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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1554372 since 2021-11-19T14:51:57Z

Published version:

DOI:10.1111/vco.12184

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02 May 2024

Prognostic significance of Ki67 evaluated by flow cytometry in dogs with high grade B-cell lymphoma

Running headline: Ki67 prognostic role in canine lymphoma

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Abstract

Ki67 can discriminate between high and low grade canine lymphomas, but its prognostic role in specific subtypes of the neoplasm is unknown. We assessed the prognostic significance of Ki67% (percentage of Ki67-positive cells), evaluated by flow cytometry, in 40 dogs with high grade B-cell lymphoma, treated with a modified Wisconsin-Madison protocol (UW-25). The following variables were investigated for association with lymphoma specific survival (LSS) and relapse free interval (RFI): Ki67%, breed, sex, age, stage, substage, complete remission (CR). By

multivariate analysis, Ki67% (P=0.009) and achievement of CR (P=0.001) were independent prognostic factors for LSS. Dogs with intermediate Ki67% (20.1-40%) presented longer LSS and RFI (median=866 and 428 days, respectively) than dogs with low (median=42 days, P<0.001; median=159 days, P=0.014) or high (median=173 days, P=0.038; median=100 days, P=0.126) values. Determination of Ki67 is a prognostic tool that improves the clinical usefulness of flow cytometric analysis in canine high grade B-cell lymphoma.

Keywords: dog, lymphoma, flow cytometry, Ki67, prognosis

Introduction

Canine lymphoma represents a heterogeneous group of neoplasms arising from the malignant transformation of lymphoid cells and is characterized by a broad range of clinical presentations and potential outcomes.

Depending upon the grade of malignancy, lymphomas are cytologically grouped into two main categories. The most commonly encountered forms are high grade lymphomas, clinically aggressive and typically fatal within a short period of time when treatment is not instituted. Conversely, low grade lymphomas are rare and are characterized by an indolent disease course.[1,2] Defining the immunophenotype is also reported to be important in predicting prognosis.[3-7] In fact multicentric T-cell forms, when compared with B-cell forms, seem to be associated with similar initial response rates, but have significantly lower response durability, even following an appropriate chemotherapy protocol.[2]

Moreover, although several prognostic factors that independently influence the response rate and the survival time have been identified, the clinical outcome remains variable between identically

48 treated lymphomas.[4] In fact, dogs with similar signalment, stage and substage of disease,
49 immunophenotype and tumour anatomic location may respond differently to the same treatment.[8]
50 In recent years, many studies have stressed the prognostic significance of the evaluation of
51 tumour biology and, in this context, the role of proliferative activity has received special
52 attention. One of the most frequently used methods to evaluate the growth fraction of neoplastic
53 populations is the detection of the Ki67 antigen.[9] This proliferation-associated nuclear protein is
54 expressed in all the active phases of the cell cycle (G1, S, G2 and mitosis), but it is absent in
55 resting cells (G0).[10,11] This exclusive expression in proliferating cells has made the antibodies
56 raised against the Ki67 antigen an invaluable diagnostic tool for grading, assessing clinical
57 behavior and determining outcome in various human malignancies.[12-15]
58 In particular, in non-Hodgkin's lymphoma, the human counterpart of canine lymphoma,[16] Ki67
59 has been found to be an independent prognostic factor.[17-19] However, contradictory results have
60 been reported, mainly due to the heterogeneity within and among the different subtypes of the
61 disease.[13,20,21]
62 The proliferative activity has also been evaluated in few studies on canine lymphoma and, while
63 Ki67 expression has shown a significant correlation with the grade of malignancy,[22,23] its
64 reliability as a prognostic marker remains unclear.[24,25] In all of these studies, the determination of
65 Ki67 has been performed through immunohistochemistry in bioptic specimens. Moreover
66 recently, our group has demonstrated that the flow cytometric detection of Ki67 is a powerful and
67 non-invasive alternative method able to discriminate between high and low grade canine
68 lymphomas.[26]
69 The aim of the present study was to assess the prognostic significance of Ki67, evaluated by flow
70 cytometry, in dogs with high grade B-cell lymphoma being treated with the same multidrug
71 chemotherapy protocol.

