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Analytical variability and uncertainty in canine leukocyte ratios obtained with manual counts

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Abstract

Background: This study aimed to determine the analytical imprecision of calculated neutrophil to lymphocyte (NLR) and lymphocyte to monocyte ratios generated from manual differential white blood cell (WBC) counts in peripheral blood smears, and to describe how to report the uncertainty around a single WBC ratio result. No information on the analytical imprecision of WBC ratios in dogs is available.

Methods: Coefficient of variations (CVs) of paired readings of one operator on 105 smears (intraoperator variability) and of three operators on 301 smears (interoperator variability) were calculated. The interoperator agreement was examined with the Fleiss' kappa coefficient (κ). Observed total errors (TEos), expanded measurement of uncertainty (EMU) and reporting intervals (RIs) were also calculated.

Results: Median CVs ranged from 3.14 to 28.28 (intraoperator) and from 5.39 to 53.85 (interoperator). No agreement among operators was found around the cut-offs. TEos were higher than allowable total errors in 32%–88% of smears. EMU ranged from 0.10 to 1.13. According to the RI, the calculated WBC ratios should be rounded to the nearest 10.

Conclusion: WBC ratios should be interpreted cautiously in dogs. The EMU should be reported to make the clinician aware of the uncertainty of these parameters. For example, an NLR result of 17 is needed to have high confidence that the result is above a cut-off of 6.

KEYWORDS
analytical variability, leukocytes, lymphocyte to monocyte ratio, neutrophil to lymphocyte ratio, white blood cell ratio

INTRODUCTION

White blood cell (WBC) ratios (i.e., the ratios between the counts of two different leukocyte populations) are simple parameters derivable from a routinely performed complete blood count. In human medicine, many studies have reported the value of WBC ratios in predicting disease severity and survival outcomes in inflammatory and neoplastic conditions and, recently, an increasing body of literature has developed in veterinary medicine on the same topic.[1–9]

In dogs, the prognostic or diagnostic properties of neutrophil to lymphocyte ratio (NLR) were evaluated in oncological patients[2–6,9] and in septic conditions.[7,8] The prognostic utility of the lymphocyte to monocyte ratio (LMR) was evaluated only in studies on oncological dogs,[1,5,6] whereas only one study evaluated the prognostic role of the neutrophil to eosinophil ratio in dogs with mast cell tumours.[5] Most of these studies advocate the ability of a single spot result at clinical presentation to provide diagnostic information when above or below the established cut-off.[1–7]

In contrast to human medicine, where the reported leukocyte differential counts and the derived WBC ratios are mostly obtained using automated techniques, in veterinary laboratory settings (both for in-clinic and reference laboratories) the inaccuracy of automated instruments for leukocyte classification

mean that the review of blood slides by trained personnel is necessary.[10] According to American Society for Veterinary Clinical Pathology (ASVCP) guidelines, the maximum level of acceptable analytical error (total allowable error, TEa) for the manual differential count is considered to be 15% for neutrophils and lymphocytes and up to 50%–60% for monocytes.[10] To our knowledge, no studies concerning the WBC ratio's analytical uncertainty and its effect on the operators classifications are available in the literature. Even if infrequently used in veterinary practice, two recognised ways to communicate to clinicians the amount of uncertainty around a result are the expanded measurement uncertainty (EMU) and the reporting intervals (RIs).[11] EMU is the number that must be added and subtracted to a single result in order to provide an estimate of the interval of values that the result actually represents at a given statistical confidence level.[11] RI is the rounding unit that should be used for reporting purposes in order to not include unnecessary information linked to the uncertainty of the measurement.[11]

Therefore, the aims of this study were:

- to determine the inter- and intraoperator imprecision of the WBC ratios more frequently recommended in veterinary medicine (NLR and LMR) and of the manual leukocyte count from which they derive (neutrophils, lymphocytes and monocytes);
- to determine the possible influence of the interoperator variability of NLR and LMR on the potential diagnostic and prognostic classification at different cut-offs;[1,3–7]
- to describe how the variability should be reported according to the uncertainty calculated in the present study.

