Analytical variability in the enumeration of neutrophil subpopulations in canine blood

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;
**Introduction**

In normal conditions, the proliferation and maturation of neutrophils occurs in bone marrow, where precursors evolve to mature polymorphonuclear granulocytes (PMNs), with segmented nuclei (Seg-PMNs). A pool of Seg-PMNs remains in the bone marrow while the majority of Seg-PMNs is released in the blood vessels. In the vessels, part of the Seg-PMNs released by the bone marrow adhere to the endothelium (marginal pool), and the remaining cells circulate in the blood stream (circulating pool).\(^1\)

When neutrophils are recruited in inflammatory foci, in order to provide an adequate support of efficient and mature neutrophils to sustain the inflammatory reaction, the cytokine- and cortisol-mediated release of the marginal pool induces a rapid increase of the number of circulating Seg-PMNs. Also the bone marrow pool of Seg-PMNs may be mobilized to increase the number of circulating mature cells. Then, immature neutrophils are released in blood only if the peripheral consumption exceeds the capability of the marginal and bone marrow pool to replace the cells recruited in inflamed tissues.\(^2\)

Therefore, detecting immature neutrophils in blood smears (left shift) is an important marker of inflammation. Moreover an exaggerated left shift may suggest an excessive peripheral consumption of mature neutrophils exists, thus working as a negative prognostic factor in inflammatory diseases.\(^3,5\)

The widely accepted approach to quantify the magnitude of the left shift is the enumeration of non segmented (band) neutrophils.\(^1,2\) However, this approach has two limitations: first, the correct identification of these cells may be subjective. A consensus statement on the definition of a band neutrophil has not been established in veterinary medicine and several definitions of bands are available in literature: according to the textbooks, PMNs are classified as bands “if the nucleus of the cell hasn’t any constrictions or has constrictions that are less than half of
the diameter of the remainder of the nucleus” or as “a neutrophil with no area of the nucleus less than two-thirds the diameter of any other area of the nucleus”. Therefore, there is an inherent subjective component to the microscopic definition of a band, and that it requires some expertise. Nevertheless the inter- or intra-observer variability of microscopic classification of bands is not known. The second limitation is that the simple classification of PMNs into the two main categories of “mature” and “bands” does not provide a complete overview of the magnitude of the left shift, since between these two categories there is a series of intermediate stages of less mature, “hyposegmented” neutrophils (Hypo-PMNs), that already lost the band morphology (i.e. that have a nuclear constriction that, however, is more than half of the remainder of the nucleus) but are not yet completely segmented (i.e. still not have 2 or more separate lobes).

The hypothesis of this study is that the inclusion of Hypo-PMNs in the pool of immature neutrophils may decrease the inter- and intra-observed imprecision of counts.

Therefore, the aims of this study was to assess the inter- and intra-operator variability in manual count of Bands and Hypo-PMNs, counted separately or in a single group of Young-PMNs (Bands + Hypo-PMNs).

**Materials and Methods**

*Retrospective search in the database and selection of cases*

The database of our diagnostic laboratory was retrospectively searched to select the cases that were likely characterized by immature neutrophils in blood. To this aim, from the general database we selected all the canine samples submitted from January to December 2014 that had data regarding the CBC and at least one slide stored in the archive of the laboratory, and 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\textsuperscript{8} or
generated in our laboratory according to ASVCP guidelines\textsuperscript{9>):

1) Leukocytosis (>19.5 WBC x10\(^3\)/µL) or leukopenia (<6.0 WBC x10\(^3\)/µL).

2) Neutrophilia (>11.5 neutrophils x10\(^3\)/µL) or neutropenia (<3.0 neutrophils x10\(^3\)/µL).

3) Presence of neutrophilic left shift or toxic changes in the report prepared at the time of

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.\textsuperscript{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.\textsuperscript{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
Time Diagnostic System, Viterbo, Italy) based on the biuret method and an immunoturbidimetric kit (Canine CRP, Randox Laboratories Limited, Country Antrim, UK), already validated in dogs.

Glass slides review

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

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

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

In addition to these two main population, we classified as “Hypo-PMNs” the neutrophils characterized by variable size (10-16 μm) and by an U-, C-, L- or S-shaped nucleus with a variable chromatin pattern, and a tendency to form two separate lobes, on which, however, the...
constrictions between the lobes was slightly less than half of the diameter of the remainder of the nucleus.

For further statistical analyses, data from these cell populations were considered separately or merged in the following groups:

- “non-Bands”, that includes Seg-PMNs and Hypo-PMNs, simulating what happens in routine hemograms, were all the “non-Band” cells are considered mature neutrophils.

- “Young-PMNs”, that includes Hypo-PMNs and Bands, to assess whether the inclusion of hyposegmented neutrophils in the immature pool ameliorates both the analytical variability and the possibility to diagnose acute inflammation.

