



ALMA MATER STUDIORUM  
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE  
DELLA RICERCA

## Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Prognostic value of peripheral blood and bone marrow infiltration assessed by flow cytometry in dogs with de novo nodal peripheral T-cell lymphoma receiving alkylating-rich chemotherapy

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Marconato, L., Comazzi, S., Agnoli, C., Aresu, L., Stefanello, D., Riondato, F., et al. (2024). Prognostic value of peripheral blood and bone marrow infiltration assessed by flow cytometry in dogs with de novo nodal peripheral T-cell lymphoma receiving alkylating-rich chemotherapy. *THE VETERINARY JOURNAL*, 303, 1-5 [10.1016/j.tvjl.2023.106057].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/954533> since: 2024-10-28

*Published:*

DOI: <http://doi.org/10.1016/j.tvjl.2023.106057>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

# The Veterinary Journal

## Prognostic value of peripheral blood and bone marrow infiltration assessed by flow cytometry in dogs with de novo nodal peripheral T-cell lymphoma receiving alkylating-rich chemotherapy --Manuscript Draft--

<b>Manuscript Number:</b>	YTVJL-D-23-00391R2
<b>Article Type:</b>	Original article
<b>Keywords:</b>	Bone marrow; Dog; Peripheral blood; Peripheral T-cell lymphoma; Prognosis
<b>Corresponding Author:</b>	laura marconato, DVM, DECVIM-CA (Oncology) University of Bologna Department of Veterinary Medical Sciences Sasso Marconi, ITALY
<b>First Author:</b>	laura marconato, DVM, DECVIM-CA (Oncology)
<b>Order of Authors:</b>	laura marconato, DVM, DECVIM-CA (Oncology) Stefano Comazzi Chiara Agnoli Luca Aresu Damiano Stefanello Fulvio Riondato Lorenzo Gamberini Silvia Sabattini
<b>Abstract:</b>	<p>Peripheral T-cell lymphoma (PTCL) is highly aggressive in dogs and demonstrates a poor response to traditional chemotherapy. The aim of this retrospective study was to assess the prognostic significance of peripheral blood (PB) and bone marrow (BM) infiltration evaluated by flow cytometry (FC) in dogs with treatment-naïve and histologically confirmed PTCL. To be included, dogs had to undergo complete staging, including FC on lymph nodes, PB and BM samples. Additionally, dogs had to receive an alkylating-rich protocol and have a complete follow-up. Treatment response was evaluated based on RECIST criteria at each chemotherapy session, and the end-staging was conducted at the completion of treatment. Endpoints were time to progression (TTP) and lymphoma-specific survival (LSS). The relationship between TTP/LSS and the percentage of PB and BM infiltration, categorized as &gt;1%, &gt;3%, &gt;5%, &gt;10%, &gt;15% and &gt;20% was investigated.</p> <p>Fifty dogs were included: based on imaging and FC, 78.0% had stage V disease, 14.0% had stage IV, 6.0% had stage III and 2.0% had stage I. By multivariable analysis, the CD4-negative phenotype was the only factor associated with a shorter TTP (P=0.049), while BM infiltration was significantly associated with LSS (P=0.037). Dogs with BM infiltration &gt;5% had shorter median LSS (114 days; 95%CI: 0-240) compared to dogs with BM infiltration ≤5% (178 days; 95%CI: 145-211). Lack of complete response (P=0.039) and administration of corticosteroids before chemotherapy (P=0.026) also significantly worsened LSS. BM flow cytometric evaluation could be considered an essential part of staging work-up for dogs with PTCL and has prognostic relevance.</p>

1 **Original Article**

2

3

4 **Prognostic value of peripheral blood and bone marrow infiltration assessed by**  
5 **flow cytometry in dogs with de novo nodal peripheral T-cell lymphoma receiving**  
6 **alkylating-rich chemotherapy**

7

8 L. Marconato <sup>a</sup> \*, S. Comazzi <sup>b</sup>, C. Agnoli <sup>a</sup>, L. Aresu <sup>c</sup>, D. Stefanello <sup>b</sup>, F. Riondato <sup>c</sup>, L.  
9 Gamberini <sup>a</sup>, S. Sabattini <sup>a</sup>

10

11 <sup>a</sup> *Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50,*  
12 *40064 Ozzano dell'Emilia (Bo), Italy*

13 <sup>b</sup> *Department of Veterinary Medical Sciences, University of Milano, Via dell'Università 6,*  
14 *26900 Lodi (LO), Italy*

15 <sup>c</sup> *Department of Veterinary Medical Sciences, University of Torino, Largo P. Braccini 2,*  
16 *10095 Grugliasco (TO), Italy*

17

18

19

20

21 \* Corresponding author. Tel.: [39 051 2097950](tel:390512097950)

22 E-mail address: [laura.marconato@unibo.it](mailto:laura.marconato@unibo.it) (L. Marconato).

23

**Commented [L1]:** Line 4 of YTVJL-D-23-00391. Deleted-  
role

**Commented [L2]:** Line 4 of YTVJL-D-23-00391R1. Deleted-  
flow cytometric

24 **Abstract**

25 Peripheral T-cell lymphoma (PTCL) is highly aggressive in dogs and  
26 demonstrates a poor response to traditional chemotherapy. The aim of this retrospective  
27 study was to assess the prognostic significance of peripheral blood (PB) and bone  
28 marrow (BM) infiltration evaluated by flow cytometry (FC) in dogs with treatment-  
29 naïve and histologically confirmed PTCL. To be included, dogs had to undergo  
30 complete staging, including FC on lymph nodes, PB and BM samples. Additionally,  
31 dogs had to receive an alkylating-rich protocol and have a complete follow-up.  
32 Treatment response was evaluated based on RECIST criteria at each chemotherapy  
33 session, and the end-staging was conducted at the completion of treatment. Endpoints  
34 were time to progression (TTP) and lymphoma-specific survival (LSS). The relationship  
35 between TTP/LSS and the percentage of PB and BM infiltration, categorized as >1%,  
36 >3%, >5%, >10%, >15% and >20% was investigated.