MATERIAL AND METHODS

Case selection

This was a retrospective study on peripheral blood smears available in the archive of the Clinical Pathology Laboratory of the Veterinary Teaching Hospital (VTH) of the University of Milan. Smears prepared from blood samples collected from dogs during routine diagnostic activities in the period between January and May 2017 were selected based on the following exclusion criteria:

- missing smears;
- smears deemed unacceptable for reading (smears with scratches, not uniformly stained, too short, excessively thick, without an evident monolayer);
- smears from samples with reported pre-analytical or biological factor potentially influencing the readings (haemolysis, lipaemia or presence of unclassified cells).

All the smears were obtained from peripheral blood samples collected in EDTA from dogs referred to the VTH for routine diagnostic or wellness visits. Therefore, according to our institution (University of Milan) guidelines, a formal approval from the Institutional Animal Care and Use Committee was not required (Ethic Committee decision 29 October 2012, renewed with the protocol no. 02-2016). All the smears were prepared according to the laboratory standard operative procedures. In detail, one small drop (approximately 2 μl) of peripheral blood was placed on a clean glass slide, and the smear was obtained by touching and then pushing the drop with another clean glass at about 45° angle in order to produce a thin layer of blood. All the slides were stained with a Romanowsky-type stain Hemacolor (Merck, Darmstadt, Germany) and coverslipped using a mounting medium.

Leukocyte counts and WBC ratio calculation

In a blind manner, for each slide, three operators (a trained veterinary medicine student, a resident of the European College of Veterinary Clinical Pathology and a practitioner with many years of experience in the field) performed a 100 cells differential count (according to the laboratory standard operating procedure). In order to speed up the counting work, only neutrophils (including segmented, non-segmented and band forms), lymphocytes and monocytes were included in the differential counts. The first 105 smears (100 plus 5% to prevent data loss) selected according to the criteria above were read in duplicate from one operator.

NLR and LMR were calculated for each slide from the relative number of neutrophils, lymphocyte and monocytes according to the following formulas: NLR = neutrophils (%)/lymphocytes (%); LMR = lymphocytes (%)/monocytes (%).

Statistical analysis

Statistics were run on an Excel spreadsheet with the Analyse-it set of macroinstructions (Analyse-it, version 4.97; Analyse-it Software, Leeds, UK).

Intraoperator variability

After the Anderson-Darling normality test and inspection of data distribution, results of paired readings were used to calculate the mean values and the standard deviations (SD). Coefficient of variations (CVs) were calculated according to the formula CV = SD/mean × 100. In order to highlight the differences between the intensity of variability for each parameter, the median CVs from each parameter were compared using the Kruskal–Wallis test (p-value < 0.05 considered significant). When this test revealed significant differences, after Bonferroni's correction of the p-value (p-value after correction was

0.01), the Mann–Whitney *U* test was used as post hoc procedure. When the ratio calculation was impossible due to a zero leukocyte count in at least one of the reading sessions, that smear was excluded from the statistical comparison.

Interoperator variability

For each smear, after the Anderson-Darling normality test and data distribution inspection, mean, SD and CV were calculated from the readings of the three operators for the WBC populations and ratios, as described above. Moreover, median CVs were compared to each other as already described for the intraoperator variability. When the ratio calculation was impossible due to a zero leukocyte count in at least one of the reading sessions, that smear was excluded from the statistical comparison.

Agreement between operators

Only for this part of the study, in order to reduce the number of excluded data, an arbitrary value of 100 was assigned to the NLR and LMR, when no lymphocyte or monocytes were counted from an operator. Agreement between the three operators to classify each sample as 'positive' or 'negative' was calculated using the following cut-offs reported in the literature—NLR: 3.50,[5] 4.52,[3] 5.67,[4] 6.00,[7] 9.44[6]; LMR: 1.20,[1] 1.43,[6] 3.00.[5] In each of these settings, results above the cut-off were considered as 'positive' or 'negative' according to the literature[1,3–7]; the agreement was calculated using the Fleiss' kappa coefficient (κ).[12] The strength of agreement was considered almost perfect if $\kappa > 0.81$, substantial if $\kappa = 0.61$–0.80, moderate if $\kappa = 0.41$–0.60, fair if $\kappa = 0.20$–0.40, slight if $\kappa = 0.00$–0.20 and poor for $\kappa < 0.00$.[13] For all tests a *p*-value of <0.05 was considered significant. The agreement was evaluated on two different data sets, one including all the selected smear (the complete set of data) and another including only the slides with WBC ratios results near the cut-offs (only results including the cut-offs ± the mean CV of that WBC ratio).

