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IDENTIFICATION OF PERIPHERAL BLOOD INVOLVEMENT IN DOGS WITH LARGE B-CELL LYMPHOMA: COMPARISON OF DIFFERENT METHODS

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Abstract

Stage V lymphoma is defined as the presence of neoplastic cells in peripheral blood (PB), bone marrow, or any other non lymphoid tissue. Still, official guidelines do not specify which technique should be used to assess infiltration.

We assessed the agreement among flow cytometry (FC), blood smear evaluation, and ADVIA120 (LUC and BASO) to quantify PB infiltration in 100 dogs with large B-cell lymphoma (LBCL).

Significant errors were found for all methods compared to FC. A moderate agreement was present between FC and blood smear evaluation, whereas LUC and BASO had excellent specificity but unsatisfactory sensitivity in detecting FC infiltrated PB samples.

The different techniques should not be used alternatively. We support the use of LUC/BASO as a speedy preliminary test to detect infiltrated samples, and the joined use of blood smear evaluation and FC to quantify definitively the infiltration. Our results are valid only within canine LBCL staging workup, once the diagnosis has been confirmed.

Introduction

Lymphoma, the most common hematopoietic neoplasm in dogs, comprises many different entities. Accordingly, different classification schemes have been developed: in particular, the World Health Organization (WHO) scheme is mainly based on the histopathological characteristics (Valli et al. 2002; Valli et al. 2011), whereas the updated Kiel scheme is mainly based on cyto-morphological characteristics (Fournel-Fleury et al., 1997; Ponce et al., 2010); phenotype assessment is also required by both classification schemes. Regardless of the classification scheme used, the most frequently diagnosed forms share the B phenotype and the medium to large size of the cells (Aresu et al., 2013; Ponce et al., 2010; Teske et al., 1994; Valli et al., 2011) and can be referred to as Large B cell Lymphomas (LBCL) (Rout and Avery 2017). Irrespective of the tumor subtype, however, all lymphoma-bearing dogs are routinely staged according to the TNM system provided by the WHO (Owen, 1980). This system provides five stages for classifying the spread of the tumor, ranging from involvement of a single node or lymphoid tissue in a single organ (stage I) to generalized lymph node involvement with infiltration of liver and/or spleen (stage IV). When neoplastic cells are detected in the peripheral blood (PB), bone marrow (BM) and/or other organs in addition to the primary solid tumor, dogs are classified as having stage V disease. To date, different techniques have been used to assess PB and/or BM infiltration: namely, blood smear evaluation (Abbo and Lucroy, 2007; Flory et al., 2007; Graff et al., 2014; Ponce et al., 2004), flow cytometry (FC) (Marconato et al., 2013; Martini et al., 2015; Riondato et al., 2016), or PCR for Antigen Receptor Rearrangement (PARR) (Lana et al., 2016). This lack of consistency may affect staging results (Flory et al., 2007) and prevents the comparison of the data obtained by different studies.

64 Our research group already demonstrated the excellent analytical and diagnostic

65 performances of FC, in detecting and quantifying PB and BM infiltration by LBCL

66 cells (Riondato et al., 2016). Although PARR is more sensitive than FC in detecting

67 the presence of neoplastic cells (Aresu et al., 2014), it is unable to quantify the extent

68 of infiltration. The combined used of this two techniques may improve the accuracy of

69 clinical staging of lymphoma in dogs.

70 However, clinicians may occasionally prefer to use less expensive and time-

71 consuming techniques, such as automated CBC or blood smear evaluation (Regan

72 et al., 2013). Today, a number of different hematology analyzers with specific

73 veterinary software are commercially available. In particular, the laser-based

74 hematology analyzer ADVIA 120 (Siemens Healthcare Diagnostics Inc., Deerfield, IL,

75 USA) provides a differential leukocyte count in the PEROX-channel based on the

76 complexity and peroxidase content of the cells, enumerating neutrophils, eosinophils,

77 lymphocytes, monocytes and large unstained cells (LUC, consisting of activated

78 lymphocytes and blasts) both as percentage and absolute number. In addition, in the

79 BASO-channel, an acidic reagent and a surfactant disrupt platelets and erythrocytes

80 and strip membranes from all other white blood cells except basophils. ADVIA

81 basophil count is not accurate in dogs and other lyse-resistant cells may fall within

82 the gate, including blasts (Lilliehöök and Tvedten, 2011). This phenomenon is called

83 'pseudobasophilia' and it has been described also in humans (Gibbs et al., 2009). In

84 particular, the presence of blasts may be suspected when cells spread from the

85 mononuclear or polymorph nuclear gate into the lyse-resistant cells area in the

86 BASO-channel, or a "blast-nose" in the BASO cytogram is found, with cells creating a

87 tip at the left bottom of the mononuclear area (Stirn et al., 2014). Thus, although

88 never reported in the veterinary literature, LUC and lyse-resistant cells in the BASO

89 channel (BASO) may be used to assess PB infiltration in dogs with lymphoma.

90 The aim of the present study was to compare retrospectively different methods and

91 parameters (blood smear evaluation, LUC, BASO and FC) for detecting PB infiltration

92 in dogs with LBCL.

93 Other methods can be used to stage lymphomas and our intention was not to define

94 which of the available techniques has to be considered the gold standard. Our

95 ultimate goal was to determine whether the techniques and parameters evaluated in

96 our study are interchangeable and equally informative for the clinician, or not.

97

98 **Materials and methods**

99 The FC database of the laboratory of the Department of Veterinary Sciences

100 (University of Turin, Turin, Italy) was interrogated and consecutive cases from

101 January 2012 to February 2014 fulfilling the following inclusion criteria were selected:

102 1) a final diagnosis of LBCL based on cytology, FC and possibly histopathology of an

103 enlarged peripheral lymph node (LN); 2) availability of FC data of lymph node (LN)

104 and PB; 3) availability of a CBC performed via ADVIA120 and/or of a good quality PB

105 smear stained with May Grünwald-Giemsa for a cytological review. All cases were

106 classified according to the updated Kiel classification. Acute lymphoid leukemia was

107 excluded based on bone marrow examination and/or number and degree of

108 hematological alterations, clinical history and signs and follow up data. Cases in

109 which acute leukemia could not be definitively ruled out were not included in the

110 study. No case had been previously treated with corticosteroids or chemotherapy.

