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Prognostic significance of peripheral blood and bone marrow infiltration in newly-diagnosed canine nodal marginal zone lymphoma

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1 **Original article**

2  
3 **Prognostic significance of peripheral blood and bone marrow infiltration in newly-**  
4 **diagnosed canine nodal marginal zone lymphoma**

5  
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## 21   **Abstract**

22           Canine nodal marginal zone lymphoma (nMZL) is infrequent and is typically  
23   diagnosed at an advanced disease stage. However, it is currently unknown whether different  
24   levels of peripheral blood (PB) and bone marrow (BM) infiltration may provide prognostic  
25   stratification in dogs with nMZL.

26           The aims of the present prospective study were to assess the influence of PB and BM  
27   infiltration detected by flow cytometry (FC) on time to progression (TTP) and lymphoma-  
28   specific survival (LSS) in dogs with newly-diagnosed multicentric nMZL, and to establish a  
29   cut-off value of prognostic significance.

30           Forty-five completely staged and treatment-naïf dogs with histologically-confirmed  
31   nMZL were enrolled. After staging, dogs received chemo-immunotherapy or chemotherapy.  
32   PB infiltration was significantly associated with TTP ( $p=0.001$ ): dogs with PB infiltration  
33    $<30\%$  had a median TTP of 186 days, whereas dogs with PB infiltration  $\geq 30\%$  had a median  
34   TTP of 43 days. Additionally, vaccinated dogs had a significantly ( $p=0.012$ ) longer TTP (399  
35   days) compared with dogs receiving chemotherapy only (211 days).

36           BM infiltration was significantly associated with LSS ( $p<0.001$ ): dogs with BM  
37   infiltration  $<1\%$  had a median LSS of 1403 days, those with BM infiltration 1-20% of 337  
38   days, and those with BM infiltration  $\geq 20\%$  of 188 days. Normal LDH levels and the  
39   administration of chemo-immunotherapy also significantly improved LSS (560 vs 211 days,  
40   and 399 vs 211 days, respectively;  $p<0.001$ ).

41           PB and BM flow cytometric evaluation is an integral part of staging work-up in dogs  
42   with nMZL and has prognostic relevance.

43

44

45   *Keywords:* Dog; Bone marrow; Cut-off; Flow cytometry; Marginal zone lymphoma

46

## 47 **Introduction**

48 In dogs, nodal marginal zone lymphoma (nMZL) is infrequent, representing  
49 approximately 10% of all lymphoma histotypes diagnosed in this species (Valli et al., 2011).  
50 It was recently documented that nMZL is characterized by generalized lymphadenopathy and  
51 is diagnosed at an advanced disease stage; thus, despite the indolent designation, the  
52 prognosis is guarded (Cozzi et al., 2018).

53 Adequate management of canine lymphoma requires accurate histological diagnosis  
54 and comprehensive staging, which includes assessment of bone marrow (BM) involvement.  
55 Therapeutic options rely mainly on the results of these procedures (Marconato et al., 2017).  
56

57 In dogs with diffuse large B-cell lymphoma (DLBCL), it has been shown that 3% BM  
58 infiltration evaluated by flow cytometry (FC) identified cases with an unfavorable prognosis  
59 (Marconato et al., 2013). According to a recent study, peripheral blood (PB) and BM  
60 infiltration occurred in 97.1% and 57.1% of canine nMZL cases, respectively (Cozzi et al.,  
61 2018). However, it is currently unknown whether different levels of PB and BM infiltration  
62 may provide prognostic stratification in dogs with nMZL.

63  
64 The aims of the present prospective study were to assess the influence of different  
65 levels of PB and BM infiltration, detected by FC, on the duration of the first remission and  
66 survival in dogs with newly-diagnosed multicentric nMZL, and to establish a cut-off value of  
67 prognostic significance.

68

## 69 **Material and methods**

### 70 *Inclusion criteria*

71 A prospective analysis of dogs with multicentric nMZL was performed. To be enrolled  
72 in the study, dogs were required to have complete clinicopathological data for analysis, and be

73 treatment-naïf. Corticosteroids before admission were permitted.