37  
38 Fifty dogs were included: based on imaging and FC, 78.0% had stage V disease,  
39 14.0% had stage IV, 6.0% had stage III and 2.0% had stage I. By multivariable analysis,  
40 the CD4-negative phenotype was the only factor associated with a shorter TTP  
41 ( $P=0.049$ ), while BM infiltration was significantly associated with LSS ( $P=0.037$ ).  
42 Dogs with BM infiltration >5% had shorter median LSS (114 days; 95%CI: 0-240)  
43 compared to dogs with BM infiltration  $\leq$ 5% (178 days; 95%CI: 145-211). Lack of  
44 complete response ( $P=0.039$ ) and administration of corticosteroids before  
45 chemotherapy ( $P=0.026$ ) also significantly worsened LSS. BM flow cytometric  
46 evaluation could be considered an essential part of staging work-up for dogs with PTCL  
47 and has prognostic relevance.

48

**Commented [L3]:** Line 27 of YTVJL-D-23-00391R1. Deleted- by flow cytometry (FC)

**Commented [L4]:** Line 28 of YTVJL-D-23-00391. Deleted- flow cytometry

**Commented [L5]:** Line 34 of YTVJL-D-23-00391. Deleted- and

**Commented [L6]:** Line 37 of YTVJL-D-23-00391. Deleted- multivariate

49 *Keywords:* Bone marrow; Dog; Peripheral blood; Peripheral T-cell lymphoma;

50 Prognosis

51

52 **Introduction**

53 According to the World Health Organization classification (Valli et al., 2011),  
54 peripheral T-cell lymphoma (PTCL) is categorized as an aggressive lymphoma,  
55 constituting around 5-15% of all canine lymphoid neoplasms (Ito et al., 2014). For all  
56 lymphoma subtypes, a comprehensive initial work-up, including  
57 cytologic/histopathologic evaluations, flow cytometry (FC), and radiologic  
58 examinations, is crucial for accurately assessing disease status and predicting the risk in  
59 dogs with lymphoma (Marconato, 2011). The clinical stage of lymphoma, determined  
60 by organ involvement, is closely linked to clinical outcomes (Valli et al., 2013).  
61 However, the frequency, extent, and prognostic significance of peripheral blood (PB)  
62 and bone marrow (BM) involvement vary significantly among different lymphoma  
63 subtypes, which reflects the variations in the underlying biology [and the different](#)  
64 [techniques used to assess it](#). As an example, in dogs with diffuse large B-cell lymphoma  
65 (DLBCL) and marginal zone lymphoma (MZL), BM involvement is common and has a  
66 detrimental effect on prognosis (Marconato et al., 2013; Marconato et al., 2019a).  
67 Nevertheless, the prognostic cut-off differs between these two subtypes. In DLBCL, a  
68 cut-off of 3% [assessed by FC](#) has been established (Marconato et al., 2013), whereas in  
69 MZL, a cut-off of 20% is used (Marconato et al., 2019b). Another example pertains to  
70 T-zone lymphoma (TZL), where BM infiltration is frequently observed, but it does not  
71 negatively affect prognosis (Martini et al., 2016). There are also considerations for PB.  
72 While no prognostic cut-off has been identified for DLBCL (Marconato et al., 2013),  
73 the same cannot be said for MZL. Notably, dogs with PB infiltration  $\geq 30\%$  had a  
74 significantly shorter time to progression (TTP) than those with infiltration  $< 30\%$   
75 (Marconato et al., 2019b). Conversely, for TZL, despite it being leukemic in over 90%

76 of cases, no correlation has been documented between the percentage of infiltration and  
77 outcome (Seelig et al., 2014; Martini et al., 2016).

78  
79 On clinical grounds, PTCLs exhibit limited response to traditional chemotherapy  
80 and poor survival rates (Purzycka et al., 2020; Blaxill et al., 2022). However, the  
81 frequency of PB and BM infiltration in dogs with PTCL and its prognostic significance  
82 have yet to be determined. Here, we hypothesized that PB and BM infiltration is a poor  
83 prognostic indicator, impacting outcome. The aims of the current retrospective research  
84 were twofold: 1) to investigate the **distribution** of PB and BM infiltration in dogs with  
85 PTCL; 2) to evaluate the impact of different levels of PB and BM infiltration, detected  
86 via FC, on the duration of first remission and survival.

87

## 88 **Materials and methods**

89 Medical records of two Oncology Referral Centers were reviewed to identify  
90 dogs with treatment-naïve and histologically confirmed PTCL. Corticosteroids before  
91 admission were permitted. To be included in the analysis, dogs had to undergo a  
92 complete staging and lymphadenectomy of a peripheral enlarged lymph node (LN) with  
93 a final diagnosis of PTCL (Valli et al., 2011), and not to be lost to follow-up.

94

95 Information on clinical stage was obtained by means of hematologic and  
96 biochemical analysis (including ionized calcium and serum lactate dehydrogenase,  
97 LDH), thoracic radiographs, abdominal ultrasound, fine-needle aspiration of spleen and  
98 liver, FC on LN, PB and BM samples. The same LN that was aspirated for obtaining a  
99 cytologic diagnosis and for FC was then surgically removed.

100

Commented [SS7]: Line 327 of YTVJL-D-23-00391.  
Deleted- prevalence

101 For FC analysis, LN aspirates were obtained with 22-gauge needles and  
102 collected in tubes containing RPMI1640 (Sigma Aldrich). BM aspirates were sampled  
103 with 16- or 18-gauge Illinois needles from the iliac crest. PB and BM samples were  
104 placed in EDTA tubes.

105  
106 Information on substage was based on the presence of clinical signs, with dogs  
107 showing weight loss >10%, fever, unexplained resting tachypnoea, vomiting and/or  
108 diarrhea, or polyuria/polydipsia attributable to hypercalcemia being classified as  
109 substage b (Škor et al., 2021).

110  
111 The care of the dogs was in accordance with institutional guidelines. therefore,  
112 ethical approval was waived for this study. All specimens were obtained under the  
113 formal consent from the owners.