Statistical analysis

Statistical analysis was performed using the software Analyse-it v. 2.1 (Analyse-it Ltd, Leeds, UK) that works on Excel spreadsheets. The level of significance was set at P<0.05. The following statistical analyses were performed:

Evaluation of intra-observer analytical variability: for each neutrophil subpopulation, alone or combined to each other, the results obtained by each operator in the two sets of 100 cells were used to calculate mean and standard deviations and the coefficient of variation using the formula: CV = SD/mean x 100. The correlation between the magnitude of the CV and the mean value of each subpopulation was assessed through the Spearman correlation test.

Evaluation of inter-observer analytical variability for each neutrophil subpopulation, alone or combined to each other, the mean result obtained by each operator in the 200 cell counts were used to calculate mean and standard deviations and the CV with the formula mentioned above. The agreement between the two investigators was assessed using a Passing Bablok and a Bland Altman test. Moreover, in order to assess the possible impact of inter-observer
variability, the differences between the counts of the two observers were assessed using the non parametric Wilcoxon signed rank test.

Results

Caseload

Among the 1249 samples from dogs included in the database, 271 fulfilled the inclusion criteria. In 4 of these, however, the quality of the stored slide was not adequate. Therefore, 267 cases were available for the analysis of intra- and inter-observer variability. These samples were collected from 209 dogs, 58 of which were sampled 2 to 10 times during the follow up. The following inclusion criteria were present in the 267 samples included in this study: leukocytosis or neutrophilia (n=148, in 3 of these cases toxic changes were found in PMNs at the time of admission), leukopenia or neutropenia (n=69), hyperproteinemia or electrophoretic changes not associated with leukocytosis or leukopenia (n=35, 14 of which characterized by hyperproteinemia and increases of one or more globulin fractions, 10 by normoproteinemia and increases of one or more globulin fractions, 5 by hyperproteinemia, 3 by hypoalbuminemia, 3 by hypoalbuminemia and increases of one or more globulin fractions), altered leukogram (i.e. leukocytosis and neutrophilia or neutropenia) associated with hyperproteinemia or electrophoretic changes (n=10), leukocytosis and neutropenia (n=3), increased CRP without changes in serum protein electrophoresis or in leukocyte/neutrophil counts (n=2).

Intra- and inter-observer variability

The data regarding the intra-observer variability (i.e. the variability of two counts of 100 cells of the same operator) and inter-observer variability (i.e. the variability of the 200-cell counts generated by the two operators) are summarized in table 1.
As shown in the table, the CVs, in terms of median values and interquartile ranges were particularly low for subpopulation of PMNs that were abundant in blood (e.g. Seg-PMNs, non-Bands) and notably higher for cell populations that were poorly represented (Hypo-PMNs, Young-PMNs), with the exception of Bands. For this cell population the median CV was 0.0%, but this CV has been calculated on the whole caseload, that included a high proportion of cases on which none of the observer detected bands in blood smears. This variability increases if only samples in which Bands have been observed are considered: for example, when the CV is calculated based on the 133 samples in which at least one observer detected Bands in the slide, the median value rises to 141.4% (min-max: 0.0-141.4%) while the median values of the 253 samples on which at least one Hypo-PMN was observed remains around 60% (63.4%; min-max: 0.0-141.4%) and further decreases in the 253 samples on which at least one Young-PMN was counted (median: 52.7%; min-max: 0.0-141.4%). However, for all the populations the min-max range was very wide, likely depending on the wide range of percentage of each cell population, that induced highest CVs in those cases with low percentages, and vice versa. The Spearmann test confirmed the presence of a weak but negative correlation between the magnitude of CVs and the percentage of PMNs (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 inter-observer CVs) or the percentages of non-Bands (P<0.001, r = -0.56 for observer 1, P<0.001, r = -0.52 for observer 2, P<0.001, r = -0.59 for inter-observer CVs). Conversely for populations that were virtually absent from blood, correlations were not statistically significant (e.g. intra-observer variability of Hypo-PMNs, Young-PMNs) weakly negative (e.g. inter-observer variability of Hypo-PMNs and Young-PMNs, respectively P<0.001, r = -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 = 0.69 for observer 2, P<0.001, r = -0.77 for inter-observer CVs).
The highest CVs were recorded for inter-observer variability and inter-observer differences in the proportion of smears with detectable bands or Hypo-PMNs were also present: according to the first observer, in the whole caseload hypo-PMNs were detectable in 230 smears, 57 of which had also Bands. Therefore, according to this observer, only in 39 cases no Bands or Hypo-PMNs were visible. Conversely, according to the second observer, Bands were present in 118 cases and Hypo-PMNs in 214 cases, some of which had no Bands detectable on the smears. Therefore, according to this observer, in 49 cases no Bands or Hypo-PMNs were visible. This lead also to some significant difference between the two observers. The percentage of Hypo-PMNs, of non-Bands and of Young-PMNs were significantly higher for the observer 1 compared with the observer 2, while the percentage of Bands was significantly higher for the observer 2 compared with the observer 1 (figure 2), and results of agreement tests (figure 3) demonstrated the presence of a significant absolute bias for all the subpopulation of neutrophils, except for Seg-PMNs (table 2). However, no constant errors were found by the agreement test and a proportional error was present only for Hypo-PMNs and Bands.

