis in dogs is associated with multiple hematological and serum chemistry abnormalities, and with a higher mortality rate, compared to ill, negative controls. The currently used hematology analyzers in veterinary medicine do not provide accurate information regarding to the absolute and relative nRBC counts in dogs. Thus, routinely assessing the peripheral blood smears for their presence, as part of the CBC, a simple and cost- and time-efficient procedure, is highly advised. Presence of rubricytosis can serve as a potentially useful diagnostic aid in the assessment of the dog's status, and as an early negative prognostic indicator, especially if it is marked.

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