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ORIGINAL ARTICLE

Evaluation of a novel moving threshold gating strategy for assessment of reticulated platelets in dogs using the ADVIA 2120 analyzer

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Abstract

Background: A novel method using a moving threshold (r-PLTmt) to determine canine r-PLTs (reticulated platelets) has been introduced for ADVIA 2120 software v6.11.7.

Objectives: We aimed to evaluate absolute (ar-PLTmt) and percent (%r-PLTmt) prior to and after visual inspection of scattergrams (ar-PLTmtv, %rPLTmtv) compared with flow cytometry (flow) and to determine reference intervals (RIs) in 120 dogs.

Methods: For method comparison, 42 blood samples of healthy and thrombocytopenic dogs were included. Calculation of Spearman's rho, Bland–Altman, and Passing-Bablok analysis was performed. Coefficients of variation (CVs) were determined for three concentration levels.

Results: Moderate correlations between %r-PLTmt and %r-PLTmtv (r_s 0.75–0.76) were seen compared with flow cytometry. The CV for medium %r-PLTs counts assessed with flow cytometry was 12.9%. Comparable CVs were obtained for ar-PLTmt (14.4%) and %r-PLTmt (15.7%), and ar-PLTmtv and %r-PLTmtv (10.9% and 12.9%, respectively). At low and high concentration levels, CVs for % and absolute r-PLTmt/rPLTmtv ranged between 23%–30% and 15%–20%. In patients with microcytic hypochromic erythrocytes, CVs for ar-PLTmt and %r-PLTmt were 36%–66%. Visual inspection of scattergrams resulted in a marked decrease in CV ranging between 15% and 20%. A proportional bias of 10.8% between %r-PLTmt and flow cytometry became lower (9.7%) after visual validation of scattergrams. Passing-Bablok analysis showed proportional and constant error. RIs for r-PLTmt and r-PLTmtv were 0.2%–3.8% and 0.6–10.2 × 10^9/L and 0.3%–4.5% and 1.1–10.3 × 10^9/L, respectively. Median values for %r-PLTmtv were higher in young adults (≤2 years) than in older dogs ($P = 0.03$).

Conclusions: r-PLTmt and r-PLTmtv were moderately correlated with flow cytometry. Visual inspection of scattergrams is recommended.

KEYWORDS
automated measurement, canine, flow cytometry, hematology, immature platelets, scattergram

1 | INTRODUCTION

Reticulated platelets were first described in 1969 by Ingram and Coopersmith while examining blood smears stained with methylene blue in dogs with acute blood loss. Because the stained, coarse material visible in the platelets appeared analogous to that seen in reticulated red blood cells, they were subsequently referred to as reticulated platelets.[1]

Under physiologic conditions, an early flow cytometric study in people using thiazole orange showed that approximately 8% of circulating platelets were reticulated.[2] The number of reticulated platelets (r-PLT) in bone marrow is, on average, 2–3 times higher than that in peripheral blood, and r-PLT has been shown to correlate with the number of megakaryocytes in the bone marrow.[3] Mature platelets in dogs have a lifespan of 6 days in the peripheral blood,[4] whereas reticulated platelets are only detectable for 1 day.[5]

Kienast and Schmitz[2] recognized the diagnostic potential of reticulated platelets (r-PLT) as a marker of thrombopoiesis. They hypothesized that r-PLTs, like reticulocytes, reflect bone marrow thrombopoiesis activity. Moreover, Kienast and Schmitz demonstrated that the percentage of r-PLT (%r-PLTs) measurements showed a sensitivity and specificity of 95% for differentiating between reduced thrombopoiesis at the bone marrow level or increased thrombopoiesis with destruction or PLT loss in the periphery.[2] To determine the underlying etiology of thrombocytopenia and predict an imminent increase in PLTs in the peripheral blood, measurement of %r-PLT is preferred over ar-PLT measurements by many authors. In both human[2,6–10] and veterinary medicine,[11–16]%r-PLT measurements have been used to differentiate between reduced thrombopoiesis and increased consumption or demand as flow cytometry served traditionally as the reference method for the detection of r-PLTs. However, platelet staining with thiazole orange and monoclonal antibodies is both labor-intensive and time-consuming. A major advantage of using automated hematology analyzers compared with flow cytometry to detect r-PLTs is the cost and time savings and minimization of sources of error, demonstrating practical usability in routine diagnostics.[17]

In veterinary medicine, the measurement of r-PLTs using automated hematology devices was established in 2009 with the Sysmex XT-2000iV.[12] In addition, reference intervals (RIs) for canine r-PLTs were determined on the Sysmex XT-2000iV, although manual gates needed to be set in the user software to accurately determine r-PLT numbers.[18] The ADVIA 2120 can determine r-PLTs using a fixed gating strategy. The software for the flow cytometric data has also been validated for use in dogs.[19] A disadvantage of the previously used fixed gating strategy was that inter-individual variations in morphology and the localization of PLT populations were not considered. Moreover, results were not easily reported during routine analysis as a separate software program was needed. Using fixed gates might also be prone to error due to changes associated with routine hematology analyzer maintenance. Recently, a novel method was introduced for the software version v6.11.7 on the ADVIA 2120 automated hematology analyzer using a moving threshold. In this way, the boundary between mature and immature platelets could be adapted with each measurement to the platelet size of each individual dog. In addition, using a moving threshold is hypothesized to allow better discrimination of reticulated platelets from erythrocytes and to minimize interferences caused by microcytic, hypochromic erythrocytes and lymphocytes.

Thus, it was the objective of our study to evaluate (1) the analytical performance of the ADVIA 2120 r-PLT measurement and (2) perform a method comparison, including comparing the novel method of r-PLT detection using a moving threshold prior to (r-PLTmt) and after visual inspection of scattergrams (r-PLTmtv) with flow cytometry (CD61/thiazol orange) serving as the reference method, whereby precision and total observed error (TE_{obs}) of all methods was determined, and (3) provide RIs for ADVIA 2120 r-PLTs determined with the novel method. We hypothesized that using software version v6.11.7 on the ADVIA 2120 with this novel gating strategy would allow more accurate detection of r-PLT than previously reported using a fixed gating strategy.