Total error, EMU and RIs

Total observed error (TEo) was calculated according to the formula:

$$TEo = 2 \times CV$$

where CV was the mean CV obtained from the interoperator variability study for *N*%, *L*%, *M*%, NLR and LMR and 2 is the rounded *z*-score of 1.96 at the 95% confidence interval.[11]

TEa for *N*%, *L*% and *M*% were obtained from the study of Nabity et al.[10] and were 15%, 15% and 60%, respectively. No TEas were found in the literature concerning WBC ratios, so TEas for these parameters were arbitrarily derived from TEa of the leukocytes involved in the formula, choosing the higher of the two. According to this, a TEa of 15% and 60% were adopted for NLR and LMR, respectively.

The EMU for NLR and LMR was calculated according to the simplified EMU equation from the study of Moore and Freeman,[11] as follows:

$$\mathrm{EMU}_{95\%} = 2 \times \left(\frac{\mathrm{CV}\%}{100}\right)$$

where CV was the mean CV obtained from the interoperator variability study for NLR and LMR and 2 was the rounded *z*-score of 1.96 at the 95% confidence interval.

In order to show the impact of EMU, results were also expressed as a range where the minimum and maximum values were stated according to the following formulas:

$$\mathrm{Min} = x_1 - \left(x_1 \times \mathrm{EMU}_{95\%}\right) \quad \mathrm{Max} = x_1 + \left(x_1 \times \mathrm{EMU}_{95\%}\right)$$

where x_1 was the measured result.

RIs were adopted according to the simplified Hawkins rules assigning a RI equal to 1 when SD < 7 and 10 when SD > 7.[11]

RESULTS

According to the inclusion and exclusion criteria, 301 slides were included in the interoperator study and 105 slides were used for the intraoperator study (Figure S1). From the data distribution inspection, *L*%, *M*%, NLR and LMR showed a left skewed, whereas *N*% showed a right skewed distribution; all parameters were found not normally distributed according to the normality test ($p < 0.05$).

Intraoperator variability

In two out of 105 blood smears, both reading sessions of operator 1 gave zero count for *M*%; in six and seven blood smears no *M*% were counted during the first and second reading sessions, respectively; in only one blood smear at the second reading no *L*% were counted. As a consequence, it was not possible to calculate NLR and LMR in one and 15 cases, respectively; therefore, for these parameters only, 104 and 90 paired results were included in the statistical analysis. Mean CVs and mean SDs are reported in Table 1, descriptive data and results from the two readings are shown in Table S1 and the distribution of CVs are shown in Figure 1a. Statistically significant differences were found between the CVs of the different leukocyte populations (*N*%, *L*% and *M*%) and ratios ($p < 0.001$). The highest median CVs were found for *M*% and LMR, whose CVs proved to be similar, followed by those of NLR and *L*%, also with similar CVs, whereas *N*% showed the lowest median CV (Figure 1a and Table S1).

TABLE 1 Number of slides finally included in the statistical analysis, mean coefficients of variation (CV), mean standard deviation (SD), total observed errors (TEo), total allowable errors (TEa) and reporting intervals (RI) obtained from the intra- and interoperator studies

	Intraoperator study (105 slides read from one operator)						Interoperator study (301 slides read from three operators)					
Parameter	***n***	**Mean CV**	**Mean SD**	**TEo (%)**	**TEa (%)**	**RI**	***n***	**Mean CV**	**Mean SD**	**TEo (%)**	**TEa (%)**	**RI**
Neutrophils%	105	4.9	6.7	9.9	15	1	301	7.3	8.5	14.6	15	10
Lymphocytes%	105	19.3	21.9	38.6	15	10	301	25.3	19.1	50.5	15	10
Monocytes%	105	42.7	43.4	85.4	60	10	301	51.6	30.2	103.1	60	10
NLR	104	22.6	19.6	45.1	15	10	299	31.7	20.8	63.4	15	10
LMR	90	37.1	29.3	74.2	60	10	275	56.7	28.8	113.4	60	10

Abbreviations: LMR, lymphocyte to monocyte ratio; NLR, neutrophil to lymphocyte ratio.