All PB samples were collected in EDTA tubes and processed within 24 hours from collection. When not provided along with the EDTA tube, PB smears were prepared at time of sample delivery to the laboratory.

Our FC service receives samples from both the internal oncology service and many different private veterinarians outside the Institution itself making uneven the collection of clinical and histopathological data. These data are not useful for the specific purposes of the present study and are not reported.

All dogs were privately owned and sampled for diagnostic purposes with a written informed consent of the owners. Thus, a formal approval of the Institution Committee for Animal Care of the University of Turin was not necessary.

Both a skilled hematologist (Operator 1) and a recently graduated student (Operator 2) reviewed all PB smears. Both operators were aware of the confirmed LBCL diagnosis, but blinded to the FC results and to the results obtained by the other operator. A 200-cells manual differential count was done and the percentage of immature lymphoid cells was recorded: these cells had to be medium to large in size (nucleus diameter ≥2 RBCs), with scant to moderate basophilic cytoplasm and a round nucleus with dispersed or finely granular chromatin and possibly one or more nucleoli. The presence of other atypical lymphocytes was also recorded when cells with a combination of the following features were detected: nucleus diameter <2 RBCs, moderate basophilic cytoplasm, round nucleus with clumped chromatin, no nucleoli. These cells were considered reactive and excluded from the calculation of infiltrating cells. According to Flory et al. (2007), samples were considered infiltrated if any immature lymphoid cell was present (0.5%).

LUC and BASO values were recorded for each case. Samples were considered infiltrated if LUC or BASO exceeded the upper reference limit calculated using data from 49 healthy dogs (2.4% and 1.6%, respectively).

Sample processing for FC was performed as previously described (Riondato et al., 2016). Samples were acquired with a BD Accuri C6 (Becton Dickinson, San Josè, CA, USA) and data were analyzed with the specific software CFlow Plus (Becton Dickinson). PB infiltration was defined as the percentage of large CD21 positive cells out of total CD45-positive cells and samples were considered infiltrated if the percentage was ≥0.56% (Riondato et al., 2016).

All methods was compared to FC, since diagnostic performances of this latter technique have already been described in our laboratory (Riondato et al., 2016).

A Shapiro-Wilk test was performed to assess whether the data obtained by Operator 1 and 2 through blood smear evaluation were normally distributed. A non-parametric test (Spearman test) was then performed to assess the possible correlation between the infiltration percentages reported by the two operators.

Passing-Bablok regression analysis and Bland-Altman plots (Analyze-it, Analyze-it Software Ltd, Leeds, UK) were used to assess agreement between percentages of infiltration obtained by FC and blood smear evaluation from either operator, LUC and BASO, respectively.

Contingency tables were prepared to compare results from the different techniques and to estimate the diagnostic accuracy of each test, including concordance (κ), sensitivity (Se) and specificity (Sp), positive and negative likelihood ratio (LR+ and LR-, respectively), diagnostic odds (DO), positive and negative predictive value (PPV and NPV, respectively). The website http://www.quantitativeskills.com/sisa was used for these calculations.

159 Finally, since different cutoffs have been used in the literature (Flory et al., 2007;

160 Graff et al., 2014) a Receiver Operating Characteristic (ROC) curve was drawn, to

161 identify the cytological percentage of immature lymphoid cells best discriminating

162 between FC infiltrated and not infiltrated PB samples. Also, ROC curves coordinates

163 were used to assess the sensitivity and specificity of the 10% cutoff described in the

164 literature for cytological assessment of PB infiltration in dogs with large cell

165 lymphoma (Graff et al., 2014). Only results obtained by the most experienced

166 operator (Operator 1) were used to this aim.

167 The statistical software SPSS v19.0 (SPSS Inc, Chicago, IL, USA) was used for

168 Shapiro-Wilk and Spearman tests and to draw ROC curves. Significance was set at

169 $p \leq 0.05$.

170 **Results**

171 Overall, samples from 100 dogs with LBCL were included in the study. The diagnosis

172 of LBCL was made based on cytology and FC of an enlarged LN in all cases; in 31

173 cases, this diagnosis was also confirmed via histopathology. FC was available for all

174 PB samples. In addition, 87 (87%) cases had both blood smear evaluation performed

175 and ADVIA data available, 11 (11%) had blood smear evaluation performed but

176 lacked ADVIA data, and 2 (2%) cases had ADVIA data but the blood smear was not

177 available for review.

178 Based on FC results, overall mean large B-cells percentage was 7.23±10.03%

179 (median, 2.75%; min-max 0-52%). In particular, 27 samples (27%) were not

180 infiltrated. For the remaining 73 samples (73%), mean PB infiltration was

181 9.84±10.62% (median, 7%; min-max, 0.6-52%).

182 PB smears were available for 98 cases. According to Operator 1, overall mean

183 immature lymphoid cells percentage was 4.7±9.5% (median, 0%; min-max 0-60%):

184   48 samples (49%) were infiltrated, with a mean immature lymphoid cells percentage

185   of 9.5±11.7% (median, 6%; min-max, 1-60%). According to Operator 2, overall mean

186   immature lymphoid cells percentage was 8.1±10.0% (median, 3%; min-max 0-55%):

187   87 samples (88.8%) were infiltrated, with a mean immature lymphoid cells

188   percentage of 8.8±10.3% (median, 5%; min-max 1-55%). A significant correlation

189   between operators was found (p=0.000, r=0.740).

190   A CBC performed with ADVIA 120 was available for 89 samples. Overall mean LUC

191   percentage was 2.43±3.44% (median, 1.5%; min-max 0-26.9%): 29 samples (32.6%)

192   were LUC-positive, with a mean percentage of 5.31±4.87% (median, 3.6%; min-max,

193   2.5-26.9%). Overall mean BASO percentage was 0.67±0.63% (median 0.5%; min-

194   max 0-3.1%): 8 samples (9%) were BASO-positive, with a mean percentage of

195   2.3%±0.49% (median, 2.2%; min-max, 1.7-3.1%). Five FC infiltrated cases (median,

196   5%; min-max, 1-14%) were negative at blood smear evaluation by both operators

197   and positive at LUC (median, 3.4%; min-max, 3.1-3.6%); 1 out of these 5 (FC = 1%)

198   was positive both at LUC (3.1%) and BASO (1.7%).

