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Minimal residual disease in lymph nodes after achievement of complete remission predicts time to relapse in dogs with large B-cell lymphoma

Abstract

Most dogs with large B-cell lymphoma (LBCL) that undergo chemotherapy and achieve clinical complete remission (CR) eventually relapse. However, time to relapse (TTR) is unpredictable. The aims of this prospective study were to assess the influence of post-chemotherapy lymph node (LN) infiltration by large CD21+ cells using flow cytometry (FC) on TTR, and to establish a cut-off value of prognostic significance.

Dogs with newly-diagnosed, completely staged LBCL in CR after treatment were enrolled. Minimal residual disease (MRD) analysis by FC was performed on LN aspirates. TTR was calculated between MRD and relapse.

Thirty-one dogs were enrolled: 4% had stage V disease, and DLBCL was the most common histotype (74%). Based on LN infiltration at MRD evaluation, 3 groups were created: 1) acellular samples; 2) $\leq 0.5\%$ infiltration; and 3) $> 0.5\%$ infiltration. Overall median TTR was 154 days (range, 31-1974): 22 (71%) dogs relapsed during the study period, whereas 9 (29%) dogs did not. The difference among the 3 groups was significant ($p=0.042$ log-rank test): median TTR was not reached for dogs with LN infiltration $\leq 0.5\%$ (range, 195-

22 429 days), 164 days (range 63-1974) for dogs with acellular LN samples, and
23 118 days (range, 31-232) for dogs with LN infiltration >0.5%.
24 These results demonstrate that MRD assessment by FC on LN aspirates in
25 dogs with LBCL in clinical CR predicts TTR. LN infiltration by >0.5% large
26 CD21+ cells after treatment is an unfavorable prognostic factor.

27

28 **Keywords**

29 canine, end-staging, flow cytometry, lymphoma, prognosis, relapse

30

31 **Introduction**

32

33 In dogs, multicentric lymphoma is most frequently diagnosed, and in
34 approximately 70% of cases it is of B-cell origin.¹⁻⁴ Large B-cell lymphoma
35 (LBCL) is characterized by medium or large-sized cells that express CD21 by
36 flow cytometry (FC).^{5,6}

37 Despite significant improvements in terms of prognosis due to better
38 treatments for LBCL, most dogs that undergo first-line chemotherapy and
39 achieve clinical complete remission (CR) will ultimately relapse. The CHOP-
40 based maintenance-free chemotherapeutic protocol including Prednisolone,
41 Vincristine, Cyclophosphamide, Doxorubicin +/- L-Asparaginase is
42 considered to be the gold standard treatment for dogs with LBCL.⁷⁻¹⁰

43 According to the published literature, CR rate and median first remission
44 duration range between 80-85% and 87-330 days, respectively.^{8,11-17} In dogs
45 with multicentric lymphoma the measurement of peripheral lymph nodes
46 (LNs) size after chemotherapy is used to document treatment response.¹⁸

47 According to the Veterinary Cooperative Oncology Group (VCOG) guidelines,
48 clinical CR is defined as the regression of all affected peripheral LNs to a size
49 considered normal by physical examination using LN palpation and calipers.¹⁸

50 Following clinical remission, the residual population of tumor cells can be
51 referred to as the minimal residual disease (MRD), which is implicated as the
52 source of tumor relapse. Therefore, measurement of MRD can provide

53 information regarding the presence of neoplastic cells even in the clinical CR
54 phase.

55 Several techniques, including FC and polymerase chain reaction amplification
56 of antigen receptor genes (PARR), have been used to detect MRD in LN,
57 peripheral blood (PB) and bone marrow (BM) samples in dogs with LBCL.^{19,20}

58 Molecular techniques for detection of MRD in PB samples during or following
59 treatment can be used as an objective parameter for the determination of
60 treatment efficacy and to select dogs that might benefit from consolidation
61 chemotherapy despite clinical CR.²¹⁻²⁶ However, the universal primers
62 previously designed for PARR showed a low sensitivity for MRD evaluation.¹⁹
63 In the current study FC was tested as an alternative method for detection of
64 MRD in the routine clinical practice.