2 | MATERIALS AND METHODS

2.1 | Method comparison

The method validation study included a comparison between the r-PLT analysis using novel v6.11.7 software with a moving threshold (r-PLTmt) on an ADVIA 2120 analyzer (prior to and after visual inspection of scattergrams) and flow cytometry.

2.1.1 | Study population

For the method validation part, 42 residual EDTA blood samples were collected from 41 dogs that were healthy ($n = 12$) or diseased ($n = 29$). Of the diseased dogs, 22 were thrombocytopenic. Blood samples from healthy dogs were taken as part of a pre-anesthetic examination for X-ray examinations for hip joint/elbow dysplasia or a general health check. Diseased dogs were presented for diagnostic workup or follow-up examinations mainly due to internal medical conditions. The inclusion criterion was sufficient sample volume to perform a flow cytometric analysis and valid flow cytometric results.

2.1.2 | Flow Cytometry

Fluorescent staining of platelets

K3-EDTA tubes (Sarstedt, Numbrecht, Germany) whole blood samples were analyzed within 4 hours after collection using a modified protocol according to Pankraz et al.[12] For platelet labeling, a CD61 antibody (Mouse Anti Pig CD61, clone JM2E5, product number MCA 2263GA, Bio-Rad Laboratories GmbH, Munich, Germany, dilution 1:400) was used, which shows cross-reactivity for canine cells based on the manufacturer's datasheet. Using a conjugation kit (Lynx rapid

APC Antibody Conjugation Kit, LNK031APC, Bio-Rad), the primary antibody was coupled with Allophycocyanin (APC). The residual RNA of the platelets was represented by TO (thiazole orange) staining. Titration experiments were performed to determine an appropriate working dilution of 1:400 (2.45 μg/ml) for the coupled antibody. A 50 μl sample of whole blood was mixed with 450 μl phosphate-buffered saline wash buffer. All buffers were filtered through 0.1-μm syringe filters (Whatman Puradisc 25, Sigma Aldrich) prior to use. The mixture was transferred to 5 ml polystyrene round-bottom tubes with an integrated cell strainer and thoroughly mixed. Then, 50 μl of the solution (containing approximately 1–2 × 10^6 platelets in healthy dogs) was transferred to Eppendorf reaction tubes, and 20 μl of the CD61 antibody conjugated with APC was added to the solution and incubated for 20 minutes in room temperature (22°C) in the dark. Then, 500 μl TO (BD Retic-Count - 349 204, Becton, Dickinson, and Company) and 500 μl wash buffer were added, followed by incubation for 60 minutes at 22°C in the dark. Prior to flow cytometric analysis, samples were diluted 1:10 with wash buffer to avoid agglutination within the instrument.

Mounts without TO and with the corresponding isotype control (Mouse IgG1 negative Control, low Endotoxin, MCA1209EL, Bio-Rad, also labeled with APC) to CD61 antibody served as negative controls.

Flow cytometric analysis

Flow cytometric detection of r-PLT was performed on the BD Accuri C6 flow cytometer (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) equipped with BD Accuri C6 Analysis Software for data analysis. Prior to measuring patient samples, internal quality control was performed using validation beads distributed by the instrument manufacturer (Spherotech 8-Peak Validation Bead—PN 653144, Spherotech 6-Peak Validation Bead—PN 653145). In the first step, the platelet population was identified based on their characteristic size and granularity in a forward and side light-scatter dot plot with logarithmic amplification. From each sample, 30 000 events were analyzed at a slow flow rate (14 μl/min). Thiazole orange fluorescence was collected through a 530/30-nm bandpass filter with logarithmic amplification, and CD61-APC fluorescence was collected through a 675/25-nm bandpass filter with logarithmic amplification. The corresponding detector for CD61-APC was in fluorescence channel 4 (FL-4) of the instrument, and the corresponding detector for TO was in fluorescence channel 1 (FL-1). To restrict the analysis to platelets, a combination of their light scatter properties and CD61 expression was used. Thus, in the second step, the CD61-APC-positive events were displayed in the forward and side light-scatter dot plot, and the gate around the platelet cloud was verified and adjusted. Only events that had the light scatter properties of platelets were further analyzed. The percentage of r-PLT was determined from the subset of FL-1/FL-4 positive platelets in relation to the total number of FL-4 positive platelets. The threshold for TO and APC-positive events was determined with the help of negative and isotype controls. A maximum of 1% positive events were allowed to occur in the r-PLT area of the negative controls for all measurements. If the negative control showed positive events in the r-PLT gate, these were subtracted from the results. The applicability of the fixed gate was thus checked for all measurements. In healthy individuals, a maximum percentage of 10% reticulated platelets was expected.[20] All tests were carried out in duplicates, and the mean value was used for statistical analysis.

2.1.3 | ADVIA 2120

Blood analysis was carried out on an ADVIA 2120 laser-based automated hematology analyzer (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) with novel multispecies system software v6.11.7 for ADVIA 2120 and 2120i using K3-EDTA whole blood samples. The hematology analyzer worked with the dog set to produce a differential blood count with reticulocytes (CBC/DIFF/RETIC). The routine calibration of the devices was carried out by the manufacturer. Maintenance was performed in accordance with the manufacturer's specifications. Prior to the measurement of patient samples, internal quality control (QC) was performed using QC material at three concentration levels (low, medium, and high) provided by the manufacturer (ADVIA Testpoint, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Erythrocytes (RBC) and platelets (PLT) were simultaneously detected in the ADVIA 2120 RBC/PLT channel. Based on cellular volume and cellular complexity, PLTs were displayed in the ADVIA 2120 PLT scattergram (Figure 1A). In contrast, R-PLTs were detected in the reticulocyte channel after staining with the nucleic acid dye oxazine 750. Optical measurements were performed at 670 nm. The scattered light was collected by detectors at low (2°–3°) and high angles (5°–15°) and as absorption. Light absorption was proportional to the RNA content of the cells. Low and high angle light scatter was proportional to cell size and refractive index, respectively. The absorption and scattered light measurements are displayed in the RETIC scattered light absorption cytogram (Figure 1B,C, gate rPLT, thereafter named PLT absorption scattergram).

