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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

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

3  
4  
5 **Abstract**

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

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

15 Thirty-one dogs were enrolled: 4% had stage V disease, and DLBCL was the  
16 most common histotype (74%). Based on LN infiltration at MRD evaluation, 3  
17 groups were created: 1) acellular samples; 2)  $\leq 0.5\%$  infiltration; and 3)  $> 0.5\%$   
18 infiltration. Overall median TTR was 154 days (range, 31-1974): 22 (71%)  
19 dogs relapsed during the study period, whereas 9 (29%) dogs did not. The  
20 difference among the 3 groups was significant ( $p=0.042$  log-rank test):  
21 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.<sup>1-4</sup> Large B-cell lymphoma  
35 (LBCL) is characterized by medium or large-sized cells that express CD21 by  
36 flow cytometry (FC).<sup>5,6</sup>

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.<sup>7-10</sup>  
43 According to the published literature, CR rate and median first remission  
44 duration range between 80-85% and 87-330 days, respectively.<sup>8,11-17</sup> In dogs  
45 with multicentric lymphoma the measurement of peripheral lymph nodes  
46 (LNs) size after chemotherapy is used to document treatment response.<sup>18</sup>

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.<sup>18</sup>

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.<sup>19,20</sup>

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.<sup>21-26</sup> However, the universal primers  
62 previously designed for PARR showed a low sensitivity for MRD evaluation.<sup>19</sup>  
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.<sup>27</sup> 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.<sup>13</sup>

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  $1 \times 10^3$  nucleated cells/ $\mu$ l were considered  
108 necessary for the evaluation of residual disease in the LN samples (which  
109 means a total of  $1 \times 10^6$  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 $\mu$ 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/ $\mu$ 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 ( $\geq 300$  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.<sup>28</sup>

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 treatment<sup>18</sup>, 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<sup>5,6,13,29,30</sup>, 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  $1 \times 10^6$  intact cells ( $\geq 1 \times 10^3$  nucleated cells/ $\mu\text{l}$ )

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.<sup>19</sup> 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

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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
<b>Breed</b> - purebred (24) - crossbred (7)	154 (31-911) 144 (42-1974)	0.542	0.538
<b>Sex</b> - male (18) - female (13)	126 (42-449) 157 (31-1974)	0.317	0.311
<b>Age</b> - <10 years (19) - ≥10 years (12)	126 (31-911) 175 (75-1974)	0.317	0.339
<b>Body weight</b> - <10 kg (5) - ≥10 kg (26)	449 (31-449) 154 (42-1974)	0.378	0.370
<b>Stage</b> - III (4) - IV (14) - V (13)	154 (63-1974) 145 (42-449) 154 (31-911)	0.530	0.509

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.

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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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