65

66 The first aim of this prospective study was to assess the influence on time to
67 relapse (TTR) of LN infiltration by large CD21+ cells quantified by FC in dogs
68 with LBCL in clinical CR after treatment. The second aim was to establish a
69 cut-off value of prognostic significance. It was hypothesized that the detection
70 and quantification of a certain amount of neoplastic cells using FC on LNs
71 samples at the time of MRD monitoring could help predicting early relapse.

72

73

74 **Material and methods**

75

76 Patient selection and inclusion criteria

77 Client-owned dogs with newly-diagnosed, previously untreated multicentric
78 LBCL were prospectively enrolled. To be eligible for recruitment dogs were
79 required to undergo a complete initial staging work-up (T0), consisting of
80 history and physical examination, complete blood cell count with differential,
81 serum biochemistry profile, thoracic radiographs, abdominal ultrasound,
82 cytological evaluation of liver and spleen regardless of their sonographic
83 appearance, FC on LN, PB and BM. Dogs were required to be diagnosed
84 with LBCL based on the presence of large-sized CD21+ cells using cytology
85 and FC. Dogs also underwent surgical removal of a peripheral LN to obtain a
86 histopathological diagnosis. Only dogs with a histopathological diagnosis of
87 Diffuse Large B-cell Lymphoma (DLBCL) or Marginal Zone Lymphoma (MZL)
88 were enrolled in the study. All dogs received a CHOP-based dose-intense
89 chemotherapy protocol with an autologous vaccine.²⁷ Dogs in clinical CR
90 following treatment based on clinical, radiological, ultrasonographic and
91 cytological investigations were the subject of the analysis and underwent
92 MRD assessment using FC on post-chemotherapy LNs (T1).

93 Written informed consent was obtained from all owners.

94

95 Evaluation of MRD by FC

96 MRD evaluation by FC was performed on LN aspirates, PB and BM samples
97 2-4 weeks after the completion of the treatment protocol if dogs were
98 documented to be in clinical CR. Multiple peripheral LNs were aspirated; in
99 case of non-palpable nodes, ultrasound was used as a guide.

100 LN samples were collected into tubes containing 1 ml of RPMI 1640. PB and
101 BM samples were collected in K3-EDTA tubes. For each matrix, a cytological
102 specimen obtained from the same aspirate was also prepared.

103 Samples were delivered to the laboratory and processed as previously
104 described within 24 hours from sampling.¹³

105 The cellularity of all matrices was tested with an automated haematology
106 analyser (Sysmex XT-2000iV, Sysmex, Kobe, Japan). According to internal
107 standard procedures, at least 1×10^3 nucleated cells/ μ l were considered
108 necessary for the evaluation of residual disease in the LN samples (which
109 means a total of 1×10^6 nucleated cells in the sample), otherwise the sample
110 was reported as “acellular” and not further processed.

111 Two tubes for FC were prepared for each matrix: one tube with unstained
112 cells served as negative control. In the second tube, cells were labelled with
113 anti-CD45-FITC (clone YKIX716.13, AbD Serotec, Oxford, UK) and anti-
114 CD21-AF647 (clone CA2.1D6, AbD Serotec) antibodies. After incubation with
115 antibodies, PB and BM samples underwent RBC lysis by means of a solution
116 containing 8% ammonium chloride. Tubes were finally washed and re-
117 suspended in 500 μ l of PBS and acquired with a BD FACScalibur flow

118 cytometer (Becton Dickinson, San José, CA). For each tube, 10,000
119 nucleated cells were acquired. Analyses were performed with the specific
120 software CellQuest (Becton Dickinson). A first gate was set in the
121 morphological scattergram to exclude debris and platelets, and a second one
122 to include only CD45-positive cells. For each matrix the residual disease was
123 quantified based on the percentage of large-sized CD21-positive cells out of
124 the total CD45-positive cells (Figure 1). As cut-off value, the mean FSC value
125 of the neutrophil population (ranging between 350 and 400 based on our
126 experimental condition) was used.

