1 **Analytical variability in the enumeration of neutrophil subpopulations in canine blood**

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4 **Running title: Imprecision of neutrophil counts in dogs**

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## Abstract

**Background.** Conventional differential leukocyte counts does not enumerate hyposegmented neutrophils (Hypo-PMNs), i.e. immature neutrophils that already lost the band morphology but are not yet completely segmented, that may early identify acute inflammation.

**Objectives.** To evaluate the analytical variability of counts of Bands, Hypo-PMNs, Young-PMNs (Bands + Hypo-PMNs), mature neutrophils (Seg-PMNs), non-Bands (Seg-PMNs + Hypo-PMNs); to assess if Hypo- or Young-PMNs identify inflammation better than Bands.

**Methods.** Neutrophil subpopulations were counted by 2 observers on 2 sets of 100 cells in 267 samples from dogs with changes potentially consistent with inflammation, to calculate the intra- and inter-observer variability.

**Results.** Median intra-observer CVs were <5.0% for Seg-PMNs and non-Bands, 20.0% to 28.0% for Hypo-PMNs and Young-PMNs; Median inter-observer CVs for Seg-PMNs, non-Bands, Hypo-PMNs, Young-PMNs were 4.6%, 5.0%, 60.0%, 47.1% respectively. Median CVs of Bands on samples on which these cells were visible were 141%.

**Conclusions.** The analytical variability of Hypo- and Young-PMNs is lower than that of Bands. This retrospective study did not allow us to investigate the diagnostic potential or the clinical relevance of these cells. However, the low inter- and intra-observer variability with these cell populations suggest that the count of Hypo- or Young-PMNs may better identify acute inflammation than the count of Bands.



*Keywords*: Dog; Inflammation; left shift; leukogram; neutrophilia;

36 **Introduction**

37

38 In normal conditions, the proliferation and maturation of neutrophils occurs in bone marrow,
39 where precursors evolve to mature polymorphonuclear granulocytes (PMNs), with segmented
40 nuclei (Seg-PMNs). A pool of Seg-PMNs remains in the bone marrow while the majority of
41 Seg-PMNs is released in the blood vessels. In the vessels, part of the Seg-PMNs released by
42 the bone marrow adhere to the endothelium (marginal pool), and the remaining cells circulate
43 in the blood stream (circulating pool).[1]
44 When neutrophils are recruited in inflammatory foci, in order to provide an adequate support
45 of efficient and mature neutrophils to sustain the inflammatory reaction, the cytokine- and
46 cortisol-mediated release of the marginal pool induces a rapid increase of the number of
47 circulating Seg-PMNs. Also the bone marrow pool of Seg-PMNs may be mobilized to
48 increase the number of circulating mature cells. Then, immature neutrophils are released in
49 blood only if the peripheral consumption exceeds the capability of the marginal and bone
50 marrow pool to replace the cells recruited in inflamed tissues.[2]
51 Therefore, detecting immature neutrophils in blood smears (left shift) is an important marker
52 of inflammation. Moreover an exaggerated left shift may suggest an excessive peripheral
53 consumption of mature neutrophils exists, thus working as a negative prognostic factor in
54 inflammatory diseases.[3-5]
55 The widely accepted approach to quantify the magnitude of the left shift is the enumeration of
56 non segmented (band) neutrophils.[1,2] However, this approach has two limitations: first, the
57 correct identification of these cells may be subjective. A consensus statement on the definition
58 of a band neutrophil has not been established in veterinary medicine and several definitions of
59 bands are available in literature: according to the textbooks, PMNs are classified as bands "if
60 the nucleus of the cell hasn't any constrictions or has constrictions that are less than half of