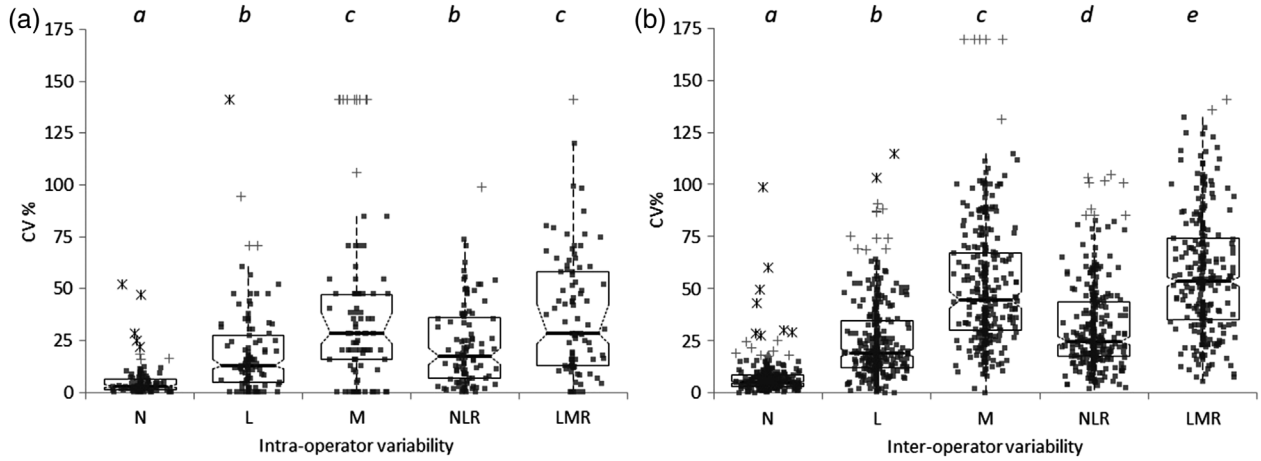

FIGURE 1 Distributions of coefficient of variations (CVs) from the (a) intraoperator and (b) interoperator studies for the percentage of neutrophils (*N*), lymphocytes (*L*) or monocytes (*M*), and for neutrophil to lymphocyte (NLR) and lymphocyte to monocyte (LMR) ratios. Boxes indicate the I–III interquartile range (IQR), the horizontal line corresponds to the median, vertical lines are the limits of observations within the I quartile minus 1.5 × IQR or within the III quartile plus 1.5 × IQR. Near outliers (>1.5 IQR) and far outliers (>3 IQR) are indicated with a cross or an asterisk, respectively. Different lowercase letter on the top of dot-plots corresponds to statistically different median CVs, within each study (all p-value < 0.001, except for *M* versus NLR, intraoperator study, and *M* versus LMR, interoperator study, that showed p-values < 0.01). Refer also to the main text for more details

Interoperator variability

Due to a zero *L*% and *M*% counts, it was not possible to calculate NLR in two cases out of 301 (one for operator 1 and one for operator 2) and LMR in 31 cases out of 301 (15 for operator 1, 13 for operator 2 and three for operator 3). In five of these cases, two operators out of three observed no monocytes on the same blood smear. Mean CV and mean SD are reported in Table 1. Descriptive data concerning the readings of the three operators are shown in Table S2. Differences were found in the median CVs among the parameters ($p < 0.001$). The *N*% showed the lowest variability, followed by *L*%, NLR, *M*% and LMR.