127

128 Follow-up

129 Dogs had to undergo follow-up evaluations to assess the remission status
130 consisting of physical examination, peripheral LNs size measurement and
131 cytological evaluation every 4 weeks during the first year, and every other
132 month thereafter. Relapse was defined as the clinical reappearance and
133 cytological evidence of lymphoma with or without FC confirmation in any
134 anatomical site in dogs having experienced CR. Once relapse was confirmed,
135 a complete restaging work-up was undertaken, and a second round of
136 chemotherapy was offered.

137

138 **Statistical analysis**

139

140 TTR was calculated from the assessment of MRD to relapse. Dogs lost to
141 follow-up, dead for lymphoma-unrelated causes before relapse, or those still
142 in CR at the end of the study, were censored for TTR analysis.

143 In order to identify the best promising cut-off to discriminate 2 prognostic
144 groups based on LN infiltration at MRD assessment, Kaplan-Meier curves
145 were drawn and visually inspected. The following values were arbitrarily
146 selected and checked to this aim: 0.5%, 1.0%, 3.0% and 5.0%.

147 Based on data distribution, only the 0.5% cut-off was tested on PB and BM
148 samples.

149 Once the best cut-off was identified, a Cox's proportional hazard regression
150 analysis was performed, to assess the possible association of the following
151 variables with TTR: breed (purebred or crossbred), sex (male or female), age
152 (< or $\geq$ 10 years), body weight (< or $\geq$ 10 kg), initial stage (I to V according to
153 WHO), substage (a or b), extranodal site involvement (yes or no), anemia
154 (hematocrit < 35%, yes or no), thrombocytopenia (platelets <200,000/ μ L, yes
155 or no), serum LDH activity (< or $\geq$ 300 IU/L) histotype (DLBCL, MZL), LN
156 group (acellular, $\leq$ 0.5% infiltrated, >0.5% infiltrated), residual disease in PB
157 (%) and BM (%).For categorical variables, Kaplan Meier curves were drawn
158 and compared with log-rank test to assess possible variations in median TTR.

159 All statistical analyses were performed using a standard statistical software
160 (SPSS 20.0 for Windows). Significance was set at $p \leq 0.05$ for all tests.

161

162

163 **Results**

164 *Demographics*

165 Thirty-one dogs met the study inclusion criteria and were enrolled. There
166 were 7 (22.6%) crossbreed, and the remaining 24 (77.4%) represented 20
167 different breeds. Among the purebred dogs, there were 3 (9.7%) German
168 shepherds, 2 (6.4%) Rottweilers, 2 (6.4%) Poodles, and one (3.2%) each of
169 the following: Dachshund, Beagle, English bulldog, Shih-tzu, Dogo Argentino,
170 Doberman, Basset hound, Border collie, Shar Pei, Bernese Mountain dog,
171 Boxer, German shorthaired pointer, Akita Inu, French bulldog, American
172 staffordshire, Pomeranian, West Highland White Terrier.

173 Eighteen (58.1%) dogs were males (4 castrated) and 13 (41.9%) were
174 females (11 spayed). The median age at diagnosis was 7 years (range, 4-12
175 years), whereas the median weight was 27.3 kg (range, 3.9-59.7 kg).

176

177 *Clinico-pathological features*

178 Two (6.5%) dogs were anemic, and 7 (22.6%) were thrombocytopenic. LDH
179 activity was increased in 7 (23.3%) dogs (≥ 300 IU/L) out of 30 dogs tested,
180 whereas serum ionized calcium was within normal limits in all cases.

181 Four (12.9%) dogs had stage III (substage a) disease, 14 (45.2%) had stage
182 IV (substage a) disease, and 13 (41.9%) had stage V disease (n=8 substage

183 a, and n=5 substage b). In 2 (6.5%) cases, extranodal (pulmonary) sites were
184 involved.

185 There were 23 (74.2%) DLBCL and 8 (25.8%) MZL.

186

187 *MRD assessment on LN, PB and BM*

188 At the time of MRD assessment, all dogs were in clinical CR. A LN aspirate
189 was obtained in all of them and submitted for FC analysis. The aspirates
190 obtained from 13 (41.9%) dogs were acellular. Among the 18 processed
191 samples, median infiltration by large CD21+ cells was 1.3% (mean
192 $5.7 \pm 13.0\%$; min-max 0.3-53.5%). In particular, it was $\leq 0.5\%$ in 3 (9.7%)
193 cases, $\leq 1.0\%$ in 7 (22.6%), $\leq 3.0\%$ in 13 (41.9%), and $\leq 5.0\%$ in 15 (48.4%)
194 dogs.