61 the diameter of the remainder of the nucleus”[6] or as “a neutrophil with no area of the nucleus
62 less than two-thirds the diameter of any other area of the nucleus”.[7] Therefore, there is an
63 inherent subjective component to the microscopic definition of a band, and that it requires
64 some expertise. Nevertheless the inter- or intra-observer variability of microscopic
65 classification of bands is not known. The second limitation is that the simple classification of
66 PMNs into the two main categories of “mature” and “bands” does not provide a complete
67 overview of the magnitude of the left shift, since between these two categories there is a
68 series of intermediate stages of less mature, “hyposegmented” neutrophils (Hypo-PMNs), that
69 already lost the band morphology (i.e. that have a nuclear constriction that, however, is more
70 than half of the remainder of the nucleus) but are not yet completely segmented (i.e. still not
71 have 2 or more separate lobes).
72 The hypothesis of this study is that the inclusion of Hypo-PMNs in the pool of immature
73 neutrophils may decrease the inter- and intra-observed imprecision of counts.
74 Therefore, the aims of this study was to assess the inter- and intra-operator variability in
75 manual count of Bands and Hypo-PMNs, counted separately or in a single group of Young-
76 PMNs (Bands + Hypo-PMNs).
77
78 **Materials and Methods**
79
80 *Retrospective search in the database and selection of cases*
81 The database of our diagnostic laboratory was retrospectively searched to select the cases that
82 were likely characterized by immature neutrophils in blood. To this aim, from the general
83 database we selected all the canine samples submitted from January to December 2014 that
84 had data regarding the CBC and at least one slide stored in the archive of the laboratory, and
85 that fulfilled one or more of the following inclusion criteria (the reference intervals reported

in the following lines were validated from literature with the transference method[8] or generated in our laboratory according to ASVCP guidelines[9]):

1) Leukocytosis (>19.5 WBC x$10^3$/µL) or leukopenia (<6.0 WBC x$10^3$/µL).
2) Neutrophilia (>11.5 neutrophils x$10^3$/µL) or neutropenia (<3.0 neutrophils x$10^3$/µL).
3) Presence of neutrophilic left shift or toxic changes in the report prepared at the time of submission for the routine diagnostic analyses.
4) Presence of hyperproteinemia (total protein >7.50 g/dL).
5) Concentration of CRP >10.0 mg/dL.
6) Electrophoretic results consistent with inflammation, i.e. characterized by one or more of the following findings: hypoalbuminemia (albumin <23.9 g/L) hyperglobulinemia (>44.8 g/L), increased $\alpha_1$-globulin (>2.8 g/L), $\alpha_2$-globulin (>13.0 g/L), $\beta_1$-globulin (>6.6 g/L), $\gamma$-globulin (>24.0 g/L) or decreased A /G ratio (<0.60).

Information on the final diagnosis, when present in the database, was also recorded.

All the samples have been collected for diagnostic purposes under an informed consent of the owner. Therefore, according to the regulations of our Institution, a formal approval of the Informed Ethical Committee was not needed.

In all cases, the laboratory work-up was performed with the same laboratory instruments and methods, as follows: hematological data were generated using an automated analyzer (Sysmex XT-2000iV) validated in dogs.[10-11] Differential leukocyte counts were microscopically verified on May-Grunwald Giemsa stained smears. Serum protein electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Florence, Italy) and the specific manufacturer's reagents (Hydragel 30 PROTEIN(E) Sebia Italia Srl), as described in a previous study.[12] The concentration of total protein and CRP were measured using an automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) using respectively a commercially available kit (Real

111 Time Diagnostic System, Viterbo, Italy) based on the biuret method and an
112 immunoturbidimetric kit (Canine CRP, Randox Laboratories Limited, Country Antrim, UK),
113 already validated in dogs.[13]

114

115 *Glass slides review*

116 The glass slides selected as described above were independently reviewed by the two Authors
117 (one Board certified clinical pathologist and one graduate student) in a blind manner (i.e.
118 without information on the final diagnosis or on the cell counts performed by the other
119 observer). The two observers counted two sets of 100 cells on each slide and performed a 7
120 populations differential. This extended leukogram included the 6 populations routinely
121 counted in diagnostic leukograms: segmented neutrophils (Seg-PMNs), band neutrophils
122 (Bands), eosinophils, basophils, lymphocytes and monocytes. The seventh population was
123 named as hyposegmented neutrophils (Hypo-PMNs). The criteria used to differentiate PMNs
124 and Bands were those described by the Schalm's hematology textbook (figure 1):[6]

125 - Seg-PMNs: neutrophils with a diameter of 10-12 μm, characterized by a nucleus
126 composed by tightly condensed chromatin, with distinct lobes (2 to 5), clearly
127 separated by constricted areas and abundant clear to slightly eosinophilic cytoplasm
128 containing several faint granulations.