2.1.4 | Automated reticulated platelet measurements using v6.11.7 software on the ADVIA 2120

Based on their absorption characteristics, the oxazine 750-stained platelets were displayed as a histogram. The maximum absorption peak of PLT histograms in each dog was automatically detected; a constant internal calculation unit of the software was added to obtain the moving threshold to differentiate between mature and reticulated platelets (Figure 1). An additional moving threshold was then automatically calculated to allow for optimal discrimination of reticulated platelets from erythrocytes. The calculation was based on several algorithms to minimize interference caused by hypochromic erythrocytes and lymphocytes. In this way, for each measurement, the boundary between mature and immature platelets was

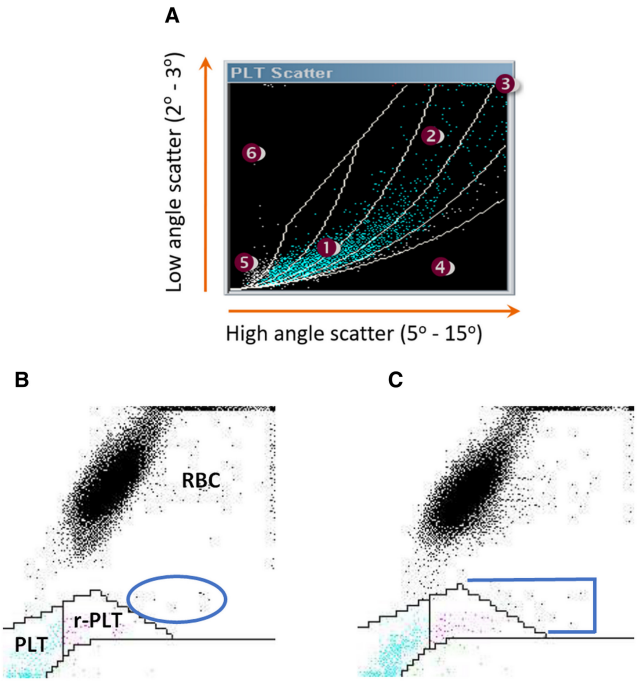

FIGURE 1 A, ADVIA 2120 PLT platelet scattergrams showing (1) platelets, (2) large platelets, (3) red blood cells, (4) RBC fragments, (5) debris, and (6) RBC ghosts. B, ADVIA 2120 platelet absorption cytogram (dog) showing the relative positions of red blood cells (RBC), platelets (PLT), and reticulated platelets (r-PLT). The blue oval region includes events directly extending from the r-PLT population located within the gate optimized for people, such that they were thought to represent canine r-PLTs. C, Example of a platelet absorption scattergram (dog) of an ADVIA 2120 scattergram with the r-PLT showing the validation of the gate (blue line) to include those r-PLTs located outside the automatically set human r-PLT gate.

adapted to the distribution of the platelet population in each dog. Based on the analyzer's automated gating, the absolute number of signals consistent with r-PLTmt was determined within the platelet absorption scattergram (Figure 1, gate rPLT).

Then, the %r-PLTmt was calculated using the number of r-PLTmt signals (sig r-PLT, unitless) located within the platelet absorption scattergram (Figure 1A, gate rPLT) and the number of signals from total platelets (sig-PLT, unitless) located within the platelet scattergram (Figure 1A). The absolute reticulated platelet count (ar-PLTmt, unit 10^9/L) was then determined by multiplying the absolute PLT count (unit 10^9/L) obtained from the ADVIA 2120 RBC/PLT channel and %r-PLTmt:

$$\text{ar} - \text{PLTmt} = \text{PLT} / 100 \times \%\,\text{r} - \text{PLTmt}$$

2.1.5 | Visual validation of automated canine reticulated platelet measurements on the ADVIA 2120

During the early stages of the study, we frequently observed that events extended beyond the automatically set r-PLT absorption gate (Figure 1A, blue oval region). As the events directly extended from the r-PLTmt population, we considered the events to be r-PLTs. Thus, we included a visual inspection step for every r-PLT absorption scattergram in our study protocol, and the r-PLT numbers were reported prior to the r-PLTmt counts, and after visual inspection and manual adjustment of r-PLT counts.

For manual adjustment of sig r-PLT counts, the reticulated platelet gate was extended on the *x* axis so that signals thought to be reticulated platelets but were excluded by the automated gate were now included. The included signals were counted manually and added to the automatically measured signals. Then, the percentage and absolute r-PLTmt were re-determined using the validated gate. Results obtained after visual inspection of the r-PLTmt counts were designated as r-PLTmtv (Figure 1B). The degree of manual adjustment was classified as mild (4–10 signals), moderate (11–20 signals), and marked (>21 signals), respectively, using an arbitrary grading scheme.

2.1.6 | Precision

The 10-run intra-assay precision of blood samples with low %rPLT (for this method) was assessed with the BD Accuri C6. For The ADVIA 2120, 10-run intra-assay precision was determined in a blood sample with low, medium, and high %rPLTs. Furthermore, 11-fold and 9-fold repeat measurements were carried out in two respective samples, each containing high numbers of microcytic and hypochromic erythrocytes as a potential source of interference.

The intra-assay CV was calculated as follows based on the mean (M) and standard deviation (SD):

$$\text{CV}(\%) = \frac{\text{SD}}{\text{M}} \times 100$$

To date, no quality requirements for the desired canine r-PLT measurement CV are available. The observed (analytical) CV (CV_a) was compared with quality requirements derived from biologic variation CVs, which were represented by an intraindividual CV [CV_i] of 25%, as described for human %r-PLT measurements and represented here as the % immature PLT fraction (IPF) obtained with the Sysmex XN analyzer. Quality requirements were fulfilled if:

$$CV_a < 0.5 \times CV_i; \text{ie}, CV_a < < 4\%.$$

2.1.7 | Total observed error

The total observed error (TE_{obs}) was calculated from the intra-assay-CV and % bias ($TE_{obs} = 2 \times CV\,[\%] + \text{bias}[\%]$) for comparisons between r-PLTmt/r-PLTmtv and flow cytometry. Due to the absence of r-PLT quality requirements in veterinary medicine, TE_{obs} were compared with the total allowable error (TE_a) of 18.4% (range 14.6%–24.7%), which was established for the analysis of human% r-PLTs (%IPF assessed with the Sysmex XN hematology analyzer) based on biologic variation.[21]