199   When comparing blood smear evaluation and FC, significant proportional error and

200   bias were found for Operator 1 but not for Operator 2 (Figure 1 and 2). Operator 1

201   performance was as follow: accuracy = 0.724, κ = 0.454, Se = 0.648, Sp = 0.926,

202   LR+ = 8.746, LR- = 0.38, DO = 23, PPV = 0.958, NPV = 0.5 (Table 1). Operator 2

203   performance was as follow: accuracy = 0.786, κ = 0.252, Se = 0.934, Sp = 0.273,

204   LR+ = 1.285, LR- = 0.24, DO = 5.3, PPV = 0.816, NPV = 0.5 (Table 2). The ROC

205   curve (drawn based on Operator 1 results) identified 0.5% of immature lymphoid cells

206   as the best cutoff for blood smear evaluation to discriminate between FC infiltrated

207   and not infiltrated PB samples, with a 67.6% sensitivity and a 92.6% specificity

208 (AUC=0.819). Finally, sensitivity and specificity for the 10% cutoff described in the

209 literature (Graff et al., 2014) were 18.3% and 100%, respectively.

210 When comparing LUC and FC, significant bias with constant and proportional errors

211 were found (Figure 3). LUC performance was as follow: accuracy = 0.573, κ = 0.274,

212 Se = 0.433, Sp = 1, LR- = 0.567, PPV = 1, NPV = 0.367 (Table 3).

213 When comparing BASO and FC, significant bias with constant and proportional

214 errors were found (Figure 4). BASO performance was as follow: accuracy = 0.337, κ

215 = 0.063, Se = 0.119, Sp = 1, LR- = 0.881, PPV = 1, NPV = 0.272 (Table 4).

216 LR+ and DO for LUC and BASO could not be calculated because no false positive

217 result was obtained.

218 The combined use of blood smear evaluation (Operator 1), LUC and BASO

219 (infiltration detected if at least one out of three was positive) reported the following

220 performance: accuracy = 0.816, κ = 0.588, Se = 0.785, Sp = 0.909, LR+ = 8.631, LR-

221 = 0.237, PPV = 0.962, NPV = 0.588 (Table 5).

222

223 **Discussion**

224 An official staging system for canine lymphoma has been created many years ago

225 (Owen, 1980). In spite of this, staging procedures vary largely among veterinarians

226 (Regan et al., 2013) and among published studies (Abbo and Lucroy, 2007; Flory et

227 al., 2007; Graff et al., 2014; Lana et al., 2006; Marconato et al., 2013; Martini et al.,

228 2015; Ponce et al., 2004; Riondato et al., 2016). Far from defining the best staging

229 system, or the "gold standard", we retrospectively analyzed PB samples from 100

230 dogs with LBCL, and discovered only a moderate agreement between blood smear

231 evaluation and FC in detecting and quantifying infiltration. Furthermore, LUC and

232 BASO results provided by ADVIA 120 are reliable in detecting PB infiltration in dogs

233 with large B-cell lymphoma only in case of LUC and/or BASO positive results. We

234 conclude that these techniques provide contrasting results and are not

235 interchangeable.

236 Discriminating reactive and neoplastic lymphoid cells via cytological evaluation alone

237 may be challenging, as they can present with similar morphological features,

238 including increased size, more abundant basophilic cytoplasm and cleaved or bilobed

239 nuclei (Stockham and Scott, 2008). Misclassification of reactive lymphocytes would

240 bring to an erroneous overestimation of PB infiltration by neoplastic cells, possibly

241 driving the clinicians toward a worse prognosis (Jagielski et al., 2002). Therefore,

242 skilled hematologist may be more prone to classify cells of ambiguous nature (not

243 completely corresponding to the description provided above) as reactive lymphocytes

244 than as neoplastic cells, in order to avoid false positive results. This hypothesis is

245 also supported by the different results obtained by the two operators involved in the

246 present study: the unskilled operator provided data with a slightly higher sensitivity,

247 but an 8-fold higher percentage of false-positive results. Still, we strongly agree that

248 cytological evaluation should always be done in conjunction with FC, in order to

249 support and confirm FC results, and to assess possible abnormalities of the other

250 cellular populations.

251 Blood smear evaluation has been used previously to assess PB infiltration in dogs

252 with lymphoma, and different cutoffs have been proposed to define positive samples.

253 On one hand, Flory and colleagues considered as positive PB samples when any

254 lymphoblast was present (Flory et al., 2007). On the other hand, in a more recent

255 study by Graff et al. (2014) a threshold of 10% large (neoplastic) lymphocytes was

256 adopted, claiming that any clinical pathologist would reproducibly identify neoplastic

257 cells present in such a high percentage. In the present study, we applied the same

258 cutoff used by Flory et al, and found only a moderate agreement with FC, mainly due

259 to the low sensitivity of blood smear evaluation. Raising the cutoff value toward 10%

260 improved specificity, but unfortunately sensitivity fell down to unacceptable values.

261 These results support our assumption that lower cutoffs work better. Since the cutoff

262 with the best sensitivity and specificity in the ROC curve corresponds with detection

263 limit of the 200-cells differential count (0.5% = 1 blast out of 200 leukocytes), it

264 remains to be determined if counting more leukocytes would increase the sensitivity

265 providing a lower detection limit.

266 We also investigated the potential value of ADVIA 120 LUC and BASO in quantifying

267 PB infiltration. The cutoff we adopted to define positive samples is the upper

268 reference limit obtained using samples from healthy dogs analyzed with our

269 instrument in our laboratory. These values (both LUC and BASO) are higher than the

270 reference limits previously reported (Moritz et al., 2004). This discrepancy once again

271 highlights the importance of in-house made reference ranges for any laboratory test.

272 However, adopting lower cutoff values could be beneficial in case of diagnosed LBCL

273 and it has to be further investigated.