114  
115 *Flow cytometry*

116 All samples were refrigerated and processed within 24h of sampling. A panel of  
117 antibodies, obtained from Bio-rad laboratories (Hercules, CA, US), was employed in a  
118 multi-color approach as previously outlined (Gelain et al., 2008), and included: CD45  
119 (clone YKIX716.13, all leukocytes), CD5 (clone YKIX322.3, T-cells), CD3 (clone  
120 CA17.2A12, T-cells), CD4 (clone YKIX302.9, T-helper), CD8 (clone YCATE55.9, T-  
121 cytotoxic), MHC II (clone YKIX334.2, B-cells and T-cells). Samples were acquired  
122 either with a Mindray BryCyte E6 flow cytometer (Mindray, Shenzhen, China) or BD  
123 Accuri C6 (Becton Dickinson), and analyzed with dedicated softwares (MRFlow,  
124 Mindray; CFlow Plus).

125

**Commented [L8]:** Line 116 of YTVJL-D-23-00391. Deleted-CD3-12



126 The extent of PB and BM infiltration was reported as the percentage of cells  
127 with the same scatter properties and antigen expression than those found in the LN out  
128 of total CD45+ events (all leukocytes). Dogs with a minimum of 1% of infiltration in  
129 PB and/or BM were arbitrarily classified as having stage V disease.

Commented [L9]: Line 123 of YTVJL-D-23-00391. Deleted forward

130  
131 *Histology*

132 Hematoxylin-eosin glass slides and immunohistochemistry (IHC) findings were  
133 reviewed by one pathologist to confirm the diagnosis (LA). For each case, serial  
134 paraffin slide sections of the extirpated LN were stained with hematoxylin and eosin  
135 and immunohistochemically labeled for CD3 and CD20. Immunohistochemistry was  
136 performed following the guidelines established by the American Association of  
137 Veterinary Diagnosticians Subcommittee on Standardization of Immunohistochemistry.  
138 Briefly, sections were processed using an automatic immunostainer (Ventana  
139 Benchmark XT, Ventana Medical Systems Inc.). Two antibodies were used, including  
140 anti-CD3 (clone F7.2.38, monoclonal mouse, 1:50; Dako Italia, Milan, Italy) and anti-  
141 CD20 (clone RB-9013-P, epitope specific rabbit, 1:800; Thermo Fisher Scientific,  
142 Ashford, UK), (Aresu et al., 2015). A normal canine LN was used as positive control.

143  
144 *Treatment and response evaluation*

145 The treatment protocol was in keeping with approved standards. All dogs  
146 received one of the following alkylating-rich protocols: VELCAP-TSC or CCNU-  
147 CHOP (Sauerbrey et al., 2007; Marconato et al., 2014; Goodman et al., 2016; Limmer  
148 et al., 2022), based on clinician's and owner's preference. Response was classified as  
149 complete remission (CR), partial remission (PR), stable disease (SD), or progressive  
150 disease (PD) based on previously published criteria (Nguyen et al., 2015). Response

151 was evaluated at each chemotherapy session by measuring peripheral LNs with or  
152 without confirmatory cytology and was required to last for  $\geq 28$  days. Relapse was  
153 defined as clinical reappearance and cytologic evidence of lymphoma in any anatomical  
154 site in dogs having experienced CR, whereas relapse for animals with PR was defined  
155 as progression.

156

157 End-staging was carried out at the end of treatment, and every clinical,  
158 radiologic, ultrasonographic, or laboratory investigation that disclosed abnormalities at  
159 pre-treatment staging was repeated. BM and PB were re-evaluated in all cases by FC.

160

#### 161 *Statistical analysis*

162 The extent of PB and BM infiltration was compared among the most prevalent  
163 phenotype-defined categories using the Mann-Whitney U test.

164 Time to progression was calculated from treatment initiation to disease  
165 progression. If progression did not occur, dogs were censored for TTP analysis.

166 Lymphoma-specific survival (LSS) was measured as the interval between treatment  
167 initiation and death for lymphoma. Dogs alive at data analysis closure or dead due to

168 causes other than lymphoma were censored for LSS analysis. Univariable Cox's  
169 proportional hazard regression analysis was performed to explore potential associations

170 between selected variables and tumor progression and tumor-related death. Variables  
171 significant at univariable analysis were then included in a multivariable analysis model

172 using the enter method. The independent variables included in the analyses were age  
173 (median value used as cut-off point), sex (male or female), weight (median value used  
174 as cut-off point), corticosteroids before admission, extranodal (other than PB and BM)  
175 involvement, substage b, anemia (hematocrit value lower than the laboratory reference

**Commented [L10]:** Line 160 of YTVJL-D-23-00391.  
Deleted- lost to follow-up

**Commented [L11]:** Line 161 of YTVJL-D-23-00391.  
Deleted- forward

**Commented [SS12]:** Line 171 of YTVJL-D-23-00391.  
Deleted- a backward elimination

176 interval, RI), thrombocytopenia (platelet concentration lower than the laboratory RI and  
177 absence of platelets aggregates), hypercalcemia (ionized calcium above the laboratory  
178 RI), increased LDH (LDH above the laboratory RI), CD5, CD3, CD4, CD8 and MHCII  
179 immunophenotype (positive or negative), chemotherapy protocol (VELCAP-TSC or  
180 CCNU-CHOP) and lack of CR (evaluated only for LSS). The prognostic relevance of  
181 the extent of PB and BM infiltration were further assessed by subdividing cases in two  
182 groups based on arbitrarily selected infiltration cut-offs (1%, 3%, 5%, 10%, 15%, 20%).  
183 The same cut-offs were also assessed by categorizing cases into various subgroups  
184 based on the immunophenotype category.

185 [When necessary, survival curves were obtained with the Kaplan-Meier method](#)  
186 [and compared with the log-rank test.](#)

187 All analyses were performed with a standard software (SPSS v20.0), and  
188 significance was set at  $P \leq 0.05$ .

189

## 190 **Results**

191 Between 2012-2022, 64 dogs with multicentric PTCL were identified. Ten dogs  
192 were excluded because they received a non-alkylating rich protocol ( $n=8$ ) or no  
193 treatment ( $n=2$ ), 3 because their BM sample was not suitable for analysis, and one  
194 because lost to follow-up. A total of 50 dogs were included in the analysis.

