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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

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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--

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Abstract:	<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 >1%, >3%, >5%, >10%, >15% and >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 >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**

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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

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23

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role

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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

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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

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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

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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.

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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.

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 ≥ 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

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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-

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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

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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.

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

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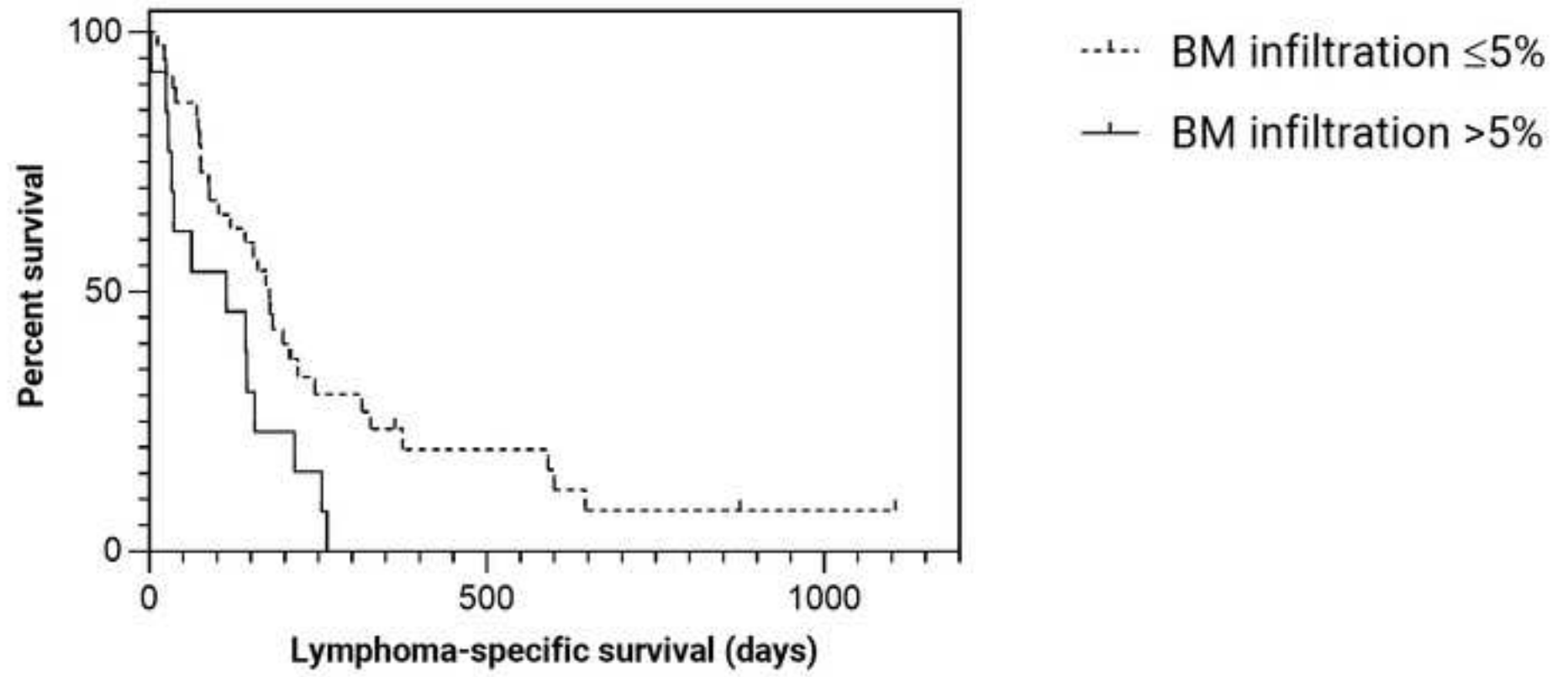
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512 **Figure legends**

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.