Received: 18 October 2019 | Revised: 20 April 2020 | Accepted: 27 April 2020

DOI: 10.1111/vcp.12925

ORIGINAL ARTICLE

Veterinary Clinical Pathology
An International Journal of Laboratory Medicine
WILEY

Evaluation of reticulocyte hemoglobin content (RETIC-HGB) for the diagnosis of iron-limited erythropoiesis in cats

Miriam Keiner[1] | Jannika Fuchs[2] | Natali Bauer[2] | Andreas Moritz[1,2]

[1]Small Animal Clinic, Internal Medicine, Justus-Liebig-University, Giessen, Germany

[2]Department of Veterinary Clinical Sciences, Clinical Pathology and Clinical Pathophysiology, Justus-Liebig-University, Giessen, Germany

Correspondence
Miriam Keiner, Justus-Liebig-University, Giessen, Frankfurter Straße 114, 35392 Giessen, Germany.
Email: miriam.keiner@vetmed.uni-giessen.de

Abstract

Background: Decreased reticulocyte hemoglobin content (CHr) (Siemens ADVIA 2120) reflects iron-limited erythropoiesis (ILE). RETIC-HGB (IDEXX ProCyte Dx) is a novel marker of ILE for veterinary use.

Objectives: We aimed to evaluate reference intervals (RIs) and the utility of RETIC-HGB and CHr in the diagnosis of feline ILE.

Materials and Methods: RIs were established in 59 healthy cats. Intra-assay coefficients of variation (CVs) and correlations between RETIC-HGB and CHr were assessed. Two hundred and seventy-five cats were classified as having ILE or not based on low plasma iron or low transferrin saturation along with anemia and/or altered RBC indices. CHr, RETIC-HGB, and serum amyloid A (SAA) were compared between the groups. The sensitivity and specificity of RETIC-HGB and CHr to diagnose ILE were analyzed to determine the RI lower limits.

Results: RIs for RETIC-HGB and CHr were 12.5-18.0 and 14.0-19.9 pg, respectively. The CV was 3% for both variables. RETIC-HGB and CHr were moderately correlated ($r_s = 0.59$) with a bias of −1.2 picograms (pgs). Twenty of the 275 cats were classified as having ILE. Compared with non-ILE cats, ILE cats had significantly lower median RETIC-HGB (14.3 vs 15.2 pg, $P = .0046$) and mean CHr (14.7 vs 16.5 pg, $P < .0001$) values and significantly increased median SAA (44.6 vs 2.3 μg/dl, $P < .0001$) values. Using the lower RI limits resulted in a low sensitivity and relatively high specificity to diagnose ILE in cats.

Conclusions: ILE was characterized by decreased CHr and RETIC-HGB; however, sensitivity was low. The moderate correlation between RETIC-HGB and CHr is likely due to species differences and different methodology.

KEYWORDS

ADVIA 2120, feline, hematology, ProCyte Dx, reference interval

1 | INTRODUCTION

In people, absolute iron deficiency (AID) is primarily caused by excessive blood loss or nutritional iron deficiency (ID) and remains the most common cause of anemia.[1] Nutritional ID is very rare in dogs and cats receiving commercial diets,[2] and AID occurs mainly due to chronic bleeding.[3,4] Functional iron deficiency (FID) represents the second most common cause of ID in human and veterinary medicine. It arises from strong erythropoietic stimuli (eg, erythropoiesis-stimulating agents)[1,5] or (chronic) inflammatory conditions[6-8] and has been associated with portosystemic shunts (PSS) in dogs.[9]

Microcytic-hypochromic anemia is considered a main hallmark of iron-limited erythropoiesis (ILE).[4] Conventional RBC indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC]) mainly characterize mature erythrocytes. Due to the long life span of erythrocytes (120 days in people and dogs, 70 days in cats), ID has to be present for weeks to months before changes in these indices even become apparent.[6,10,11] Thus, conventional RBC indices are relatively insensitive during the early stages of iron-deficient states, and acute or subacute ID can consequently go unrecognized.[11,12]

Reticulocyte indices reflect functional iron available for erythropoiesis over the past two to four days.[6,11-14] Several reticulocyte variables have been investigated and introduced for ILE diagnoses in recent years, with reticulocyte hemoglobin content becoming increasingly important. Reticulocyte hemoglobin content has gained particular significance in the early detection of iron-deficient states in children and adults.[15,16] Various studies performed in dogs have shown that reticulocyte hemoglobin content alone is insufficient for the diagnosis of ID but can be used as a complementary marker for the early detection of ILE.[4,9,17]

Depending on various methodologies, different terminologies are used for reticulocyte hemoglobin content (Table 1). Siemens hematology analyzers determine both the volume and hemoglobin concentration of mature erythrocytes and reticulocytes. CHr is calculated as the product of the mean reticulocyte volume (MCVr) and mean corpuscular hemoglobin concentration (CHCMr) of reticulocytes.[4,10,14] Hematology analyzers manufactured by Sysmex and IDEXX use the mean value of forward scattered light of mature erythrocytes (RBC-Y) and reticulocytes (RET-Y)[10] to determine the hemoglobin equivalent of erythrocytes (RBC-He) and reticulocytes (RET-He/RETIC-HGB).[6,10,18]

RETIC-HGB (IDEXX ProCyte Dx) has recently been introduced for veterinary use. This variable has been shown to be an early indicator of ILE in dogs but failed to provide a specific distinction between different disease complexes.[19] With regard to cats, only one study has been published, evaluating a reference interval (RI) (0.88-1.22 fmol/14.2-19.7 pg) and cut-off values for CHr.[17] At a cut-off value of 0.88 fmol (14.2 pg), sensitivity and specificity to detect ILE were 93.3% and 76.9%, respectively.[17]

The objective of this study was to evaluate RETIC-HGB on the IDEXX ProCyte Dx hematology analyzer with the goals of establishing an RI, assessing RETIC-HGB stability at room temperature, and determining its precision and comparability with CHr values. Furthermore, we evaluated the utility of RETIC-HGB for the diagnosis of feline ILE compared with that of CHr.

Our hypothesis was that RETIC-HGB can be used as an early indicator of ILE in cats and compares well with CHr.