195 PB was tested for MRD by FC in all 31 cases. Median infiltration was 0.3%
196 (mean $0.41 \pm 0.34\%$; min-max 0.1-1.5%). In particular, it was $\leq 0.5\%$ in 21
197 (67.7%) dogs.

198 BM was tested for MRD by FC in 25 (80.6%) cases. Median infiltration was
199 0.3% (mean $0.51 \pm 0.49\%$; min-max 0.1-1.9%). In particular, it was $\leq 0.5\%$ in
200 16 (64%) dogs.

201

202 *Outcome and prognostic groups based on LN infiltration*

203 Overall median TTR was 154 days (range 31-1974 days). Twenty-two (71%)
204 dogs relapsed during the study period, whereas 9 (29%) were censored for

205 TTR (8 were still alive and in CR at data analysis closure and 1 died from
206 unrelated causes still being in CR for lymphoma after 61 days).

207 Among the 4 cut-offs tested for LN infiltration, only 0.5% discriminated among
208 prognostic groups (Figure 2). Thus, three groups were created for statistical
209 analysis, based on LN infiltration at MRD assessment: 1) acellular LN
210 sample; 2) $\leq 0.5\%$ infiltration; and 3) $> 0.5\%$ infiltration. Conversely, no
211 discriminating cut-off was identified for PB and BM samples.

212 The three LN groups significantly differed for median TTR according to log-
213 rank test ($p=0.042$), but not according to Cox analysis ($p=0.067$).

214 Nevertheless, significant results were obtained with Cox analysis when LN
215 groups 1 and 2 were coupled together and compared with samples $> 0.5\%$
216 infiltration ($p=0.030$, Figure 3). None of the remaining variables significantly
217 influenced TTR by either test. Median TTR and p-values for all variables are
218 listed in Table 1.

219
220

221 **Discussion**

222

223 Monitoring MRD is crucial for dogs with LBCL undergoing treatment. Relapse
224 is the major cause of treatment failure and decrease in survival rate, and it
225 emerges from the outgrowth of residual treatment-resistant neoplastic cells.²⁸
226 While diagnosing an overt relapse is quite straightforward, recognizing the

227 presence of residual neoplastic cells if peripheral LNs are not enlarged is
228 challenging. The current prospective study shows that MRD assessment by
229 FC on LN samples in dogs with LBCL in clinical CR may predict TTR, thereby
230 representing a useful tool for disease monitoring after therapy completion. In
231 detail, dogs with >0.5% LN infiltration were more likely to relapse despite the
232 clinical CR.

233 According to the VCOG guidelines, all dogs in the present study were in
234 clinical CR at the end of treatment¹⁸, yet 50% of them had detectable MRD
235 based on FC, ultimately leading to early relapse. Thus, FC may play a role
236 not only in the initial diagnostic work up^{5,6,13,29,30}, but also in post-treatment
237 surveillance. In fact, cytology and even imaging may occasionally detect
238 clinically occult relapse, but the benefit of these techniques has not been
239 demonstrated in LBCL. Whether the detection of relapse ahead of clinical
240 symptoms translates into a clinical benefit was not investigated in the current
241 study, therefore it is currently unknown whether early MRD-based
242 intervention can affect outcome. Nevertheless, these results allow at least the
243 question to be addressed in clinical trials, by including MRD assessment at
244 the end of treatment to document the presence or absence of subclinical
245 disease.

246

247 The second aim of this study was to establish a cut-off value of prognostic
248 significance for TTR. The identification of a MRD cut-off value might

249 represent an objective and repeatable tool for risk assessment, by identifying
250 those dogs that are at risk of relapse, yet clinically in remission phase.

251 In the current study 22 (71%) dogs relapsed during the study at different time
252 points (31-1974 days). By applying a cut-off value of 0.5%, two prognostic
253 groups were identified: an early relapse group (median TTR 118 days) and a
254 late relapse group (median TTR not reached).