74

75 All dogs were staged according to the World Health Organization system (Owen,  
76 1980), comprising history, physical examination, haematology, serum biochemistry  
77 (including serum lactate dehydrogenase, LDH), thoracic radiographs, abdominal ultrasound,  
78 and cytological evaluation of a fine-needle aspirate of an enlarged peripheral lymph node  
79 (LN). Dogs also underwent LN, PB and BM sampling for FC evaluation, and  
80 lymphadenectomy of an enlarged peripheral LN, having histopathological evaluation and  
81 immunohistochemistry (CD3, CD20) as a part of their initial staging work-up (Aresu et al,  
82 2015). The same LN that was aspirated for obtaining a cytological diagnosis and for FC was  
83 then surgically removed. The diagnosis of nMZL late-stage was confirmed according to the  
84 WHO classification (Valli et al., 2011).

85 For the specific aims of the present research, at the end of the study, FC data were  
86 blindly re-analyzed to minimize possible interpretation biases among different readers over  
87 time. [Only the re-analyzed results have been considered for the study.](#)

88

89 The care of the dogs enrolled in the study was in accordance with institutional  
90 guidelines. All owners provided written informed consent.

91

## 92 *Flow cytometry*

93 Flow cytometric immunophenotyping was performed on LN aspirates obtained with  
94 22-gauge needles and collected in tubes containing RPMI1640 (Sigma Aldrich) and on PB  
95 and BM samples collected into EDTA tubes. All samples were kept refrigerated and  
96 processed within 24h of sampling. A panel of antibodies was used for LN labeling using a  
97 multi-colour approach as previously described (Gelain et al., 2008), and included: CD45  
98 (clone YKIX716.13, pan-leukocyte), CD5 (clone YKIX322.3, T-cells), CD21 (clone

99 CA2.1D6, B-cells), and CD34 (clone 1H6, precursor cells). PB and BM samples were stained  
100 with CD45, CD5 and CD21 antibodies. All antibodies but CD34 were provided by Bio-Rad  
101 (formerly AbD Serotec; Oxford, UK); CD34 was provided by BD Pharmingen (San Diego,  
102 CA, USA). Erythrocyte lysis was not necessary on LN samples, since haemodilution was  
103 minimal, and double labeling using CD45 easily enabled the distinction between lymphoid  
104 cells and debris or erythrocytes. Conversely, erythrocyte lysis was performed on PB and BM  
105 samples by means of an erythrocyte lysis buffer containing 8% ammonium chloride, after  
106 incubation with antibodies. All samples were acquired with a BD FACScalibur flow  
107 cytometer (Becton Dickinson, San José, CA, USA) and analyzed with CellQuest software  
108 (Becton Dickinson).

109

#### 110 *Determination of PB and BM involvement*

111 PB and BM aspirates were obtained in all cases at the time of the initial staging work-  
112 up. BM was sampled with 16- or 18-gauge Illinois needles from the iliac crest. PB and BM  
113 samples were placed in EDTA tubes for FC analysis.

114 The extent of PB and BM infiltration by large B-cells was reported as the percentage  
115 of medium-large CD21-positive cells out of the total CD45-positive cells (leucocytes and  
116 their precursors). The threshold for cell size was set based on the FSC of non-neoplastic T-  
117 lymphocytes from the same subject. Normal circulating B-cells show the lowest FSC among  
118 lymphocytes, being smaller than normal T-cells. Reactive medium-sized B-cells present FSC  
119 properties partially overlapping those of T-cells. Thus, most of reactive medium-sized B-cells  
120 were not included in the count of infiltrating neoplastic cells with our gating strategy.

121

#### 122 *Treatment and response evaluation*

123 The treatment protocol was in keeping with approved standards. Dogs whose owners  
124 wished to pursue immunotherapy received a 20-week dose-intense chemotherapy regimen,

125 consisting of L-Asparaginase, Vincristine, Cyclophosphamide, Doxorubicin, Lomustine, and  
126 prednisone (Table 1). These dogs also received an intradermal injection of 0.5 ml autologous  
127 vaccine on weeks 4, 5, 6, 7, 12, 16, 20, and 24, as previously described (Marconato et al.,  
128 2014).

129 Dogs treated with chemotherapy only, received the following protocol, consisting of  
130 L-Asparaginase, Vincristine, Cyclophosphamide, Doxorubicin, Lomustine, and Prednisone  
131 (Table 1).