Agreement among operators

When the results from the 301 blood smears were considered, the three operators showed a substantial (NLR) and moderate (LMR) agreement in classifying smears according to the different cut-offs ($p < 0.001$; Table 2). However, when only the slides with results near the cut-offs were included, the κ was not statistically different from zero and thus the agreement was interpreted as poor at all cut-off levels of NLR and LMR ratios ($p > 0.05$; Table 3). This was confirmed by the low frequencies of fully concordant results at the different cut-offs of NLR (from 26.3% to 35.2%) and LMR (from 25.9% to 27.5%). An example of the different classification of results for NLR and LMR according to different operators is given in Table 4.

TEo, EMU, RI and comparison with TEa

TEos, respectively, for *N*%, *L*%, *M*%, NLR and LMR, were 9.9, 38.6, 85.4, 45.1, and 74.2 in the intraoperator study and 14.6, 50.5, 103.1, 63.4 and 113.4 in the interoperator study (Table 1). TEo was higher than the TEa for all parameters except for *N*% in both intra- and interoperator study. The number (%) of slides that showed a TEo outside the TEa, respectively, for *N*%, *L*%, *M*%, NLR and LMR, were 21/105 (20%), 73/105 (70%), 50/103 (49%), 77/104 (74%), and 44/90 (49%) in the intraoperator study and 96/301 (32%), 266/301

TABLE 2 Results of the agreement between the three operators at different cut-off values including all the slides ($n = 301$)

Cut-off	Fleiss' kappa (p-value)	Number of fully concordant cases (percentage on total cases)[a]	Number of discordant cases (percentage on total cases)[b]
NLR min–max of mean ratios: 0.02–86 ($n = 301$)			
3.50[5]	0.69 (<0.001)	231 (76.7%)	70 (23.3%)
4.52[3]	0.69 (<0.001)	231 (76.7%)	70 (23.3%)
5.67[4]	0.68 (<0.001)	234 (77.7%)	67 (22.3%)
6.00[7]	0.68 (<0.001)	236 (78.4%)	65 (21.6%)
9.44[6]	0.68 (<0.001)	253 (84%)	48 (16%)
LMR min–max of mean ratios: 0.04–99 ($n = 301$)			
1.20[1]	0.48 (<0.001)	246 (81.7%)	55 (18.3%)
1.43[6]	0.52 (<0.001)	236 (78.4%)	65 (21.6%)
3.00[5]	0.53 (<0.001)	189 (62.8%)	112 (37.2%)

Note: Superscript numbers refer to the references.
Abbreviations: LMR, lymphocyte to monocyte ratio; NLR, neutrophil to lymphocyte ratio.
[a]Fully concordant cases derive from slides were all the three operators obtained a ratio above or below the cut-off at the same time.
[b]Discordant cases derive from slides where one or two operators out of three obtained a discordant result.

TABLE 3 Results of the agreement between the three operators at different cut-off values including only slides with mean of the ratio results obtained from the three operators within the cut-off ± the mean coefficient of variation (CV) of the ratio

Cut-off	Min–max mean of ratios included	Fleiss' kappa (p-value)	Number of fully concordant cases (percentage on total cases)[a]	Number of discordant cases (percentage on total cases)[b]
NLR (mean CV = 31.72%)				
3.50[5]	2.39–4.61 ($n = 84$)	0.05 (0.412)	27 (32.2%)	57 (67.8%)
4.52[3]	3.09–5.95 ($n = 91$)	0.09 (0.151)	32 (35.2%)	59 (64.8%)
5.67[4]	3.87–7.47 ($n = 79$)	−0.02 (1.207)	23 (29.1%)	56 (70.9%)
6.00[7]	4.10–7.90 ($n = 76$)	−0.07 (1.685)	20 (26.3%)	56 (73.7%)
9.44[6]	6.45–12.43 $n = 43$)	0.05 (0.548)	14 (32.6%)	29 (67.4%)
LMR (mean CV = 56.72%)				
1.20[1]	0.62–2.24 ($n = 54$)	−0.03 (1.276)	14 (25.9%)	40 (74.1%)
1.43[6]	0.52–1.88 ($n = 62$)	−0.03 (1.268)	17 (27.4%)	45 (72.6%)
3.00[5]	1.30–4.70 ($n = 103$)	0.00 (0.996)	27 (26.2%)	76 (73.8%)

Note: Superscript numbers refer to the references.
Abbreviations: LMR, lymphocyte to monocyte ratio; NLR, neutrophil to lymphocyte ratio.
[a]Fully concordant cases derive from slides were all the three operators obtained a ratio above or below the cut-off.
[b]Discordant cases derive from slides where one or two operators out of three obtained a discordant result.