195

### 196 *Tumor and dogs' characteristics*

197 There were 23 (46.0%) males (5 neutered) and 27 (54.0%) females (17 spayed).  
198 Median age was 7 years (range, 4–14), and median weight was 27 kg (range, 6–58).  
199 There were 17 (34.0%) mixed breeds and 33 (66.0%) pure-breed dogs. Among these,  
200 there were 9 (18.0%) Boxers, 3 (6.0%) Beagle, 3 (6.0%) Cane Corso, 2 (4.0%) Dogue

201 de Bordeaux, 2 (4.0%) German shepherd, 2 (4.0%) Rhodesian ridgeback. Other breeds  
202 were represented once. Overall, 39 (78.0%) dogs had stage V disease, 7 (14.0%) had  
203 stage IV disease, 3 (6.0%) had stage III and 1 (2.0%) had stage I. Eighteen dogs  
204 (36.0%) were asymptomatic (substage a), while 32 (64.0%) dogs had substage b.  
205 Anemia and thrombocytopenia were registered in 5 (10.0%) and 14 (28.0%) dogs,  
206 respectively. Results of calcium concentration and serum LDH activity were available  
207 for 48 and 38 cases respectively, with elevated levels observed in 23 (47.9%) cases for  
208 calcium and 19 (50.0%) cases for LDH.

209

#### 210 *Flow cytometry*

211 Based on CD4 and CD8 expression, the following phenotype categories were  
212 detected: CD4+/CD8-, n=30 (60.0%); CD4-/CD8-, n=7 (14.0%); CD4-/CD8+, n=7  
213 (14.0%); CD4+/CD8+, n=6 (12.0%). CD5 was expressed in 28/44 (63.6%), CD3 in  
214 33/40 (82.5%), and MHCII in 5/24 (20.8%) cases.

215

216 FC results for PB and BM were available for all cases. Overall, 31 (62%) dogs  
217 had PB infiltration, and 29 (58%) dogs had BM involvement. Three dogs with BM  
218 involvement had no circulating neoplastic cells, whereas 5 dogs with PB involvement  
219 had no BM infiltration. Median PB infiltration at diagnosis was 2.6% (range, 0.0-31.0).

220 It was >1% in 33 (66.0%) dogs, >3% in 21 (42.0%), >5% in 13 (26.0%), >10% in 3  
221 (6.0%). Median BM infiltration at diagnosis was 1.3% (range, 0.1-66.5). It was >1% in  
222 28 (56%) dogs, >3% in 20 (40.0%), >5% in 13 (26.0%), >10% in 6 (12.0%), >15% in 4  
223 (8.0%), and >20% in 4 (8.0%). In the CD4+/CD8- category, BM infiltration (3.4%;  
224 range, 0.6-66.5) was significantly higher than in the remaining cases (0.9%; range, 0.1-

**Commented [SS13]:** Line 209 of YTVJL-D-23-00391.  
Deleted- 95% confidence interval [CI], 1.1-3.6

**Commented [SS14]:** Line 211 of YTVJL-D-23-00391.  
Deleted- 95%CI, 0.9-3.6

**Commented [L15]:** Line 214 of YTVJL-D-23-00391.  
Deleted- others

225 20.5;  $P=0.015$ ). No significant differences were detected for PB infiltration in the same  
226 two groups.

227

#### 228 *Treatment and outcome*

229 Thirteen (26.0%) dogs received prednisolone before diagnosis. After staging, all  
230 dogs received chemotherapy: 28 (56.0%) CCNU-CHOP and 22 (44%) VELCAP-TSC.  
231 Overall, 32 (64.0%) dogs achieved CR, 14 (28.0%) PR, while 4 (8.0%) progressed. At  
232 relapse, 18 (36.0%) dogs received a rescue protocol.

233

234 Median TTP was 90 days (95%CI 53-127). At data analysis closure, 3 (6.0%)  
235 dogs were alive after a median follow-up of 334 days (range, 180-845), while 47 (94%)  
236 had died. Cause of death was attributable to lymphoma in 44 dogs and to tumor-  
237 unrelated causes in 3. Median LSS was 154 days (95%CI 118-190).

238

#### 239 *Analysis of prognostic factors*

240 At univariable analysis, variables significantly associated with an increased risk  
241 of tumor progression were weight <27 kg (hazard ratio [HR]=2.1; 95%CI 1.1-3.9;  
242  $P=0.021$ ), administration of corticosteroids before diagnosis (HR=2.0; 95%CI 1.1-4.1;  
243  $P=0.032$ ), extranodal involvement (HR=2.2; 95%CI 1.1-4.4;  $P=0.029$ ), and CD4-  
244 phenotype (HR=3.0; 95%CI 1.4-6.6;  $P=0.005$ ). At multivariable analysis only the CD4-  
245 phenotype retained prognostic significance (HR=2.4; 95%CI 1.0-5.6;  $P=0.049$ ).

246

247 Variables significantly associated with an increased risk of lymphoma-related  
248 death included administration of corticosteroids before diagnosis (HR=2.5; 95%CI 1.3-  
249 4.9;  $P=0.009$ ), extranodal involvement (HR=2.4; 95%CI 1.2-4.8;  $P=0.012$ ), CD4-

250 phenotype (HR=2.4; 95%CI 1.2-4.9;  $P=0.017$ ), BM infiltration >5% (HR=2.2; 95%CI  
251 1.1-4.3;  $P=0.023$ ), CCNU-CHOP protocol (HR=2.0; 95%CI 1.1-3.8;  $P=0.034$ ), and lack  
252 of CR (HR=2.7; 95%CI 1.3-5.4;  $P=0.006$ ). At multivariable analysis administration of  
253 corticosteroids (HR=2.6; 95%CI 1.1-5.9;  $P=0.026$ ), BM infiltration >5% (HR=2.5;  
254 95%CI 1.1-5.8,  $P=0.037$ ) and lack of CR (HR=2.5; 95%CI 1.0-5.8;  $P=0.039$ ) retained  
255 prognostic significance. Dogs with BM infiltration >5% exhibited a significantly lower  
256 median LSS (114 days; 95%CI 0-240), compared to those with an infiltration  $\leq 5\%$  (178  
257 days; 95%CI 145-211;  $P=0.020$ ; Fig. 1).

258

259 The prognostic significance of BM and PB cut-offs was further evaluated within  
260 the most prevalent phenotype category (CD4+/CD8-). The significant BM cut-offs were  
261 >10% (HR=3.5; 95%CI 1.1-11.3;  $P=0.035$ ) for tumor progression and >5% (HR=2.5;  
262 95%CI 1.1-5.7;  $P=0.033$ ) for tumor-related death. No prognostic cut-off was identified  
263 for PB infiltration.