Materials and methods

Case selection

Dogs with multicentric high grade B-cell lymphoma diagnosed at the Veterinary Teaching Hospital of the University of Turin between April 2011 and September 2014 were considered. The diagnosis was based on clinical presentation (lymph node enlargement), cytological examination of lymph nodes and flow cytometric analysis.

Inclusion criteria for the study were: cytological diagnosis of high grade lymphoma according to the updated Kiel classification,[27,28] presence of flow cytometric B-cell immunophenotype, flow cytometric Ki67 determination, treatment with a modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25)[29] and the availability of follow up data. Dogs previously treated with corticosteroid or chemotherapy agents were excluded. For each included dog, signalment data (breed, sex and age), when available, were retrieved and clinical stage (I-V) and substage (a or b) were assigned according to the World Health Organization (WHO) system.[30] In particular, stage V was assigned when the neoplastic population, detected by flow cytometry, was ≥ 3% in peripheral blood and/or bone marrow.

Flow cytometric immunophenotyping and Ki67 determination

At time of initial staging, flow cytometric immunophenotyping was performed on lymph node fine-needle aspirate biopsies (FNABs), peripheral blood samples and/or bone marrow aspirates within 24 h from collection as previously reported.[31] The following panel of monoclonal antibodies (mAbs) was used: CD45-Alexa647 (pan-leucocyte marker; clone YKIX716.1, AbD Serotec, Oxford, UK), CD3-FITC (T-cells marker; clone CA17.2A12, AbD Serotec), CD5-FITC

(T-cells marker; clone YKIX322.3, AbD Serotec), CD4-Alexa647 (T-helper marker; clone YKIX302.9, AbD Serotec), CD8-PE (T-cytotoxic/suppressor marker; clone YCATE55.9, AbD Serotec), CD21-PE (B-cells marker; clone CA21D6, AbD Serotec), CD79b-FITC (B-cells marker; clone AT107-2, AbD Serotec) and CD34-PE (precursor cells marker; clone 1H6, Pharmingen, Becton Dickinson, San Jose, CA, USA).

The proliferative activity was determined using the same lymph node FNABs utilized for immunophenotyping. Cells were labelled with anti-Ki67-FITC monoclonal antibody (clone MIB-1, DAKO, Glostrup, DK) using a fixation and permeabilization method with methanol, as described previously.[26] A minimum of 10,000 events were acquired both for immunophenotype and Ki67 determination on BD Accuri C6 flow cytometer (Becton Dickinson). Data were analyzed using CFlow Plus software (Becton Dickinson). A gate of analysis was depicted on forward (FSC) versus side scatter (SSC) plot to exclude debris and background. The proliferative activity was expressed as the percentage of Ki67 positive cells (Ki67%) calculated on SSC versus fluorescence intensity plot.

Cytological evaluation

Smears obtained by FNABs of enlarged lymph nodes were air-dried, fixed and stained with May-Grünwald-Giemsa. Each case was classified according to the updated Kiel classification[27,28] and allocated to a specific grade of malignancy and cytological subtype.

Follow up

Information pertaining to the achievement of remission, occurrence of relapse, survival at the end of first chemotherapy protocol, lymphoma specific survival (LSS), relapse-free interval (RFI), date and cause of death was collected.

Responses were classified as follows: complete remission (CR), which indicated ~~100%~~ reduction to normal size ~~in the size~~ of all measurable lymph nodes; partial remission (PR), which indicated more than 50% but less than 100% reduction of all measurable lesions and stable disease (SD), which indicated less than 50% reduction or no change in the size of all measurable lesions. Relapse was defined as clinical reappearance and cytological evidence of lymphoma in any anatomic site in dogs having experienced CR. RFI was defined as the time in days from when a dog achieved CR until relapse. LSS was defined as the interval in days between the date on which chemotherapy was started and the date of death due to lymphoma related causes.