(88%), 227/301 (75%), 282/299 (94%), and 221/275 (80%) in the interoperator study.

According to the intraoperator study, EMU was 0.10, 0.39, 0.85, 0.45 and 0.74 for *N*%, *L*%, *M*%, NLR and LMR, respectively and, according to the interoperator study, 0.15, 0.51, 1.03, 0.63, and 1.13, respectively. RIs for all the parameters was 10 (results rounded to tens), except for neutrophils in the intraoperator study where an RI of 1 (no decimal points) was found. In Table 5 are shown examples of the uncertainty ranges obtainable applying the EMU as well as how those results should in practice be reported according to EMU and RI for some possible NLR and LMR results.

DISCUSSION

According to the results of the current study, the analytical imprecision of the differential counts was higher for monocytes, and progressively lower for lymphocytes and neutrophils, both when the same operator (intraoperator study) or different operators (interoperator study) read the slides. The analytical variability occurring when different operators read the same slide may be likely due to different classification of leukocytes (i.e., monocytes interpreted as lymphocytes) or to different slides areas evaluated, as leukocytes may be uneven distributed onto the slides. Conversely, differences observed for the repeated reading of the same slide from the same operator may be due predominantly on the evaluation of a different area of the slide at the second reading.[14]

Concerning ratios, the highest variability was observed for LMR in comparison to NLR, in both studies. This was expected as the analytical variability of a WBC ratio is the result of the combined analytical variability of the leukocytes populations involved in

TABLE 4 Contingency tables of the agreement between each pair of the three operators for the classification of smears according to the neutrophil to lymphocyte ratio (NLR) cut-off of 6 and the lymphocyte to monocyte ratio (LMR) cut-off of 1.43

Operator 1	Operator 2			Operator 1	Operator 3			Operator 2	Operator 3		
	NLR < 6	**NLR ≥ 6**	**Total**		**NLR < 6**	**NLR ≥ 6**	**Total**		**NLR < 6**	**NLR ≥ 6**	**Total**
NLR < 6	31 (40.8%)	23 (30.3%)	54 (71.1%)	NLR < 6	34 (44.7%)	20 (26.3%)	54 (71.1%)	NLR < 6	25 (32.9%)	19 (25%)	44 (57.9%)
NLR ≥ 6	13 (17.1%)	9 (11.8%)	22 (28.9%)	NLR ≥ 6	14 (18.4%)	8 (10.5%)	22 (28.9%)	NLR ≥ 6	23 (30.3%)	9 (11.8%)	32 (42.1%)
Total	44 (57.9%)	32 (42.1%)	76 (100%)	Total	48 (63.2%)	28 (36.8%)	76 (100%)	Total	48 (63.2%)	28 (36.8%)	76 (100%)
Operator 1	**Operator 2**			**Operator 1**	**Operator 3**			**Operator 2**	**Operator 3**		
	LMR < 1.43	**LMR ≥ 1.43**	**Total**		**LMR < 1.43**	**LMR ≥ 1.43**	**Total**		**LMR < 1.43**	**LMR ≥ 1.43**	**Total**
LMR < 1.43	22 (35.5%)	14 (22.6%)	36 (58.1%)	LMR < 1.43	21 (33.9%)	15 (24.2%)	36 (58.1%)	LMR < 1.43	27 (43.5%)	12 (19.4%)	39 (62.9%)
LMR ≥ 1.43	17 (27.4%)	9 (14.5%)	26 (41.9%)	LMR ≥ 1.43	19 (30.6%)	7 (11.3%)	26 (41.9%)	LMR ≥ 1.43	13 (21%)	10 (16.1%)	23 (37.1%)
Total	39 (62.9%)	23 (37.1%)	62 (100%)	Total	40 (64.5%)	22 (35.5%)	62 (100%)	Total	40 (64.5%)	22 (35.5%)	62 (100%)

Note: Only slides with mean of the ratio results obtained from the three operators within the cut-off ± the mean coefficient of variation (CV) of the ratio were included.

their calculations, and LMR includes the two leukocytes population with the highest CVs observed in the study. Moreover, in the intraoperator study, CVs of NLR and LMR were similar to CVs of *L*% and *M*%, respectively, whereas in the interoperator study they were higher. This may be explained by the lower variability observed between the repeated counts of operator 1 that, as expected, was more consistent in leukocytes classification during the repeated reading of the slides.