129 - Bands: slightly larger than PMNs (12-16 μm or rarely up to 18-20 μm), with a U-, C-,
130 L- or S-shaped nucleus, with less condensed chromatin, that has parallel sides lacking
131 any discrete nuclear constrictions or having constrictions smaller than half of the
132 diameter of the remainder of the nucleus.

133 In addition to these two main population, we classified as "Hypo-PMNs" the neutrophils
134 characterized by variable size (10-16 μm) and by an U-, C-, L- or S-shaped nucleus with a
135 variable chromatin pattern, and a tendency to form two separate lobes, on which, however, the

136 constrictions between the lobes was slightly less than half of the diameter of the remainder of
137 the nucleus.
138 For further statistical analyses, data from these cell populations were considered separately or
139 merged in the following groups:
140 - "non-Bands", that includes Seg-PMNs and Hypo-PMNs, simulating what happens in
141 routine hemograms, were all the "non-Band" cells are considered mature neutrophils.
142 - "Young-PMNs", that includes Hypo-PMNs and Bands, to assess whether the inclusion
143 of hyposegmented neutrophils in the immature pool ameliorates both the analytical
144 variability and the possibility to diagnose acute inflammation.
145
146 *Statistical analysis*
147 Statistical analysis was performed using the software Analyse-it v. 2.1 (Analyse-it Ltd, Leeds,
148 UK) that works on Excel spreadsheets. The level of significance was set at $P<0.05$. The
149 following statistical analyses were performed:
150 *Evaluation of intra-observer analytical variability:* for each neutrophil subpopulation, alone
151 or combined to each other, the results obtained by each operator in the two sets of 100 cells
152 were used to calculate mean and standard deviations and the coefficient of variation using the
153 formula: CV = SD/mean x 100. The correlation between the magnitude of the CV and the
154 mean value of each subpopulation was assessed through the Spearman correlation test.
155 *Evaluation of inter-observer analytical variability* for each neutrophil subpopulation, alone or
156 combined to each other, the mean result obtained by each operator in the 200 cell counts were
157 used to calculate mean and standard deviations and the CV with the formula mentioned
158 above. The agreement between the two investigators was assessed using a Passing Bablok and
159 a Bland Altman test. Moreover, in order to assess the possible impact of inter-observer

160 variability, the differences between the counts of the two observers were assessed using the
161 non parametric Wilcoxon signed rank test.
162
163 **Results**
164 *Caseload*
165 Among the 1249 samples from dogs included in the database, 271 fulfilled the inclusion
166 criteria. In 4 of these, however, the quality of the stored slide was not adequate. Therefore,
167 267 cases were available for the analysis of intra- and inter-observer variability. These
168 samples were collected from 209 dogs, 58 of which were sampled 2 to 10 times during the
169 follow up. The following inclusion criteria were present in the 267 samples included in this
170 study: leukocytosis or neutrophilia (n=148, in 3 of these cases toxic changes were found in
171 PMNs at the time of admission), leukopenia or neutropenia (n=69), hyperproteinemia or
172 electrophoretic changes not associated with leukocytosis or leukopenia (n=35, 14 of which
173 characterized by hyperproteinemia and increases of one or more globulin fractions, 10 by
174 normoproteinemia and increases of one or more globulin fractions, 5 by hyperproteinemia, 3
175 by hypoalbuminemia, 3 by hypoalbuminemia and increases of one or more globulin
176 fractions), altered leukogram (i.e. leukocytosis and neutrophilia or neutropenia) associated
177 with hyperproteinemia or electrophoretic changes (n=10), leukocytosis and neutropenia (n=3),
178 increased CRP without changes in serum protein electrophoresis or in leukocyte/neutrophil
179 counts (n=2).
180
181 *Intra- and inter-observer variability*
182 The data regarding the intra-observer variability (i.e. the variability of two counts of 100 cells
183 of the same operator) and inter-observer variability (i.e. the variability of the 200-cell counts
184 generated by the two operators) are summarized in table 1.