264

## 265 Discussion

266 This study represents the first comprehensive analysis of the prognostic impact  
267 of PB and BM infiltration on the outcome of dogs with de novo PTCL. Within this  
268 series, PB infiltration did not show any association with worse TTP or LSS. However,  
269 BM involvement was significantly associated with shorter LSS when the infiltration  
270 level exceeded 5%.

271

272 BM is the most common site of extranodal involvement in lymphoid  
273 malignancies and the frequency of BM involvement varies according to the specific  
274 lymphoma subtype. However, studies specifically examining BM infiltration and its

275 prognostic significance have primarily focused on DLBCL, MZL and TZL (Marconato  
276 et al., 2013; Martini et al., 2016; Marconato et al., 2019b).

277

278         In the current series, we present data regarding 50 PTCL dogs undergoing  
279 complete staging and treated with alkylating-rich protocols. Based on the initial work-  
280 up, most dogs (78%) had stage V disease and, of these, 74% exhibited some degree of  
281 BM infiltration (encompassing 58% of all included dogs). Previous studies reporting on  
282 BM involvement in PTCL have shown varying frequencies, ranging from 22%  
283 (Purzycka et al., 2020) to 63% (Aresu et al., 2015) of dogs with suspected or confirmed  
284 BM infiltration. However, some of these studies included dogs with different types of  
285 aggressive T-cell lymphomas in addition to PTCL. Moreover, the number of dogs  
286 undergoing BM evaluation was relatively small, and in certain cases, FC analysis of BM  
287 aspirates was not consistently performed. This limitation restricted the diagnosis to  
288 morphologic evaluation (Aresu et al., 2015; Purzycka et al., 2020), making it  
289 challenging to draw definitive conclusions. The findings of the present study indicate  
290 that a BM infiltration level exceeding 5% independently influences LSS. This  
291 significant result holds clinical relevance and supports the recommendation to include  
292 BM [flow cytometric](#) evaluation as part of the comprehensive work-up for all dogs  
293 diagnosed with multicentric PTCL. Additionally, the data suggests that the clinical  
294 behavior of lymphoma is more closely associated with the degree of infiltration rather  
295 than the mere presence or absence of infiltration alone, highlighting the limitations of  
296 relying solely on BM morphologic evaluation and emphasizing the importance of  
297 quantifying neoplastic cells through FC.

298

299 We detected a significantly higher percentage of BM infiltration in the  
300 CD4+/CD8- immunophenotype category compared to the other cases. This finding  
301 suggests a possible biological distinction among the PTCL subtypes or might be related  
302 to a different FC sensitivity within the evaluated immunophenotypic categories.  
303

304 In the context of the most prevalent phenotype category (CD4+/CD8-), a BM  
305 cut-off >5% was significantly associated to an unfavorable prognosis, substantiating the  
306 previously observed data. A cut-off of BM infiltration >10% was found to have a  
307 notable impact on TTP, though not on LSS. The interpretation of these results might  
308 have been influenced by the fact that during the assessment of LSS, 3 dogs succumbed  
309 to treatment-related complications while in remission. While these cases were  
310 categorized as tumor-related deaths, their presence might add complexity to the  
311 interpretation of our findings.  
312

313 In addition to BM infiltration, other variables that independently distinguished  
314 subgroups of dogs with varying LSS included the failure to achieve CR during  
315 chemotherapy and the prior administration of corticosteroids. It is not surprising that  
316 dogs failing to achieve CR or progressing during initial chemotherapy had a worse  
317 prognosis (Bennett et al., 2023). It is recognized that PTCL exhibits high expression of  
318 ABC transporter proteins, which contribute to early chemoresistance (Zandvliet, 2015).  
319 While not specifically explored in the dogs included in the present study, it is plausible  
320 that factors such as resistance to initial therapy, early relapses, ineffective salvage  
321 therapies, and overall compromised performance status may have contributed to the  
322 unfavorable prognosis observed in non-responders. Treatment with steroids prior to  
323 initiating chemotherapy repeatedly has been reported to negatively impact prognosis of

**Commented [L16]:** Line 308 of YTVJL-D-23-00391.  
Deleted- 2016



324 dogs with lymphoma, possibly due to multidrug resistance induction through  
325 upregulation of the drug efflux pump P-glycoprotein (Price et al., 1991; Teske et al.,  
326 1994; Bergman et al., 2003; Marconato, 2011; Limmer et al., 2022). In agreement with  
327 a recent study on nodal aggressive T-cell lymphomas (Purzycka et al., 2020), we  
328 confirm here that prior steroids had a deleterious effect on survival time.

329  
330 In the present series, BM infiltration had no prognostic role in determining TTP,  
331 which was found to be more influenced by other variables such as the CD4- phenotype.  
332 Previous studies have documented that specific immunotypes of multicentric PTCL are  
333 correlated with longer progression-free intervals (CD4+/CD8-/MHCII+, CD4-  
334 /CD8+/MHCII-, CD4-/CD8-/MHCII+) or improved survival (CD4+/CD8-/MHCII+,  
335 CD4-/CD8+/MHCII+, CD4-/CD8+/MHCII-) (Deravi et al 2017). According to another  
336 study, dogs with the less common CD8+ or CD4-/CD8- aggressive T-cell lymphoma  
337 had a more unfavorable clinical course (Harris et al., 2020). In our **univariable analysis**,  
338 the CD4- phenotype was validated as a negative prognostic factor for both TTP and  
339 LSS and retained prognostic significance for TTP in **multivariable analysis**. However,  
340 due to the heterogeneity among dogs expressing different combinations of CD4, CD8  
341 and MHCII, extracting additional insights from this data was unattainable. and  
342 conducting a direct comparison with Deravi's study was not feasible, **firstly** because the  
343 latter included various T-cell lymphomas, including TZL.

344  
345 This study has limitations, inherent to its retrospective nature. Also, CD3, CD5  
346 and MHCII were not simultaneously assessed in all samples. Thus, aside from the  
347 categorization derived from the CD4 and CD8 subset antigens, the cases encompassed  
348 in this study could reflect a spectrum of phenotypes with distinct biological behaviors.