According to the present study, agreements between operators were found only when all the slides of the study were included. However, when only the slides with a result near the cut-offs were considered, therefore including a narrower analytical range, substantial disagreements were found. In other words, a substantial number of samples with results close to the cut-offs can be classified differently, as 'positive' or 'negative', by different operators. This essentially highlights how, near the cut-offs proposed to classify different clinical conditions in dogs,[1–7] the analytical variability is too high to obtain certainty of the result for diagnostic purposes when performing a manual differential count. These findings were also endorsed by the frequency of unacceptable performance for manual differential of leukocytes counts. Only for *N*% an acceptable TEo was obtained. However, also in this case, the TEa was exceeded in more than 30% of the slides evaluated in the interoperator study and the CVs substantially decreased only when more than 50% of leukocytes were represented by neutrophils on a slide.

The unacceptable performance of lymphocytes and monocytes counts were clearly highlighted in the present study. In fact, even if in the present study the TEo calculation did not include the method analytical bias, possibly leading to underestimation of the actual TEo, the frequency of unacceptable results (i.e., slides with TEo higher than TEa) was very high. This may be due to the manual method used to obtain leukocytes percentages in the present study. However, even if the inherent imprecision of manual WBC differential counts is higher than that of automated methods, the review of a blood smear is always recommended to confirm automated results and becomes mandatory when abnormalities in WBC morphology are present.[10] The occurrence of morphology abnormalities is higher when blood samples belong to dogs with oncological or inflammatory conditions (i.e., atypical lymphocytes, activated monocytes and immature or toxic neutrophils). Those are the target patients of the WBC ratio cut-offs proposed to differentiate some clinical conditions and disease stages.[1–7] Thus, it is likely that even adopting automated methods, the degree of analytical error remains similar. The WBC ratio cut-offs included in the present paper to test the agreement of the operators were obtained by means of manual methods,[3,4] by means of instruments with[5] or without[1,7] crosschecking with a manual method or with a not specified method.[6] Thus, the different analytical variability must be considered when adopting cut-offs generated with different methods as this may have impacted on the generated cut-off.

TABLE 5 Uncertainty interval around different results of neutrophil to lymphocyte ratio (NLR) and lymphocyte to monocyte ratio (LMR)

NLR result	Uncertainty interval according to EMU	How to report according to EMU and RI	LMR result	Uncertainty interval according to EMU	How to report according to EMU and RI
1	0.37–1.63	0–0	0.1	−0.01 to 0.21	0–0
2	0.74–3.26	0–0	0.2	−0.03 to 0.43	0–0
3	1.11–4.89	0–0	0.3	−0.04 to 0.64	0–0
4	1.48–6.52	0–10	0.4	−0.05 to 0.85	0–0
5	1.85–8.15	0–10	0.5	−0.06 to 1.07	0–0
6	2.22–9.78	0–10	0.6	−0.08 to 1.28	0–0
–	–	–	0.7	−0.09 to 1.49	0–0
15	5.55–24.45	10–20	0.8	−0.10 to 1.70	0–0
16	5.92–26.08	10–30	–	–	–
17	6.29–27.71	10–30	10	−1.30 to 21.30	0–20
18	6.66–29.34	10–30	11	−1.43 to 23.43	0–20

Note: Expanded measurement uncertainty (EMU) and reporting intervals (RI) were obtained from the interoperator study. The grey areas represent the range of results where is not possible to have certainty of the result for the NLR cut-off of 6 (on the left) and the LMR cut-off of 1.43 (on the right).

Future studies are needed to compare the analytical variability of automated method for ratio calculation, and this should be considered in the design of studies including WBC ratios.