132 Response was classified as complete remission (CR), partial remission (PR), stable disease  
133 (SD), or progressive disease (PD) based on previously published criteria (Marconato et al.,  
134 2013). Responses were required to last for  $\geq 28$  days.

135

136 Relapse was defined as clinical reappearance and cytological evidence of lymphoma in  
137 any anatomical site in dogs having experienced CR, whereas relapse for animals with PR was  
138 defined as progression.

139 Response was evaluated at each chemotherapy session by measurement of peripheral  
140 LNs. End-staging was carried out at the end of treatment, and every clinical, radiological,  
141 ultrasonographic, or laboratory investigation that disclosed abnormalities at pre-treatment  
142 staging was repeated. BM and PB were re-evaluated in all cases by FC. The end-staging  
143 results were necessary to assess treatment response.

144

#### 145 *Statistical analysis*

146 Time to progression (TTP) was calculated from the start of treatment to disease  
147 progression (Vail et al., 2010). Dogs lost to follow-up or dead for lymphoma-unrelated causes  
148 before disease progression, as well as those in CR at the end of the study, were censored for  
149 TTP analysis.

150 Lymphoma-specific survival (LSS) was measured as the interval between the start of



151 treatment and death for lymphoma (Vail et al., 2010). Dogs alive at the end of the study, lost  
152 to follow-up or dead due to causes other than lymphoma were censored for LSS analysis.

153

154 To detect possible associations between TTP and LSS, and PB and BM infiltration,  
155 cases were subdivided into two groups based on the arbitrarily selected infiltration cut-offs of  
156 1.0%, 3.0%, 5.0%, 10.0%, 20.0% and 30.0%. Thereafter, Kaplan-Meier curves were drawn  
157 for each cut-off and compared using the log-rank test. Based on data distribution, the 30.0%  
158 cut-off was not tested on BM samples. As two different BM cut-offs gave significant results  
159 for LSS analysis, these were coupled to stratify the study population into three groups, which  
160 were further tested by Kaplan-Meier curves and log-rank test.

161

162 Univariate Cox's proportional hazard regression analysis was performed to determine  
163 a possible association between selected variables and TTP and LSS, respectively. Variables  
164 with  $p \leq 0.3$  at univariate analysis were then included in a backward elimination multivariate  
165 analysis. For categorical variables, Kaplan-Meier curves were drawn and compared by log-  
166 rank test.

167

168 The independent variables included in the analyses were: breed (pure or mixed), age  
169 ( $< \text{or} \geq 8$  years), sex (male or female), weight ( $< \text{or} \geq 22.5$  kg), clinical stage (I-V), substage  
170 (a or b), anemia (presence or absence), thrombocytopenia (presence or absence), LDH (within  
171 or outside the reference interval [0-170 U/l]), FC PB infiltration (%), PB infiltration group  
172 ( $< 30\%$  or  $\geq 30\%$ ), FC BM infiltration (%), BM infiltration group ( $< 1\%$ ,  $1\% - 20\%$ , or  $\geq 20\%$ ),  
173 therapy (chemotherapy or chemo-immunotherapy).

174 Possible differences in the aforementioned variables between the two treatment groups  
175 were investigated with Pearson chi-squared test (for categorical variables) or with Mann-  
176 Whitney test (for continuous, non-parametric variables).

177

178 All analyses were performed with a standard software (SPSS v20.0 for Windows), and  
179 significance was set at  $p \leq 0.05$  for all analyses.

180

## 181 **Results**

182 Between 2011 and 2018, 45 cases met the inclusion criteria and were enrolled. There  
183 were 12 (26.7%) mixed-bred and 33 (73.3%) pure-breed dogs. Among these, there were 3  
184 Rottweiler, 3 Poodle, 2 jack russel terrier, 2 Australian shepherd, 2 Pomeranian, 2 Golden  
185 retriever, 2 German shepherd, 2 French Bulldog, 2 Labrador retriever, and one each of the  
186 following: Bassethound, dachshund, shih-tzu, beagle, Petit Bleu, Yorkshire terrier, Border  
187 collie, Bernese Mountain dog, Pinscher, Akita Inu, Dobermann, Boxer, and German Hound.