Statistical analysis

LSS and RFI for all dogs were estimated using the Kaplan-Meier product limit method. Contingency tables were prepared for each of following variables: Ki67% (low, intermediate, high), breed (purebred or crossbred), sex (male or female), age (< or ≥ 10 years), stage (I-IV or V), substage (a or b), and CR (yes or no). Pearson's χ^2 with z-test for column proportion comparisons and Bonferroni adjustment for multiple comparisons were calculated to test the association between each variable and the achievement of CR and survival at the end of first chemotherapy protocol (UW-25). Dogs that died from causes other than lymphoma and dogs that had not yet completed the protocol and did not meet the event (CR or death) were excluded from the contingency tables. Ki67% cut-off values were defined rounding the thresholds of 25th and 75th percentiles to 20% and 40%, respectively, and thus generating the following groups ~~arbitrarily identified on the basis of the data distribution as follows~~: low if Ki67 ≤ 20%, intermediate if Ki67 between 20.1% and 40%, and high if Ki67>40%.

To evaluate the prognostic significance of each variable, univariate logistic regression for LSS and RFI was first used and variables with $P<0.3$ were then included in a multivariate Cox proportional hazards model progression analysis with a backward step selection. Kaplan-Meier curves were drawn for Ki67% groups and compared by log-rank test to assess the survival analysis. Dogs that were alive at the end of the study, lost to follow-up or dead due to causes other than lymphoma were censored for survival analysis. Differences were considered significant with $P<0.05$. Statistical analyses were performed using SPSS software (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA).

Results

Lymphoma cases

Forty cases met the inclusion criteria and were enrolled in the study. Data about the identification of breed were reported for 39 cases. There were 26 (66.7%) purebred dogs (three Labrador retrievers, two German shepherds, two Dobermans, two Bloodhounds, two Pit bull terriers and one each of Italian Mastiff, Great Dane, Poodle, Dachshund, Beagle, Bernese mountain dog, Cavalier King Charles Spaniel, Golden retriever, Jack Russell, Rottweiler, White Swiss shepherd, Cocker Spaniel, English Bulldog, Lagotto Romagnolo, American Staffordshire terrier) and 13 (33.3%) crossbred dogs. Sixteen dogs (42.1%) were males (1 castrated) and 22 (57.9%) were females (9 spayed), while in 2 cases the sex was unknown. The age was only reported for 37 dogs and the median age was 9 years (range, 4-15 years). The included lymphomas were cytologically classified as follows: 8 (20%) centroblastic monomorphic, 24 (60%) centroblastic polymorphic predominantly large cell, 5 (12.5%) immunoblastic, 2 (5%) lymphoblastic and 1 (2.5%)

plasmacytoid. At time of diagnosis, 27 dogs (67.5%) were in stage IV (10 substage a, 16 substage b and 1 unknown) and 13 (32.5%) in stage V (all substage b).

Response to treatment

CR was achieved in 25 (62.5%) dogs. Twelve out of these 25 (48%) relapsed (median RFI=180 days; range 28-530 days), 10 (40%) were still in CR at the end of the study (median follow up period=321 days; range 60-1005 days) and 3 (12%) died of causes unrelated to lymphoma after 34, 210 and 240 days from the beginning of chemotherapy, with lymphoma remaining in CR.

Relapses were treated with a second UW-25 or with other rescue protocols (DMAC, L-asparaginase + lomustine), depending on when the relapse occurred and owner compliance. At the end of the study 8 out of 12 relapsed dogs (66.7%) were dead because of PD (median LSS = 390 days; range 150-866 days), 3 (25%) were in PR (follow up period of 515, 800 and 1108 days) and 1 (8.3%) was in SD (follow up period=295 days).

Among the 15 dogs that did not achieve CR, 11 (73.3%) died because of PD (median LSS=42 days; range 15-1100 days), 3 (20%) were in PR at the end of the study (follow up period of 28, 157 and 653 days) and 1 (6.7%) died of causes unrelated to lymphoma after 45 days.

Estimated median RFI and LSS for all dogs were 414 days (95% CI range 228-600 days) and 442 days (95% CI range 236-648 days), respectively.