274 Overall accuracy of both ADVIA 120 parameters was low, but these unsatisfying

275 values were mostly due to the low sensitivity, whereas specificity was optimal, as no

276 false positive result was obtained when analyzing PB samples from dogs with LBCL.

277 It means that a positive LUC/BASO result is conclusive for the presence of PB

278 infiltration in dogs with previously diagnosed LBCL. Unfortunately, a large number of

279 false negative LUC/BASO results occur. LUC/BASO might therefore be used only as

280 a preliminary test and LUC/BASO negative samples should be further tested to rule

281 out minor infiltration. Combining blood smear evaluation, LUC and BASO data

282 slightly improves the results, increasing the number of cases correctly identified as

283 infiltrated. Anyway, both a constant and a proportional error were found when

284 comparing LUC/BASO and FC. Thus, it would be better to analyze by FC even

285 LUC/BASO-positive samples, in order to quantify the infiltration with higher accuracy.

286 A possible decisional algorithm is suggested in Fig.5

287 In the recent years, many upgrades have been made in the diagnostic procedures

288 and classification systems for canine lymphoma, and most recent studies are

289 focusing on specific lymphoma subtypes. Following on from this background, only

290 dogs with a confirmed diagnosis of LBCL, which is the highly prevalent subtype in

291 dogs (Aresu et al., 2013; Ponce et al., 2010; Teske et al., 1994; Valli et al., 2011),

292 were included in the present study. Therefore, our results may be not applicable to

293 other lymphoma subtypes, such as small cell or T-cell lymphomas. Further studies

294 are needed to assess whether the techniques investigated here have similar

295 performances when applied to different lymphoma entities. In addition, all our results

296 and conclusions are valid only within a staging workup, once the LBCL diagnosis has

297 been confirmed: the occasional detection of positive PB samples by any technique

298 should never be considered conclusive for LBCL if a primary lesion has not been

299 detected or investigated.

300 The main pitfall of the present study is the lack of PARR data: this analysis was not

301 performed on the included samples, due to the retrospective nature of this study.

302 Although FC has great sensitivity and specificity in identifying infiltrated blood

303 samples detected by PARR (Riondato et al., 2016) discordance between these two

304 techniques may occur in few cases (Aresu et al., 2014): further studies are warranted

305 to assess whether the concomitant use of other techniques might be of aid in this

306 subset of cases.

307 Finally, we did not investigate the clinical relevance of PB infiltration. A recent

308 prospective study highlighted a prognostic role for BM infiltration quantified by FC in

309 dogs with LBCL, but failed to recognize such a role for PB infiltration (Marconato et

310 al., 2013). Prospective studies are needed to assess if PB infiltration quantified by

311 other techniques (including PARR, blood smear evaluation, ADVIA's LUC or BASO)

312 may have any clinical or prognostic role.

313 In conclusion, the present study demonstrated that FC, blood smear evaluation and

314 ADVIA 120 LUC and BASO have only a moderate agreement in the quantification of

315 PB involvement in dogs with LBCL. Thus, results from these different methods and

316 parameters are not comparable and they should not be used alternatively. We

317 suggest the use of LUC/BASO only as a fast preliminary test having optimal

318 specificity but unsatisfactory sensitivity, and the joined use of blood smear evaluation

319 and FC to quantify definitively the degree of PB infiltration. Also, PARR analysis may

320 be of benefit in many cases. Further studies are needed to assess the prognostic role

321 of PB infiltration evaluated by these techniques.

322

323 **Conflict of interest**

324 There is no conflict of interest of any authors in relation to the submission.

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329

330 **References**

331   Abbo, A.H., Lucroy, M.D., 2007. Assessment of anemia as an independent

332   predictor of response to chemotherapy and survival in dogs with lymphoma: 96

333   cases (1993-2006). J. Am. Vet. Med. Assoc. 231, 1836-1842.

334   Aresu, L., Aricò, A., Ferraresso, S., Martini, V., Comazzi, S., Riondato, F., et al.,

335   2014. Minimal residual disease detection by flow cytometry and PARR in lymph

336   node, peripheral blood and bone marrow, following treatment of dogs with diffuse

337   large B-cell lymphoma. Vet. J. 200, 318-324.

338   Aresu, L., Martini, V., Rossi, F., Vignoli, M., Sampaolo, M., Aricò, A., et al., 2013.

339   Canine indolent and aggressive lymphoma: clinical spectrum with histologic

340   correlation. Vet. Comp. Oncol.; doi: 10.1111/vco.12048.

341   Flory, A.B., Rassnick, K.M., Stokol, T., Scrivani, P.V., Erb, H.N., 2007. Stage

342   migration in dogs with lymphoma. J. Vet. Intern. Med. 21, 1041-1047.

343   Fournel-Fleury, C., Magnol, J.P., Bricaire, P., Marchal, T., Chabanne, L.,

344   Delverdier, A., Bryon, P.A., Felman, P., 1997. Cytohistological and immunological

345   classification of canine malignant lymphomas: comparison with human non-

346   Hodgkin's lymphomas. Journal of Comparative Pathology 117, 35–59.

347   Gibbs, G., Campbell, G., Christie, I., 2009. Pseudobasophilia and the Advia 120.

348   Hematology 14,159-63.

349   Graff, E.C., Spangler, E.A., Smith, A., Denhere, M., Brauss, M., 2014.

350   Hematologic findings predictive of bone marrow disease in dogs with multicentric

351   large-cell lymphoma. Vet. Clin. Pathol. 43, 505-512.

352   Jagielski, D., Lechowski, R., Hoffmann-Jagielska, M., Winiarczyk, S., 2002. A

353   retrospective study of the incidence and prognostic factors of multicentric

354   lymphoma in dogs (1998-2000). J. Vet. Med. A Physiol. Pathol. Clin. Med. 49,

355   419-24.

356 Lana, S.E., Jackson, T.L., Burnett, R.C., Morley, P.S., Avery, A.C., 2006. Utility of
357 polymerase chain reaction for analysis of antigen receptor rearrangement in
358 staging and predicting prognosis in dogs with lymphoma. J. Vet. Intern. Med. 20,
359 329-334.