255 Because dogs with MRD >0.5% at the end of treatment had the shortest TTR,
256 it was hypothesized that MRD could not only represent the best indicator for
257 the risk of relapse, but also a substantial aid to decide the interval at which
258 dogs should be monitored, possibly leading to treatment intensification.

259 The period between clinical remission and clinical relapse was different in
260 dogs in the early relapse group. The clinical relapse occurred after a median
261 interval of 118 days of MRD assessment. This period was even shorter in
262 some dogs, thereby indicating that the evolution of disease is not completely
263 similar in all dogs despite being categorized in the same risk group.

264 Based on the above, we strongly recommend to assess the clinical remission
265 status on a biweekly basis if MRD is above the defined threshold, as a follow-
266 up interval of 2 weeks allows predicting the clinical relapse events before
267 clinical manifestations.

268

269 Conversely, dogs with MRD levels $\leq 0.5\%$ or those in which LN samples were
270 acellular were mostly associated with a good outcome. While the threshold of

271 $\leq 0.5\%$ is intuitive, one may argue the meaning of acellular samples.

272 In the current study, at least 1×10^6 intact cells ($\geq 1 \times 10^3$ nucleated cells/ μl)

273 were considered necessary for the evaluation of MRD by FC. This threshold

274 was arbitrarily set, since this amount of cells is required for the application of

275 a minimal immunophenotyping panel according to internal standard

276 procedures. Indeed, working dilutions of the antibodies have been

277 established for a concentration of 500,000 cells/tubes. While the reduced

278 panel of antibodies used here may be adapted to less cellular samples, thus

279 possibly reducing the percentage of “acellular” cases, it must be noted that

280 statistically the acellular samples tended to overlap with those with $< 0.5\%$

281 infiltration in terms of outcome. The most likely explanation for this finding is

282 that dogs achieving CR after treatment undergo LNs atrophy, as previously

283 documented.¹⁹ Fine-needle aspirates obtained from one or more peripheral

284 LNs were always evaluated by means of cytology prior to FC analysis, to

285 ensure that a LN was actually sampled. Despite having performed

286 ultrasound-guided fine-needle aspiration of multiple peripheral non-palpable

287 LNs and having obtained good cytological smears (data not shown), the

288 cellularity of atrophic LNs was inadequate for flow cytometric analysis in 13

289 dogs, yielding acellular samples. Nevertheless, dogs with acellular samples

290 at the end of treatment experienced a long TTR. Based on the above,

291 acellular samples by FC suggest that residual neoplastic cells are virtually

292 absent in the LNs, ultimately being associated to a good prognosis.

293

294 This study has some limitations.

295 While cytology and FC are commonly used for diagnosing canine lymphoma,
296 histopathology is still not routinely performed. Despite the group enrolled in
297 the current study was homogeneous as far as cytological and FC features,
298 histopathology showed that there were 23 aggressive (DLBCL) and 8 indolent
299 lymphomas (MZL). Further development and validation of MRD techniques
300 are required to confirm that FC detection of residual neoplastic cells is
301 feasible and prognostic among all lymphoma subtypes. Also, dogs underwent
302 one single MRD testing 2-4 weeks after the end of treatment. This time point
303 was arbitrarily selected. It is possible that MRD assessment at different time
304 points or incorporated in serial monitoring could add value for clinical
305 management.

306 MRD testing itself has some technical and practical limitations, as a
307 significant number of MRD-negative dogs still relapse. First, while the
308 cellularity of samples is crucial for FC analysis, the minimal amount of
309 neoplastic cells that permits sample processing has not been clearly
310 identified. Second, clonality of putative neoplastic cells cannot be tested by
311 FC. We assessed the percentage of large CD21+ cells in LNs after treatment
312 because these cells have the same FC morphological and immunophenotypic
313 features of neoplastic cells at diagnosis. Still, medium or large reactive B-
314 cells may have been included in the count, thereby falsely increasing MRD

315 values. Finally, it would be interesting to evaluate if LN aspirates from
316 different sites may influence the percentage of infiltration thus causing
317 migration from one relapse group to another. All these aspects could be
318 better elucidated in specific studies aimed to create standardized
319 recommendation for the assessment of MRD via FC in dogs with LBCL.