2.1.8 | Statistical analysis for the method comparison

Statistical evaluation for the method comparison was carried out using SPSS Statistics Version 25 for Windows (IBM) and Stata Statistics Version 16.1 for Windows (StataCorp). Evaluation of the r-PLT measurements using the automated method with a moving threshold and the modified method for dogs on the ADVIA 2120 compared with flow cytometry was carried out using the Spearman's rank correlation coefficient, and Bland–Altman and Passing-Bablok analyses. Furthermore, a test for linearity was carried out, and Lin's concordance correlation coefficient was determined. The flow cytometric determination of the r-PLTs on the BD Accuri C6 flow cytometer served as the reference method. The correlation was judged as "excellent" for a Spearman's rank correlation coefficient (r_s) = 0.93–0.99, "good" for an r_s = 0.80–0.92, "fair" for an r_s = 0.59–0.79, and "poor" for an r_s <0.59.[22]

2.2 | Establishment of reference intervals

2.2.1 | Study population

The RIs for r-PLTmt and r-PLTmtv for dogs were determined using 120 blood samples from healthy adult dogs that were presented in the clinic for small animals at the Justus Liebig University as part of an expert report for hip/elbow dysplasia, blood donation, or a general health check between March 2017 and August 2017. The dogs were healthy based on history, physical examination, and the absence of significant hematologic and clinical chemical abnormalities. Blood specimens were analyzed within 4 hours after sampling.

2.2.2 | Statistical analysis for RI development

The RIs were determined using Reference Value Advisor Version 2.1 for Microsoft Excel.[23] According to the guidelines of the ASVCP (American Society of Veterinary Clinical Pathology), the population of dogs was sufficiently large ($n \geq 120$) such that the non-parametric method could be used to calculate the reference intervals.[24] The 90% confidence interval of the reference interval was determined using the non-parametric bootstrap method. Data distribution was assessed using *Q*–*Q* plots. Dixon's range statistic and Tukey's interquartile fences were used to detect potential outliers. One outlier was detected that was not removed as it did not have an impact on the calculation of RIs using the non-parametric method. The RIs were created for both the automated and the modified ADVIA 2120 measurement method for r-PLTs. A Mann–Whitney test was used to assess the impact of age, ie, a potential difference between r-PLT numbers in young adult (>1 ≤ 2 years) and adult (>2 years) dogs. The cut-off value of 2 years to differentiate between young adult and adult dogs for reaching full adulthood, including mental adulthood, was chosen as published previously.[25] The parameters were not normally distributed and were thus described using a median and range. The significance level was set at $P < 0.05$.

3 | RESULTS

3.1 | Inter-method comparison

3.1.1 | Precision

In samples without interferences, intra-assay CV for %r-PLTmt and ar-PLTmt ranged between 15.7%–29.1% and 14.4%–29.6%, respectively (Table 1). Adaptation of r-PLT numbers after visual inspection of the scattergrams resulted in a mildly increased precision with CVs %r-PLTmt and ar-PLTmt ranging between 12.9–22.6% and 10.9–24.3%, respectively. Overall, the lowest CVs were present in samples within the medium r-PLT concentration range and the highest CVs within the low r-PLT concentration range.

As shown in Table 1, a similar intra-assay CV of 12.9% was obtained in a sample with a mean concentration of 4.4%r-PLT, considered in the low concentration range for flow cytometry. It was also found in a sample with a mean %r-PLTmtv concentration of 4%, considered the medium concentration range for the ADVIA 2120.

In patients with microcytic, hypochromic erythrocytes, intra-assay CV was 2-4-fold higher (36.0%–65.0%) than in a sample with a comparable r-PLT count and normocytic-normochromic erythrocytes (Figure 2). The CV decreased markedly after visual inspection of the scattergrams. It was similar to or slightly higher than the normocytic, normochromic samples with medium r-PLT percentages.

3.1.2 | Total observed error

The determined TE_{obs} was 69.0% for %r-PLTmt and 54.9% for %r-PLTmtv. TE_{obs} exceeded the TE_a derived from human medicine.

3.1.3 | Method comparison

A total of 56 blood samples were measured using flow cytometry. Fourteen/56 samples were excluded from the evaluation, of which eight samples failed to label cells within the platelet population, two samples had a positive isotype control, three samples had wash buffer autofluorescence, and one sample that had a contaminated buffer, which led to increased debris in the platelet population. In total, 42 samples (obtained from 41 dogs) were included for the method comparison part. Thirteen/42 blood samples were obtained from healthy dogs and the remaining 29/42 samples from diseased dogs with various underlying diseases. Of the 29 diseased dogs, 22 dogs had thrombocytopenia of varying severity (median: 98×10^9/L; range: 23–144×10^9/L).

Visual inspection of the ADVIA 2120 scattergrams revealed that in 12/42 samples (29%), no adjustment of the automated gate set

TABLE 1 Impact of concentration level and interference by microcytic, hypochromic erythrocytes on intra-assay precision of the percent and absolute numbers of r-PLTs assessed with the ADVIA 2120 analyzer. Intra-assay CV for r-PLT was assessed with flow cytometry in a sample with a low %r-PLT concentration (for this method)

Variable	Erythrocyte morphology	Concentration range	Mean ± SD (10^9/L)	CV (%)	Mean ± SD (%)	CV (%)
r-PLT flow cytometry	Normocytic, normochromic erythrocytes[a]	Low			4.4 ± 0.6	12.9
r-PLTmt	Normocytic, normochromic erythrocytes	Low	1.5 ± 0.4	29.6	0.8 ± 0.2	29.1
		Medium	5.7 ± 0.8	14.4	3.7 ± 0.6	15.7
		High	14.7 ± 2.2	14.8	20.9 ± 4.2	20.2
	Microcytic, hypochromic erythrocytes	Medium	3.1 ± 2.0	65.0	5.2 ± 3.4	65.7
		Low	1.8 ± 0.7	36.7	2.2 ± 0.8	35.8
r-PLTmtv	Normocytic, normochromic erythrocytes	Low	2.3 ± 0.6	24.3	1.2 ± 0.3	22.6
		Medium	6.2 ± 0.7	10.9	4.0 ± 0.5	12.9
		High	16.8 ± 2.3	13.5	23.8 ± 4.3	18.3
	Microcytic, hypochromic erythrocytes	Medium	6.5 ± 1.1	17.5	11.1 ± 2.2	19.8
		Low	5.9 ± 0.9	14.6	7.4 ± 1.0	13.1

Abbreviations: r-PLTmt, reticulated platelets moving threshold; r-PLTmtv, reticulated platelets after visual validation; SD, standard deviation; CV, coefficient of variation.

