Peripheral Nucleated Red Blood Cells in Cats and their Association with Age, Laboratory Findings, Diseases, Morbidity and Mortality – A Retrospective Case-Control Study

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ABSTRACT

Metarubricytosis occurs in cats in various disorders. This retrospective case-control study characterized the clinical and laboratory findings, diagnoses and prognoses of 117 cats presenting metarubricytosis, compared to 201 negative, time-matched controls. Cats with metarubricytosis were younger ($P = 0.043$) compared to the controls (median 3.5 years; range 0.1-19.0 vs. median 6.0 years; range 0.1-21.0, respectively). They had higher ($P < 0.03$) frequency of weakness, depression, dyspnea, hypothermia, shock, epistaxis and anemia compared to the controls, and presented higher ($P < 0.05$) median leukocyte count and mean corpuscular volume, lower ($P < 0.001$) hematocrit, hemoglobin concentration, RBC count and mean corpuscular hemoglobin concentration compared to the controls. Cats with metarubricytosis had higher ($P < 0.01$) serum muscle enzyme activities and hyperbilirubinemia, and lower ($P = 0.01$) total protein concentration compared to the controls. They had higher ($P = 0.01$) frequencies of traumatic disorders (i.e., hit by car, high rise syndrome, fractures, pneumothorax and lung contusions), pyothorax and hemoplasmosis-associated hemolytic anemia compared to the controls. Cats with metarubricytosis showed a higher ($P = 0.05$) mortality rate, longer hospitalization and higher treatment cost compared to the controls. Nevertheless, the absolute peripheral nucleated red blood cell count was an inaccurate outcome predictor. Metarubricytosis in cats is associated with anemia, multiple hematological and serum biochemistry abnormalities and higher morbidity and mortality. Most metarubricytosis-associated hematological abnormalities were attributed to regenerative anemia (i.e., physiologic metarubricytosis), hemolysis, and trauma. The latter likely led to metarubricytosis due to shock, inflammation and hypoxia. In cats, according to our assessment, metarubricytosis should be considered a negative prognostic indicator, warranting intensive treatment and monitoring.

Keywords: Feline; Metarubricytosis; Rubricytosis; Anemia; Hematology; Prognosis.

INTRODUCTION

Erythropoiesis in adult cats occurs mainly in the bone marrow (1). The rubriblast is the first erythroid progenitor recognizable by light microscopy. Further mitoses and differentiation lead consecutively to formation of the prorubrictye, basophilic rubricyte, polychromatophilic rubricyte, and metarubricyte, at which mitoses cease, and beyond, cells only mature. With expulsion of the nucleus and further cytoplasmic maturation, reticulocytes are formed, and eventually, with removal of cytoplasmic organelles and nuclear remnants by bone marrow and splenic macrophages, cells mature to erythrocytes (2). In cats, two reticulocyte types are recognized in new-methylene blue-stained blood smears. Aggregate reticulocytes, consisting up to 0.4% of the erythrocytes in healthy cats, are associated with erythroid regeneration (3, 4), and are correlated with polychromato-

phils observed using Romanowsky stains (1). These mature to punctate reticulocytes within the peripheral blood or the spleen within 12 hours (5). Punctuate reticulocytes, do not present polychromasia in smears stained with Romanowsky stains, and mature to normocytes over 10 days. Their presence indicates an erythroid regeneration that has occurred 2 to 4 weeks earlier (1, 4). Since aggregate reticulocytes are larger, and their hemoglobin concentration is lower compared to erythrocytes, reticulocytosis is often associated with anisocytosis, macrocytosis, polychromasia and hypochromasia (4, 5).

Erythropoiesis is regulated by multiple growth and transcription factors including erythropoietin (EPO), interleukin (IL)-3, stem cell factor, granulocyte colony-stimulating and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF, respectively) (1, 2, 4). EPO is the most important factor controlling erythropoiesis, playing a major role in determination of total erythrocyte mass, promoting mitoses and differentiation of erythroid precursors, mostly within the bone marrow (6).