**Commented [L17]:** Line 327 of YTVJL-D-23-00391.  
*Deleted- univariate*

**Commented [L18]:** Line 329 of YTVJL-D-23-00391.  
*Deleted- multivariate*

**Commented [L19]:** Line 332 of YTVJL-D-23-00391.  
*Deleted- also*

349 Future investigations focusing on the clinical outcomes of dogs diagnosed with various  
350 PTCL immunophenotypes may better clarify the prognostic significance of BM  
351 infiltration cut-offs in the most frequent PTCL subtypes.

352 [Finally, the cut-off for establishing PB and BM infiltration, set at 1%, has been](#)  
353 [arbitrarily defined in the absence of analytical validation.](#)

354  
355 Although only dogs treated with an alkylating-rich protocol were included, two  
356 different protocols were administered, possibly biasing the results. According to the  
357 literature, dogs with non-indolent T-cell lymphoma receiving VELCAP-TSC  
358 chemotherapy achieved an overall remission rate of 72.9%; median TTP and LSS were  
359 175 and 237 days, respectively (Goodman et al., 2016). Dogs treated with a CHOP-  
360 based protocol incorporating lomustine achieved an overall remission rate of 79.4%;  
361 had a median TTP of 161 days and a median survival of 210 days (Limmer et al., 2022).  
362 Therefore, while a direct comparison cannot be made, these two distinct protocols  
363 appear to have similar outcomes.

364

### 365 **Conclusions**

366 The current findings indicate that PB and BM involvement is common in dogs  
367 with PTCL, and that a BM infiltration exceeding 5% adversely affects LSS. These  
368 results highlight the need for prospective studies to be designed to further investigate  
369 the prognostic significance of PB and BM involvement in dogs with PTCL.

370

### 371 **Conflict of interest statement**

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

374

375 **References**

376

377 Aresu, L., Martini, V., Rossi, F., Vignoli, M., Sampaolo, M., Aricò, A., Laganga, P., Pierini,  
378 A., Frayssinet, P., Mantovani, R., et al., 2015. Canine indolent and aggressive  
379 lymphoma: clinical spectrum with histologic correlation. *Veterinary and Comparative*  
380 *Oncology* 13, 348-362.

381

382 Bennett, P., Williamson, P., Taylor, R., 2023. Review of canine lymphoma treated with  
383 chemotherapy-outcomes and prognostic factors. *Veterinary Sciences* 10, 342-366.

384

385 Bergman, P.J., 2003. Mechanisms of anticancer drug resistance. *The Veterinary Clinics of*  
386 *North America. Small Animal Practice* 33, 651-667.

387

388 Blaxill, J., Buzzacott, P., Finlay, J., 2022. Prognostic indicators for naïve canine non-indolent  
389 T-cell lymphoma treated with combination lomustine, vincristine, procarbazine and  
390 prednisolone chemotherapy. *Veterinary and Comparative Oncology* 20, 215-226.

391

392 Deravi, N., Berke, O., Woods, J.P., Bienzle, D., 2017. Specific immunotypes of canine T cell  
393 lymphoma are associated with different outcomes. *Veterinary Immunology and*  
394 *Immunopathology* 191, 5-13.

395

396 Garrett, L.D., Thamm, D.H., Chun, R., Dudley, R., Vail, D.M., 2002. Evaluation of a 6-month  
397 chemotherapy protocol with no maintenance therapy for dogs with lymphoma. *Journal*  
398 *of Veterinary Internal Medicine* 16, 704-709.

399

400 Gelain, M. E., Mazzilli, M., Riondato, F., Marconato, L., Comazzi, S., 2008. Aberrant  
401 phenotypes and quantitative antigen expression in different subtypes of canine  
402 lymphoma by flow cytometry. *Veterinary Immunology and Immunopathology* 121,  
403 179-188.

404

405 Goodman, I.H., Moore, A.S., Frimberger, A.E., 2016. Treatment of canine non-indolent T cell  
406 lymphoma using the VELCAP-TSC protocol: a retrospective evaluation of 70 dogs  
407 (2003-2013). *The Veterinary Journal* 211, 39-44.

408

409 Greenlee, P.G., Filippa, D.A., Quimby, F.W., Patnaik, A.K., Calvano, S.E., Matus, R.E.,  
410 Kimmel, M., Hurvitz, A.I., Lieberman, P.H., 1990. Lymphomas in dogs. A  
411 morphologic, immunologic, and clinical study. *Cancer* 66, 480-490.

412

413 Harris, L.J., Hughes, K.L., Ehrhart, E.J., Labadie, J.D., Yoshimoto, J., Avery, A.C., 2019.  
414 Canine CD4+ T-cell lymphoma identified by flow cytometry exhibits a consistent  
415 histomorphology and gene expression profile. *Veterinary and Comparative Oncology*  
416 17, 253-264.

417

418 Harris, L.J., Rout, E.D., Labadie, J.D., Avery, P.R., Fernandez, M., Yoshimoto, J., Avery,  
419 A.C., 2020. Clinical features of canine nodal T-cell lymphomas classified as CD8+ or  
420 CD4 CD8- by flow cytometry. *Veterinary and Comparative Oncology* 18, 416-427.

421

422 Ito, D., Frantz, A.M., Modiano, J.F., 2014. Canine lymphoma as a comparative model for  
423 human non-Hodgkin lymphoma: recent progress and applications. *Veterinary*  
424 *Immunology and Immunopathology* 15, 192-201.  
425

426 Keller, E-T., MacEwen, E.G., Rosenthal, R.C., Helfand, S.C., Fox, L.E., 1993. Evaluation of  
427 prognostic factors and sequential combination chemotherapy with doxorubicin for  
428 canine lymphoma. *Journal of Veterinary Internal Medicine* 7, 289-295.  
429

430 Limmer, S., Nerschbach, V., Eberle, N., Teske, E., Simon, Betz. D., 2022. Efficacy and  
431 tolerability of a 12-week combination chemotherapy followed by lomustine  
432 consolidation treatment in canine B- and T-cell lymphoma. *Acta Veterinaria*  
433 *Scandinavica* 64, 36-48.  
434

435 Marconato, L., 2011. The staging and treatment of multicentric high-grade lymphoma in  
436 dogs: a review of recent developments and future prospects. *The Veterinary Journal*  
437 188, 34-38.  
438