Proliferative activity

The mean Ki67% was 33.8% (SD=14.2%) and the median was 30.7% (range 10-67%). Six cases presented low Ki67% (≤20%), 24 were in the intermediate group (20.1-40%) and 10 were in the high group (>40%).

Survival at the end of chemotherapy protocol and achievement of CR

Survival at the end of chemotherapy protocol was significantly associated with the achievement of CR (P=0.001). In fact, 91.7% of the dogs that achieved CR were alive compared with 33.3% of dogs that did not reach CR (Table 1). Ki67% showed near-significant association with both survival (P=0.063) and achievement of CR (P=0.075) at the end of chemotherapy protocol. In fact, percentages of both survival and CR were higher for dogs with intermediate Ki67% (85.7% and 81%, respectively) compared with dogs with low (50% and 33%) and high Ki67% (50% and 60%) (Table 1).

Prognostic factors for LSS and RFI

Ki67% (P=0.007) and achievement of CR (P=0.001) significantly influenced LSS on univariate analysis and were confirmed to be independent prognostic factors for LSS (P=0.009 and P=0.001, respectively) in the multivariate analysis (Table 2). None of the variables significantly influenced RFI in the univariate analysis and none were of prognostic significance for RFI in the multivariate analysis (Table 2).

The Kaplan-Meier analysis showed that dogs with intermediate Ki67% had significantly longer LSS (median=866 days) than dogs with low (median=42 days; P<0.001) and high Ki67% (median=173 days; P=0.038) (Fig. 1). Intermediate Ki67% was a significant predictor also for one year and two years survival (P=0.001 and P=0.004 vs low and high Ki67%, respectively, at both time points) (Fig. 1).

Dogs with intermediate Ki67% reported also longer RFI (median=428 days) than dogs with low (median=159 days; P=0.014) and high Ki67% (median=100 days; P=0.126), although the difference with the high Ki67% group did not reach statistical significance (Fig. 2).

Discussion

Ki67 is one of the most widely used markers of cell proliferation. Although it is considered an important factor for grading neoplasms and predicting their biological behavior,[12,14] its clinical relevance is still being debated both in human and canine lymphomas. In previous work,[26] we assessed the feasibility of flow cytometric determination of Ki67 in canine lymphoma, and we demonstrated its association with malignancy grade, regardless of phenotype and morphology.

In this study, we investigated the prognostic significance of Ki67, as evaluated by flow cytometry in dogs with high grade B-cell lymphoma treated with the UW-25 chemotherapy protocol. We focused on the most common type of canine lymphoma to limit heterogeneity with regards to some clinical prognostic features, such as malignancy grade and immunophenotype.[4] Likewise in our case series, all dogs were treated with the same chemotherapeutic protocol to avoid treatment bias in the response, although the LSS of relapsed dogs may have been influenced by receiving multiple reinduction or rescue protocols.

The achievement of CR and the intermediate Ki67% values were associated with the survival at the end of chemotherapy protocol, suggesting their prognostic role, even though the association with the Ki67 didn't reach a statistical significance. Based on multivariate analysis, Ki67% and CR were found to be independent prognostic factors for LSS, while none of the investigated variables had prognostic significance for RFI. Moreover, the Kaplan-Meier analysis showed that an intermediate Ki67% was associated with a better prognosis, with longer LSS and RFI compared to dogs with a low or high Ki67%.

These findings are discordant with the results of previous studies that evaluated the prognostic significance of Ki67 in dogs with lymphoma. In the work by Kiupel et al.[25] Ki67 expression showed no prognostic value, while Phillips et al.[24] reported that Ki67 was predictive for duration of first RFI but not overall survival. Differences in inclusion criteria and method of Ki67