The blood-bone marrow-barrier (BBMB) is composed of specialized reticular cells (barrier cells), their protrusions, and collagen fibers creating a crowded mesh, to which the hematopoietic progenitors bind through specific receptors, thereby preventing nucleated red blood cells (nRBCs) release into the peripheral circulation (7). As erythroid progenitors mature, they gradually lose their adhesion properties to the BBMB, due to changes in membranous receptor structure and number, enabling their release into the peripheral circulation (7, 8). In regenerative anemia, with increased red blood cell (RBC) demand, a decrease in the surface of the reticular cells cover occurs, allowing release of nRBC, even if the BBMB is intact and changes in cellular receptors do not occur (7). This resultant metarubricytosis, associated with erythroid regeneration, is physiologic (appropriate), characterized by a reticulocyte:nRBCs (R/nRBC) ratio ≥ 1 (4, 9). Conversely, pathologic metarubricytosis, characterized by a R/nRBC ratio < 1, occurs in absence of erythroid regeneration, with functional and structural BBMB changes, promoting premature nRBCs release, or due to extramedullary, mostly splenic, erythropoiesis (EMH), because the spleen has no barrier similar to the BBMB (4, 6, 9-11). Pathologic (inappropriate) metarubricytosis might occur due to inflammation, infection (e.g., feline leukemia virus [FeLV] subtype C), necrosis, hypoxia, thrombosis, infarction, hyperthermia, poisoning, myelodysplasia, lympho- and myelo-proliferative disorders and other neoplastic diseases (4, 11, 12). Additionally, since splenic macrophages play a role in eliminating peripheral nRBCs (pnRBC) nuclei, hyposplenism, due to trauma, neoplasia or inflammation, or splenectomy, might result in pathologic metarubricytosis, even in absence of erythroid regeneration (4, 9, 11).

In cats, pnRBCs counts of 1/100 leukocytes are considered normal. The pnRBC count in cats has to be performed manually, because feline pnRBCs are counted by most hematology analyzers as leukocytes, mostly as lymphocytes (11, 12). Presence of pnRBCs in adult humans (termed normoblasthemia or eryrhroblastosis) is uncommon, and is mostly pathologic (13). Several studies in adult human patients, under different clinical settings, showed that normoblasthemia carries poor prognosis, with high mortality rates (15-18), while in human infants, the pnRBC count is an accurate predictor of the short-term outcome (19).

There are very few studies investigating pnRBCs in cats in general. In a single retrospective, uncontrolled study, metarubricytosis, defined as absolute pnRBC counts (anRBC) > 100/μL, was recorded in 20/313 (6.3%) ill cats. Metarubricytosis was associated with hemoplasmosis (4/20), hepatic lipidosis (4/20), acute trauma (3/20), upper respiratory infection (2/20) and myelo-proliferative disorders (2/20) (11). In 9/20 cats (45%), metarubricytosis was not associated with presence of other immature blood cells in the peripheral blood, while in the remaining 11 (55%), immature myeloid cell numbers were increased (12). The small number of cats with metarubricytosis in the above study (12) precludes drawing robust general conclusions as to the association of metarubricytosis with the primary diagnoses and with other laboratory findings.

The aim of this retrospective case-control study was to investigate the association of metarubricytosis with the signalment, history, clinical and laboratory findings, diagnoses, morbidity and mortality, in a general population of ill cats, and to assess its utility as a prognostic marker. We hypothesized that metarubricytosis in cats is mostly physiologic, associated with erythroid regeneration, and those that present metarubricytosis have a poorer prognosis compared to ill negative controls, as reported in human patients.

MATERIALS AND METHODS

Selection of cases and data collection

The medical records of cats presented to the Hebrew University Veterinary Teaching Hospital (HUVTH) between 1999 and 2001 were reviewed retrospectively. Ill cats, presenting pnRBCs (> 1/100 leukocytes) in smears stained with modified-Wright's stain were consecutively included in the study (nRBC) group, while time-matched cats, presented within a period of 2 weeks before or after a corresponding study cat, in which pNRBC were absent in stained blood smears, were consecutively included in the control group. When several consecutive complete blood counts (CBCs) were performed in a cat, in either group, only the first CBC was selected.

Data collected from the medical records included signalment, history, physical examination, laboratory findings, diagnoses, length of hospitalization, treatment-cost and survival. Non-survivors included cats that died or were euthanized within 30 days from discharge. The final diagnoses were divided into categories based on the DAMN-IT system; developmental, degenerative, anatomical, allergic, metabolic, nutritional, neoplastic, inflammatory, infectious (subdivided into bacterial, viral or parasitic), iatrogenic, idiopathic, toxic and traumatic. In order to investigate the association between the putative mechanism of anemia and the intensity of metarubricytosis, anemic cats were divided according to the following etiologies: inflammation, chronic kidney disease (CKD), blood loss or hemolysis (i.e., regenerative anemia), or combination of these etiologies (defined as 'other' causes).

Laboratory tests

Blood samples for CBC and differential count were collected at presentation in potassium-ethylenediaminotetraacetic acid (EDTA) tubes, and analyzed within 30 minutes using automated impedance cell analysers (Abacus or Arcus, Diatron, Wien, Austria). Manual 200-cell white blood cell (WBC) differential and nRBC counts and morphological blood cell evaluation were performed by a single clinician (IA) in modified-Wright's stained blood smears. Nucleated RBCs were counted as nRBCs/100 WBCs, and when present, the WBC was corrected accordingly, and the absolute pnRBC count (anRBC; as nRBC/μL) was calculated (20). The polychromatophil:pnRBC (P/nRBC) ratio was determined in most cases. The packed cell volume (PCV) was measured by centrifugation (12,000g X 3 min) of whole blood in heparinized capillaries. Total plasma protein (TPP) was measured by refractometry.