**Discussion**

The aim of this study was to provide an estimate of intra- and inter-observer variability of microscopical counts of Bands and of Hypo-PMNs (i.e. hyosegmented neutrophils that already lost the “band morphology” but do not have yet the peculiar segmentations that characterizes mature neutrophils), in order to assess whether the analytical variability of Hypo-PMNs is lower than that of Bands and of other PMNs subpopulation, thus providing a more reliable tool for the identification of acute inflammation.

To this aim, slides stored in our archive were selected based on inclusion criteria that allowed us to increase the likelihood to have a wide range of percentages of immature neutrophils.
This selection process included also samples repeatedly collected after treatment from the same dog. This approach allowed us to increase the caseload and minimally influenced the interpretation of the results since the study was focused on the analytical variability of the different cell counts and not on the diagnostic or prognostic role of the different subpopulation of neutrophils.

The intra-observer CVs recorded by each of the observers in the 2 sets of 100 cells were always similar to each other. This suggests that each single observer well standardized the criteria to correctly classify the cells. Nevertheless, the CVs were often high, likely depending on the low percentage of some cell population. On this regards it should be stressed that in our caseload the range of percentages of each cell type was very wide also for the cell populations that are usually abundant in blood such as segmented PMNs. This was due to the inclusion in the caseload of cases with acute inflammation, characterized by high percentages of PMNs, of cases characterized by leuko- neutropenia and of leukemic cases, where neutrophils are virtually absent since the percentage of neoplastic cells may approach 100%. This wide range, however, allowed us to demonstrate that the intra-and inter-individual variability are at least in part influenced by the percentage of cells, as demonstrated by the negative correlation between the abundance of cell populations and the CVs, that, although weak, confirms that the lower is the percentage of cells in blood, the higher is the variability. This is not surprising since this inverse relationship is well known\(^\text{14}\) and may affect the differential leukocyte counts,\(^\text{15}\) for which is also known that the inter-observer variability decreases if the number of counted cells increases.\(^\text{16}\) Therefore, in all the conditions on which it may be important to precisely quantify cell populations that are poorly represented in blood, such as the neutrophil subpopulations investigated in this study, it may be appropriate to count higher number of cells than the 100 cells usually performed for manual differential.\(^\text{15}\) The CVs were even higher for inter-observer variability, especially for cell populations that were poorly
represented in blood, as expected.\textsuperscript{15,16} This led to significant differences between the two observers for almost all the neutrophil subpopulations except for segmented PMNs likely due to an intrinsic difference in the visual perception of the operators, or to the different level of experience of the operators, (and subsequent misclassification of Hypo-PMNs as Bands or mature PMNs) rather than on other variables potentially inducing inter-observer variations (e.g. different distribution of cells in different areas of the slides). Looking at the distribution of data, however, these significant differences are probably not relevant on a clinical standpoint for the subpopulations that are abundant in blood (mature PMNs, non-Bands) but it may be relevant for cell populations poorly represented (Bands, Hypo-PMNs and Young-PMNs), for which even a deference of a few cells in two different counts may be relevant. From this perspective, it should be noted that, when samples without Bands or Hypo-PMNs are excluded from the calculation, the inter-observer variability is lower for Hypo-PMNs than for Bands and further decreases when Bands and Hypo-PMNs are merged in a single category of Young-PMNs. This higher reliability in the results may be relevant in the evaluation of acute inflammation. This has not been investigated in this study, but the possible diagnostic advantage of the use of less strict criteria for the identification of left shift (i.e. the inclusion in the count of “bands” of cells that do not perfectly fit the definition of bands based on the proportion between thinner and ticker parts of the nucleus) has been demonstrated in species other than dogs, such as marine mammals, on which immature cells with the classical band morphology are not frequent.\textsuperscript{17} However, the lack of precise clinical information is a limitation of this study. The lack of this information did not allow us to define the possible clinical relevance of the enumeration of the different neutrophils population. Another limitation of the study is the inherent subjectivity of classification of Bands vs Hypo-PMNs. However, based on the current results,
this subjectivity decreases, as demonstrated by the decreased magnitude of the CVs, when Bands and Hypo-PMNs are merged in a single group of Young-PMNs.