2 | MATERIALS AND METHODS

2.1 | Study population

2.1.1 | Healthy population

The control population consisted of 59 healthy, nonanemic, client-owned adult cats that presented for health checkups (n = 12), preanesthetic controls (n = 2), or blood donations (n = 45) between October 2017 and October 2018. Eleven blood donors (11/45) presented for repeated blood donation (more than one blood donation, last blood donation performed more than three months before the inclusion into this study). A normal health status was established if no abnormalities were found from the historical information, physical examination, and CBC and biochemistry profiles. The hematocrits (HCTs), hemoglobin concentrations (HGBs), and RBC counts also needed to be within normal limits (ProCyte Dx, manufacturer's RIs[20]; ADVIA 2120, internal laboratory RIs [Department of Veterinary Clinical Pathology, Justus-Liebig-University Giessen, Germany]).

2.2 | Diseased population

The diseased population comprised a total number of 216 diseased cats. These animals were grouped according to their diagnosed disorder. The most probable etiopathogenesis of ILE (ie, hematologic diseases, inflammatory diseases, miscellaneous diseases, neoplastic diseases, portosystemic shunts, and renal diseases) was based on historical information, clinical examinations, and CBC and biochemical analyses of blood samples (EDTA, heparin plasma, serum) and

TABLE 1 Terminology used for the determination of reticulocyte hemoglobin content

Siemens ADVIA 120 or 2120/2120i Siemens Healthcare GmbH, Erlangen, Germany	Sysmex XE-2100, XE-5000, XN-9000 Sysmex Corporation, Kobe, Japan	IDEXX ProCyte Dx IDEXX Laboratories, Maine, USA
CHr	Ret-He	RETIC-HGB (previously also Ret-He)

additional diagnostic modalities (eg, liver function tests, diagnostic imaging). Cats diagnosed with inflammatory disorders (eg, sepsis, feline infectious peritonitis, pneumonia) were assigned to the inflammatory disease group. Irrespective of the underlying etiopathogenesis of ILE, the evidence of inflammation was of interest in all disease groups. Therefore, cats were diagnosed as having inflammation if pyrexia, and/or inflammatory leukograms and/or increased SAA concentrations were present.

The grouping was performed by a first-year Internal Medicine Resident (MK) and an ECVCP Diplomate (NB).

2.3 | RIs and the imprecision of limits for RETIC-HGB and CHr

RIs for RETIC-HGB and CHr were calculated using Reference Value Advisor,[21] a freeware add-in for Microsoft Excel 2010-2013 (Microsoft Corporation). As recommended by Reference Value Advisor[21] and Friedrichs et al,[22] the robust method was used ($40 \leq n \leq 120$). Tukey's test was performed to detect possible outliers. The imprecision of limits was estimated by the ratio of the width of the 90% confidence interval (WCI) and the width of the respective reference interval (WRI).[23] Imprecision of the limits was considered acceptable if the ratio of WCI/WRI was <0.2.[22]

A calibration of both the Siemens ADVIA 2120 and the ProCyte Dx hematology analyzer was performed by Siemens and IDEXX personnel during the annual maintenance service. Internal quality control (QC) was performed daily using three levels of commercially available QC material (IDEXX ProCyte Dx, IDEXX ProCyte Dx Quality Control; Siemens ADVIA 2120, ADVIA Testpoint Hematology Controls).

2.4 | Stability of RETIC-HGB

Because agglutination as a result of incorrect sample handling, storage-related swelling of cells, or hyperosmolarity may lead to macrocytosis[24] and an erroneous increase of RETIC-HGB may occur with this condition,[25] the stability of RETIC-HGB over 48 hours was assessed by measuring ten EDTA blood samples after storage at room temperature for four different time periods. The first measurement was performed within one hour after collection, then after six hours, 12, 24, and 48 hours, respectively. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software). Because distribution did not pass the Shapiro-Wilk test for normality, a nonparametric Friedman test was used to assess RETIC-HGB stability. Significance was set at $P < .05$.

2.5 | Intra-assay coefficient of variation for RETIC-HGB and CHr

The intra-assay coefficient of variation (CV) for RETIC-HGB was calculated from repeated measurements (n = 25) of one feline sample in the middle range (RETIC-HGB = 15.6 pg) of the RI according to the following formula: CV (%) = standard deviation/mean × 100. CV for CHr was calculated from repeated measurements (n = 20) of one feline sample in the middle range (CHr = 15.9 pg) of the RI. All analyses were performed in succession (within a one-hour time frame; both analyzers (ProCyte Dx and Siemens ADVIA 2120) need less than 2 minutes for each analysis).

2.6 | Comparison of RETIC-HGB and CHr in cats

Correlations between RETIC-HGB and CHr were assessed using leftover EDTA blood samples (n = 275) of feline patients admitted to our clinic (Small Animal Clinic, Justus-Liebig University Giessen). Samples were randomly analyzed on either one of the analyzers first to avoid bias. A special method for randomization was not used. For statistical analyses, Spearman's coefficient of rank correlation (r_s), Bland-Altman plots, Cusum tests, and Passing-Bablok regression analyses were performed using MedCalc Version 15.11.4 (MedCalc Software).

2.7 | An evaluation of the diagnostic utility of RETIC-HGB and CHr for diagnosing iron-limited erythropoiesis in cats

Overall, CBCs, biochemistry profiles, iron panels, SAA values, and additional patient data from 216 diseased cats and 59 healthy cats were included. For the diagnosis of ILE, animals did not have to be anemic. The algorithm used for an ILE diagnosis is shown in Figure 1. In the case of an ILE diagnosis, the most relevant underlying etiology was of interest. Thus, cats with ILE were separated into groups according to their diagnosis and the most probable etiopathogenesis of ID (ie, hematologic diseases, inflammatory diseases, miscellaneous diseases, neoplastic diseases, portosystemic shunt, and renal diseases).

Serum iron was measured on the ABX Pentra C400 (Horiba ABX SAS) clinical chemistry analyzer using a direct colorimetric method (ferene method). For the determination of %TfS, total iron-binding capacity (TIBC) indirectly reflecting transferrin concentration had to be assessed first. Samples were pretreated with a kit for TIBC (bioanalytic GmbH) and were then analyzed just like samples for iron measurement resulting in the unbound iron-binding capacity (UIBC). TIBC was consistent with the sum of UIBC and serum iron concentration. %TfS was then calculated from plasma iron and TIBC as follows: %TfS = serum iron/TIBC × 100.

SAA was measured using a latex agglutination reaction previously evaluated for cats[26] (LZ Test "Eiken" SAA, Mast Diagnostica GmbH) on the ABX Pentra C400.