Blood samples for serum biochemistry were 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, performed within 24 hours from collection, using a wet chemistry autoanalyzer (Cobas-Mira, Roche, Mannheim, Germany, at 37°C). Whole blood samples obtained in potassium-EDTA were also used for serological testing for FeLV antigen and feline immunodeficiency virus (FIV) antibodies using a commercial in-house ELISA kit (SNAP FeLV antigen/ FIV antibody Combo test, Idexx Laboratories. Westbrook, Maine USA).

Statistical analysis

The distribution pattern of continuous parameters was examined using the Shapiro-Wilk test. Continuous parameters were compared between the two groups using Student's *t*-test and Mann-Whitney *U*-test, for normally and non-normally distributed parameters respectively. Comparison of more than two groups was done using analysis of variance or Kruskal-Wallis test, for normally and non-normally distributed variables, respectively, and when results were significant, *post-hoc* comparison of group pairs was done using Student's *t*- or Mann-Whitney *U*-tests, respectively. Fisher's exact or chi-square tests were used to compare categorical variables and between two groups. Logistic regression was performed to assess the relationship between various variables and mortality. The predictive performance of pnRBC of mortality was also assessed using the receiver operator characteristics (ROC) curve, with its area under the curve (AUC) and 95% confidence interval ($CI_{95\%}$). For statistical analysis purpose, the nRBC group was divided into four subgroups, based on anRBC count quartiles as following: quartile 1. anRBC > 0 and ≤ 0.129 $x10^9$/L; quartile 2. anRBC > 0.129 and ≤ 0.311$x10^9$/L; quartile 3. anRBC > 0.311$x10^9$/L and ≤ 0.80$x10^9$/L and quartile 4. anRBC > 0.80$x10^9$/L. Each quartile was then treated as a categorical variable, and was compared, using logistic regression, to the negative control group, used as a reference category. In addition, study cats were divided into two groups based on the anRBC count; those with anRBC < 0.2$x10^3$/μL, which is the upper HUVTH Laboratory reference limit in cats, and those with

anRBC ≥ 0.2x10^3/μL, and were therefore considered with absolute metarubricytosis. All tests were two-tailed, and a *P* value ≤ 0.05 was considered statistically significant. Statistical analyses were done using a statistical software package (SPSS 17.0, SPSS Inc., Chicago, IL).

RESULTS

The study included 117 cats with metarubricytosis and 201 negative control cats. Most were domestic shorthair cats (71%), and the rest included Persian (10%), domestic longhair (8%), Siamese (5%) and other pure-breed cats (6%). There were no statistical group differences between in sex, breed or body weight. Cats with metarubricytosis were younger (*P* > 0.043) compared to the controls (median 3.5 years; range 0.1-19.0 vs. median 6.0 years; range 0.1-21.0, respectively). The proportion of cats aged ≤ 1 year was higher, albeit insignificantly (*P* = 0.09) in the metarubricytosis group.

Cats with metarubricytosis showed higher occurrence of weakness (*P* = 0.009), depression (*P* = 0.003), dyspnea (*P* = 0.001), shock (*P* < 0.001), epistaxis (*P* = 0.027), skeletal fractures (*P* < 0.001) and lameness (*P* = 0.004) at presentation compared to the controls, while anorexia (*P* = 0.041) and cachexia (*P* = 0.0002) were more common in the control group. Cats with metarubricytosis had lower (*P* = 0.004) rectal temperature compared to the controls (median 37.8°C; range 32.3-41.0 vs. median 38.2°C; range 32.5-41.2, respectively) and higher occurrence of hypothermia (*P* = 0.002) compared to the controls.

Cats with metarubricytosis had lower median RBC count, hematocrit, hemoglobin concentration, PCV and TPP (*P* < 0.001 for all), higher median mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (*P* = 0.01 for all), higher WBC count (*P* = 0.023), and higher frequency of anemia (*P* = 0.003) compared to the controls (Table 1). Hypochromasia and polychromasia were significantly more common in the metarubricytosis group compared to the controls (*P* < 0.002 for all), while anisocytosis was more common in the control group (*P* < 0.001) (Table 2). When the nRBC group was divided into quartiles based on anRBC count, an increase in the anRBC quartile was significantly and positively associated with the proportions of increased polychromasia or hypochromasia (*P* = 0.006 and *P* = 0.03, respectively).