[a]normocytic, normochromic erythrocytes—low: considered for flow cytometry.

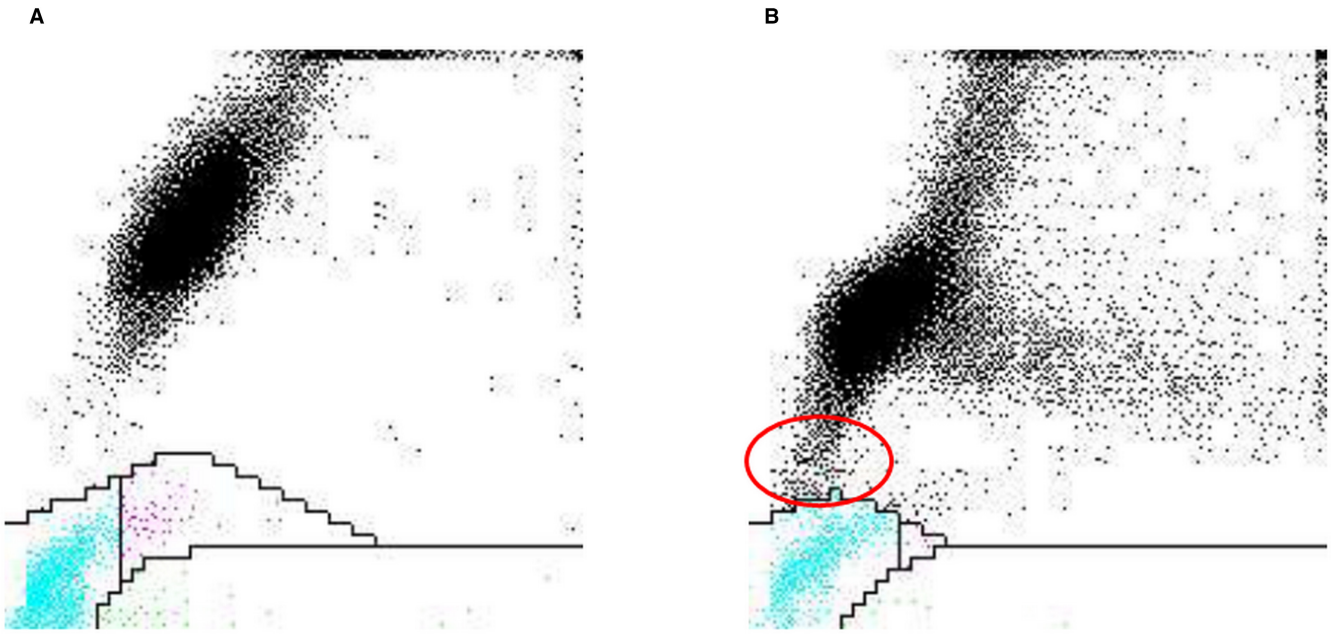

FIGURE 2 A, Example of an ADVIA 2120 platelet absorption cytogram (dog) with medium reticulated platelet counts (with normocytic, normochromic erythrocytes) on the left and (B) high interference ('Patient 1') (microcytic, hypochromic erythrocytes) on the right.

by the analyzer was necessary. In the majority of cases (30/42 samples; 71%); however, an adjustment of the automatically set gate was required. In the majority of measurements (21/30 samples, 70%), a mild adjustment was performed. In 8/30 samples (27%), the adjustment was moderate, and in 1/30 samples (dog with coumarin intoxication), a marked adjustment of 35 signals was carried out. In 5/42 samples, microcytic, hypochromic erythrocytes were present. The specimens with microcytic, hypochromic erythrocytes were mainly obtained from dogs with portosystemic shunts (4/5). One sample out of five with microcytic, hypochromic erythrocytes was from a dog with sepsis and subsequent disseminated intravascular coagulation (without known etiology for microcytosis). Mild and moderate manual adjustment of the automated gate was needed in 2/5 samples each. In 1/5 samples with microcytic, hypochromic erythrocytes, no adjustment of the automated gate was required. Microcytic, hypochromic erythrocytes interfered within the r-PLTmt gate in 1/5 cases.

The results of Spearman's correlation showed a fair correlation with the flow cytometry reference method for both r-PLTmt (r_s = 0.75) and r-PLTmtv (r_s = 0.76). The Passing-Bablok analysis

revealed moderate agreement between the two methods with wide 95% confidence intervals. The slope of the regression line was significantly < 1 for both measurement methods (0.34; 90% CI: 0.24–0.45 and 0.43; 90% CI: 0.30–0.54), indicative of significant proportional error, which increased for higher r-PLT values. The *y*-intercept was also significantly < 0 for both methods (0.64; 90% CI: −1.57 – −0.02 and 1.08; 90% CI: −1.84 – −0.34) indicative of significant constant error (Figure 3A and C). The Bland-Altman analysis revealed a proportional positive bias of 10.8% between %r-PLTmt and the reference method, which was slightly lower (9.7%) after visual validation of the scattergrams (%r-PLTmtv) (Figure 3B,D). Three/42 (Figure 3B) and 2/42 (Figure 3D) values exceeded the 95% agreement levels.