Two levels of QC samples (serum iron, TIBC, SAA) were analyzed daily using control samples provided by the manufacturer (Horiba ABX SAS, ABX Pentra N Control). All results met the performance goals.

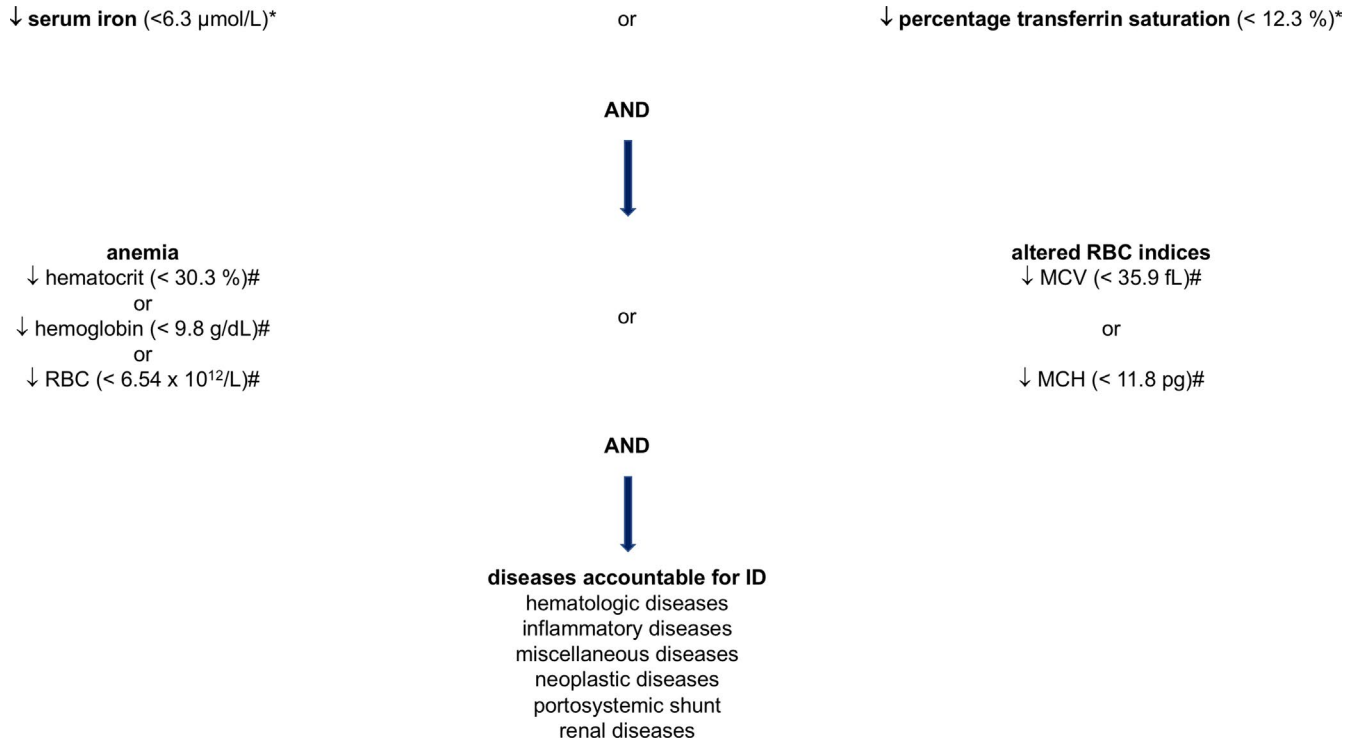

FIGURE 1 An algorithm for diagnosing iron-limited erythropoiesis (ILE). *,Cut-off values for serum iron and %TfS, which had previously been established by our working group in 59 healthy cats (unpublished data); #,Lower end of the reference interval of the IDEXX ProCyte Dx[20] hematology analyzer; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume

Reticulocyte hemoglobin content (RETIC-HGB, CHr) and SAA were compared in cats with and without ILE using an unpaired *t* test or the nonparametric Mann-Whitney U test, whereby commercially available statistical software was applied (GraphPad Prism 8). Normality was assessed with the Shapiro-Wilk test. The diagnostic utility of a decreased RETIC-HGB and CHr to diagnose ILE was analyzed using the lower limit of the established RIs as the cut-off value. MedCalc Version 15.11.4 software was used to plot receiver operating characteristic (ROC) curves and calculate the area under the ROC curve (AUC-ROC), which compared both ROC curves and assessed sensitivities and specificities. A $P < .05$ was considered significant for all tests.

2.8 | Study approval registration and owner consent

The study was performed according to the German Animal Welfare Act (article 8) and was approved by the responsible ethics committee (Regierungspräsidium Giessen, Dezernat 54, Wetzlar, Germany, ethics committee number #V 54-19 c 20 15 h 02 GI 18/17 kTV 11/2017; 10/09/2017). Client consent was obtained through the conditions of admission at the Small Animal Clinic, Justus-Liebig-University Giessen, Germany, which included the owners giving their agreement that specimens (eg, blood) could be sent to internal and external laboratories for special diagnostics. For all parts of the study, no further sampling was required, and only leftover blood (EDTA, heparin plasma, serum) was used.

3 | RESULTS

3.1 | Study population

3.1.1 | Healthy population

The most represented breeds within the healthy cat population were Domestic Shorthair (n = 39/59), British Shorthair (n = 6/59), and Maine Coon (n = 4/59) cats, followed by crossbred cats (n = 3/59), Chartreux (n = 2/59), Turkish Angora (n = 2/59), and one of each British Longhair, Burma, and Scottish Fold cats. These animals, including 35 males (two intact) and 24 females (two intact), were between four months and 18 years of age (median age 5.2 years).