Cats with metarubricytosis had higher (*P* < 0.05) median serum activities of aspartate aminotransferase (AST), creatine

Table 1: Complete blood count results of 107 cats with metarubricytosis and 201 negative control cats

Parameter	Control				nRBC[1]				*P* value[3]	*P* value[4]
	n	Median (range)	% below RI[2]	% above RI[2]	n	Median (range)	% below RI[2]	% above RI[2]		
White blood cell count (10^9/L) ^	200	10.43 (2.10-53.20)	8.0	36.0	116	12.70 (0.50-69.30)	10.3	43.6	0.023	0.232
Red blood cell count (10^{12}/L) *	200	8.5 (2.6-19.1)	5.5	45.5	116	7.1 (1.2-14.0)	25.9	22.4	<0.001	<0.001
Hematocrit (%)*	199	37.6 (12-79)	13.6	21.6	117	32.2 (6-55)	32.5	9.4	<0.001	<0.001
Hemoglobin (g/dl)*	200	11.8 (3.4-22.0)	12.5	14.7	117	10.3 (1.5-17.7)	28.2	7.7	0.001	0.002
Mean corpuscular volume (fL) ^	200	42.50 (29-68)	14.5	0.5	117	46 (14-91)	10.3	9.4	<0.001	0.0002
MCH[5] (g/dl) ^	199	13.2 (9.2-42)	26.6	2.0	117	14.3 (9.9-31.1)	18.8	12.0	<0.001	0.001
MCHC[6] (g/dl) ^	199	30.90 (14-39.7)	29.6	1.0	117	31.50 (17.3-41.5)	23.1	6.0	0.01	0.023
Packed cell volume (%) ^	198	33 (12-53)	23.2	0.0	116	29 (6-50)	39.7	0.0	0.001	0.003
Total plasma protein (g/dL) ^	198	8.2 (3.1-12.0)	3.5	48.5	114	8.0 (5.0-12.0)	3.5	29.8	<0.001	0.003

1. Nucleated red blood cells; 2. Reference interval; 3. Comparing group medians; 4. Comparing proportions of abnormalities between groups; 5. Mean Corpuscular Hemoglobin; 6. Mean Corpuscular Hemoglobin Concentration; * Normal distribution; ^ Non-normal distribution.

Table 2: Frequency of anisocytosis, hypochromasia and polychromasia in 107 cats with metarubricytosis and 201 negative control cats

Parameter		Control n (%)	nRBC[1] n (%)	*P* value
Anisocytosis	Absent	19 (17.8%)	1 (0.5%)	<0.001
	Mild	88 (43.8%)	20 (17.1%)	
	Moderate	83 (41.3%)	38 (32.5%)	
	Marked	29 (14.4%)	21 (17.9%)	
	Total	201 (100.0%)	107 (100.0%)	
Hypochromasia	Absent	171 (85.1%)	82 (76.6%)	0.002
	Very mild	5 (2.5%)	0 (0.0%)	
	Mild	23 (11.4%)	15 (14.0%)	
	Moderate	2 (1.0%)	7 (6.5%)	
	Marked	0 (0.0%)	3 (2.8%)	
	Total	201 (100.0%)	107 (100.0%)	
Polychromasia	Absent	171 (85.1%)	41 (38.3%)	<0.001
	Very mild	6 (3.0%)	4 (3.7%)	
	Mild	19 (9.5%)	36 (33.6%)	
	Moderate	4 (2.0%)	14 (14.0%)	
	Marked	1 (0.5%)	11 (10.3%)	
	Total	201 (100.0%)	107 (100.0%)	

1. Nucleated red blood cells

kinase (CK) and lactate dehydrogenase (LDH), higher median concentrations of total serum bilirubin ($P<0.001$) and triglycerides ($P=0.001$), and lower ($P<0.05$) median concentrations of total serum protein (TP), potassium, sodium, total and ionized calcium as well as γ-glutamyltransferase (GGT) and amylase activities compared to the controls (Table 3). Hypertriglyceridemia ($P=0.008$), hyperbilirubinemia ($P=0.0003$), hypocalcemia ($P=0.001$) and ionized calcium hypocalcemia ($P=0.008$) were more common in the metarubricytosis group compared to the controls (Table 3).

Cats with metarubricytosis had a higher ($P<0.03$) occurrence of hemoplasmosis, skeletal fractures, head trauma, high rise syndrome (HRS), hit by car (HBC), lung contusions, pleural effusion, pneumothorax, pyothorax and bacterial and traumatic diseases compared to the controls, while in the latter, CKD, mammary tumors, and allergic, viral and metabolic diseases were more common ($P<0.05$) compared to the metarubricytosis group. The frequency of inflammatory ($P=0.058$) and neoplastic diseases ($P=0.082$) tended to be higher in the control group (Tables 4 and 5).