360 Lilliehöök, L., Tvedten, H.W., 2011. Errors in basophile enumeration with 3
361 veterinary hematology systems and observations on the occurrence of basophiles
362 in dogs. Vet. Clin. Pathol. 40, 450-458.

363 Marconato, L., Martini, V., Aresu, L., Sampaolo, M., Valentini, F., Rinaldi, V., et
364 al., 2013 Assessment of bone marrow infiltration diagnosed by flow cytometry in
365 canine large B-cell lymphoma: prognostic significance and proposal of a cutoff
366 value. Vet. J. 197, 776-781.

367 Martini, V., Melzi, E., Comazzi, S., Gelain, M.E., 2015. Peripheral blood
368 abnormalities and bone marrow infiltration in canine large B-cell lymphoma: is
369 there a link? Vet. Comp. Oncol. 13, 117-123.

370 Moritz, A., Fickenscher, Y., Meyer, K., Failing, K., Weiss, D.J., 2004. Canine and
371 feline hematology reference values for the ADVIA 120 hematology system. Vet.
372 Clin. Path. 33, 32-38.

373 Owen, L., 1980. TNM Classification of Tumors in Domestic Animals. World Health
374 Organization, Geneva, Switzerland.

375 Ponce, F., Magnol, J.P., Ledieu, D., Marchal, T., Turinelli, V., Chalvet-Monfray, K.,
376 et al., 2004. Prognostic significance of morphological subtypes in canine
377 malignant lymphomas during chemotherapy. Vet. J. 167, 158-166.

378 Ponce, F., Marchal, T., Magnol, J.P., Turinelli, V., Ledieu, D., Bonnefont, C., et
379 al., 2010. A morphological study of 608 cases of canine malignant lymphoma in

France with a focus on comparative similarities between canine and human lymphoma morphology. Vet.Pathol 47, 414-433.

Regan, R.C., Kaplan, M.S., Bailey, D.B., 2013. Diagnostic evaluation and treatment recommendations for dogs with substage-a high-grade multicentric lymphoma: results of a survey of veterinarians. Vet. Comp. Oncol. 11, 287-295.

Riondato, F., Miniscalco, B., Poggi, A., Aricò, A., Aresu, L., Comazzi, S., et al., 2016. Analytical and diagnostic validation of a flow cytometric strategy to quantify blood and marrow infiltration in dogs with large B-cell lymphoma. Cytometry B Clin. Cytom. 90, 525-530.

Rout, ED and Avery, PR, 2017. Lymphoid Neoplasia: Correlations Between Morphology and Flow Cytometry. Vet Clin North Am Small Anim Pract. 47,53-70.

Stirn, M., Moritz, A., Bauer, N., 2014. Rate of manual leukocyte differentials in dog, cat and horse blood samples using ADVIA 120 cytograms. BMC Vet. Res. 10, 125.

Stockham, S.L., Scott, M.A., 2008. Fundamentals of Veterinary Clinical Pathology. second ed. Wiley-Blackwell, Ames, IA.

Teske, E., Wisman, P., Moore, P.F., Van Heerde, P., 1994. Histologic classification and immunophenotyping of canine non-Hodgkin's lymphomas: unexpected high frequency of T cell lymphomas with B cell morphology. Exp. Hematol. 22, 1179-1187.

Valli, V.E., Jacobs, R.M., Parodi, A.L., Vernau, W., Moore, P.F., 2002. Histological classification of hematopoietic tumors of domestic animals. World Health Organization. Second Series, vol. 8. Armed Forces Institute of Pathology, Washington, DC, USA.

404   Valli, V.E., San Myint, M., Barthel, A., Bienzle, D., Caswell, J., Colbatzky, F., et

405   al., 2011. Classification of canine malignant lymphomas according to the World

406   Health Organization criteria. Vet.Pathol. 48, 198-211.

407

408   **Table 1:** contingency table showing the distribution of 98 dogs diagnosed with large

409   B-cell lymphoma, according to the peripheral blood infiltration status, assessed via

410   flow cytometry (reference method) and blood smear evaluation by a skilled

411   hematologist (PB smear Op.1).

	Flow cytometry		
	negative	positive	Total
PB smear Op.1 **negative**	25	25	50
positive	2	46	48
Total	27	71	98

412

413

414   **Table 2:** contingency table showing the distribution of 98 dogs diagnosed with large

415   B-cell lymphoma, according to the peripheral blood infiltration status, assessed via

416   flow cytometry (reference method) and blood smear evaluation by an unskilled

417   hematologist (PB smear Op.2).

	Flow cytometry		
	negative	positive	Total
PB smear Op.2 negative	6	5	11
positive	16	71	87
Total	22	76	98

418

419

420 **Table 3**: contingency table showing the distribution of 89 dogs diagnosed with large

421 B-cell lymphoma, according to peripheral blood infiltration status, assessed via flow

422 cytometry (reference method) and large unstained cells (LUC) detected with ADVIA

423 120 hematology analyzer.

	Flow cytometry		
	negative	positive	Total
LUC **negative**	22	38	60
positive	0	29	29
Total	22	67	89

424

425

426 **Table 4**: contingency table showing the distribution of 89 dogs diagnosed with large

427 B-cell lymphoma, according to peripheral blood infiltration status, assessed via flow

428 cytometry (reference method) and lyse-resistant cells (BASO) detected with ADVIA

429 120 hematology analyzer.