185 As shown in the table, the CVs, in terms of median values and interquartile ranges were
186 particularly low for subpopulation of PMNs that were abundant in blood (e.g. Seg-PMNs,
187 non-Bands) and notably higher for cell populations that were poorly represented (Hypo-
188 PMNs, Young-PMNs), with the exception of Bands. For this cell population the median CV
189 was 0.0%, but this CV has been calculated on the whole caseload, that included a high
190 proportion of cases on which none of the observer detected bands in blood smears. This
191 variability increases if only samples in which Bands have been observed are considered: for
192 example, when the CV is calculated based on the 133 samples in which at least one observer
193 detected Bands in the slide, the median value rises to 141.4% (min-max: 0.0-141.4%) while
194 the median values of the 253 samples on which at least one Hypo-PMN was observed remains
195 around 60% (63.4%; min-max: 0.0-141.4%) and further decreases in the 253 samples on
196 which at least one Young-PMN was counted (median: 52.7%; min-max: 0.0-141.4%).
197 However, for all the populations the min-max range was very wide, likely depending on the
198 wide range of percentage of each cell population, that induced highest CVs in those cases
199 with low percentages, and vice versa. The Spearmann test confirmed the presence of a weak
200 but negative correlation between the magnitude of CVs and the percentage of PMNs
201 ($P<0.001$, $r = -0.48$ for observer 1, $P<0.001$, $r = -0.46$ for observer 2, $P<0.001$, $r = -0.53$ for
202 inter-observer CVs) or the percentages of non-Bands ($P<0.001$, $r = -0.56$ for observer 1,
203 $P<0.001$, $r = -0.52$ for observer 2, $P<0.001$, $r = -0.59$ for inter-observer CVs). Conversely for
204 populations that were virtually absent from blood, correlations were not statistically
205 significant (e.g. intra-observer variability of Hypo-PMNs, Young-PMNs) weakly negative
206 (e.g. inter-observer variability of Hypo-PMNs and Young-PMNs, respectively $P<0.001$, r = -
207 0.26 and $P<0.001$, $r = -0.25$) or positive (Bands; $P<0.001$, $r = 0.86$ for observer 1, $P<0.001$, r
208 = 0.69 for observer 2, $P<0.001$, $r = -0.77$ for inter-observer CVs).

209 The highest CVs were recorded for inter-observer variability and inter-observer differences in
210 the proportion of smears with detectable bands or Hypo-PMNs were also present: according
211 to the first observer, in the whole caseload hypo-PMNs were detectable in 230 smears, 57 of
212 which had also Bands. Therefore, according to this observer, only in 39 cases no Bands or
213 Hypo-PMNs were visible. Conversely, according to the second observer, Bands were present
214 in 118 cases and Hypo-PMNs in 214 cases, some of which had no Bands detectable on the
215 smears. Therefore, according to this observer, in 49 cases no Bands or Hypo-PMNs were
216 visible. This lead also to some significant difference between the two observers. The
217 percentage of Hypo-PMNs, of non-Bands and of Young-PMNs were significantly higher for
218 the observer 1 compared with the observer 2, while the percentage of Bands was significantly
219 higher for the observer 2 compared with the observer 1 (figure 2), and results of agreement
220 tests (figure 3) demonstrated the presence of a significant absolute bias for all the
221 subpopulation of neutrophils, except for Seg-PMNs (table 2). However, no constant errors
222 were found by the agreement test and a proportional error was present only for Hypo-PMNs
223 and Bands.
224
225 **Discussion**
226 The aim of this study was to provide an estimate of intra- and inter-observer variability of
227 microscopical counts of Bands and of Hypo-PMNs (i.e. hyposegmented neutrophils that
228 already lost the "band morphology" but do not have yet the peculiar segmentations that
229 characterizes mature neutrophils), in order to assess whether the analytical variability of
230 Hypo-PMNs is lower than that of Bands and of other PMNs subpopulation, thus providing a
231 more reliable tool for the identification of acute inflammation.
232 To this aim, slides stored in our archive were selected based on inclusion criteria that allowed
233 us to increase the likelihood to have a wide range of percentages of immature neutrophils.