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

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgements:

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References


Table 1

Percentage values recorded by each observer on a 200 cell count performed on the 267 smears included in this study, and overall percentage recorded by the 2 observers, with the corresponding intra- and inter-observer CV. Data are reported as median value, I-III interquartile range (between parenthesis) and min-max value (in italic).

<table>
<thead>
<tr>
<th></th>
<th>Observer 1 (%)</th>
<th>Observer 2 (%)</th>
<th>Mean of the 2 observers (%)</th>
<th>CV intra-observer 1 (%)</th>
<th>CV intra-observer 2 (%)</th>
<th>CV inter-observer (%)</th>
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<tbody>
<tr>
<td>Seg-PMNs</td>
<td>72.0 (60.5-79.9)</td>
<td>71.0 (58.6-80.0)</td>
<td>70.8 (59.3-79.5)</td>
<td>3.7 (1.7-6.8)</td>
<td>3.9 (1.7-7.4)</td>
<td>4.6 (2.1-9.8)</td>
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<tr>
<td>Bands</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>0.3 (0.0-0.8)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-47.1)</td>
<td>0.0 (0.0-141.0)</td>
</tr>
<tr>
<td>Hypo-PMNs</td>
<td>3.0 (1.0-5.5)</td>
<td>1.5 (0.5-3.9)</td>
<td>2.3 (1.0-4.7)</td>
<td>20.2 (0.0-47.1)</td>
<td>28.3 (0.0-70.7)</td>
<td>60.6 (23.6-124.7)</td>
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<tr>
<td></td>
<td>non-Bands</td>
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<td></td>
<td>76.5</td>
<td>74.0</td>
<td>74.0</td>
<td>3.3</td>
<td>3.6</td>
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<td>(64.5-86.0)</td>
<td>(61.5-83.4)</td>
<td>(63.5-84.5)</td>
<td>(1.6-6.5)</td>
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<td></td>
<td>0.5-96.5</td>
<td>7.0-97.0</td>
<td>7.0-96.0</td>
<td>0.0-141.4</td>
<td>0.0-60.6</td>
<td>0.0-135.3</td>
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<tr>
<td>Young-PMNs</td>
<td>3.0</td>
<td>2.0</td>
<td>2.5</td>
<td>20.2</td>
<td>28.3</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td>(1.0-6.0)</td>
<td>(0.5-5.0)</td>
<td>(1.0-5.5)</td>
<td>(0.0-44.8)</td>
<td>(0.0-70.7)</td>
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<td>0.0-34.5</td>
<td>0.0-43.0</td>
<td>0.0-36.2</td>
<td>0.0-141.4</td>
<td>0.0-141.4</td>
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Table 2: Details of the Passing Bablok coefficients and of the absolute bias recorded by Bland Altman analysis regarding the comparison between the percentage of cells belonging to each neutrophil subpopulation recorded by the two observers on the whole caseload (267 canine blood smears). (Seg-PMNs = segmented neutrophils; Bands = band neutrophils; Hypo-PMNs = hypo.segmented neutrophils; Non Bands = Hypo-PMNs + Seg-PMNs; Young PMNs = Bands + Hypo-PMNs) The 95% confidence intervals are reported in brackets.

<table>
<thead>
<tr>
<th>Neutrophil subpopulation</th>
<th>Passing Bablok</th>
<th>Bland Altman</th>
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<tr>
<td></td>
<td>Intercept</td>
<td>Slope</td>
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<tr>
<td>Seg-PMNs</td>
<td>-3.12 (-8.54 to 1.17)</td>
<td>1.04 (0.98 to 1.11)</td>
</tr>
<tr>
<td>Bands</td>
<td>0.00 (0.00 to 0.00)</td>
<td>1.00 (1.00 to 1.00)</td>
</tr>
<tr>
<td>Hypo-PMNs</td>
<td>-0.01 (-0.29 to 0.00)</td>
<td>0.60 (0.50 to 0.71)</td>
</tr>
<tr>
<td>Non-Bands</td>
<td>-2.99 (-9.06 to 1.50)</td>
<td>1.01 (0.95 to 1.09)</td>
</tr>
<tr>
<td>Young PMNs</td>
<td>-0.33 (-0.50 to 0.00)</td>
<td>0.83 (0.69 to 1.00)</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1**
Examples of non segmented neutrophils (Bands; A), Hypossegmented neutrophils (Hypo-Seg, B and C) and mature segmented neutrophils (Seg-PMNs, D). In the Band neutrophil in A no constriction are visible in the “U” shaped nucleus and the diameter of the nucleus is homogeneous; in the Hypo-Segs in B and C the diameter of the nuclei where constrictions are present (black arrows) is more than half of the diameter of the thicker parts of the nucleus (grey arrows) but no clearly separate lobes are present; In the Seg-PMN in D the nucleus shows an evident constriction that forms a distinct lobe. Dog, blood smear, May Grünwald-Giemsa stain. Bars = 10 µm

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

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