3.2 | Diseased population

Diseased cats were categorized as having miscellaneous (eg, cardiorespiratory disorders) (n = 131/216), neoplastic (n = 23/216), hematopoietic (n = 17/216), inflammatory (n = 16/216), and renal (n = 15/216) diseases, or portosystemic shunts (n = 14/216). The most represented breeds within the diseased cat population were Domestic Shorthair (n = 137/216), crossbred (n = 20/216), British Shorthair (n = 19/216), and Maine Coon (17/216) cats, followed by 23 cats of different breeds (eg, Bengal, British Longhair, Burmese, Domestic Longhair, Persian). These animals, including

TABLE 2 Reference intervals for reticulocyte hemoglobin content (CHr, RETIC-HGB) in healthy, nonanemic cats (N = 59)

Variables	n	Units	Mean	SD	Median	Min	Max	RI LRL (90% CI)	RI URL (90% CI)	Dist	Meth
RETIC-HGB	59	pg	15.4	1.3	15.7	12.6	18.4	12.5 (12.0-13.2)	18.0 (17.5-18.4)	NG	Rb
CHr	59	pg	16.9	1.5	17.1	13.9	19.7	14.0 (13.4-14.5)	19.9 (19.5-20.4)	NG	Rb

Abbreviations: CHr, reticulocyte hemoglobin content (Siemens ADVIA 2120); CI, confidence interval; Dist, distribution; LRL, lower reference limit; max, maximum; Meth, method; min, minimum; n, number of valid observations; NG, non-Gaussian; pg, picogram; RETIC-HGB, reticulocyte hemoglobin content (IDEXX ProCyte Dx); RI, reference interval; Rob, robust method; SD, standard deviation; URL, upper reference limit.

TABLE 3 Reference interval accuracies estimated by the ratio of the width of the 90% confidence interval and the width of the reference interval (WCI/WRI)

	RI	WRI	90% CI (LRL)	WCI_L	WCI_L/WCI	90% CI (URL)	WCI_U	WCI_U/WRI
RETIC-HGB (pg)	12.5-18.0	5.5	12.0-13.2	1.2	0.22	17.5-18.4	0.9	0.16
CHr (pg)	14.0-19.9	5.9	13.4-14.5	1.1	0.19	19.5-20.4	0.9	0.15

Abbreviations: CI, confidence interval; LRL, lower reference limit; RI, reference interval; URL, upper reference limit; WCI_L, width of the 90% confidence interval of the lower reference limit; WCI_u, width of the 90% confidence interval of the upper reference limit.

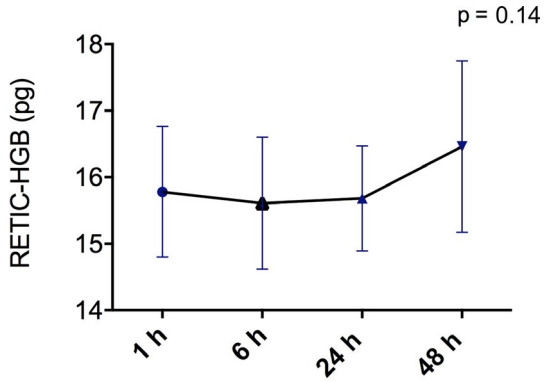

FIGURE 2 The stability of reticulocyte hemoglobin content (RETIC-HGB) over 48 h (n = 10) in feline EDTA blood samples. Results are shown as the median and range. $P < .05$ indicates significant differences between the storage periods. RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx); *P*, level of significance

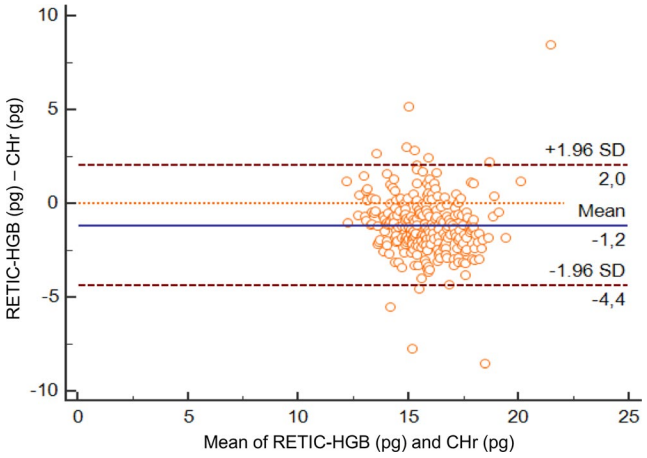

FIGURE 3 A Bland-Altman plot of reticulocyte hemoglobin content (RETIC-HGB and CHr) (n = 275) with lines for the mean difference (blue solid line) and for the 95% limits of agreement (red dashed lines), as well as the line of equality (orange dotted line). CHr, reticulocyte hemoglobin content (Siemens ADVIA 120 or 2120/2120i); RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx)

127 males (18 intact) and 89 females (eight intact), were between three months and 19 years of age (median age 8.17 years).

3.3 | RIS and the imprecision of limits for RETIC-HGB and CHr

Results are presented in Tables 2 and 3. Outliers were not detected for either variable.

3.4 | Stability of RETIC-HGB

RETIC-HGB values did not change significantly over time, although a slight upward trend was noted (Figure 2). Median values increased slightly by 4% from 15.50 pg after 1 hour to 16.15 pg after 48 hours. All RETIC-HGB values remained within the RI.

3.5 | Intra-assay coefficient of variation for RETIC-HGB and CHr

The 25-run intra-assay CV for RETIC-HGB was 3.32% (0.52 pg/15.66 pg × 100). The 20-run intra-assay CV for CHr was 3.01% (0.48 pg/15.95 pg × 100).

3.6 | Comparisons of RETIC-HGB and CHr in cats

To estimate the correlation between RETIC-HGB and CHr in cats, 275 feline samples with RETIC-HGB ranging from 11.3 to 26.7 pg and CHr ranging from 11.6 to 22.7 pg were evaluated. Spearman's

coefficient of rank correlation was moderate[27] (r_s = 0.59; P < .0001; 95% CI 0.50-0.66). The Bland-Altman plot revealed a small negative systematic bias of −1.2 pg (Figure 3). The Cusum test did not show a significant deviation from linearity (P = .65). The Passing-Bablok regression analysis showed both small constant and proportional errors (Figure 4). The regression equation was $y = 1.31 + 0.84x$, with an intercept of 1.31 (95% CI −0.53-2.89) representing constant error and a slope of 0.84 (95% CI 0.75-0.96) representing proportional error.