Anemia of inflammation was more common ($P<0.001$)

Table 3: Serum biochemistry results of 117 cats with metarubricytosis and 201 negative control cats

Parameter	Control				nRBC[1]				*P* value[3]	*P* value[4]
	n	Median (range)	% below RI[2]	% above RI[2]	n	Median (range)	% below RI[2]	% above RI[2]		
Alanine aminotransferase (U/L)^	201	55 (9-2349)	11.9	20.4	69	65 (18-1273)	11.6	29	0.089	0.33
Albumin (g/dL)*	200	2.79 (1.4-4.6)	64.0	0.0	60	2.7 (1.1-3.9)	76.7	0.0	0.08	0.085
Alkaline phosphatase (U/L)^	196	45 (5-2190)	66.3	7.3	59	34 (7-3030)	71.2	11.9	0.111	0.2
Amylase (U/L)^	201	1083 (107-4180)	7.0	16.4	58	831.5 (242-3980)	19.0	10.3	0.001	0.022
Aspartate aminotransferase (U/L)^	201	42 (6-1500)	0.5	40.8	59	66 (18-1381)	0.0	66	<0.001	0.001
Cholesterol (mg/dL)^	201	138 (50-471)	1.5	30.3	59	139 (70-342)	0.0	30.5	0.806	1.00
Creatine kinase (U/L)^	201	406 (36-506870)	2	51.2	57	853 (28-84461)	1.8	66.7	0.023	0.069
Globulins (g/dL)^	201	4.3 (1.4-9.0)	0.5	42.3	58	4.1 (1.1–6.1)	1.7	32.8	0.085	0.205
γ-glutamyltransferase (U/L)^	163	6 (0-51)	NA	78.0	46	3 (0-232)	NA	57	0.003	0.008
Ionized calcium (mmol/L)^	156	1.23 (0.53-1.85)	19.2	39.7	58	1.15 (0.77-1.46)	32.8	19	0.001	0.008
Lactate dehydrogenase (U/L)^	26	658 (143-4289)	NA	76.9	18	1076 (430-4755)	100.0	NA	0.036	0.067
Potassium (mmol/L)^	184	4.3 (2.2-8.3)	5.4	12.0	71	4.0 (2.4-5.3)	19.7	4.2	<0.001	0.001
Sodium (mmol/L)^	185	154 (114-188)	1.1	84.3	60	150 (131-182.8)	0.0	62.5	<0.001	0.0002
Total bilirubin (mg/dL)^	199	0.27 (0-43.4)	NA	31.7	60	0.5 (0.11-18)	NA	58.3	<0.001	0.0003
Total calcium (mg/dL)^	197	9.1 (5-13.4)	26.4	2.5	58	8.8 (6.6-10.3)	36.2	0.0	0.018	0.214
Total protein (g/dL)*	201	7.1 (3.4-11)	6.0	23.9	58	6.6 (3.4-9)	8.6	13.8	0.011	0.209
Triglycerides (mg/dL)^	188	76.5 (8-980)	1.6	35.1	54	129 (9-2330)	1.9	57.4	0.001	0.008

1. Nucleated red blood cells; 2. Reference interval; 3. Comparing group medians; 4. Comparing proportions of abnormalities between groups;
* normal distribution; ^ non-normal distribution; NA. not applicable.

Table 4: Frequency of diseases and conditions (diagnosed in ≥ 5% of the cats) in 107 cats with metarubricytosis and 201 negative controls.

Diagnosis / Abnormality	Control n (%)	nRBC[1] n (%)	Total n (%)	*P* value
Anemia (all kinds)[2]	27 (13.6%)	38 (32.5%)	65 (20.6%)	<0.001
Icterus	27 (13.4%)	20 (17.1%)	47 (14.8%)	0.414
High rise syndrome	2 (1.0%)	26 (24.3%)	28 (8.8%)	<0.001
Chronic kidney disease	23 (11.4%)	3 (2.6%)	26 (8.2%)	0.005
Skeletal fracture	4 (2%)	20 (17.1%)	24 (7.5%)	<0.001
Pancreatitis	14 (7%)	7 (6.0%)	21 (6.6%)	0.818
Enteritis and gastroenteritis	17 (8.5%)	4 (3.4%)	21 (6.6%)	0.102
Hepatic lipidosis	11 (5.5%)	9 (7.7%)	20 (6.3%)	0.476
Pleural effusion	7 (3.5%)	11 (9.4%)	18 (5.7%)	0.042
FIV[3] infection	10 (5.0%)	5 (4.3%)	15 (4.7%)	1.00
Hypertrophic cardiomyopathy	7 (3.5%)	8 (6.8%)	15 (4.7%)	0.182
Mycoplasma hemofelis infection	4 (2.0%)	10 (8.5%)	14 (4.4%)	0.00002
Hit by car	1 (0.5%)	8 (6.8%)	9 (2.8%)	0.002
Mammary neoplasia	8 (4%)	0 (0.0%)	8 (2.5%)	0.029
Pneumothorax	0 (0.0%)	8 (6.8%)	8 (2.5%)	<0.001
Lung contusions	1 (0.5%)	6 (5.1%)	7 (2.2%)	0.011
Head trauma	0 (0.0%)	6 (5.1%)	6 (1.9%)	0.002
Pyothorax	1 (0.5%)	5 (4.3%)	6 (1.9%)	0.027