Data concerning the acceptable limit for the analytical variability (i.e., TEa) of WBC ratios are not available in the literature. In the present study, TEa for *L*% and *M*% were deliberately used as tolerable limit for NLR and LMR, respectively, choosing the higher TEa from those of the leukocytes populations included into the formula. However, for all the cut-offs included, substantial disagreement was constantly present.

According to the EMU calculated in the present study, the uncertainty around a single WBC ratio result, obtained by manual method, likely precludes the use of any of the cut-offs extrapolated from the literature, as the high uncertainty around the results determines that any result represents a wide range of possible results (see also Table 5). For example, if NLR cut-off is recognised to identify a systemic inflammatory condition when equal or higher than 6.00, a result equal or higher than 17 would be necessary, since, based on the calculation of the uncertainty, this result actually corresponds to a number between 6.29 and 27.71. On the contrary, to have the certainty that the range of possible results is below 6.00 (healthy animals in this hypothesis), a single NLR result equal or lower than 3.00 would be necessary (the uncertainty range in this case would be 1.11–4.89). For this cut-off, the NLR results between 4 and 16 represent a very wide 'grey zone', in which a clinical decision based solely on a WBC ratio result should not be performed. An even higher uncertainty was showed for LMR. For example, assuming we use a LMR cut-off supposed to predict a worse prognosis for lymphoma in dogs when below 1.43, only with results below 0.60 it is possible to have a realistic certainty that the result effectively includes a value below this cut-off. Whereas, as negative values are always included into the possible range of values due to the high analytical imprecision, it is not possible to obtain a result with a range that includes only values higher than the cut-off. For this WBC ratio, the 'grey zone' includes almost all the possible ranges of the expected results obtainable from a canine population. This highlights the need to always interpret these laboratory results taking into consideration the clinical information and presentation when a prognostic or diagnostic decision should be formulated, mostly when results fall within the 'grey zone' for that WBC ratio. The uncertainty around this result is even more emphasised when the proper RI is considered. According to our study, WBC ratio results obtained with manual methods should be always rounded at tens. This means that the laboratory should report the results of NLR and LMR between 0 and 5 as 0 and those between 5 and 15 as 10. This even more highlights how the use of the WBC ratio for diagnostic and monitoring purposes is extremely unreliable.

Finally, data concerning the variability of eosinophil counts were not evaluated in the present study. Even if the neutrophils to eosinophil ratio is reported in one study,[5] eosinophils are rarely represented in routine samples referred to laboratories; thus, in order to speed up the operators work, it was deliberately chosen to exclude this population from the study. However, due to the low percentage of eosinophils in the peripheral blood, an even greater analytical error than those recorded for WBC ratios in this study is expected. Another limitation of the present study is that results are derived from a limited number of operators (one for the intraoperator and three for the interoperator study). This approach does not make the results generalisable to facilities where more than three operators take turns on the routine work. Moreover, despite the fact that counting a higher number of cells per slides would likely have reduced both the intra- and interoperator variability, a 100 cells

differential count was performed in the present study in order to reproduce what occurs in a real context of a laboratory routine, as this is the most frequently adopted method in diagnostic laboratories.[10]

In conclusion, the results of the present study showed that WBC ratios obtained with manual counts are extremely imprecise and should not be used for the clinical classification of patients. When used, results should be reported taking into account the EMU and using the proper RI (results rounded to 10), to make the clinicians aware of the amount of analytical error.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

All the four authors contributed to the design and implementation of the study. Pierangelo Moretti, Roberta Franchi and Teresa Maria Poluzzi performed data collection. Saverio Paltrinieri and Pierangelo Moretti performed statistical analyses and data interpretation. All authors discussed the results, gave critical feedback, took the lead in writing and were responsible for the overall content of the manuscript.

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ETHICS STATEMENT

All peripheral blood samples used in this study were collected from dogs referred to our hospital for routine diagnostic or wellness visits. Therefore, according to our institution (University of Milan) guidelines, a formal approval from the Institutional Animal Care and Use Committee was not required (Ethic Committee decision 29 October 2012, renewed with the protocol no. 02-2016).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article and from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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