determination could account for these discrepancies. In fact, in the previous studies,[24,25] both high and low grade lymphomas and both B and T-cell immunophenotypes were included. Moreover, Ki67 detection was carried out through immunohistochemistry on bioptic specimens, while we used flow cytometric analysis of FNABs. Furthermore, the different selection of the cut-off values to define groups may have influenced the results. In our work we used an approach similar to that of Phillips et al.[24], using median and 75th percentile to differentiate two prognostic groups and we get near significant results with the latter (data not shown). Observing that the longest survival times were associated with intermediate Ki67% values, we assessed the prognostic significance of Ki67% dividing cases in three different groups using quartiles. Furthermore, we rounded the quartile cut-off values to 20% and 40% in order to simplify the use in clinical practice. Unfortunately, a direct comparison of our cut-off values with those assessed by Phillips et al is not possible because they did not reported the actual percentages that define quartiles in their caseload. However this comparison, although interesting, would presumably be limited by the different method used to measure Ki67 expression. In this regard, Kiupel et al.[25] did not get significant results despite the application of thresholds similar to ours (Ki67≤20%; 21-40%; 41-60%; >60%)

Contradictory results on prognostic significance of Ki67 have also been reported in human non-Hodgkin's lymphoma, due to the heterogeneity of the different subtypes of this disease.[19] Many studies have assessed Ki67 in aggressive diffuse large B-cell lymphomas, with a wide range of expression.[21] In accordance with our results, Jerkeman et al.[32] found that patients with either low (<60%) or high Ki67 (>90%) expression demonstrated a trend toward overall lower survival than patients with moderate expression (60-90%). Moreover, a low proliferation index was associated with a low level of failure-free survival compared with moderate or high indexes. This behavior is likely because lymphomas with a low proliferation rate exhibit resistance to cycle specific

cytotoxic chemotherapy, given that the majority of cells are resting in the G0 phase of the cell cycle. Conversely, in cases with high proliferation rates, treatment failure may be caused through regrowth or by the increasing the likelihood of further mutations.

In addition to the proliferative activity, we also found that achievement of CR was an independent prognostic factor for LSS, as reported in previous studies, where obtaining CR led to prolonged survival for dogs with aggressive lymphoma.[7,33,34] Stage and substage did not show prognostic significance for LSS or RFI, in contrast with some authors,[8,35,36] but in accordance with others.[37,38] These differences may be due to the inclusion of different types of lymphoma, different therapeutic strategies and different methods and cut-offs used to stage the disease.

Major limits of this work are its retrospective nature and the limited number of cases. Prospective studies considering a larger number of lymphomas are required to confirm the clinical usefulness of a Ki67-based stratification of patients.

In conclusion flow cytometric determination of Ki67 was found to be an independent predictor for LSS in treated high grade B-cell lymphomas; intermediate values were associated with the best prognosis. We previously demonstrated that this determination is useful in discriminating between low and high grade lymphomas. Thus, we suggest the introduction of Ki67 in the routine panel of labeling to add diagnostic and prognostic value to the flow cytometric analysis.

Acknowledgements

This work was supported by Progetti di Ricerca di Interesse Nazionale (PRIN 2008), Ministry of Education, University and Research, Italy.

References

1. Teske E. Canine malignant lymphoma: a review and comparison with human non-Hondgkin's lymphomas. *Veterinary Quarterly* 1994; **16**: 209–219.
2. Vail DM, Pinkerton ME and Young KM. Canine lymphoma and lymphoid leukemia. In: *Withrow and MacEwen's Small Animal Clinical Oncology*. 5th edn., St Louis, Saunders Elseviers, 2012; 608–638.
3. Greenlee PG, Filippa DA, Quimby FW, Patnaik AK, Calvano SE, Matus, *et al*. Lymphomas in dogs. A morphologic, immunologic and clinical study. *Cancer* 1990; **66**: 480–490.
4. Teske E, van Heerde P, Rutteman GR, Kurzman ID, Moore PF and MacEwen EG. Prognostic factors of treatment of malignant lymphoma in dogs. *Journal of the American Veterinary Medical Association* 1994; **205**: 1722–1728.
5. Ruslander DA, Gebhard DH, Tompkins MB, Grindem CB and Page RL. Immunophenotypic characterization of canine lymphoproliferative disorders. *In Vivo* 1997; **11**: 169–172.
6. Dobson JM, Blackwood LB, McInnes EF, Bostock DE, Nicholls P, Hoather TM, *et al*. Prognostic variables in canine multicentric lymphosarcoma. *Journal of Small Animal Practice* 2001; **42**: 377–384.
7. Simon D, Moreno SN, Hirschberger J, Moritz A, Kohn B, Neumann S, *et al*. Efficacy of a continuous, multiagent chemotherapeutic protocol versus a short-term single-agent protocol in dogs with lymphoma. *Journal of the American Veterinary Medical Association* 2008; **232**: 879–885.
8. Marconato L, Stefanello D, Valenti P, Bonfanti U, Comazzi S, Roccabianca P, *et al*. Predictors of long-term survival in dogs with high-grade multicentric lymphoma. *Journal of the American Veterinary Medical Association* 2011; **238**: 480–485.

9. Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, *et al*. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *The Journal of Cell Biology* 1993; **123**: 513–522.
10. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H. Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki-67. *The Journal of Immunology* 1984; **133**: 1710–1715.
11. Bruno S and Darzynkiewicz Z. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Proliferation* 1992; **25**: 31–40.
12. Brown DC and Gatter KC. Monoclonal antibody Ki-67: Its use in histopathology. *Histopathology* 1990; **17**: 489–503.
13. Brown DC and Gatter KC. Ki67 protein: the immaculate deception? *Histopathology* 2000; **40**: 2–11.
14. Scholzen T and Gerdes J. The Ki-67 protein: from the known and the unknown. *Journal of Cellular Physiology* 2000; **182**: 311–322.
15. Endl E and Gerdes J. The Ki-67 protein: fascinating forms and an unknown function. *Experimental Cell Research* 2000; **257**: 231–237.
16. MacEwen EG. Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. *Cancer and Metastasis Reviews* 1990; **9**: 125–36.
17. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS, *et al*. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. *Blood* 1988; **71**: 1157–1160.

18. Miller TP, Grogan TM, Dahlberg S, Spier CM, Braziel RM, Banks PM, *et al.* Prognostic significance of the Ki-67 associated proliferative antigen in aggressive non-Hodgkin's lymphomas: a prospective Southwest Oncology Group trial. *Blood* 1994; **83**: 1460–1466.
19. Broyde A, Boycov O, Strenov Y, Okon E, Shpilberg O and Bairey O. Role and prognostic significance of the Ki-67 index in non-Hodgkin's lymphoma. *American Journal of Hematology* 2009; **84**: 338–343.
20. Palestro G, Pich A, Chiusa L, Geuna M, Ponti R, Kerim S, *et al.* Biological heterogeneity of diffuse mixed small and large cell non-Hodgkin's lymphomas assessed by DNA flow cytometry and Ki67. *Leukemia and Lymphoma* 1995; **19**: 467–472.
21. Bairey O. Role and prognostic significance of the Ki-67 index in non Hodgkin's lymphoma. *European Journal of Clinical & Medical Oncology* 2010; **2**: 113– 119.
22. Fournel-Fleury C, Magnol JP, Chabanne L, Ghernati I, Marchal T, Bonnefond C, *et al.* Growth fractions in canine non-Hodgkin's lymphomas as determined in situ by the expression of the Ki-67 antigen. *Journal of Comparative Pathology* 1997; **117**: 61–72.
23. Patruno R, Zizzo N, Zito AF, Catalano V, Valerio P, Pellecchia V, *et al.* Microvascular density and endothelial area correlate with Ki-67 proliferative rate in the canine non-Hodgkin's lymphoma spontaneous model. *Leukemia and Lymphoma* 2006; **47**: 1138–1143.
24. Phillips BS, Kass PH, Naydan DK, Winthrop MD, Griffey SM and Madewell BR. Apoptotic and proliferation indexes in canine lymphoma. J*ournal of Veterinary Diagnostic Investigation* 2000; **12**: 111–117.
25. Kiupel M, Teske E and Bostock D. Prognostic factors for treated canine malignant lymphoma. *Veterinary Pathology* 1999; **36**: 292–300.