320

321 In conclusion, the present study indicates that MRD assessment by FC on LN
322 aspirates is a useful tool for assessing the presence of subclinical disease in
323 dogs with LBCL treated with chemo-immunotherapy. Relapse occurrence
324 could be efficiently predicted through FC prior to clinical relapse diagnosis,
325 and the value $>0.5\%$ was associated with early recurrence. Further trials
326 randomizing dogs according to their MRD-status are required to assess
327 whether MRD-guided management of dogs with LBCL will ultimately lead to
328 improved outcome and personalized care.

329

330

331 **Conflict of interest**

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

335

336

References

1. Ponce F, Marchal T, Magnol JP, et al. 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* 2010; 47:414-433.
2. Vezzali E, Parodi AL, Marcato PS, Bettini G. Histopathologic classification of 171 cases of canine and feline non-Hodgkin lymphoma according to the WHO. *Vet Comp Oncol* 2010; 8:38-49.
3. Jacobs, R.M., Messick, J.B., Valli, V.E. Tumors of the hemolymphatic system. In: Tumors in Domestic Animals, Meuten. DJ, 4th ed. Ames, IA: Iowa State Press; 2002:119-142.
4. Teske E. Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. *Vet Q* 1994; 16:209-219.
5. Riondato F, Martini V, Poggi A, et al. Identification of peripheral blood involvement in dogs with large B-cell lymphoma: Comparison of different methods. *Res Vet Sci* 2017; 115:288-293.
6. Rout ED, Avery PR. Lymphoid Neoplasia: Correlations Between Morphology and Flow Cytometry. *Vet Clin North Am Small Anim Pract* 2017; 47:53-70.
7. Vail DM, Young KM. Canine lymphoma and lymphoid leukemia. In: Withrow SJ, Vail DM, eds. Withrow & MacEwen's Small Animal Clinical Oncology. 4th ed. Philadelphia: WB Saunders Co; 2007:699–722.

- 359 8. Simon D, Nolte I, Eberle N, Abbrederis N, Killich M, Hirschberger J.
360 Treatment of Dogs with Lymphoma Using a 12-Week, Maintenance-Free
361 Combination Chemotherapy Protocol. *J Vet intern Med* 2006; 20:948–954.
- 362 9. Garrett LD, Thamm DH, Chun R, Dudley R, Vail DM. Evaluation of a 6-
363 month chemotherapy protocol with no maintenance therapy for dogs with
364 lymphoma. *J Vet Intern Med* 2002; 16:704–709.
- 365 10. Moore AS, Cotter SM, Rand WM, et al. Evaluation of a discontinuous
366 treatment protocol (VELCAP-S) for canine lymphoma. *J Vet Intern Med* 2001;
367 15:348–354.
- 368 11. Childress MO, Ramos-Vara JA, Ruple A. Retrospective analysis of
369 factors affecting clinical outcome following CHOP-based chemotherapy in
370 dogs with primary nodal diffuse large B-cell lymphoma. *Vet Comp Oncol*
371 2018; 16:159-168.
- 372 12. Cozzi M, Marconato L, Martini V, et al. Canine nodal marginal zone
373 lymphoma: descriptive insight into the biological behavior. *Vet Comp Oncol*
374 2018; 16:246-252.
- 375 13. Marconato L, Martini V, Aresu L, et al. Assessment of bone marrow
376 infiltration diagnosed by flow cytometry in canine large B cell lymphoma:
377 prognostic significance and proposal of a cut-off value. *Vet J* 2013; 197: 776–
378 781.