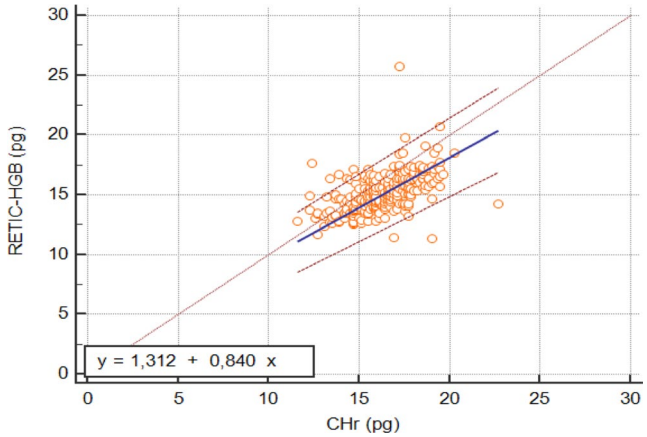

FIGURE 4 A Passing-Bablok plot of RETIC-HGB and CHr (n = 275) with a regression line (solid blue line), a 95% confidence interval for the regression line (red dashed line), and an identity line (orange dotted line). CHr, reticulocyte hemoglobin content (Siemens ADVIA 120 or 2120/2120i); RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx)

3.7 | An evaluation of the diagnostic utility of RETIC-HGB for diagnosing iron-limited erythropoiesis in cats

Overall, ILE occurred in 20/275 cats (7.3%), accounting for 9.3% of the diseased cat population (n = 20/216). The most represented breeds within the ILE group were Domestic Shorthair (n = 12/20), crossbred (n = 3/20), and British Shorthair (n = 2/20) cats, followed by three cats of different breeds (eg, Maine Coon). These animals, including 14 males (one intact) and six females (one intact), were between three months and 18 years of age (median age 9.2 years).

Nineteen of 20 cats showed decreased serum iron concentrations (<6.3 µmol/L), 12/20 had decreased percent transferrin saturation (%TfS; <12.3%), and 11/20 cats had both decreased serum iron as well as decreased %TfS. A decrease in CHr (<14.0 pg) was detected in 6/20 cats, whereas a decrease in RETIC-HGB (<12.5 pg) was found in only 1/20 cats. Eighteen out of 20 cats showed an SAA concentration (>3.9 µg/mL[26]). Inflammatory diseases accounted for the largest subpopulation (8/20, 40%) in ILE cats. The second most common causes of ILE were neoplastic and miscellaneous diseases (each 4/20, 20%). In the neoplasia group, 3/4 cats had concurrent evidence of inflammation, whereas all of the cats included in the group with miscellaneous diseases had a concomitant inflammatory reaction. Hematopoietic disorders were present in 2/20 cases (10%), PSS and renal disorders accounted for 1/20 (5%) each. Leukocytosis, as well as neutrophilia, were detected in one cat each in the hematopoietic (1/2) and renal disease (1/1) groups. In total, 17/20 cats showed evidence of inflammation.

Compared with the non-ILE cats, ILE cats had significantly lower median RETIC-HGB and mean CHr values. Median SAA concentrations were significantly higher in ILE cats (Table 4).

TABLE 4 A comparison of reticulocyte hemoglobin content values, RBC variables, iron metabolism variables, and SAA measurements between ILE and non-ILE cats. Results are depicted as the median and range (*CHr, mean and standard deviation)

	Median value and range		
Variable	**ILE (n = 20)**	**Non-ILE (n = 255)**	***P*-value**
RETIC-HGB (pg)	14.25 (11.70-16.30)	15.15 (12.24-18.20)	**.0046**
CHr (pg)*	14.69 ± 1.57	16.49 ± 1.68	**<.0001**
RBC (×10^{12}/L)	6.86 (1.49-11.50)	9.33 (1.02-13.87)	#
Hematocrit (L/L)	26.60 (7.80-38.90)	41.00 (5.20-66.20)	#
Hemoglobin (g/dL)	8.65 (1.90-12.70)	12.70 (1.00-18.10)	#
MCV (fL)	39.80 (33.80-52.30)	43.45 (30.50-71.20)	#
MCH (pg)	12.65 (10.70-15.00)	13.80 (9.10-22.80)	#
Iron (µmol/L)	5.10 (1.80-7.00)	11.85 (1.40-58.60)	#
TIBC (µmol/L)	42.80 (26.73-85.05)	49.82 (21.06-104.50)	#
%TfS	11.20 (3.29-22.96)	24.36 (2.70-157.41)	#
SAA (µg/dL)	44.60 (1.40-187.90)	2.30 (0.10-309.10)	**<.0001**

Note: P < .05 indicates significant differences between both groups. Significant results are shown in bold numbers.

Abbreviations: %TfS, transferrin saturation; CHr, reticulocyte hemoglobin content (Siemens ADVIA 120 or 2120/2120i); ILE, iron-limited erythropoiesis; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; p, level of significance; RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx); SAA, serum amyloid A; TIBC, total iron-binding capacity, #*P*-values not reported as these variables were nondependent.

TABLE 5 Sensitivity, specificity, and area under the ROC curve (AUC-ROC) for the RETIC-HGB cut-off value of 12.5 pg and for the CHr cut-off value of 14.0 pg in cats with iron-limited erythropoiesis (ILE)

	Sensitivity (95% CI)	Specificity (95% CI)	AUC-ROC (95% CI)
RETIC-HGB (12.5 pg)	5% (0.2-30.2)	97% (94.5-98.9)	0.66 (0.55-0.76)
CHr (14.0 pg)	35% (18.1-56.7)	87% (82.6-90.9)	0.74 (0.63-0.84)

Abbreviations: AUC-ROC, area under the receiver operating characteristic (ROC) curve; CHr, reticulocyte hemoglobin content (Siemens ADVIA 120 or 2120/2120i); CI, confidence interval; RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx).

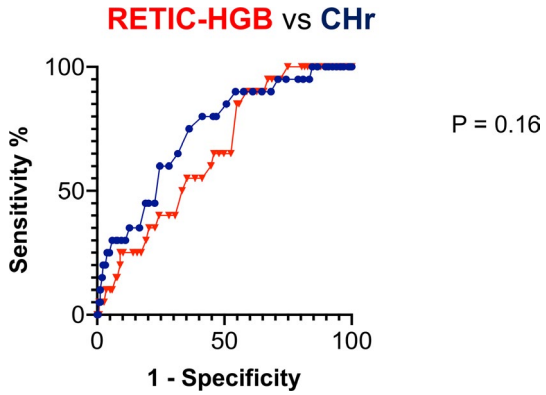

FIGURE 5 A receiver operating characteristic (ROC) curve analysis regarding the diagnostic use of reticulocyte hemoglobin equivalents (RETIC-HGB) and reticulocyte hemoglobin content (CHr) in diagnosing iron-limited erythropoiesis (ILE). CHr, reticulocyte hemoglobin content (Siemens ADVIA 120 or 2120/2120i); RETIC-HGB, reticulocyte hemoglobin equivalent (IDEXX ProCyte Dx); *P*, level of significance

The sensitivity, specificity, and AUC-ROCs of the RETIC-HGB and CHr cut-off values for the diagnosis of ILE are shown in Table 5 and Figure 5. The AUC-ROCs revealed low (RETIC-HGB) and moderate (CHr) accuracy,[28] respectively. Using the lower limit of the RI as the cut-off value resulted in a low sensitivity and relatively high specificity in being able to diagnose ILE in cats. The AUC-ROC had a broad 95% confidence interval.

