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Abstract:	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. 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.

Original Article

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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Abstract

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.

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.

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

Introduction

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

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

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

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Materials and methods

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

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

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

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

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

Flow cytometry

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

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

Histology

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

Treatment and response evaluation

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

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

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

Statistical analysis

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

Time to progression was calculated from treatment initiation to disease progression. If progression did not occur, dogs were censored for TTP analysis. Lymphoma-specific survival (LSS) was measured as the interval between treatment initation and death for lymphoma. Dogs alive at data analysis closure or dead due to causes other than lymphoma were censored for LSS analysis. Univariable Cox's proportional hazard regression analysis was performed to explore potential associations between selected variables and tumor progression and tumor-related death. Variables significant at univariable analysis were then included in a multivariable analysis model using the enter method. The independent variables included in the analyses were age (median value used as cut-off point), sex (male or female), weight (median value used as cut-off point), corticosteroids before admission, extranodal (other than PB and BM) involvement, substage b, anemia (hematocrit value lower than the laboratory reference

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

When necessary, survival curves were obtained with the Kaplan-Meier method and compared with the log-rank test.

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

Results

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

Tumor and dogs' characteristics

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

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

Flow cytometry

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

FC results for PB and BM were available for all cases. Overall, 31 (62%) dogs had PB infiltration, and 29 (58%) dogs had BM involvement. Three dogs with BM involvement had no circulating neoplastic cells, whereas 5 dogs with PB involvement had no BM infiltration. Median PB infiltration at diagnosis was 2.6% (range, 0.0-31.0). It was >1% in 33 (66.0%) dogs, >3% in 21 (42.0%), >5% in 13 (26.0%), >10% in 3 (6.0%). Median BM infiltration at diagnosis was 1.3% (range, 0.1-66.5). It was >1% in 28 (56%) dogs, >3% in 20 (40.0%), >5% in 13 (26.0%), >10% in 6 (12.0%), >15% in 4 (8.0%), and >20% in 4 (8.0%). In the CD4+/CD8- category, BM infiltration (3.4%; range, 0.6-66.5) was significantly higher than in the remaining cases (0.9%; range, 0.1-

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

Treatment and outcome

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

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

Analysis of prognostic factors

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Finally, the cut-off for establishing PB and BM infiltration, set at 1%, has been arbitrarily defined in the absence of analytical validation.

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

Conclusions

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

Conflict of interest statement

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

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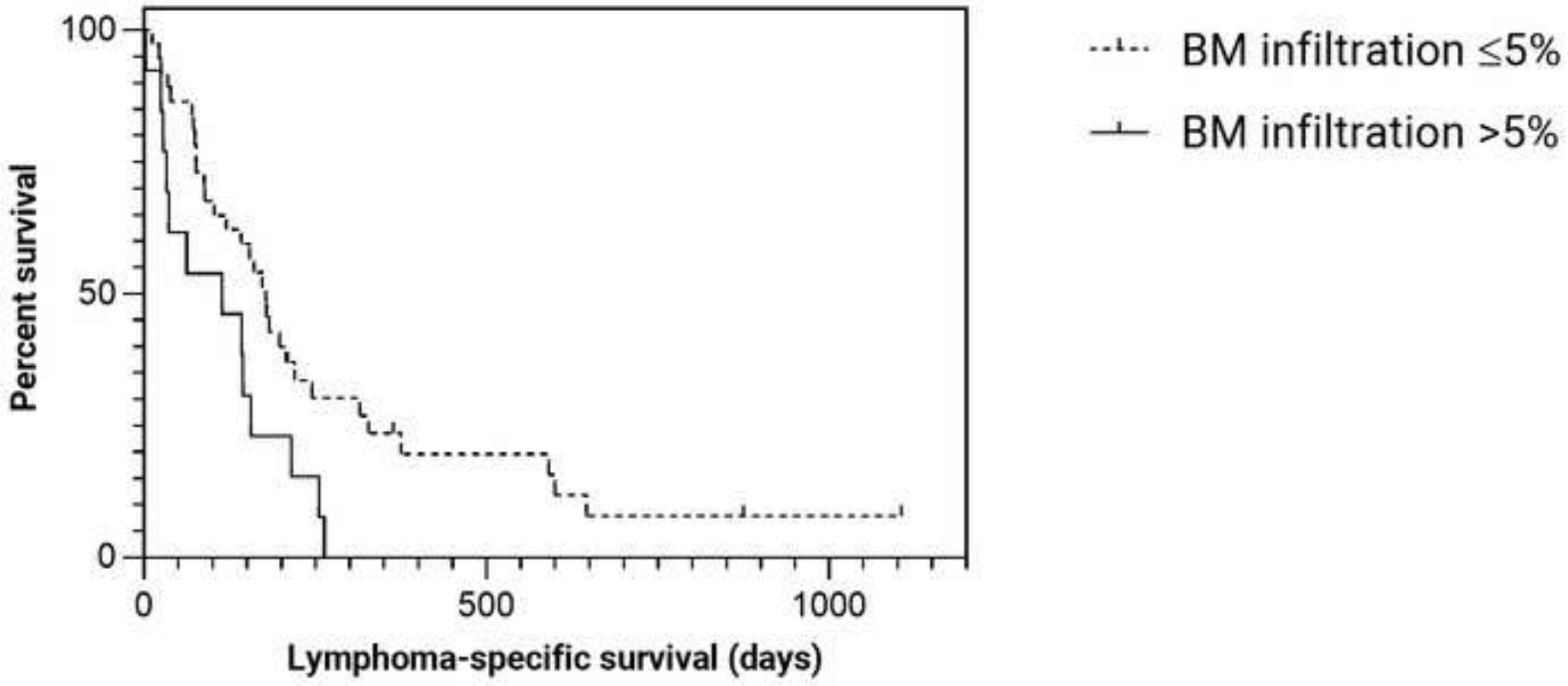
Commented [L20]: Line 495 of *YTVJL-D-23-00391. Deleted*-Zandvliet, M., 2016. Canine lymphoma: a review. The Veterinary Quarterly 36, 76-104.

Figure legends

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

Figure 1



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 top be place 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.