1. Nucleated red blood cells; 2. Based on hematocrit < 27%;
3. Feline immunodeficiency virus.

Table 5: Frequency of disease categories in 107 cats with metarubricytosis and 201 negative controls

Disease category	Control n (%)	nRBC[1] n (%)	All cats n (%)	*P* value
Metabolic	57 (28.4%)	20 (17.1%)	77 (24.2%)	0.03
Traumatic	13 (6.5%)	42 (35.9%)	55 (17.3%)	<0.001
Infectious bacterial	15 (7.5%)	26 (22.2%)	41 (12.9%)	<0.001
Inflammatory	26 (12.9%)	7 (6.0%)	33 (10.4%)	0.058
Neoplastic	25 (12.4%)	7 (6.0%)	32 (10.1%)	0.082
Idiopathic	14 (7.0%)	11 (9.4%)	25 (7.9%)	0.518
Infectious viral	20 (10.0%)	4 (3.4%)	24 (7.5%)	0.046
Infectious parasitic	6 (3.0%)	6 (5.1%)	12 (3.8%)	0.369
Toxic	5 (2.5%)	6 (5.1%)	11 (3.5%)	0.222
Vascular	7 (3.5%)	4 (3.4%)	11 (3.5%)	1.00
Anatomic	8 (4.0%)	3 (2.6%)	11 (3.5%)	0.752
Allergic	7 (3.5%)	0 (0.0%)	7 (2.2%)	0.05
Developmental	4 (2.0%)	1 (0.9%)	5 (1.6%)	0.655
Degenerative	1 (0.5%)	2 (1.7%)	3 (0.9%)	0.557
Iatrogenic	0 (0.0%)	2 (1.0%)	2 (0.6%)	0.533
Nutritional	2 (1.0%)	0 (0.0%)	2 (0.6%)	0.533

1. Nucleated red blood cells

in the control group, while anemia secondary to blood loss (P < 0.001) or hemolysis ($P = 0.02$) were both more frequent in the metarubricytosis group. When the metarubricytosis group was divided into two sub-groups based on upper reference limit of anRBC in cats, anRBC > $0.2x10^3$ cells/μL and anRBC < $0.2x10^3$ cells/μL, anemia was more common ($P = 0.002$) in those with absolute metarubricytosis (51.4% vs. 20.9%, respectively).

Cats with metarubricytosis had longer ($P = 0.017$) hospitalization time (median 2 days; range 0-21 days, vs. median 1 day; range 0-28 days in the controls), higher ($P = 0.013$), treatment cost (median 1648 NIS; range 208-11986 vs. median 1499 NIS; range 320-5900 NIS in the controls) and required surgery more often ($P = 0.007$, 24% in the metarubricytosis group vs. 12% in the controls) compared to the control group. The outcome (died, euthanized or survived 30 days post discharge) was recorded in 314 cats. Survival rate was higher ($P = 0.05$) in the control group compared to the metarubricytosis group (75.5% vs. 64.9%, respectively). ROC analysis of anRBC as a predictor of death had an AUC of 0.53 ($CI_{95\%}$ 0.41-0.64). When the association of metarubricytosis with mortality was investigated in certain diseases (in which were the number of cats was > 15), the odds ratio (OR) for death of cats with metarubricytosis were significantly increased in CKD (OR 1.67, $CI_{95\%}$ 1.01-2.67; $P = 0.047$), FIV infection (OR 1.67, $CI_{95\%}$ 1.01-2.67; $P = 0.045$) and congestive heart failure (OR 1.67, $CI_{95\%}$ 1.01-2.67; $P = 0.047$) compared to those in which metarubricytosis was absent. increased in CKD, FIV infection and congestive heart failure (OR 1.67, $CI_{95\%}$ 1.01-2.80, for all) compared to those in which metarubricytosis was absent.

In order to investigate the type of metarubricytosis (i.e., physiologic vs. pathologic), the metarubricytosis group (n = 104) was subdivided into two groups, those with physiologic metarubricytosis (P/nRBC ratio ≥ 1) and those with pathologic metarubricytosis (P/nRBC ratio < 1). There was no association between the type of metarubricytosis and the outcome. Inflammatory diseases were significantly ($P = 0.013$) associated with pathologic metarubricytosis (85% of these cats), while traumatic diseases were significantly ($P = 0.01$) associated with physiologic metarubricytosis (78.4% of such cats). In 29/37 cats (69%) with metarubricytosis and trauma-related injury metarubricytosis was physiologic, while only 8 (31%) showed pathologic metarubricytosis. Conversely, metarubricytosis was pathologic in 6/7 cats (86%) with metarubricytosis and inflammatory conditions.