188 Twenty (44.4%) dogs were females (11 spayed) and 25 (55.6%) were males (3  
189 neutered). Mean age was  $7.9 \pm 3.2$  years (median 8 years, range 3-15 years), with 21 (46.7%)  
190 dogs being <8 year-old and 24 (53.3%)  $\geq 8$  year-old. Mean body weight was  $22.5 \pm 12.5$  kg  
191 (median 24.4 kg, range 3.0-44.4 kg); the body weight was <22.5 kg in 21 (46.7%) dogs and  
192  $\geq 22.5$  kg in 24 (53.3%).

193 One (2.2%) dog had stage IIIa disease, 6 (13.3%) had stage IV disease (4 substage a, 2  
194 substage b), and 38 (84.4%) had stage V disease (24 substage a, 14 substage b). Anemia and  
195 thrombocytopenia were present in 4 (8.9%) and 6 (13.3%) dogs, respectively. LDH activity  
196 was tested in 41 dogs and was increased in 17 (41.5%).

197

198 Mean PB infiltration at diagnosis was  $10.43 \pm 12.82\%$  (median 5.0%, range 0.2-53.5%).  
199 It was <1% in 8 (17.8%) dogs, <3% in 15 (33.3%), <5% in 20 (44.4%), <10% in 32 (31.1%),  
200 <20% in 36 (80.0%), <30% in 41 (91.1%), and  $\geq 30\%$  in 4 (8.9%).

201 Mean BM infiltration at diagnosis was  $6.86 \pm 9.39\%$  (median 3.0%, range 0.2-51.6%).  
202 It was <1% in 9 (20.0%) dogs, <3% in 22 (48.9%), <5% in 25 (55.6%), <10% in 36 (80.0%),

203 <20% in 42 (93.3%), <30% in 44 (97.8%), and  $\geq 30\%$  in 1 (2.2%).

204

205 Twenty-four (53.3%) dogs received chemo-immunotherapy, and 21 (46.7%) were  
206 treated with chemotherapy. Twenty (83.3%) of 24 dogs treated with chemo-immunotherapy  
207 and 13 (61.9%) of 21 dogs treated with chemotherapy achieved CR, confirmed by a complete  
208 end-staging. However, 17 (85%) of the 20 dogs treated with chemo-immunotherapy and all  
209 (100%) dogs treated with chemotherapy eventually relapsed.

210 The two treatment cohorts were homogeneous for all investigated variables.

211

212 Median TTP was 179 days (range, 1-1295 days). In particular, 40 (88.9%) dogs  
213 progressed during the study period, whereas 4 (8.9%) died for lymphoma-unrelated causes  
214 after 93, 151, 181 and 237 days, respectively, and one (2.2%) dog was still alive and in CR  
215 after 1295 days.

216 Median LSS was 337 days (range, 5-1403 days). In particular, 33 (73.3%) dogs died  
217 for their lymphoma during the study period, 8 (17.8%) were still alive at data analysis closure  
218 with a median follow-up of 701 days (range 281-1295 days), and 4 (8.9%) died for  
219 lymphoma-unrelated causes after 93, 151, 181 and 237 days, respectively.

220

221 The results obtained for the different PB and BM infiltration cut-offs are shown in  
222 Table 2. According to these results, cases were subdivided into two PB infiltration groups  
223 (<30% and  $\geq 30\%$ ) and into three BM infiltration groups (<1%, 1-20%, and  $\geq 20\%$ ).

224

225 Concerning TTP, univariate Cox's analysis and log-rank test gave significant results  
226 for substage ( $p=0.020$  and  $p=0.017$ , respectively), treatment ( $p=0.037$  and  $p=0.033$ ,  
227 respectively) and PB infiltration groups ( $p=0.006$  and  $p=0.002$ , respectively) (Fig 1).  
228 Multivariate analysis showed significant results for treatment ( $p=0.012$ ) and PB infiltration

229 groups ( $p=0.001$ ).

230 Median TTP for significant variables is shown in Table 3.

231

232 Concerning LSS, univariate Cox's analysis and log-rank test gave significant results  
233 for substage ( $p=0.002$  and  $p=0.001$ , respectively), LDH ( $p=0.050$  and  $p=0.044$ , respectively),  
234 PB infiltration groups ( $p=0.015$  and  $p=0.008$ , respectively) and BM infiltration groups  
235 ( $p=0.035$  and  $p=0.022$ , respectively) (Fig 2). Multivariate analysis gave significant results for  
236 LDH ( $p<0.001$ ), treatment ( $p=0.002$ ) and BM infiltration groups ( $p<0.001$ ). In particular,  
237 dogs with BM infiltration  $<1\%$  had a median LSS of 1403 days, whereas dogs with BM  
238 infiltration of  $1-20\%$  and  $\geq 20\%$  had a median LSS of 337 and 188 days, respectively.