3.2 | Establishment of reference intervals

Measurements from 120 healthy dogs were available to establish the RIs. Forty-seven dogs were presented as part of an expert opinion for hip joint/elbow dysplasia, 45 dogs for blood donation, and 28 dogs for a general health check. Of the 120 dogs, 57 were female (female intact n = 46; female spayed n = 11) and 63 were male (male intact n = 41; male neutered n = 22). The animals were between 1 and 14 years old, the median age was 2 years. Overall, mixed breed dogs (n = 26), Labrador Retrievers (n = 22), German Shepherds (17), and Golden Retrievers (n = 14); were most frequently represented. Less frequently, Rhodesian Ridgebacks (n = 6), Bearded Collies (n = 5), Great Danes (n = 3), Beagle dogs (n = 2), Bordeaux Mastiffs (n = 2),

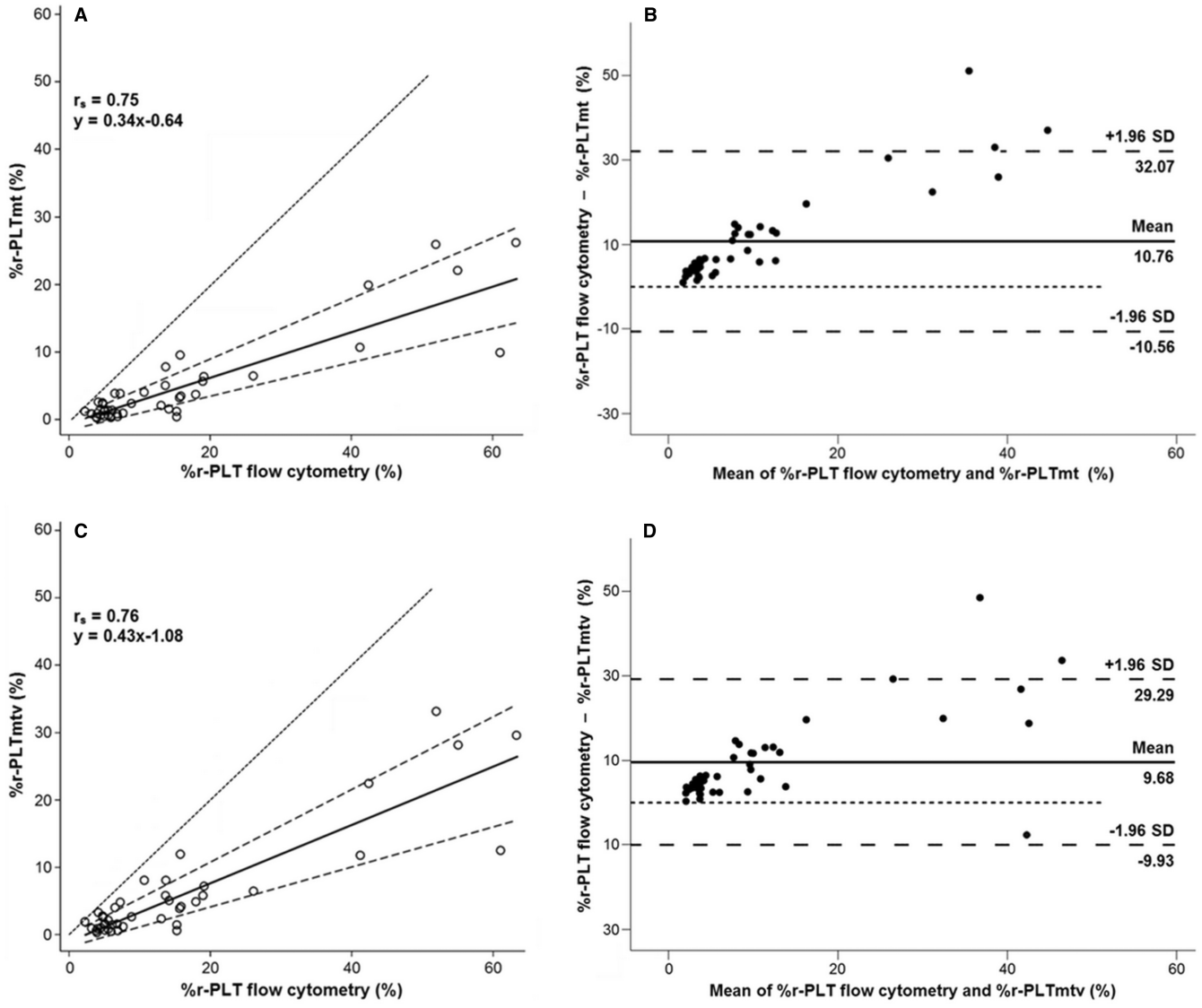

FIGURE 3 A, Passing-Bablok analysis and (B) Bland–Altman analysis of the automated measurement method (%r-PLTmt) on the ADVIA 2120; (C) Passing-Bablok, and (D) Bland–Altman analysis after visual validation (%r-PLTmtv). Abbreviations: SD = standard deviation; r-PLT = reticulated platelets; mt = moving threshold. Passing-Bablok analysis: thick line = the regression line with a 95% confidence interval (dashed lines); thin line = the identity line. Bland–Altman analysis: the dotted line = line of identity; thick line = absolute bias; dashed lines = 95% confidence intervals.

Border Collies (n = 2), and Cocker Spaniels (n = 2) were included. The remainder 19/120 dogs were of various breeds. RIs for r-PLTmt and r-PLTmtv are shown in Table 2.

In the majority of samples, a mild automated gate adjustment was usually required (96/120 samples; 80%). The required adjustment was mild in 76/96 samples (79.17%), moderate in 19/96 samples (19.79%), and marked in 1/96 samples (1.04%). In 24/120 samples (20%), no adjustment of the automated gate was needed after visual inspection of the scattergrams.

Overall, the group of healthy dogs included 67/120 young, adult dogs ≤2 years, and 53 older dogs >2 years.

Median %r-PLTmtv values were significantly higher in young adult dogs than in older dogs (Table 3).