4 | DISCUSSION

Except for the manufacturer's operating guidelines,[20] current literature does not provide any information regarding RETIC-HGB RIs in cats. Compared with the manufacturer's RI (13.2-20.8 pg), the RI determined in this study (12.5-18.0 pg) was slightly narrower and had a marginally lower upper limit. Moreover, the RI was well below the RIs published for dogs (ie, 22.3-29.6 pg[20] and 22.2-28.6 pg[29]) and had a slightly narrower range. Similar results have been found for CHr (1.43-1.71 fmol [23.0-27.6 pg] in dogs and 0.88-1.22 fmol [14.2-19.7 pg] in cats).[17] The smaller cellular volume of feline compared with canine reticulocytes could explain the lower feline RI. Currently, there is a paucity of information available for feline RIs of CHr. Our CHr RI shows a high agreement with a previously published RI[17] (ie, 14.0-19.9 pg in our study vs 14.2-19.7 pg in the previous study, if the units are converted into pg). The conversion factors used (pg = fmol × 16.11) were primarily established for human blood samples, and the application of this conversion must be regarded as a certain limitation of this study. Additionally, IDEXX Laboratories,[20] as well as PRINS et al[17] established their RIs using a larger population (n = 145, personal communication Dennis DeNicola, 2019 and n = 150, respectively).

Based on the WCI/WRI ratio, the imprecision of limits of the RIs, and thus, the number of reference samples used in this study could be considered suitable. For RETIC-HGB, the WCI/WRI ratio of the lower reference limit exceeded 0.2. However, given the slight exceedance of 0.02, the RI might still be considered useable.

Since the German cat population mainly consists of Domestic Shorthair cats, determination of RIs based on a population predominantly consisting of this breed (n = 39/59; 66.1%) should be considered suitable. However, an influence of certain breeds (ie, pedigree cats) has been demonstrated for some hematologic and clinical chemistry variables[30] and has to be taken into consideration. The age distribution of our healthy cat population could be considered representative as similar distributions were found in demographic studies of English and Australian cat populations.[31,32]

The overall stability of RETIC-HGB (Figure 2) is in accordance with human[18] and canine studies.[17,29] In contrast to dogs, human RETIC-HGB increased over time without statistical significance (*P* = .14). Consequently, RETIC-HGB might be falsely increased in older blood samples (≥2 days). Accordingly, storage-related RBC and reticulocyte swelling can lead to physiologic or even increased RETIC-HGB values, despite the presence of ID,[25] possibly contributing to a misinterpretation of the actual iron status. As the ProCyte Dx represents an in-house hematology analyzer, sample storage at room temperature for more than 24 hours is unlikely.[29] Prolonged sample storage should, therefore, be considered a negligible factor.

The intra-assay CV of 3.3% reflected a precise and repeatable RETIC-HGB measurement and is in accordance with human studies.[18] In cats, the number of punctate reticulocytes is much greater than in dogs.[33] As the distinction between aggregate and punctate reticulocytes is often difficult using automated hematology analyzers, a higher CV (3.3%) is plausible in cats than in dogs (CV 1.8%[27,34]) that only have aggregate reticulocytes. Additionally, as reported for CHr, a higher CV might be explained by lower reticulocyte counts in cats.[17]

The correlation between RETIC-HGB and CHr was moderate (r_s = 0.59). This result is interesting in the face of similar RIs obtained for both variables and might be explained by the different methodologies

of both hematology analyzers.[29] CHr is calculated as the product of MCVr and CHCMr (CHr = MCVr × CHCMr). The ProCyte Dx uses the mean value of the forward scattered light of reticulocytes labeled with fluorescent dyes (RET-Y) to determine the hemoglobin equivalent of reticulocytes (RET-He, new term RETIC-HGB).[10] Finally, the correlation of RET-Y with MCH of the respective cell population (reticulocytes) results in a mathematical transformation into RET-He/RETIC-HGB.[29] Thus, cellular variables affecting light scattering, such as individual cellular sizes, likely resulted in greater RETIC-HGB changes. For example, false-low RETIC-HGB levels could be found in cases of microcytosis, whereas they could be falsely high with macrocytosis.[25] Thus, macrocytic anemias can lead to physiologic or even increased RETIC-HGB values, despite the presence of ID. Due to the relationship described above, RETIC-HGB is not suitable for assessing iron status in macrocytic anemia.[29] AID or FID are predominantly associated with the presence of microcytic-hypochromic anemias, resulting in decreased RETIC-HGB concentrations due to both a decrease in cellular size and a decrease in hemoglobin content.[29] It can thus be assumed that RETIC-HGB can be used as an indicator of iron status in the presence of both AID and FID.

Automated hematology analyzers primarily determine aggregate but not punctate reticulocytes.[35] Therefore, the reticulocyte count reported reflects aggregate reticulocytes. However, a small overestimation of the reticulocyte count has been reported for feline samples analyzed on Sysmex analyzers. A possible explanation for this finding is the inclusion of a low number (approximately 6%) of punctate reticulocytes into the analyzer's aggregate reticulocyte count.[35] The low proportion of punctate reticulocytes included might potentially vary between different analyzers and methodologies and is another potential reason for the relatively low correlation between RETIC-HGB and CHr (r_s = 0.59 in cats vs r_s = 0.74 in dogs[29]). The low proportion of punctate reticulocytes counted could further contribute to a possible misinterpretation of RETIC-HGB in cats. Punctate reticulocytes are more mature than aggregate reticulocytes and reflect iron status from the past two weeks rather than the iron status of the past two to four days, as in the case of aggregate reticulocytes.[36]

Criteria for diagnosis of ILE included either the presence of anemia and/or decreased MCH/MCV. Therefore, a bias due to these inclusion criteria has to be taken into consideration. Based on the significant decreases in RETIC-HGB and CHr in ILE cats, it is likely that reticulocyte hemoglobin content is a suitable variable for the diagnosis of ILE.[4]