- 379 14. Elliott JW, Cripps P, Marrington AM, Grant AI, Blackwood L. Epirubicin as
380 part of multi-agent chemotherapy protocol for canine lymphoma. *Vet Comp*
381 *Oncol* 2013; 11:185-198.
- 382 15. Chun R, Garrett LD, Vail DM. Evaluation of high-dose chemotherapy
383 protocol with no maintenance therapy for dogs with lymphoma. *J Vet Intern*
384 *Med* 2010; 14:120-124.
- 385 16. Sorenmo K, Overley B, Krick E, Ferrara T, LaBlanc, A, Shofer F.
386 Outcome and toxicity associated with a dose-intensified, maintenance-free
387 CHOP-based chemotherapy protocol in canine lymphoma: 130 cases. *Vet*
388 *Comp Oncol* 2010; 8:196-208.
- 389 17. Turek MM, Thamm DH, Mitzey A, et al. Human granulocyte-macrophage
390 colony-stimulating factor DNA cationic-lipid complexed autologous tumour
391 cell vaccination in the treatment of canine B-cell multicentric lymphoma. *Vet*
392 *Comp Oncol* 2007; 5:219-231.
- 393 18. Vail DM, Michels GM, Khanna C, et al. Response evaluation criteria for
394 peripheral nodal lymphoma in dogs (v1.0)--a Veterinary Cooperative
395 Oncology Group (VCOG) consensus document. *Vet Comp Oncol* 2010; 8:28-
396 37.
- 397 19. Aresu L, Aricò A, Ferraresso S, et al. Minimal residual disease detection
398 by flow cytometry and PARR in lymph node, peripheral blood and bone
399 marrow, following treatment of dogs with diffuse large B-cell lymphoma. *Vet J*
400 2014; 200:318-324.

- 401 20. Thilakaratne DN, Mayer MN, MacDonald VS, Jackson ML, Trask BR,
402 Kidney BA. Clonality and phenotyping of canine lymphomas before
403 chemotherapy and during remission using polymerase chain reaction (PCR)
404 on lymph node cytologic smears and peripheral blood. *Can Vet J* 2010; 51:
405 79–84.
- 406 21. Yamazaki J, Takahashi M, Setoguchi A, Fujino Y, Ohno K, Tsujimoto H.
407 Monitoring of minimal residual disease after multidrug chemotherapy and its
408 correlation outcome in dogs with lymphoma a: a proof-of-concept pilot study.
409 *J Vet Intern Med* 2010; 24:897–903.
- 410 22. Yamazaki J, Baba K, Goto-Koshino Y, et al. Quantitative assessment of
411 minimal residual disease (MRD) in canine lymphoma by using real-time
412 polymerase chain reaction. *Vet Immunol Immunopathol* 2008; 126:321-331.
- 413 23. Sato M, Yamazaki J, Goto-Koshino Y, et al. Increase in minimal residual
414 disease in peripheral blood before clinical relapse in dogs with lymphoma that
415 achieved complete remission after chemotherapy. *J Vet Intern Med* 2011; 25:
416 292–296.
- 417 24. Sato M, Yamzaki J, Goto-Koshino Y, et al. The prognostic significance of
418 minimal residual disease in the early phases of chemotherapy in dogs with
419 high-grade B-cell lymphoma. *Vet J* 2013; 195:319–324.
- 420 25. Sato M, Yamazaki J, Goto-Koshino Y, et al. Minimal residual disease in
421 canine lymphoma: An objective marker to assess tumour cell burden in
422 remission. *Vet J* 2016; 215:38-42.

- 423 26. Gentilini F, Turba ME, Forni M. Retrospective monitoring of minimal
424 residual disease using hairpin-shaped clone specific primers in B-cell
425 lymphoma affected dogs. *Vet Immunol Immunopathol* 2013; 153:279-288.
- 426 27. Marconato L, Frayssinet P, Rouquet N, et al. Randomized, placebo-
427 controlled, double-blinded chemoimmunotherapy clinical trial in a pet dog
428 model of diffuse large B-cell lymphoma. *Clin Cancer Res* 2014; 20:668–677.
- 429 28. Marconato L, Stefanello D, Valenti P, et al. Predictors of long-term
430 survival in dogs with high-grade multicentric lymphoma. *J Am Vet Med Assoc*
431 2011; 238:480-485.
- 432 29. Comazzi S, Cozzi M, Bernardi S, et al. effects of pre-analytical variables
433 on flow cytometric diagnosis of canine lymphoma: A retrospective study
434 (2009-2015). *Vet J* 2018; 232:65-69.
- 435 30. Comazzi S, Gelain ME. Use of flow cytometry immunophenotyping to
436 refine cytological diagnosis of canine lymphoma. *Vet J* 2011; 188:149-155.
- 437