4 | DISCUSSION

The ADVIA 2120 v6.11.7 software uses a gating algorithm optimized for human r-PLTS. During this study, it became apparent that in dogs, some r-PLTs might be located outside of the pre-set human r-PLT gate. Visual inspection of the ADVIA 2120 r-PLT gate allowed un-gated events to be included to form a validated r-PLT count. The cause of the r-PLTs localizing further to the right in the scattergram is unknown at the time of this study. However, this phenomenon has not been observed in measurements of human blood samples (G. Prechtl, unpublished data). Therefore, a unique property of canine r-PLTs, such as a higher binding capacity of the oxazine fluorescent dye, can be assumed. Our study revealed that in 28.6% of the samples, no manual adjustment was needed; thus, a perfect delineation and inclusion of r-PLTs in the automatically generated gate occurred. However, in 71.4% of the samples, the detected signals were outside of the pre-set gate, requiring a manual adjustment of r-PLT counts. A connection to diseased animals seems unlikely since this phenomenon was also observed in 80% of the healthy dogs serving as the reference population. From an analytical point of view, the cells falling outside the automatically generated r-PLT gate are cells with increased RNA content compared with the cells within the gate. An explanation for the signals located further to

TABLE 2 Reference intervals determined for reticulated platelets (r-PLT) for all dogs, dogs ≤2 years, and dogs >2 years prior to (r-PLTmt) and after visual validation (r-PLTmtv) using an ADVIA 2120 analyzer

Parameter	Age	Unit	Lower reference limit	Upper reference limit	Lower CI (90%)	Upper CI (90%)
r-PLTmt	All	%	0.2	3.8	0.2–0.3	3.4–9.0
		10^9/L	0.6	10.2	0.4–1.0	7.1–15.0
	≤2 years	%	0.2	5.4	0.2–0.5	3.5–9.0
		10^9/L	0.5	10.3	0.4–1.0	6.9–15.0
	>2 years	%	0.2	4.0	0.2–0.3	3.2–4.0
		10^9/L	0.6	13.1	0.6–0.9	8.3–14.7
r-PLTmtv	all	%	0.3	4.5	0.2–0.6	4.0–10.3
		10^9/L	1.1	10.3	0.4–1.5	8.6–17.2
	≤2 years	%	0.3	6.2	0.2–0.7	3.8–10.3
		10^9/L	0.9	11.8	0.4–1.8	7.6–17.2
	>2 years	%	0.3	4.5	0.2–0.5	3.9–4.6
		10^9/L	0.8	14.1	0.6–1.5	8.8–16.2

Abbreviation: CI, confidence interval.

TABLE 3 Mann–Whitney U-test to evaluate significant differences in the %r-PLT prior to and after visual validation using an ADVIA 2120 analyzer with respect to age dependency between patients ≤2 years and patients >2 years

Parameter	Unit	Age (years)	Median	Range	*P*-value
r-PLTmt	%	≤2	1.17	0.19–8.97	0.21
		>2	1.05	0.22–4.01	
	10^9/L	≤2	2.77	0.42–14.98	0.43
		>2	2.44	0.58–14.68	
r-PLTmtv	%	≤2	1.61	0.19–10.29	**0.03**
		>2	1.26	0.22–4.58	
	10^9/L	≤2	4.05	0.42–17.18	0.11
		>2	3.41	0.58–16.16	

Abbreviations: r-PLT, reticulated platelets for all dogs; r-PLTmt, reticulated platelets for dogs ≤2 years and dogs >2 years before visual validation; r-PLTmtv reticulated platelets after visual validation. Significant *P*-values are marked in bold letters.

the right in the scattergram could be an individual peculiarity. For example, platelet size is individually and genetically determined,[26] which could also apply to r-PLT. In addition, platelet size is thought to be influenced by platelet turnover,[27] which may be forced, for example, by inflammatory processes.[28] The same could be true for r-PLT; undetected inflammatory processes cannot be excluded as a cause for signals further localized to the right in scattergram plots. The most likely explanation for the phenomenon that some canine r-PLTs tend to be seen outside the borders of the human gate might be that canine r-PLTs tend to be larger and have a higher platelet distribution width (PDW) than human r-PLTs. The hypothesis is well possible as the mean canine PLT platelet volume (MPV) and PDW were 10.9 fl and 57.4%,[29] respectively; thus, these values are higher than the mean human MPV of 7.1 fl and PDW of 17.3%. Further studies are needed to elucidate the phenomenon that a subpopulation of canine r-PLTs exists with relatively high oxazine binding capacity.

An evidence-based evaluation of the precision of the r-PLT parameter is challenging because there are currently no consensus guidelines in human and veterinary medicine for r-PLT. All CVs obtained here for canine r-PLT measurements markedly exceeded the 4% quality specification for human IPF as determined by biological variation.[21] However, quality specifications derived from biological variation are generally considered to be too stringent to be used for method validation studies.[30] Alternatively, quality goals for CV might be derived from TEa. For canine PLTs, in general, a total allowable error (TEa) of 20% is acceptable according to the ASVCP guidelines, and CVs should be $<0.5\times$TEa, ie, $<10\%$.[30] Using the quality specifications for canine PLTs for canine r-PLTs, only CVs for medium concentration r-PLTmtv are close to but still slightly exceed 10%. However, it is likely that the TEa for canine PLTs cannot be used as a quality specification for canine r-PLTs as the TEa for human IPF is approximately twice as high as that for human PLTs (18.4% vs 10.5%).[21] When using a CV < 20% as a quality goal for canine r-PLTs, CVs for the ADVIA 2120 medium and high r-PLT measurements fulfilled quality criteria, as well as the CV for the flow cytometric measurement. For the low range of r-PLTs, CVs ranged between 20% and –30%. Oellers et al also demonstrated higher CVs for low reticulated platelet counts in their study.[19] Ideally, the CV for flow cytometry would have also been determined for medium and high r-PLT counts. However, flow cytometry requires a certain number of PLTs for analysis. Thus, in thrombocytopenic patients, a high sample volume is needed for r-PLT determination. Since only residual blood samples could be used for the study, it was technically not possible to determine precision at high %r-PLT (and, therefore, low platelet counts). Overall, PLT and r-PLT measurements in people with CVs of 1.09% and 4.25%, respectively,[21] is more precise than in dogs with CVs of 6.5%[31] and 15.7%, respectively, but these measurements are still within the acceptable range for clinical use. Nevertheless, they should always be considered when interpreting canine r-PLT results, especially regarding follow-up measurements or the limits of the RI. Values must at least be >20% different from the previous result or reference limit to be considered as truly "different" or abnormal.