239 Median LSS for significant variables is shown in Table 4.

240

## 241 Discussion

242 Recent advances in imaging and laboratory methodology have the potential to improve  
243 disease characterization and outcome in dogs with lymphoma, possibly leading to changes in  
244 clinical practice and, eventually, in trial design. PB and BM evaluation is part of the staging  
245 work-up for canine lymphoma, and FC is essential to detect and quantify their involvement  
246 (Riondato et al., 2016; Riondato et al., 2017). However, knowledge about the prognostic value  
247 of PB and BM infiltration based on FC in canine nMZL is limited.

248 The results of the current study suggest, in a homogeneous series of 45 dogs with  
249 newly-diagnosed nMZL late-stage, that PB and BM infiltration detected by FC at diagnosis is  
250 frequent and influences the duration of first remission and survival.

251 PB involvement in nMZL as detected by FC significantly influenced TTP. The  
252 presence of circulating neoplastic cells was related to clinical stage and BM involvement, and  
253 correlated with poor disease control. Indeed, dogs with  $<30\%$  PB involvement had a median  
254 TTP of 186 days, whereas dogs with  $\geq 30\%$  PB involvement relapsed earlier (TTP 43 days).

255 Lymphoma cells in PB reflect the biological property of entry of solid tumor cells into the  
256 circulation. Relapse is due either to incomplete remission in dogs with extensive tumor  
257 burden or to chemoresistance. Dogs with marked ( $\geq 30\%$ ) PB involvement had a very short  
258 remission; therefore, it may be possible that these cells were chemoresistant or had  
259 temporarily resided at sites poorly accessible to chemotherapy.

260         It has been previously documented that indolent B-cell lymphoma appears to be  
261 associated with a higher incidence of BM infiltration compared with aggressive B-cell  
262 lymphoma (33-80% versus 18-27%, respectively) (Aresu et al, 2015; Marconato et al., 2015a,  
263 Cozzi et al., 2018), thereby confirming the need for extensive staging in all lymphoma cases.  
264 Indeed, dogs with nMZL showed a heterogeneous clinical course based on BM infiltration,  
265 which resulted to be an independent predictor for LSS. Dogs with BM infiltration  $< 1\%$  had a  
266 significantly longer survival time compared with dogs with BM infiltration in the range of 1-  
267 20% and  $\geq 20\%$ . Moreover, despite the overall poor treatment outcomes in nMZL cases with  
268 BM infiltration, dogs with less extent of BM infiltration (1-20%) had a more favorable  
269 outcome compared with dogs in the other group (12 months versus 6 months, respectively),  
270 stressing the need not only for the detection of marrow involvement, but also for neoplastic  
271 cell quantification.

272         Beside PB and BM involvement, further variables were independent predictive factors  
273 in multivariate analysis.

274         An elevated LDH activity at initial diagnosis was predictive for shorter survival. This  
275 is in agreement with a previous study, whereby it was shown that elevated LDH activity was  
276 more frequent in dogs with DLBCL and higher PB and BM infiltration, possibly indicating a  
277 marked disease activity. Also, in the same study, high LDH activity predicted a more  
278 aggressive clinical course (Marconato et al., 2013). The same may hold true for nMZL.

279         Dogs receiving chemo-immunotherapy had a significantly longer TTP and LSS  
280 compared with dogs treated with chemotherapy only. In a previous study (Marconato et al.,

281 2014), chemo-immunotherapy significantly improved TTP in dogs with indolent B-cell  
282 lymphoma when compared to dogs receiving chemotherapy only, whereas LSS only tended to  
283 be longer. The differences between these studies might be explained by sample size and the  
284 different enrollment criteria, because the earlier study included all subtypes of indolent  
285 lymphoma as one entity instead of individual subtypes.