355 26. Poggi A, Miniscalco B, Morello E, Comazzi S, Gelain ME, Aresu L, *et al.* Flow
356 cytometric evaluation of Ki67 for the determination of malignancy grade in canine
357 lymphoma. *Veterinary and Comparative Oncology* 2013; doi:10.1111/vco.12078.
358 27. Fournel-Fleury C, Magnol JP, Bricaire P, Marchal T, Chabanne L, Delverdier A, *et al.*
359 Cytohistological and immunological classification of canine malignant lymphomas:
360 comparison with human non-Hodgkin's lymphomas. *Journal of Comparative Pathology*
361 1997; **117**: 35–59.
362 28. Ponce F, Marchal T, Magnol JP, Turinelli V, Ledieu D, Bonnefont C, *et al.* A
363 morphological study of 608 cases of canine malignant lymphoma in France with a focus
364 on comparative similarities between canine and human lymphoma morphology.
365 *Veterinary Pathology* 2010; **47**: 414–433.
366 29. Garrett LD, Thamm DH, Chun R, Dudley R and Vail DM. Evaluation of a 6 month
367 chemotherapy protocol with no maintenance therapy for dogs with lymphoma. *Journal of*
368 *Veterinary Internal Medicine* 2002; **16**: 704–709.
369 30. Owen, L. TNM Classification of Tumors in Domestic Animals. 1st edn., World Health
370 Organization, Geneva, Switzerland, 1980: 46–47.
371 31. Gelain ME, Mazzilli M, Riondato F, Marconato L and Comazzi S. Aberrant phenotypes
372 and quantitative antigen expression in different subtypes of canine lymphoma by flow
373 cytometry. *Veterinary Immunology and Immunopathology* 2008; **121**: 179–188.
374 32. Jerkeman M, Anderson H, Dictor M, Kvaløy S, Akerman M and Cavallin-Ståhl E.
375 Assessment of biological prognostic factors provides clinically relevant information in
376 patients with diffuse large B-cell lymphoma. A Nordic Lymphoma Group study. *Annals*
377 *of Hematology* 2004; **83**: 414–419.

33. Ettinger SN. Principles of treatment for canine lymphoma. *Clinical Techniques in Small Animal Practice* 2003; **18**: 92–97.
34. Lurie DM, Gordon IK, Thèon AP, Rodriguez CO, Suter SE and Kent MS. Sequential low-dose rate half-body irradiation and chemotherapy for the treatment of canine multicentric lymphoma. *Journal of Veterinary Internal Medicine* 2009; **23**: 1064–1070.
35. Jagielski D, Lechowski R, Hoffmann-Jagielska M and Winiarczyk S. A retrospective study of the incidence and prognostic factors of multicentric lymphoma in dogs (1998-2000). *Journal of Veterinary Medicine* 2002; **49**: 419–424.
36. Lana SE, Jackson TL, Burnett RC, Morley PS and Avery AC. Utility of polymerase chain reaction for analysis of antigen receptor rearrangement in staging and predicting prognosis in dogs with lymphoma. *Journal of Veterinary Internal Medicine* 2006; **20**: 329–334.
37. Morrison-Collister KE, Rassnick KM, Northrup NC, Kristal O, Chretin JD, Williams LE, *et al.* A combination chemotherapy protocol with MOPP and CCNU consolidation (Tufts VELCAP-SC) for the treatment of canine lymphoma. *Veterinary and Comparative Oncology* 2003; **1**: 180–190.
38. Flory AB, Rassnick KM, Stokol T, Scrivani PV and Erb HN. Stage migration in dogs with lymphoma. *Journal of Veterinary Internal Medicine* 2007; **21**: 1041–1047.