DISCUSSION

This study is the first large-scale, case-control study investigating the association of metarubricytosis with clinical and laboratory findings and the outcome in ill cats. Metarubricytosis was associated with anemia, polychromasia and leukocytosis. Cats with metarubricytosis were younger than ill controls. Metarubricytosis was associated with hemolytic anemia, trauma and pleural and lung diseases, with hyperbilirubinemia, likely secondary to hemolysis, and with increased muscle enzyme activities, likely due to trauma. Metarubricytosis was associated with longer hospitalization, more frequent requirement of surgery and higher mortality rate compared to negative controls.

In this study, cats with metarubricytosis were significantly younger compared to the controls, possibly because the conditions associated with metarubricytosis, including fractures, head trauma and lung contusions likely resulted to HBC, HRS and pleural diseases (i.e., pyothorax, pneumothorax and pleural effusion), which occur more commonly in younger cats (21). Conversely, in dogs, the incidence of metarubricytosis increases in older age, while in humans, it is higher in neonates and in older patients (14-19). Exclusion of human patients younger than 18 years of age, however, likely contributed to some bias in the age distribution of this phenomenon in humans (15-18).

Cats with metarubricytosis presented more severe and acute clinical signs compared to the ill negative controls, with lower median rectal temperature, and higher frequency of hypothermia, most likely due to shock (21, 22), which was more common in the metarubricytosis group. The latter is common in trauma, severe metabolic diseases and poisoning cases (23-25), which were more frequent in this group. Both shock and dyspnea, more common in the metarubricytosis group, are often associated with acute life-threatening conditions in cats, and at least likely to account partially for the higher mortality rate in the metarubricytosis group. Conversely, anorexia and cachexia, often associated with more chronic conditions (e.g., CKD), were more common in the control group.

Anemia and polychromasia were more frequently documented in the metarubricytosis group, likely accounting for the significantly lower hemoglobin concentration, RBC count and hematocrit and higher MCV and proportion of macrocytosis compared to the controls. Therefore metarubricytosis in cats is mostly associated with erythroid regeneration, and is physiologic and appropriate. This was observed in most of the trauma-associated metarubricytosis cases, while in most cats with inflammatory conditions, characterized by non-regenerative anemia (26), metarubricytosis was pathologic. Cats with metabolic diseases showed no difference between the frequency of physiologic and pathologic metarubricytosis, however, the latter comparison included only 28 cats, resulting in a low statistical power of the analysis.

Previous studies in humans following trauma have demonstrated bone marrow failure, resulting in non-regenerative anemia with a decrease in bone marrow erythroid precursors and concurrent increase in their peripheral blood concentrations (27). A similar phenomenon might account for the trauma cases with pathologic metarubricytosis herein.

Cats with metarubricytosis had higher median WBC and frequencies of neutrophilia and neutrophilic left shift compared to the controls, as described in dogs (24). In cats, severe regenerative anaemia is often associated with a leukoerythroblastic response, characterized by concurrent release of nRBC and immature neutrophils from the bone marrow (12), and concentrations of both G-CSF and GM-CSF, involved in inflammation, stimulate granulopoiesis as well as erythropoiesis (4). Additionally, in certain inflammatory conditions, described in humans with increased pnRBC, IL-3 and IL-6 concentrations were noted (28, 29). Possibly, the effect of these cytokines on both erythropoiesis and myelopoiesis in the bone marrow, and on the BBMB, contributed to the concurrent metarubricytosis, neutrophilia and left shift.

Cats with metarubricytosis showed more frequent serum chemistry abnormalities compared to the controls, likely reflecting the underlying diseases, as well as the secondary effects of anemia, trauma and hypoxia. Hemolysis, more commonly noted in the metarubricytosis group, likely accounted for the higher median serum bilirubin concentration and frequency of hyperbilirubinemia in this group. The higher activities of AST, LDH and CK in the metarubricytosis group likely resulted, at least partially, from hemolysis, since RBCs contain high AST and LDH activities, while high RBC glucose-6-phosphate activity leads to spuriously increased CK activity due to interference with its assay (30). However, muscle enzyme activities were very likely increased in this group also due to muscle damage, associated with trauma, shock and hypoxia (30, 31). Conditions leading to hypoxia were recorded in 49/100 children with increased

pnRBCs, and in human neonates, this phenomenon being related mainly to chronic hypoxia and acute asphyxia (25, 32). In adult humans, inflammation and hypoxia were associated with presence of pnRBC, and EPO also may stimulate release of nRBCs during hypoxia, unrelated to anemia (4, 14). In this study, pleural and lung diseases (i.e., pneumothorax, lung contusions, pleural effusion and pyothorax), often leading to systemic hypoxia, were significantly more common among the metarubricytosis cats.