The presence of inflammatory processes (17/20 ILE cats) combined with significantly decreased RETIC-HGB values indicated cytokine-mediated iron sequestration. Lipopolysaccharides (LPS) and interleukin-6 (IL-6) induce hepcidin expression,[15] resulting in increased uptake and retention of iron within macrophages.[37] Consequently, the limited availability of iron will lead to the inhibition of erythropoiesis.[3]

Currently, there is no true gold standard for diagnosing ID.[11,17] Bone marrow (BM) aspirates have been regarded as the gold standard for diagnosing AID in people. Nevertheless, the absence of stainable iron is not necessarily associated with IDA.[38] Cats lack stainable iron (hemosiderin) in their BM,[39] and therefore, BM samples cannot be used to evaluate feline iron status. Variables of iron metabolism (ie, serum iron concentration, TIBC, %TfS) can be altered due to various pathologic mechanisms and are, thus, not always effective in detecting ILE.[9] In addition to the lack of a gold standard, the lack of further biochemical testing (eg, determination of ferritin or the soluble transferrin receptor [sTfR]) was a limiting factor of this study. sTfR has not yet been evaluated in veterinary medicine,[29] and problems with the standardization of sTfR in human medicine remain.[15] Ferritin could provide a decision-making aid in the presence of AID and FID.[8] However, there is limited availability of a species-specific ELISA suitable for assessing feline serum ferritin concentrations. Moreover, the sample volumes of leftover serum blood samples included were relatively low, so ferritin was not measured in this study.

Due to the overlapping results between individual disease groups, neither CHr nor the variables of iron metabolism are suitable to reliably differentiate between underlying diseases that ultimately result in ILE.

CHr has been shown to be an early marker of ILE and, therefore, should begin to decrease before conventional variables can identify ILE.[4,18] In this study, however, AUC-ROC values for CHr revealed only moderate test performances (AUC-ROC = 0.74). Better test performance was found in a canine study (AUC-ROC = 0.87-0.91),[11] in which ID was induced by feeding an iron-depleted diet. A study evaluating CHr cut-off values to detect ILE in anemic cats using a cut-off value of 0.88 fmol (14.2 pg if the units were converted into pg) revealed a high sensitivity (93.8%) and an acceptable specificity (76.9%).[17] Cats included in our study did not have to be anemic to be diagnosed with ILE, likely contributing to the lower sensitivity found. Moreover, the proportion of ILE cats was markedly lower than in the previous study[17] and probably contributed to the low sensitivity in this study. It has been demonstrated that the prevalence of a disease significantly influences the test sensitivity. A meta-analysis of 416 studies[40] with median disease prevalence ranging from 1% to 77% revealed that a "change in disease prevalence from the lowest to highest value"[40] resulted in a corresponding change in sensitivities and specificities of up to 40%. The AUC-ROC values for feline RETIC-HGB were well below the AUC-ROCs published in dogs[29] (AUC 0.90-0.91) using similar inclusion criteria for the diagnosis of ILE. Still, due to the lack of a gold standard for detecting ILE, a reasonable quantification of test performance is arguable and complicates the use of cut-off values.[11,17,29] Additionally, serum iron concentration and %TfS were used to classify ILE. As previously described,[29] any variation in these variables (iron, TIBC, %TfS) influences test performance.

Despite all the limitations, CHr and RETIC-HGB could be regarded as complementary markers for the detection of developing ILE, as they will be provided automatically with every hematologic analysis performed on the Siemens ADVIA and ProCyte Dx hematology analyzers. Both variables are available for routine analyses, even in a point-of-care setting,[29] and provide relatively inexpensive screening tools in suspected ILE cases. Currently, RETIC-HGB is not a specific criterion to differentiate between AID or FID.[8] Thus,

further studies are required for RETIC-HGB evaluations based on a larger and more extensive sample selection.[29]

5 | CONCLUSIONS

CHr and RETIC-HGB are precise and stable variables reflecting feline reticulocyte hemoglobin content and are significantly decreased in cats with ILE. However, the sensitivity for detecting ILE is markedly lower in cats than in dogs limiting the diagnostic usefulness of these variables. The moderate correlation between RETIC-HGB and CHr is likely due to species differences and the different methodologies.

ACKNOWLEDGEMENTS

Open access funding enabled and organized by Projekt DEAL.