Interference from microcytic hypochromic erythrocytes was observed in both cases that were used for the assessment of intra-assay CV, although precision was markedly improved after visual inspection and manual adjustment of the r-PLT gate. In our study of patients with microcytic, hypochromic erythrocytes, the CV was two to fourfold higher than in a sample with a comparable r-PLT count and normocytic, normochromic erythrocytes. The presence of microcytic, hypochromic erythrocytes interfered with the creation of the gate used to differentiate reticulated and mature platelets. The interference is due to the fact that the algorithm for creating the area of r-PLTs also includes lines that should minimize interference from hypochromic and microcytic erythrocytes presented as a valley between platelets and erythrocytes. This valley can be shifted downward by the presence of many hypochromic and microcytic erythrocytes, which in turn results in a very reduced area for r-PLT. The consequence is that some r-PLT are not covered by the gate. In these samples, small differences in area detection led to large differences in reported values. For scattergrams that create small areas for r-PLTs, visual inspection and manual adjustment is, therefore, needed.

Our method comparison study revealed a high TE_{obs} of 69.0% for %r-PLTmt and 54.9% for %r-PLTmtv that markedly exceeded TE_a derived from human medicine.[21] The high TE_{obs} was not only caused by relatively high imprecision but also by a bias of approximately 10% between the methods. Thus, r-PLT counts obtained with different methods cannot be directly compared with each other, and de novo RIs need to be determined for each method.

The RI established for the standard automated r-PLTmt parameter (0.2%–3.8%; $0.6–10.2\times10^9$/L), using samples from 120 dogs, was similar to the validated r-PLTmtv parameter (0.3%–4.5%; $1.1–10.3\times10^9$/L). Both the percentage automated r-PLT and validated r-PLT parameters on the ADVIA 2120 compared well with previously reported ranges using a Sysmex XT-2000iV of 0.2%–3.7% and 0.2%–3.9%, respectively.[18] Both RIs compared well with a published flow cytometric reference range that used a monoclonal antibody to platelet CD61 in combination with thiazole orange (0%–4.3%).[32] Absolute values for the r-PLTmt and r-PLTmtv parameter on the ADVIA 2120 ($0.6–10.2\times10^9$/L and $1.1–10.3\times10^9$/L) were slightly wider than previously reported RIs using the Sysmex XT-2000iV of $0.4–5.7\times10^9$/L and $0.4–5.6\times10^9$/L.[18] A study of healthy and diseased human subjects using an Abbott CELL-DYN Sapphire hematology analyzer produced r-PLT counts that ranged from 0.4% to 6.0% ($1.0–18.3\times10^9$/L).[33] Differences in RIs may also be due to patient selection. In human medicine, studies indicate that differences occur depending on the gender and age of the patients. Hoffmann et al[33] showed slightly higher values of r-PLT in male patients but without statistical significance. In contrast, r-PLT counts increased significantly with age.[33] MacQuinn et al also showed an impact of age on RIs,[34] and Oellers et al established RIs for a healthy Beagle group, including 153 young adult dogs with low variability in age ranging between 10 and 14 months. RIs published previously for young adult Beagle dogs were 0.5%–3.5% for r PLT% and absolute r-PLTs of $1.3–7.4\times10^9$/L.[19] In that

study, the RIs were slightly lower than demonstrated in our study for dogs in the same age category, ie, 0.3%–6.2% and 0.0–11.8 $\times 10^9$/L, respectively. The difference between our study and that previous study might be due to the breeds studied and the methodology used. As demonstrated in the previous study, RIs for % r-PLT and absolute r-PLT were much smaller in the Beagle dogs than for healthy dogs of various ages, breeds, and ages (median 4 years; range 3 months–16 years), for which the % r-PLT and absolute r-PLT ranged from 0.3% to 6.9% and from 0.8 $\times 10^9$/L to 15.4 $\times 10^9$/L, respectively.[19] We also demonstrated an impact of age and thus, thrombopoietic activity on %r-PLTmtv. Not surprisingly, % r-PLTmtv values were higher in young adult dogs ≤2 years of age than in older dogs. The age dependency of the immature PLT fraction has also been demonstrated in people, with higher percentages in neonates than in adults.[35] Follow-up studies are desirable to establish accurate age-dependent RIs for dogs.

A limitation of the study is the flow cytometric measurement of r-PLTs per se. Fourteen of 56 measurements could not be evaluated using flow cytometry, demonstrating that this method is also error-prone and, as a gold standard, it has some limitations. Many authors have noted that a true gold standard for counting r-PLTs is not currently available. A multitude of different protocols and the strongly deviating values that resulted with regard to published reference ranges could again underline the advantages of an automated measurement with the ADVIA 2120 analyzer. In addition, measurement by flow cytometry is often not practical in routine diagnostics due to the cost and time required.[20,36] As in previous studies, a further limitation of the methods for r-PLT assessment is that visual inspection of the results and adaptation of the gates in cases of suspected interferences is still necessary. A manual adjustment of the automatically set gates was further limited by the fact that the events on the right side of the automatically set r-PLT gate were not truly proven to be r-PLTs. However, as a manual adjustment of r-PLTmt counts resulted in an increase in correlations and reduction in bias between the ADVIA 2120 r-PLT values and the reference method, it is highly probable that the events detected outside the ADVIA r-PLT gate truly represent canine r-PLTs with high RNA content.

Unlike reticulocytes, immature platelets are produced in various sizes ranging from small to large. The ADVIA 2120 r-PLT count parameter uses dual-angle laser light cytometry to enumerate platelets that contain RNA independent of size. In contrast to r-PLTmt, platelet size has been shown to influence IPF, and the IPF value may be disproportionately raised in cases of congenital macrothrombocytopenia.[37] The novel r-PLT method used by the ADVIA 2120 may, therefore, offer improved clinical utility; however, further studies must be performed to verify this hypothesis.

5 | CONCLUSIONS

Overall, the ADVIA 2120 analyzer could detect r-PLT and is thus a suitable tool for routine diagnostics. A moderate correlation for the novel r-PLT parameter on the ADVIA 2120 was seen compared with flow cytometry. The Bland–Altman analysis revealed high positive bias, indicating that the r-PLT% was underestimated on the ADVIA 2120 compared with flow cytometry. As some events were extended from the pre-defined human r-PLT gate in most canine samples, visual inspection of the reticulated platelet cytogram is advised to manually re-calculate the count. The visual inspection of scattergrams improved method precision and accuracy and is recommended for all samples; it is especially relevant for samples with microcytic, hypochromic erythrocytes. Moreover, method imprecision needs to be considered.

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