Table 1. Association between different variables and survival or achievement of complete remission (CR) at the end of first chemotherapy protocol (UW-25)

		SURVIVAL			ACHIEVEMENT OF CR		
		ALIVE	**DEAD**	**P [c]**	**NO**	**YES**	**P [c]**
Breed	Crossbred	9[a]	4[a]		4[a]	9[a]	
		69.2%	30.8%	0.633	30.8%	69.2%	0.553
	Purebred	16[a]	7[a]		8[a]	15[a]	
		69.6%	30.4%		34.8%	65.2%	
	Tot	25	11		12	24	
Sex	Female	13[a]	7[a]	0.440	9[a]	11a	0.118

		65.0%	35.0%		45.0%	55.0%	
	Male	11[a]	4[a]		3[a]	12[b]	
		73.3%	26.7%		20.0%	80.0%	
	Tot	24	11		12	23	
Age	<10years	14[a]	5[a]		6[a]	13[a]	
		73.7%	26.3%	0.471	31.6%	68.4%	0.600
	≥10years	10[a]	5[a]		5[a]	10[a]	
		66.7%	33.3%		33.3%	66.7%	
	Tot	24	10		11	23	
Stage	Stage I-IV	19[a]	6[a]		7[a]	18[a]	
		76.0%	24.0%	0.235	28.0%	72.0%	0.320
	Stage V	7[a]	5[a]		5[a]	7[a]	
		58.3%	41.7%		41.7%	58.3%	
	Tot	26	11		12	25	
Substage	a	7[a]	1[a]		3[a]	6[a]	
		87.5%	12.5%	0.210	33.3%	66.7%	0.665
	b	18[a]	10[a]		9[a]	18[a]	
		64.3%	35.7%		33.3%	66.7%	
	Tot	16	10		12	24	
Ki67%	≤20%	3[a]	3[a]		4[a]	2[a]	
		50.0%	50.0%		66.7%	33.3%	
	20.1-40%	18[a]	3 [b]		4[a]	17[a]	
		85.7%	14.3%	0.063	19.0%	81.0%	0.075
	>40%	5[a]	5[a]		4[a]	6[a]	
		50.0%	50.0%		40.0%	60.0%	
	Tot	26	11		12	25	
Complete	No	4[a]	8[b]	0.001			

Remission		33.3%	66.7%	–	–	–
	Yes	22[a]	2[b]	–	–	
		91.7%	8.3%			
	Tot	26	10			

[a-b] Within each row, different superscript letters indicate a significant difference (P<0.05)

P[c]: P value for Pearson's χ^2 test

Table 2. Univariate and multivariate analyses of prognostic variables for lymphoma specific survival (LSS) and (relapse free interval) RFI in dogs with B-cell high grade lymphoma treated with the same multidrug chemotherapy protocol (UW-25)

Univariate analysis for LSS					**Univariate analysis for RFI**				**Multivariate analysis LSS**	
Variable	**Number**	**Median (days)**	**HR**	**P value**	**Number**	**Median (days)**	**HR**	**P value**	**P value**	**HR**
Breed	39				24					
Crossbred	13	866			9	428				

Purebred	26	442	1.000	1.000	15	376	0.898	0.856	–	–
Sex	38				23					
Male	16	366			12	285				
Female	22	536	0.941	0.896	11	428	0.361	0.118	–	–
Age	37				23					
<10y	20	536			13	414				
≥10y	17	1100	0.733	0.576	10	305	1.421	0.638	–	–
Stage	40				25					
I-IV	27	536			18	376				
V	13	240	1.404	0.481	7	100	2.200	0.221	–	–
Substage	39				24					
a	10	413			6	285				
b	29	442	1.124	0.826	18	414	0.991	0.990	–	–
Ki67%	40			0.007	25			0.111	0.009	
≤ 20	6	42			2	159				
20.1- 40	24	866	0.171	0.002	17	428	0.162	0.052	0.004	0.166
>40	10	173	0.535	0.301	6	100	0.593	0.575	0.740	0.817
Complete remission	37									
Yes	25	536			–	–	–	–		
No	12	42	4.669	0.001					0.001	5.707

HR: Hazard Ratio

Figure legends

Figure 1. Kaplan-Meier curves of lymphoma specific survival (LSS) in 40 dogs with high grade B-cell lymphoma treated with UW-25 stratified according to Ki67% (≤20% - low; 20.1%-40% - intermediate; >40% - high).

Figure 2. Kaplan-Meier curves of relapse free interval (RFI) in 40 dogs with high grade B-cell lymphoma treated with UW-25 stratified according to Ki67% (≤20% - low; 20.1%-40% - intermediate; >40% - high)