234 This selection process included also samples repeatedly collected after treatment from the
235 same dog. This approach allowed us to increase the caseload and minimally influenced the
236 interpretation of the results since the study was focused on the analytical variability of the
237 different cell counts and not on the diagnostic or prognostic role of the different
238 subpopulation of neutrophils.
239 The intra-observer CVs recorded by each of the observers in the 2 sets of 100 cells were
240 always similar to each other. This suggests that each single observer well standardized the
241 criteria to correctly classify the cells. Nevertheless, the CVs were often high, likely depending
242 on the low percentage of some cell population. On this regards it should be stressed that in our
243 caseload the range of percentages of each cell type was very wide also for the cell populations
244 that are usually abundant in blood such as segmented PMNs. This was due to the inclusion in
245 the caseload of cases with acute inflammation, characterized by high percentages of PMNs, of
246 cases characterized by leuko- neutropenia and of leukemic cases, where neutrophils are
247 virtually absent since the percentage of neoplastic cells may approach 100%. This wide range,
248 however, allowed us to demonstrate that the intra-and inter-individual variability are at least
249 in part influenced by the percentage of cells, as demonstrated by the negative correlation
250 between the abundance of cell populations and the CVs, that, although weak, confirms that
251 the lower is the percentage of cells in blood, the higher is the variability. This is not surprising
252 since this inverse relationship is well known[14] and may affect the differential leukocyte
253 counts,[15] for which is also known that the inter-observer variability decreases if the number of
254 counted cells increases.[16] Therefore, in all the conditions on which it may be important to
255 precisely quantify cell populations that are poorly represented in blood, such as the neutrophil
256 subpopulations investigated in this study, it may be appropriate to count higher number of
257 cells than the 100 cells usually performed for manual differential.[15] The CVs were even
258 higher for inter-observer variability, especially for cell populations that were poorly

259 represented in blood, as expected.[15,16] This led to significant differences between the two
260 observers for almost all the neutrophil subpopulations except for segmented PMNs likely due
261 to an intrinsic difference in the visual perception of the operators, or to the different level of
262 experience of the operators, (and subsequent misclassification of Hypo-PMNs as Bands or
263 mature PMNs) rather than on other variables potentially inducing inter-observer variations
264 (e.g. different distribution of cells in different areas of the slides). Looking at the distribution
265 of data, however, these significant differences are probably not relevant on a clinical
266 standpoint for the subpopulations that are abundant in blood (mature PMNs, non-Bands) but it
267 may be relevant for cell populations poorly represented (Bands, Hypo-PMNs and Young-
268 PMNs), for which even a deference of a few cells in two different counts may be relevant.
269 From this perspective, it should be noted that, when samples without Bands or Hypo-PMNs
270 are excluded from the calculation, the inter-observer variability is lower for Hypo-PMNs than
271 for Bands and further decreases when Bands and Hypo-PMNs are merged in a single category
272 of Young-PMNs. This higher reliability in the results may be relevant in the evaluation of
273 acute inflammation. This has not been investigated in this study, but the possible diagnostic
274 advantage of the use of less strict criteria for the identification of left shift (i.e. the inclusion in
275 the count of "bands" of cells that do not perfectly fit the definition of bands based on the
276 proportion between thinner and ticker parts of the nucleus) has been demonstrated in species
277 other than dogs, such as marine mammals, on which immature cells with the classical band
278 morphology are not frequent.[17]
279 However, the lack of precise clinical information is a limitation of this study. The lack of this
280 information did not allow us to define the possible clinical relevance of the enumeration of
281 the different neutrophils population. Another limitation of the study is the inherent
282 subjectivity of classification of Bands vs Hypo-PMNs. However, based on the current results,