	Flow cytometry		
	negative	positive	Total
BASO negative	22	59	81
positive	0	8	8
Total	22	67	89

430

431

432　**Table 5**: contingency table showing the distribution of 87 dogs diagnosed with large

433　B-cell lymphoma, according to peripheral blood infiltration status, assessed via flow

434　cytometry (reference method) and the concomitant evaluation of a blood smear and

435　the LUC and BASO percentages detected with ADVIA 120 hematology analyzer

436　(smear/LUC/BASO).

|	Flow cytometry		
	negative	positive	Total
Smear/LUC/BASO **negative**	20	14	34
positive	2	51	53
Total	22	65	87

437

438

439   **Figure 1**. Passing–Bablok regression analyses (left) and Bland–Altman difference

440   plots (right) for flow cytometric and smear evaluation assessment of blood infiltration

441   in dogs with large B cell lymphoma (n = 98). % FC = % of large B cells detected on

442   flow cytometric analysis; % PB smear Op.1 = % of lymphoblasts in a 200-cells

443   manual differential count on a peripheral blood smear detected by a skilled

444   hematologist

445

446   **Figure 2**. Passing–Bablok regression analyses (left) and Bland–Altman difference

447   plots (right) for flow cytometric and smear evaluation assessment of blood infiltration

448   in dogs with large B cell lymphoma (n = 98). % FC = % of large B cells detected on

449   flow cytometric analysis; % PB smear Op.2 = % of lymphoblasts in a 200-cells

450   manual differential count on a peripheral blood smear detected by an unskilled

451   hematologist

452

453   **Figure 3**. Passing–Bablok regression analyses (left) and Bland–Altman difference

454   plots (right) for flow cytometric and ADVIA120 LUC assessment of blood infiltration in

455   dogs with large B cell lymphoma (n = 89). % FC = % of large B cells detected on flow

456   cytometric analysis; % LUC = % of leukocytes in the LUC region (PEROX channel) of

457   the ADVIA120 differential count

458

459   **Figure 4**. Passing–Bablok regression analyses (left) and Bland–Altman difference

460   plots (right) for flow cytometric and ADVIA120 BASO assessment of blood infiltration

461   in dogs with large B cell lymphoma (n = 89). % FC = % of large B cells detected on

462   flow cytometric analysis; % BASO = % of leukocytes in the basophils region (BASO

463   channel) of the ADVIA120 differential count

464

465   **Figure 5**. Decisional algorithm integrating ADVIA120, peripheral blood (PB) smear

466   evaluation and flow cytometric (FC) analysis of peripheral blood infiltration in dogs