286 Interestingly, when comparing BM infiltration data with the ones previously published  
287 in DLBCL (Marconato et al., 2013), a better stratification for TTP and LSS was obtained in  
288 dogs with nMZL. This result is also emphasized by the diagnostic and biological limits that  
289 have been described so far for these two histotypes. Indeed, the histological diagnosis of  
290 nMZL late-stage is still a conundrum because of the lack of immunohistochemical markers  
291 that allow in many cases distinguishing it from DLBCL. To rule out this risk, we included  
292 only nMZLs that were histologically characterized by a diffuse infiltration of neoplastic cells  
293 with intermediate-sized nuclei, prominent single central nucleoli, abundant lightly stained  
294 cytoplasm, low mitotic rate and absence of centroblasts and immunoblasts. Moreover, two  
295 gene expression profile studies have recently shown that nMZL and DLBCL are similar at the  
296 transcriptomic level (Frantz et al., 2013), but they result two separate entities clinically,  
297 suggesting other biological mechanisms underlying tumor cells of origin.

298 This study has some limitations.

299 First, there was treatment regimens heterogeneity in the population of our study.  
300 Approximately 50% of dogs received an autologous vaccine in addition to conventional  
301 chemotherapy. Analyses of prognostic factors for TTP and LSS showed a better outcome for  
302 dogs that received chemo-immunotherapy. As of today, there are no standard recommended  
303 treatments for nMZL, which has not proven curable with classical chemotherapy (Aresu et al,  
304 2015, Marconato et al., 2015b). In the current study, the combination of chemotherapy and  
305 immunotherapy significantly improved outcome. This finding raises the question as to  
306 possible choices in terms of classical chemotherapy and whether immunotherapy should be

307 the new gold standard for first-line treatment in dogs with nMZL.

308         Second, the FC strategy used to quantify PB and BM infiltration in the present study  
309 has never been validated for canine nMZL, but was derived by the one used to stage canine  
310 large B-cell lymphomas (Riondato et al., 2016). In particular, we lowered the FSC threshold  
311 in order to include medium-sized cells in the count. This likely affects the analytical and  
312 diagnostic performances reported. In particular, the limit of detection (LOD) of medium-large  
313 CD21+ cells in non-neoplastic samples is likely higher than the one reported for large B-cells,  
314 and even higher than 1%. Nevertheless, we report relevant prognostic implications only for  
315 highly infiltrated BM samples (>20%), which is likely far from the LOD of the technique, and  
316 for samples with <1% infiltration, where neoplastic cells are virtually absent. Cases  
317 potentially located between 1% and LOD would fall in the limbo of the 1-20% BM infiltration  
318 group, which bears an intermediate prognosis and warrants examination of further prognostic  
319 parameters.

320

## 321 **Conclusions**

322         The detection of PB and BM involvement in most dogs with nMZL confirms the need  
323 for PB and BM flow cytometric evaluation as an integral part of staging work-up also in these  
324 patients. We found that PB infiltration  $\geq 30\%$  and BM infiltration  $\geq 20\%$  in dogs with nMZL  
325 are independent negative prognostic factors. More specifically, in dogs with PB infiltration  
326  $< 30\%$ , BM evaluation stratifies dogs into 3 prognostic groups: those with a poor (infiltration  
327  $\geq 20\%$ ), intermediate (infiltration 1-20%) and better (infiltration  $< 1\%$ ) prognosis.

328         Future efforts should be directed toward finding optimal treatment modalities in  
329 managing nMZL dogs based on the extent of PB and BM infiltration.

330

331

## 332 **Conflict of interest statement**

333           None of the authors has any financial or personal relationships that could inappropriately  
334 influence or bias the content of the paper.

335

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405 **Table 1**

406 Therapeutic protocols administered to 45 dogs with Marginal Zone Lymphoma (MZL).

		Week number																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Chemo-immunotherapy protocol																				
L-ASP	X																			
VCR		X	X	X										X						
CYCLO		X												X						
DOXO								X									X			
CCNU											X									X
Prednisolone administered at 1 mg/kg daily from week 1 to week 4; then 0.5 mg/kg daily until the end of treatment																				
Chemotherapy protocol																				
L-ASP	X																			
VCR		X				X						X						X		
CYCLO				X				X						X					X	
DOXO					X			X						X						X
CCNU										X						X				
Prednisolone administered at 1 mg/kg daily from week 1 to week 4; then 0.5 mg/kg daily until the end of treatment																				