283 this subjectivity decreases, as demonstrated by the decreased magnitude of the CVs, when
284 Bands and Hypo-PMNs are merged in a single group of Young-PMNs.
285 In conclusion, this study demonstrated that both the intra- and inter-observer variability of
286 Hypo-PMNs and of Young-PMNs (i.e. Hypo-PMNs and Bands) is lower than that of Bands,
287 although when all the cell populations are rare in blood, the coefficient of variation are still
288 higher than 50%. The retrospective nature of this study did not allow to standardize the
289 inclusion criteria to classify dogs as affected by acute or chronic inflammation or to evaluate
290 the diagnostic or prognostic power of WBC morphology through the assessment of the
291 clinical outcome. However, these results are encouraging to design future prospective studies
292 focused to investigate the diagnostic performances of Hypo-PMNs or of Young-PMNs in
293 selected acute inflammatory diseases and/or to assess whether changes in these populations
294 occurs earlier in the course of inflammatory diseases than those of Bands or of Seg-PMNs.
295

296 **Conflict of interest statement**
297 None of the authors of this paper has a financial or personal relationship with other people or
298 organizations that could inappropriately influence or bias the content of the paper.
299

300 **Acknowledgements:**
301 This study did not receive a specific grant. The Authors are grateful to the staff of the
302 Diagnostic laboratory of the Department, that was involved in routine sample reception and
303 processing
304

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373

374 **Table 1**

375 Percentage values recorded by each observer on a 200 cell count performed on the 267 smears included in this study, and overall percentage

376 recorded by the 2 observers, with the corresponding intra- and inter-observer CV. Data are reported as median value, I-III interquartile range

377 (between parenthesis) and min-max value (in italic).

| | Observer 1 (%) | Observer 2 (%) | Mean of the 2 observers (%) | CV intra-observer 1 (%) | CV intra-observer 2 (%) | CV inter-observer (%) |
|---|---|---|---|---|---|---|
| Seg-PMNs | 72.0<br>(60.5-79.9)<br>*0.5-96.0* | 71.0<br>(58.6-80.0)<br>*7.0-95.5* | 70.8<br>(59.3-79.5)<br>*5.3-94.8* | 3.7<br>(1.7-6.8)<br>*0.0-141.4* | 3.9<br>(1.7-7.4)<br>*0.0-141.4* | 4.6<br>(2.1-9.8)<br>*0.0-128.0* |
| Bands | 0.0<br>(0.0-0.0)<br>*0.0-9.5* | 0.0<br>(0.0-1.0)<br>*0.0-19.0* | 0.3<br>(0.0-0.8)<br>*0.0-12.3* | 0.0<br>(0.0-0.0)<br>*0.0-141.4* | 0.0<br>(0.0-47.1)<br>*0.0-141.4* | 0.0<br>(0.0-141.0)<br>*0.0-141.0* |
| Hypo-PMNs | 3.0<br>(1.0-5.5)<br>*0.0-25.0* | 1.5<br>(0.5-3.9)<br>*0.0-27.0* | 2.3<br>(1.0-4.7)<br>*0.0-24.0* | 20.2<br>(0.0-47.1)<br>*0.0-141.4* | 28.3<br>(0.0-70.7)<br>*0.0-141.4* | 60.6<br>(23.6-124.7)<br>*0.0-141.4* |

| | | | | | | |
|---|---|---|---|---|---|---|
| non-Bands | 76.5<br>(64.5-86.0)<br>*0.5-96.5* | 74.0<br>(61.5-83.4)<br>*7.0-97.0* | 74.0<br>(63.5-84.5)<br>*7.0-96.0* | 3.3<br>(1.6-6.5)<br>*0.0-141.4* | 3.6<br>(1.6-6.6)<br>*0.0-60.6* | 5.0<br>(2.1-9.9)<br>*0.0-135.3* |
| Young-<br>PMNs | 3.0<br>(1.0-6.0)<br>*0.0-34.5* | 2.0<br>(0.5-5.0)<br>*0.0-43.0* | 2.5<br>(1.0-5.5)<br>*0.0-36.2* | 20.2<br>(0.0-44.8)<br>*0.0-141.4* | 28.3<br>(0.0-70.7)<br>*0.0-141.4* | 47.1<br>(20.2-113.1)<br>*0.0-141.4* |