ORCID

Miriam Keiner https://orcid.org/0000-0001-5025-9174

REFERENCES

1. Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. *Clin Chem*. 2003;49(10):1573-1578.
2. Case LP, Daristotle L, Hayek MG, Raasch MF. Chapter 6: Minerals. In: Case LP, Daristotle L, Hayek MG, Rausch MF, eds. *Canine and Feline Nutrition: A Resource for Companion Animal Professionals*. 3rd ed. Maryland Heights, MO: Elsevier Mosby; 2010:40-41.
3. Weiss G. Anemia of chronic disease. *N Engl J Med*. 2005;134.
4. Steinberg JD, Olver CS. Hematologic and biochemical abnormalities indicating iron deficiency are associated with decreased reticulocyte hemoglobin content (CHr) and reticulocyte volume (rMCV) in dogs. *Vet Clin Pathol*. 2005;34(1):23-27.
5. Major A, Mathez-Loic F, Rohling R, Gautschi K, Brugnara C. The effect of intravenous iron on the reticulocyte response to recombinant human erythropoietin. *Br J Haematol*. 1997;98(2):292-294.
6. Briggs C. Quality counts: new parameters in blood cell counting. *Int J Lab Hematol*. 2009;31(3):277-297.
7. Goodnough LT. Iron deficiency syndromes and iron-restricted erythropoiesis (CME). *Transfusion*. 2012;52(7):1584-1592.
8. Meléndez-Lazo A, Tvarijonaviciute A, Cerón JJ, Planellas M, Pastor J. Evaluation of the relationship between selected reticulocyte parameters and inflammation determined by plasma C-reactive protein in dogs. *J Comp Pathol*. 2015;152(4):304-312.
9. Schaefer DMW, Stokol T. The utility of reticulocyte indices in distinguishing iron deficiency anemia from anemia of inflammatory disease, portosystemic shunting, and breed-associated microcytosis in dogs. *Vet Clin Pathol*. 2015;44(1):109-119.
10. Thomas C, Thomas L. Anemia of chronic disease: pathophysiology and laboratory diagnosis. *Lab Hematol*. 2005;11(1):14-23.
11. Fry MM, Kirk CA. Reticulocyte indices in a canine model of nutritional iron deficiency. *Vet Clin Pathol*. 2006;35(2):172-181.
12. Thomas DW, Hinchliffe RF, Briggs C, Macdougall IC, Littlewood T, Cavill I. Guideline for the laboratory diagnosis of functional iron deficiency. *Br J Haematol*. 2013;161(5):639-648.
13. Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M.M. Wintrobes's original 1934 classification to the third millennium. *Curr Opin Hematol*. 2013;20(3):222-230.
14. Piva E, Brugnara C, Chiandetti L, Plebani M. Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis. *Clin Chem Lab Med*. 2010;48(10):1369-1380.
15. Petkova NY, Raynov JI, Petrova DY, Ramsheva ZN, Petrov BA. Diagnostic significance of biomarkers of iron deficiency for anemia in clinical practice. *Folia Med*. 2019;61(2):223-230.
16. Vázquez-López MA, López-Ruzafa E, Ibáñez-Alcalde M, Martín-González M, Bonillo-Perales A, Lendínez-Molinos F. The usefulness of reticulocyte haemoglobin content, serum transferrin receptor and the sTfR-ferritin index to identify iron deficiency in healthy children aged 1–16 years. *Eur J Pediatr*. 2019;178(1):41-49.
17. Prins M, van Leeuwen MW, Teske E. Stability and reproducibility of ADVIA 120-measured red blood cell and platelet parameters in dogs, cats, and horses, and the use of reticulocyte haemoglobin content (CHR) in the diagnosis of iron deficiency. *Tijdschr Diergeneeskd*. 2009;134(7):272-278.
18. Brugnara C, Schiller B, Moran J. Reticulocyte hemoglobin equivalent (Ret He) and assessment of iron-deficient states. *Clin Lab Haematol*. 2006;28(5):303-308.
19. Fuchs J, Moritz A, Grußendorf E, et al. Canine reticulocyte hemoglobin content (RET-He) in different types of iron-deficient erythropoiesis. *Vet Clin Pathol*. 2017;46(3):422-429.
20. IDEXX Laboratories. Reference intervals for the IDEXX ProCyte Dx*. Hematology Analyzer. 2019. https://www.idexx.com/files/procyte-dx-reference-ranges-en.pdf
21. Geffré A, Concordet D, Braun J-P, Trumel C. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel: Reference Value Advisor. *Vet Clin Pathol*. 2011;40(1):107-112.
22. Friedrichs KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 2012;41(4):441-453.
23. Harris EK, Boyd JC. On dividing reference data into subgroups to produce separate reference ranges. *Clin Chem*. 1990;36(2):265-270.
24. Cowgill ES, Neel JA, Grindem CB. Clinical application of reticulocyte counts in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2003;33(6):1223-1244.
25. David O, Grillo A, Ceoloni B, et al. Analysis of red cell parameters on the Sysmex XE 2100 and ADVIA 120 in iron deficiency and in uraemic chronic disease. *Scand J Clin Lab Invest*. 2006;66(2):113-120.
26. Hansen AE, Schaap MK, Kjelgaard-Hansen M. Evaluation of a commercially available human serum amyloid A (SAA) turbidimetric immunoassay for determination of feline SAA concentration. *Vet Res Commun*. 2006;30(8):863-872.
27. Bauer N, Nakagawa J, Dunker C, Failing K, Moritz A. Evaluation of the automated hematology analyzer Sysmex XT-2000 i VTM compared to the ADVIA® 2120 for its use in dogs, cats, and horses. Part II: Accuracy of leukocyte differential and reticulocyte count, impact of anticoagulant and sample aging. *J Vet Diagn Invest*. 2012;24(1):74-89.
28. Swets JA. Measuring the accuracy of diagnostic systems. *Science*. 1988;240(4857):1285-1293.
29. Fuchs J, Moritz A, Grußendorf E, et al. Evaluation of reticulocyte hemoglobin content (RET-He) in the diagnosis of iron-deficient erythropoiesis in dogs. *Vet Clin Pathol*. 2017;46(4):558-568.
30. Reynolds BS, Concordet D, Germain CA, Daste T, Boudet KG, Lefebvre HP. Breed dependency of reference intervals for plasma biochemical values in cats: reference intervals for cats. *J Vet Intern Med*. 2010;24(4):809-818.
31. Toribio J-ALM, Norris JM, White JD, Dhand NK, Hamilton SA, Malik R. Demographics and husbandry of pet cats living in Sydney, Australia: results of cross-sectional survey of pet ownership. *J Feline Med Surg*. 2009;11(6):449-461.
32. Sánchez-Vizcaíno F, Noble P-JM, Jones PH, et al. Demographics of dogs, cats, and rabbits attending veterinary practices in Great Britain as recorded in their electronic health records. *BMC Vet Res*. 2017;13(1):218.

33. Alsaker RD, Laber J, Stevens J, Perman V. A comparison of polychromasia and reticulocyte counts in assessing erythrocytic regenerative response in the cat. *J Am Vet Med Assoc*. 1977;170(1):39-41.
34. Nabity MB, Harr KE, Camus MS, Flatland B, Vap LM. ASVCP guidelines: allowable total error hematology. *Vet Clin Pathol*. 2018;47(1):9-21. https://doi.org/10.1111/vcp.12583
35. Lilliehöök I, Tvedten H. Validation of the Sysmex XT-2000iV hematology system for dogs, cats, and horses. I. Erythrocytes, platelets, and total leukocyte counts. *Vet Clin Pathol*. 2009;38(2):163-174.
36. Reagan WJ, Vap LM, Weiser MG. Flow cytometric analysis of feline reticulocytes. *Vet Pathol*. 1992;29(6):503-508.
37. Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood*. 2003;101:4148-4154.
38. Ganti AK, Moazzam N, Laroia S, Tendulkar K, Potti A, Mehdi SA. Predictive value of absent bone marrow iron stores in the clinical diagnosis of iron deficiency anemia. *In Vivo*. 2003;17(5):389-392.
39. Bohn AA. Diagnosis of disorders of iron metabolism in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2013;43(6):1319-1330.
40. Leeflang MMG, Rutjes AWS, Reitsma JB, Hooft L, Bossuyt PMM. Variation of a test's sensitivity and specificity with disease prevalence. *CMAJ*. 2013;185(11):537-544.

How to cite this article: Keiner M, Fuchs J, Bauer N, Moritz A. Evaluation of reticulocyte hemoglobin content (RETIC-HGB) for the diagnosis of iron-limited erythropoiesis in cats. *Vet Clin Pathol*. 2020;49:557–566. https://doi.org/10.1111/vcp.12925