439 Marconato, L., Stefanello, D., Valenti, P., Bonfanti, U., Comazzi, S., Roccabianca, P.,  
440 Caniatti, M., Romanelli, G., Massari, F., Zini, E., 2011. Predictors of long-term  
441 survival in dogs with high-grade multicentric lymphoma. *Journal of the American*  
442 *Veterinary Medical Association* 238, 480-485.  
443

444 Marconato, L., Martini, V., Aresu, L., Sampaolo, M., Valentini, F., Rinaldi, V., Comazzi, S.,  
445 2013. Assessment of bone marrow infiltration diagnosed by flow cytometry in canine  
446 large B cell lymphoma: prognostic significance and proposal of a cut-off value. *The*  
447 *Veterinary Journal* 197, 776-781.  
448

449 Marconato, L., Frayssinet, P., Rouquet, N., Comazzi, S., Leone, V.F., Laganga, P., Rossi, F.,  
450 Vignoli, M., Pezzoli, L., Aresu, L., 2014. Randomized, placebo-controlled, double-  
451 blinded chemoimmunotherapy clinical trial in a pet dog model of diffuse large B-cell  
452 lymphoma. *Clinical Cancer Research* 20, 668-677.  
453

454 Marconato, L., Aresu, L., Stefanello, D., Comazzi, S., Martini, V., Ferrari, R., Riondato, F.,  
455 Rouquet, N., Frayssinet, P., Sabattini, S., 2019a. Opportunities and challenges of  
456 active immunotherapy in dogs with B-cell lymphoma: a 5-year experience in two  
457 veterinary oncology centers. *The Journal of Immunotherapy of Cancer* 7, 146-155.  
458

459 Marconato, L., Comazzi, S., Aresu, L., Riondato, F., Stefanello, D., Ferrari, R., Martini, V.,  
460 2019b. Prognostic significance of peripheral blood and bone marrow infiltration in  
461 newly-diagnosed canine nodal marginal zone lymphoma. *The Veterinary Journal* 246,  
462 78-84.  
463

464 Martini, V., Marconato, L., Poggi, A., Riondato, F., Aresu, L., Cozzi, M., Comazzi, S., 2016.  
465 Canine small clear cell/T-zone lymphoma: clinical presentation and outcome in a  
466 retrospective case series. *Veterinary and Comparative Oncology* 14 Suppl 1, 117-126.  
467

468 Nguyen, S. M., Thamm, D.H., Vail, D.M., London, C.A., 2015. Response evaluation criteria  
469 for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG)  
470 consensus document. *Veterinary and Comparative Oncology* 13, 176-183.  
471

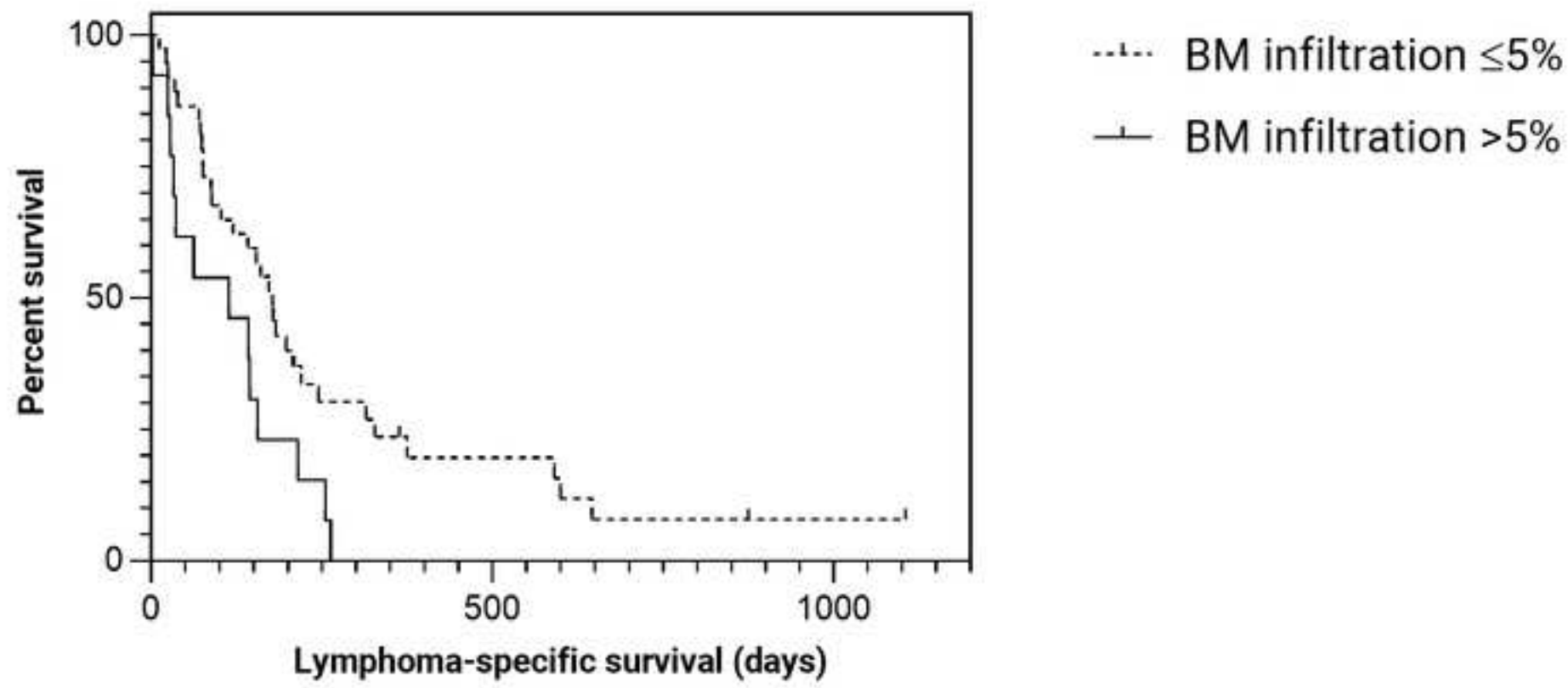
472 Price, G.S., Page, R.L., Fischer, B.M., Levine, J.F., Gerig, T.M., 1991. Efficacy and toxicity  
473 of doxorubicin/cyclophosphamide maintenance therapy in dogs with multicentric  
474 lymphosarcoma. *Journal of Veterinary Internal Medicine* 5, 259-262.  
475  
476 Purzycka, K., Peters, L.M., Desmas, I., Davies, O., Chang, Y.M., Lara-Garcia A., 2020.  
477 Clinicopathological characteristics and prognostic factors for canine multicentric non-  
478 indolent T-cell lymphoma: 107 cases. *Veterinary and Comparative Oncology* 18, 656-  
479 663.  
480  
481 Sauerbrey, M.L., Mullins, M.N., Bannink, E.O., Van Dorp, T.E., Kaneene, J.B., Obradovich,  
482 J.E., 2007. Lomustine and prednisone as a first-line treatment for dogs with  
483 multicentric lymphoma: 17 cases (2004-2005). *Journal of American Veterinary*  
484 *Medical Association* 230, 1866-1869.  
485  
486 Seelig, D.M., Avery, P., Webb, T., Yoshimoto, J., Bromberek, J., Ehrhart, E.J., Avery, A.C.,  
487 2014. Canine T-zone lymphoma: unique immunophenotypic features, outcome, and  
488 population characteristics. *Journal of Veterinary Internal Medicine* 28, 878-886.  
489  
490 Škor, O., Bicanová, L., Wolfesberger, B., Fuchs-Baumgartinger, A., Ruetgen, B., Štěrbová,  
491 M., Schwendenwein, I., Kleiter, M., 2021. Are B-symptoms more reliable prognostic  
492 indicators than substage in canine nodal diffuse large B-cell lymphoma. *Veterinary*  
493 *and Comparative Oncology* 19, 201-208.  
494  
495 Teske, E., 1994. Canine malignant lymphoma: a review and comparison with human non-  
496 hodgkin's lymphoma. *The Veterinary Quarterly* 16, 209-219.  
497  
498 Valli, V. E., San Myint, M., Barthel, A., Bienzle, D., Caswell, J., Colbatzky, F., Durham, A.,  
499 Ehrhart, E.J., Johnson, Y., Jones, et al., 2011. Classification of canine malignant  
500 lymphomas according to the World Health Organization criteria. *Veterinary Pathology*  
501 48, 198-211.  
502  
503 Valli, V.E., Kass, P.H., San Myint, M., Scott, F., 2013. Canine lymphomas: association of  
504 classification type, disease stage, tumor subtype, mitotic rate, and treatment with  
505 survival. *Veterinary Pathology* 50, 738-748.  
506  
507 Zandvliet, M., Teske, E., Schrickx, J.A., Mol, J.A., 2015. A longitudinal study of ABC  
508 transporter expression in canine multicentric lymphoma. *The Veterinary Journal* 205,  
509 263-271.  
510  
511