378 **Table 2:** Details of the Passing Bablok coefficients and of the absolute bias recorded by
379 Bland Altman analysis regarding the comparison between the percentage of cells belonging to
380 each neutrophil subpopulation recorded by the two observers on the whole caseload (267
381 canine blood smears). (Seg-PMNs = segmented neutrophils; Bands = band neutrophils; Hypo-
382 PMNs = hypo.segmented neutrophils; Non Bands = .Hypo-PMNs + Seg-PMNs; Young
383 PMNs = Bands + Hypo-PMNs) The 95% confidence intervals are reported in brackets.

| Neutrophil subpopulation | Passing Bablok | | Bland Altman | |
|---|---|---|---|---|
| | Intercept | Slope | Bias (Obs 2 – Obs 1) | P |
| Seg-PMNs | -3.12 (-8.54 to 1.17) | 1.04 (0.98 to 1.11) | -0.28% (-1.57% to 1.00%) | ns |
| Bands | 0.00 (0.00 to 0.00) | 1.00 (1.00 to 1.00) | -0.09% (-0.23% to 0.05%) | <0.001 |
| Hypo-PMNs | -0.01 (-0.29 to 0.00) | 0.60 (0.50 to 0.71) | -1.50% (-1.92% to -1.08%) | <0.001 |
| Non-Bands | -2.99 (-9.06 to 1.50) | 1.01 (0.95 to 1.09) | -1.78% (-3.11% to 0.45%) | 0.008 |
| Young PMNs | -0.33 (-0.50 to 0.00) | 0.83 (0.69 to 1.00) | -0.66% (-1.18% to -0.13%) | 0.014 |

384

385 **Figure legends**

386 Figure 1

387 Examples of non segmented neutrophils (Bands; A), Hyposegmented neutrophils (Hypo-Seg,
388 B and C) and mature segmented neutrophils (Seg-PMNs, D). In the Band neutrophil in A no
389 constriction are visible in the "U" shaped nucleus and the diameter of the nucleus is
390 homogeneous; in the Hypo-Segs in B and C the diameter of the nuclei where constrictions are
391 present (black arrows) is more than half of the diameter of the thicker parts of the nucleus
392 (grey arrows) but no clearly separate lobes are present; In the Seg-PMN in D the nucleus
393 shows an evident constriction that forms a distinct lobe. Dog, blood smear, May Grünwald-
394 Giemsa stain. Bars = 10 µm

395

396 Figure 2: Percentages of the different neutrophil subpopulation recorded by the two observers
397 on the 267 canine blood smears. Boxes indicate the I-III interquartile interval, the horizontal
398 line corresponds to the median value, the vertical lines are the limits of outlier distribution
399 according to the Tukey rule. Near outliers are indicated by open circles and far outliers with
400 the black circles. The asterisks within the boxes indicate significant differences (*** =
401 P<0.001) between observers.

402

403 Figure 3: Results of Passing Bablok (upper row of plots) and Bland Altman (lower row of
404 plots) tests regarding the comparison between the percentage of cells belonging to each
405 neutrophil subpopulation recorded by the two observers on the whole caseload (267 canine
406 blood smears). The blue lines indicate the Passing Bablok fit in the Passing Bablok plot and
407 the bias in the Bland Altman plot and the grey line indicate, in both the plots, the identity line:
408 the dotted lines indicate the 95% Confidence Interval of the Passing Bablok fir and of the
409 bias.