In ill adult humans with increased pnRBC, serum concentrations of EPO, IL-3 and IL-6 are higher compared to negative controls, while in human neonates presenting pnRBC, serum EPO and IL-6 are increased (28, 29). Both serum EPO and IL-6 concentration.s increase within hours following hypoxia. EPO, IL-6 and IL-3 promote erythropoiesis, while IL-6 is also a major pro-inflammatory cytokine (4, 28, 29). EPO accelerates mitotic divisions of nRBCs within the bone marrow, and increases the bone marrow blood flow and its porous infrastructure (28). IL-6 is associated with anemia of inflammation through induction of expression of the polypeptide hormone hepcidin, which down regulates the expression of the iron export channel ferroportin, thereby blocking release of iron from storage cells, restricting iron availability for erythropoiesis (32, 33). In this study, traumatic conditions, hemolytic anemia and pleural effusion were more frequent in the metarubricytosis group. Possibly, hypoxic and inflammatory tissue damage due to these conditions induced such cytokine profiles that stimulated release nRBC from the bone marrow into the peripheral blood. The association between metarubricytosis and polychromasia, representing a physiologic metarubricytosis, supports an ongoing increased erythropoiesis in most of these states, rather than bone marrow or BBMB lesions, as a cause of metarubricytosis. This interpretation is also supported by the finding that anemia was significantly more frequent in the metarubricytosis group, and within this group, anemia was significantly more common among cats with anRBC count > 200/μL. Nevertheless, the cytokine profile in cats with metarubricytosis needs to be investigated in order to substantiate these interpretations.

In human patients, presence of pnRBC was associated with a poorer prognosis and higher mortality rates in a wide range of medical conditions (14, 16, 18, 35). In general, a pnRBC cut-off of > 200 cells/μL was associated with 80% mortality rate (14). Herein, metarubricytosis was associated with more severe diseases, as reflected by longer hospitalization period, higher treatment cost, higher requirement of surgery and a higher mortality rate compared to the controls, likely because traumatic conditions and pleural diseases (e.g., pneumothorax and pyothorax) were more frequent in this group. Nevertheless, ROC analysis showed that the anRBC is an inaccurate predictor of survival, and should not be used a sole predictor. In humans, presence of pnRBC was noted in patients during hospitalization as early as 9-14 days before death. Such time-period allows intervention with intensive care and monitoring to increase survival. It was therefore recommended that such patients should be followed using repeated, frequent CBCs during hospitalization to improve the treatment and outcome (14). This recommendation might be applicable in cats with metarubricytosis as well, because this phenomenon in cats is an indicator of potential complications and higher mortality rate, especially so in cats with FIV infection, CKD and congestive heart, in which metarubricytosis was significantly and positively associated with mortality.

This study has several limitations: First, it is retrospective, and therefore, some data were missing in the medical records, thereby decreasing the power of the statistical analyses. Second, in contrast with studies of nRBC in humans, where anRBC was based on automatic methods, herein, the pnRBC count was done manually, in stained blood smears, using light microscopy, which is probably relatively insensitive when the anRBC is low (i.e., < 200 cells/μL) (13). Nevertheless, currently, no automated counting method for pnRBC has been validated in cats. Third, although this study is a relatively large-scale case control, the population included is of ill cats in general, with heterogeneous diseases. Therefore, the number of cats in each diagnosis was limited, weakening the statistical analyses, Fourth, many comparisons were performed in this study, and therefore, possibly, some statistically significant associations occurred due to pure chance (type-I error). Fifth, ideally, regeneration should have been assessed using a reticulocyte count, which was not done. Assessment of regeneration, and therefore, of the type of metarubricytosis (i.e., physiologic vs, pathologic) was done based on polychromasia, which is less sensitive than the reticulocyte count. Sixth, assessment of hypoxemia was subjective, while arterial blood gas analysis or pulse oximetry data were missing in most cases. Seventh, this study has investigated only the CBC obtained at presentation. Based on human studies, assessment of repeated CBCs is indicated in patients with in-

creased pnRBCs. Such an investigation might have improved the performance of metarubricytosis as a prognostic indicator in cats. Lastly, in this study, we recorded only the 30-day survival rate, while longer-term survival was not assessed.

In conclusion, this is the largest study of metarubricytosis in ill cats in general, and in contrast with previous ones, it is case-controlled. Metarubricytosis in cats is mostly physiologic, associated with erythroid regeneration, secondary to blood loss, hemolysis, various inflammations, and is possibly associated with hypoxia due to anemia, trauma and shock. It occurs more commonly in cases of trauma and pleural and lung disorders. Metarubricytosis at presentation is associated with more severe acute clinical signs, longer hospitalization, higher treatment cost and lower survival rate. Metarubricytosis in cats should alert attending clinicians to provide intensive care and monitoring of ill cats to improve the outcome.

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