438 **Table 1:** Time To Relapse (TTR) in 31 dogs with large B-cell lymphoma
 439 (LBCL), according to different variables

Variables (number of dogs)	Median TTR in days (range)	p-value	
		Univariate analysis	Log-rank test
Breed		0.542	0.538
- purebred (24)	154 (31-911)		
- crossbred (7)	144 (42-1974)		
Sex		0.317	0.311
- male (18)	126 (42-449)		
- female (13)	157 (31-1974)		
Age		0.317	0.339
- <10 years (19)	126 (31-911)		
- ≥10 years (12)	175 (75-1974)		
Body weight		0.378	0.370
- <10 kg (5)	449 (31-449)		
- ≥10 kg (26)	154 (42-1974)		
Stage		0.530	0.509
- III (4)	154 (63-1974)		
- IV (14)	145 (42-449)		
- V (13)	154 (31-911)		

Substage		0.437	0.431
- a (26)	154 (31-1974)		
- b (5)	191 (42-195)		
Extranodal site involvement		0.607	0.601
- yes (2)	69 (69;429)		
- no (29)	154 (31-1974)		
Anemia		0.537	0.531
- yes (2)	42 (42;195)		
- no (29)	154 (31-1974)		
Thrombocytopenia		0.674	0.672
- yes (7)	157 (42-1206)		
- no (24)	154 (34-1974)		
Serum LDH activity		0.950	0.950
- normal (23)	154 (42-1974)		
- increased (7)	126 (31-429)		
Histotype		0.652	0.650
- DLBCL (23)	154 (31-1974)		
- MZL (8)	191 (61-429)		
Residual disease in lymph nodes		0.067	0.042
- ≤0.5% (3)	Not reached		

- >0.5% (15)	(195-429)		
- Acellular sample (13)	118 (31-232) 164 (63-1974)		
Residual disease in peripheral blood (%) (31)		0.608	
Residual disease in bone marrow (%) (25)		0.392	

440

441 **Figure 1**

442 Flow cytometric scattergrams showing Minimal Residual Disease (MRD)
443 evaluation in 3 dogs with Large B-Cell Lymphoma (LBCL). Left column: a first
444 gate in a morphological scattergram was set to exclude platelet and debris
445 (R1). Central column: only R1 events are shown; a second gate was set to
446 include only CD45-positive events. Right column: only events included in both
447 R1 and R2 are shown; MRD was calculated as the percentage of cells in the
448 upper right quadrant (large-sized CD21-positive cells). The three rows show 3
449 cases with different MRD levels.

450

451

452 **Figure 2**

453 Kaplan-Meier curves showing Time To Relapse (TTR) in 31 dogs with large
454 B-cell lymphoma (LBCL) according to flow cytometric (FC) results on post-
455 chemotherapy lymph node (LN) aspirates, peripheral blood (PB) and bone
456 marrow (BM) samples. Dashed line: large CD21+ cells $\leq$ the cut-off.
457 Continuous line: large CD21+ cells $>$ the cut-off. Dotted line: acellular
458 samples. **A-D**: cases were classified according to percentage of residual
459 disease in LN aspirates. **A**: the cut-off was set at 0.5%. **B**: the cut-off was set
460 at 1.0%. **C**: the cut-off was set at 3.0%. **D**: the cut-off was set at 5.0%. **E**:
461 cases were classified according to the percentage of residual disease in PB
462 samples; the cut-off was set at 0.5%. **F**: cases were classified according to
463 the percentage of residual disease in BM samples; the cut-off was set at
464 0.5%.

465

466 **Figure 3**

467 Kaplan-Meier curves showing Time To Relapse (TTR) in 31 dogs with large
468 B-cell lymphoma (LBCL) according to flow cytometric (FC) results on post-
469 chemotherapy lymph node (LN) aspirates. Continuous line: large CD21+ cells
470 $> 0.5\%$. Dotted line: large CD21+ cells $\leq 0.5\%$ and acellular samples. The
471 difference between the two groups was statistically significant ($p=0.030$).

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