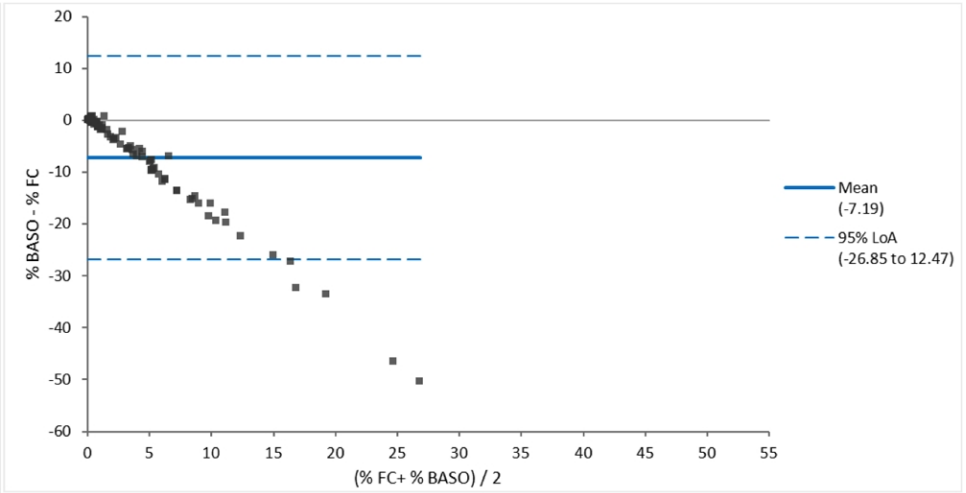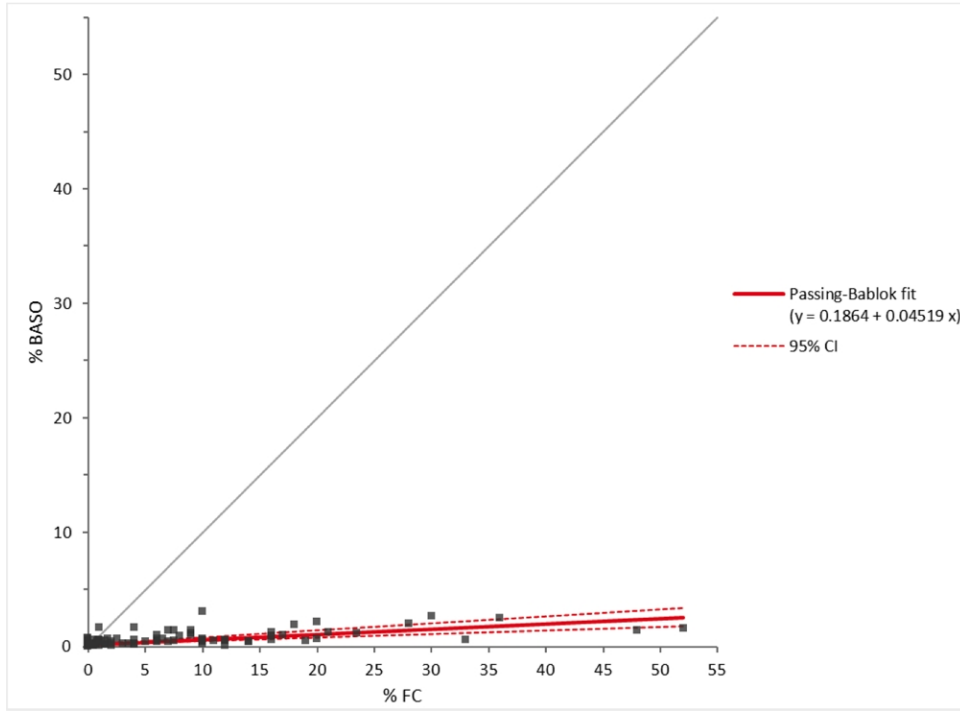467   with large B-cell lymphoma (LBCL).
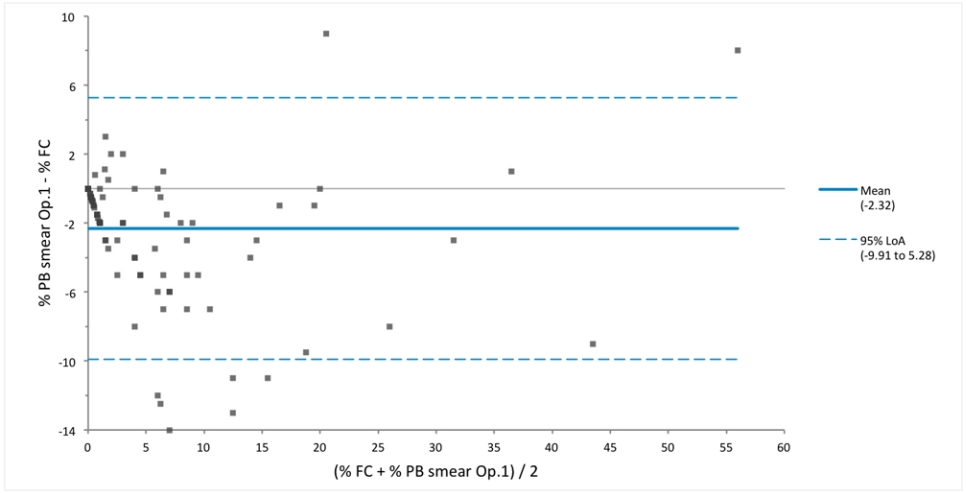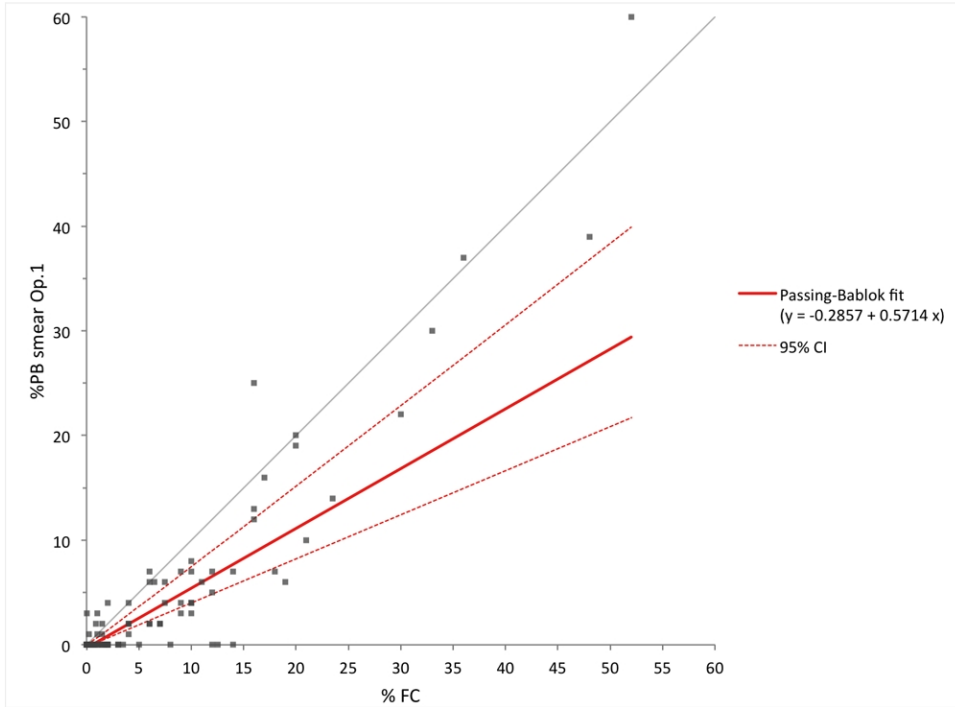
	Passing-Bablok		Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	Bias mean (95% CI)	Bias SD
FC vs PB smear Op.1	0.57 (0.42-0.77)	-0.29 (-0.38-0)	-2.32 (-3.10 - -1.54)	3.87
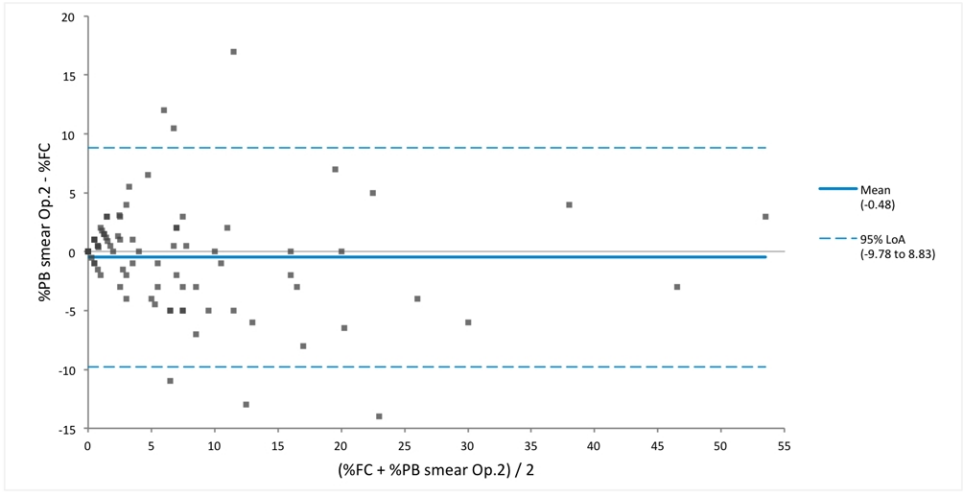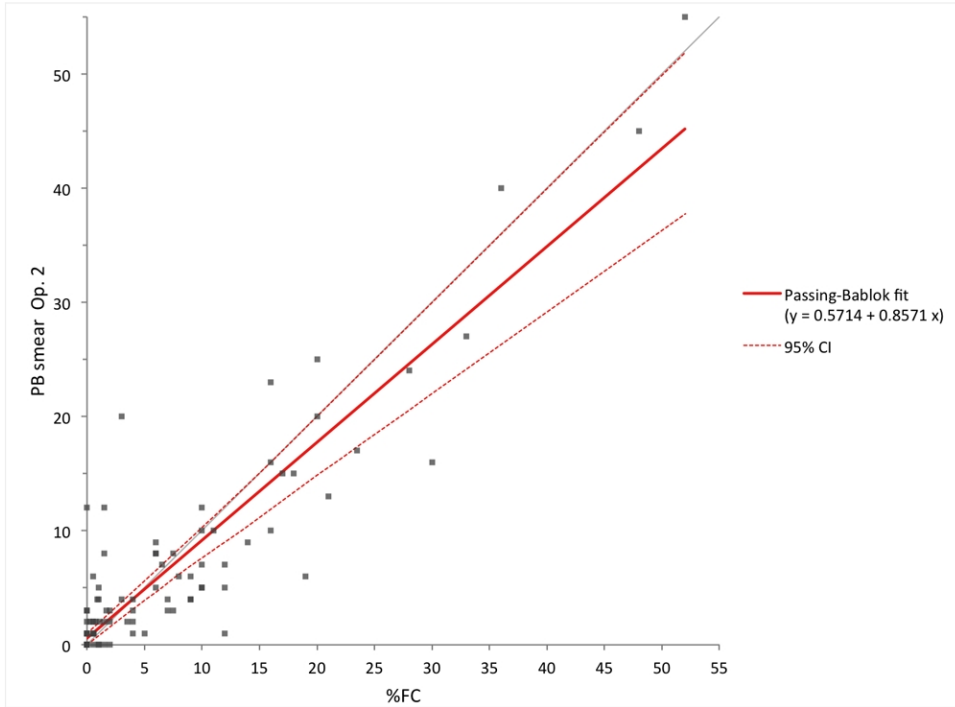
	Passing-Bablok		Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	Bias mean (95% CI)	Bias SD
FC vs PB smear Op.2	0.86 (0.71-0.99)	0.57 (0-1)	-0.48 (-1.53 – 0.57)	4.75