**Commented [L20]:** Line 495 of [YTVJL-D-23-00391](#).  
~~Deleted~~ Zandvliet, M., 2016. Canine lymphoma: a review.  
*The Veterinary Quarterly* 36, 76-104.

512 **Figure legends**

513

514 Fig.1. Lymphoma-specific survival (LSS) in 50 dogs with peripheral T-cell lymphoma  
515 (PTCL) grouped according to the extent of bone marrow (BM) involvement. Dogs with BM  
516 infiltration >5% exhibited a significantly lower median LSS (114 days; 95% CI 0-240),  
517 compared to those with an infiltration  $\leq$ 5% (114 vs 178 days, respectively;  $P=0.020$ ).



## Highlights

- Bone marrow infiltration was common in dogs with peripheral T-cell lymphoma
- Bone marrow infiltration >5% was linked to shorter median survival
- CD4-negative phenotype was the associated with a shorter time to progression
- Peripheral blood infiltration had no prognostic relevance



Reviewer #1: Thank you for addressing the comments.

My comments are limited to minor editorial changes:

1. Title: "flow cytometry infiltration" is incorrect. Use something like "infiltration as assessed by flow cytometry" or something like that.
2. Line 27: as above: "by flow cytometry" needs to be placed elsewhere in the sentence, as you did not assess by flow the prognostic significance, rather you assessed the prognostic significance of data generated by flow.

**Authors:** dear Reviewer, thank you for your feedback. According to your recommendation we have changed the title and abstract as follows.

Title: "Prognostic value of peripheral blood and bone marrow infiltration assessed by flow cytometry in dogs with de novo nodal peripheral T-cell lymphoma receiving alkylating-rich chemotherapy".

Line 27: "The aim of this retrospective study was to assess the prognostic significance of peripheral blood (PB) and bone marrow (BM) infiltration evaluated by flow cytometry (FC) in dogs with treatment-naïve and histologically confirmed PTCL".

Reviewer #2: I am sorry that there is some misunderstanding here from my part. The authors stated that the first aim was to investigate the prevalence of PB and BM infiltration in dogs with PTCL. My understanding of the term "prevalence" is used to describe the proportion of individuals in the population have certain condition and prevalence will be a value between 0 and 100%. Hence my earlier recommendation of reporting 95% confidence interval of prevalence as I assumed the values for PB and BM infiltration will be dichotomized based on some cut-off thresholds. If this is not the case and the authors want to describe the distribution of PB and BM infiltration in dogs with PTCL, then median and range would be appropriate, and the aim should be rephrased instead.

**AUTHORS:** the term "prevalence" has been replaced with "distribution". 95% CI were replaced with ranges.

If other variables have been eliminated through backward elimination process and only one predictor remained in the final model, then it means that is the only predictor of the outcome and the other variables are correlated with the CD4- phenotype (hence their significance in the univariable analysis). If model selection process is used (such as backward elimination here), then the results from the final model should be reported and in the case of tumor progression it's the same as the univariable analysis.

**AUTHORS:** we apologize for the misunderstanding. We did not use a stepwise (backward) elimination process for our analysis, we used the forced entry method, so all the pre-selected variables were entered into the model simultaneously. That is way the HR changed between univariable and multivariable analysis. This has been amended in the Materials and Methods:

“Variables significant at univariable analysis were then included in a multivariable analysis model using the enter method.”

Reviewer #3: The reviewer thanks the authors for their thorough and eloquent rebuttal. The reviewer disagrees with the authors' view on treatment response being a prognostic indicator but acknowledges (and accepts) that there are different views on this specific matter.

**AUTHORS:** we thank you for the valuable comments which contributed to manuscript improvement.