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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) ≤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 ≤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

Introduction

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

Despite significant improvements in terms of prognosis due to better treatments for LBCL, most dogs that undergo first-line chemotherapy and achieve clinical complete remission (CR) will ultimately relapse. The CHOP-based maintenance-free chemotherapeutic protocol including Prednisolone, Vincristine, Cyclophosphamide, Doxorubicin +/- L-Asparaginase is considered to be the gold standard treatment for dogs with LBCL.[7-10] According to the published literature, CR rate and median first remission duration range between 80-85% and 87-330 days, respectively.[8,11-17] In dogs with multicentric lymphoma the measurement of peripheral lymph nodes (LNs) size after chemotherapy is used to document treatment response.[18] According to the Veterinary Cooperative Oncology Group (VCOG) guidelines, clinical CR is defined as the regression of all affected peripheral LNs to a size considered normal by physical examination using LN palpation and calipers.[18] Following clinical remission, the residual population of tumor cells can be referred to as the minimal residual disease (MRD), which is implicated as the source of tumor relapse. Therefore, measurement of MRD can provide

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

Several techniques, including FC and polymerase chain reaction amplification of antigen receptor genes (PARR), have been used to detect MRD in LN, peripheral blood (PB) and bone marrow (BM) samples in dogs with LBCL.[19,20] Molecular techniques for detection of MRD in PB samples during or following treatment can be used as an objective parameter for the determination of treatment efficacy and to select dogs that might benefit from consolidation chemotherapy despite clinical CR.[21-26] However, the universal primers previously designed for PARR showed a low sensitivity for MRD evaluation.[19] In the current study FC was tested as an alternative method for detection of MRD in the routine clinical practice.

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

Material and methods

4

Patient selection and inclusion criteria

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

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/µl 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 500µl of PBS and acquired with a BD FACScalibur flow

cytometer (Becton Dickinson, San Josè, CA). For each tube, 10,000

nucleated cells were acquired. Analyses were performed with the specific

software CellQuest (Becton Dickinson). A first gate was set in the

morphological scattergram to exclude debris and platelets, and a second one

to include only CD45-positive cells. For each matrix the residual disease was

quantified based on the percentage of large-sized CD21-positive cells out of

the total CD45-positive cells (Figure 1). As cut-off value, the mean FSC value

of the neutrophil population (ranging between 350 and 400 based on our

experimental condition) was used.

Follow-up

Dogs had to undergo follow-up evaluations to assess the remission status

consisting of physical examination, peripheral LNs size measurement and

cytological evaluation every 4 weeks during the first year, and every other

month thereafter. Relapse was defined as the clinical reappearance and

cytological evidence of lymphoma with or without FC confirmation in any

anatomical site in dogs having experienced CR. Once relapse was confirmed,

a complete restaging work-up was undertaken, and a second round of

chemotherapy was offered.

Statistical analysis

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 ≥ 10 years), body weight (< or ≥ 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/μL, yes

155 or no), serum LDH activity (< or ≥ 300 IU/L) histotype (DLBCL, MZL), LN

156 group (acellular, ≤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≤0.05 for all tests.

161

Results

Demographics

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

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

Clinico-pathological features

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

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

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

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

MRD assessment on LN, PB and BM

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

PB was tested for MRD by FC in all 31 cases. Median infiltration was 0.3% (mean 0.41±0.34%; min-max 0.1-1.5%). In particular, it was ≤0.5% in 21 (67.7%) dogs.

BM was tested for MRD by FC in 25 (80.6%) cases. Median infiltration was 0.3% (mean 0.51±0.49%; min-max 0.1-1.9%). In particular, it was ≤0.5% in 16 (64%) dogs.

Outcome and prognostic groups based on LN infiltration

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

10

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) ≤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

presence of residual neoplastic cells if peripheral LNs are not enlarged is

challenging. The current prospective study shows that MRD assessment by

FC on LN samples in dogs with LBCL in clinical CR may predict TTR, thereby

representing a useful tool for disease monitoring after therapy completion. In

detail, dogs with >0.5% LN infiltration were more likely to relapse despite the

clinical CR.

According to the VCOG guidelines, all dogs in the present study were in

clinical CR at the end of treatment[18], yet 50% of them had detectable MRD

based on FC, ultimately leading to early relapse. Thus, FC may play a role

not only in the initial diagnostic work up[5,6,13,29,30], but also in post-treatment

surveillance. In fact, cytology and even imaging may occasionally detect

clinically occult relapse, but the benefit of these techniques has not been

demonstrated in LBCL. Whether the detection of relapse ahead of clinical

symptoms translates into a clinical benefit was not investigated in the current

study, therefore it is currently unknown whether early MRD-based

intervention can affect outcome. Nevertheless, these results allow at least the

question to be addressed in clinical trials, by including MRD assessment at

the end of treatment to document the presence or absence of subclinical

disease.

The second aim of this study was to establish a cut-off value of prognostic

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 ≤0.5% or those in which LN samples were

270 acellular were mostly associated with a good outcome. While the threshold of

≤0.5% is intuitive, one may argue the meaning of acellular samples.

In the current study, at least 1×10^6 intact cells ($\geq 1 \times 10^3$ nucleated cells/µl) were considered necessary for the evaluation of MRD by FC. This threshold was arbitrarily set, since this amount of cells is required for the application of a minimal immunophenotyping panel according to internal standard procedures. Indeed, working dilutions of the antibodies have been established for a concentration of 500,000 cells/tubes. While the reduced panel of antibodies used here may be adapted to less cellular samples, thus possibly reducing the percentage of "acellular" cases, it must be noted that statistically the acellular samples tended to overlap with those with <0.5% infiltration in terms of outcome. The most likely explanation for this finding is that dogs achieving CR after treatment undergo LNs atrophy, as previously documented.[19] Fine-needle aspirates obtained from one or more peripheral LNs were always evaluated by means of cytology prior to FC analysis, to ensure that a LN was actually sampled. Despite having performed ultrasound-guided fine-needle aspiration of multiple peripheral non-palpable LNs and having obtained good cytological smears (data not shown), the cellularity of atrophic LNs was inadequate for flow cytometric analysis in 13 dogs, yielding acellular samples. Nevertheless, dogs with acellular samples at the end of treatment experienced a long TTR. Based on the above, acellular samples by FC suggest that residual neoplastic cells are virtually 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

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

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

Conflict of interest

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

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437

Table 1: Time To Relapse (TTR) in 31 dogs with large B-cell lymphoma

(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

Figure 1

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

Figure 2

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

Figure 3

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

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