407 L-ASP = L-asparaginase (400 UI/kg SQ); VCR = Vincristine (0.75 mg/m<sup>2</sup> IV); CYCLO = Cyclophosphamide (250 mg/m<sup>2</sup> PO in the chemo-  
408 immunotherapy protocol, 75 mg/m<sup>2</sup> PO for 4 consecutive days in the chemotherapy protocol); DOXO = Doxorubicin (30 mg/m<sup>2</sup> IV); CCNU =  
409 Lomustine (80 mg/m<sup>2</sup> PO)

410 **Table 2**

411 P-values obtained by comparing Kaplan-Meier curves using different arbitrarily selected cut-offs  
 412 for peripheral blood and bone marrow infiltration in 45 dogs diagnosed with Marginal Zone  
 413 Lymphoma.

Matrix	Cut-off	Log-rank test p-value	
		Time to progression	Lymphoma specific survival
Peripheral blood	1%	0.416	0.419
	3%	0.515	0.614
	5%	0.315	0.463
	10%	0.510	0.824
	20%	0.183	0.572
	30% <sup>§</sup>	0.002*	0.008*
Bone marrow	1%	0.098	0.039*
	3%	0.155	0.102
	5%	0.241	0.258
	10%	0.188	0.237
	20%	0.092	0.044*
	1% and 20% <sup>§</sup>	0.077	0.022*

414 \*= significant result; §= cutoff selected for further survival analyses

415

416

417 **Table 3**

418 Time to progression (TTP) in 45 dogs with nodal Marginal Zone Lymphoma, according to specific  
419 variables

Variable (number of dogs)	Median TTP in days (range)	P-value			Hazard ratio (95% CI)
		Univariate analysis	Log- rank test	Multivariate analysis	
Substage		0.020*	0.017*	0.930	
a (29)	312 (24-1295)				Ref
b (16)	60 (1-420)				2.195 (1.131-4.263)
Therapy		0.037*	0.033*	0.012*	
Chemotherapy (21)	78 (1-1295)				1.950 (1.040-3.658)
Chemo- immunotherapy (24)	227 (29-720)				Ref
PB infiltration		0.006*	0.002*	0.001*	
<30% (41)	186 (1-1295)				Ref
≥30% (4)	43 (1-134)				4.897 (1.579-15.188)

420 \*=significant result

421

422

423 **Table 4**

424 Lymphoma specific survival (LSS) in 45 dogs with nodal Marginal Zone Lymphoma, according to  
425 specific variables.

Variable (number of dogs)	Median LSS in days (range)	P-value			Hazard ratio (95% CI)
		Univariate analysis	Log-rank test	Multivariate analysis	
Substage		0.002*	0.001*	0.905	
a (29)	544 (93-1403)				Ref
b (16)	125 (5-862)				3.375 (1.572-7.247)
Therapy		0.093	0.088	0.002*	
Chemotherapy (21)	211 (5-1403)				1.833 (0.905-3.714)
Chemo- immunotherapy (24)	399 (93-1321)				Ref
PB infiltration		0.015*	0.008*	0.175	
<30% (41)	349 (5-1403)				Ref
≥30% (4)	125 (7-215)				4.017 (1.314- 12.283)
LDH activity		0.050*	0.044*	<0.001*	
Normal (24)	560 (111-1403)				Ref

Increased (17)	211				2.172 (1.002-4.710)
	(7-730)				
BM infiltration		0.035*	0.022*	<0.001*	
<1% (9)	1403				Ref
	(45-1403)				
1%-20% (33)	337				2.754 (0.948-7.997)
	(5-1321)				
≥20% (3)	188				7.771 (1.621-
	(7-215)				37.244)

426 \*=significant result

427

428 **Fig. 1.** Kaplan-Meier curves representing time to progression of 45 dogs with nodal Marginal Zone  
429 Lymphoma with  $<30\%$  (continuous line) or  $\geq 30\%$  (dotted line) peripheral blood flow cytometric  
430 infiltration.

431

432 **Fig. 2.** Kaplan-Meier curves representing lymphoma specific survival of 45 dogs with nodal  
433 Marginal Zone Lymphoma with  $<1\%$  (continuous line), 1-20% (dashed line) or  $\geq 20\%$  (dotted line)  
434 bone marrow flow cytometric infiltration.

435