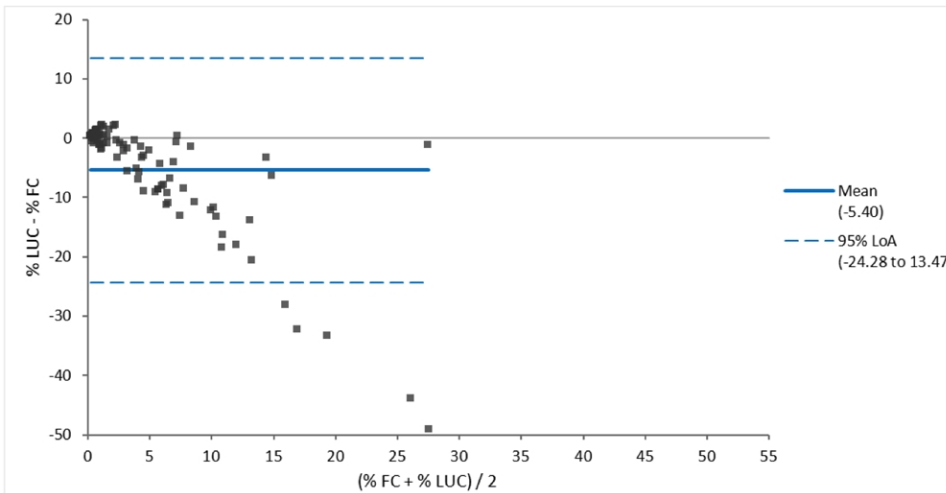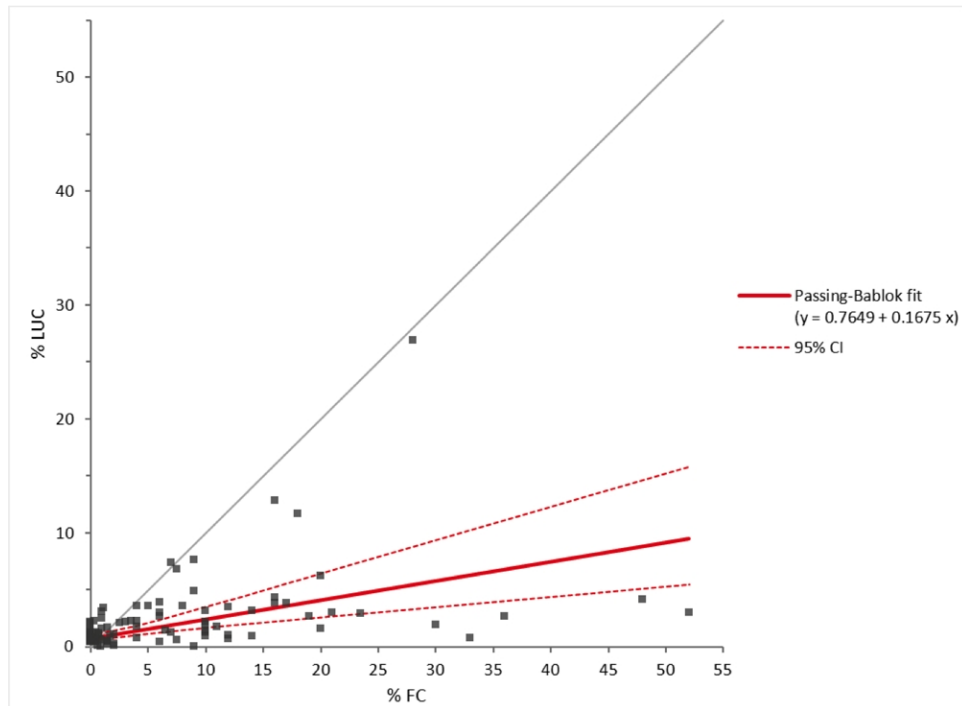
	Passing-Bablok		Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	Bias mean (95% CI)	Bias SD
FC vs LUC	0.17 (0.09-0.29)	0.76 (0.48-1.09)	-5.40 (-7.43 - -3.38)	9.63

	Passing-Bablok		Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	Bias mean (95% CI)	Bias SD
FC vs BASO	0.05 (0.03-0.06)	0.19 (0.14-0.28)	-7.19 (-9.30 - -5.08)	10.03

DOGS WITH ALREADY DIAGNOSED LBCL

LUC / BASO
(ADVIA120)

pos   neg

FC
>0.56%

PB smear
evaluation

<1%   ≥1%

Stage I-IV or
V low burden

Stage V
detection
quantification