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

(Article begins on next page)

19 April 2024

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

2 **lymphoma**

3 Running headline: Ki67 prognostic role in canine lymphoma

4

5 A. Poggi[1], B. Miniscalco[1], E. Morello[1], F. Gattino[1], A. Delaude[1], L. Ferrero Poschetto[1],

6 L. Aresu[2], M.E. Gelain[2], V. Martini[3], S. Comazzi[3], F. Riondato[1]

7

8 [1] Department of Veterinary Sciences, University of Turin, Grugliasco, Italy

9 [2] Department of Comparative Biomedicine and Food Science, University of Padua, Legnaro, Italy

10 [3] Department of Veterinary Sciences and Public Health, University of Milan, Milan, Italy

11

12 Corresponding author:

13 Fulvio Riondato, DVM, PhD

14 Department of Veterinary Sciences, University of Turin

15 Largo Braccini 2, 10095 Grugliasco, Turin, Italy

16 e-mail: fulvio.riondato@unito.it

17

18 **Abstract**

19 Ki67 can discriminate between high and low grade canine lymphomas, but its prognostic role in

20 specific subtypes of the neoplasm is unknown. We assessed the prognostic significance of Ki67%

21 (percentage of Ki67-positive cells), evaluated by flow cytometry, in 40 dogs with high grade B-

22 cell lymphoma, treated with a modified Wisconsin-Madison protocol (UW-25). The following

23 variables were investigated for association with lymphoma specific survival (LSS) and relapse

24 free interval (RFI): Ki67%, breed, sex, age, stage, substage, complete remission (CR). By

25 multivariate analysis, Ki67% (P=0.009) and achievement of CR (P=0.001) were independent

26 prognostic factors for LSS. Dogs with intermediate Ki67% (20.1-40%) presented longer LSS and

27 RFI (median=866 and 428 days, respectively) than dogs with low (median=42 days, P<0.001;

28 median=159 days, P=0.014) or high (median=173 days, P=0.038; median=100 days, P=0.126)

29 values. Determination of Ki67 is a prognostic tool that improves the clinical usefulness of flow

30 cytometric analysis in canine high grade B-cell lymphoma.

31

33

34 **Introduction**

35 Canine lymphoma represents a heterogeneous group of neoplasms arising from the malignant

36 transformation of lymphoid cells and is characterized by a broad range of clinical presentations

37 and potential outcomes.

38 Depending upon the grade of malignancy, lymphomas are cytologically grouped into two main

39 categories. The most commonly encountered forms are high grade lymphomas, clinically

40 aggressive and typically fatal within a short period of time when treatment is not instituted.

41 Conversely, low grade lymphomas are rare and are characterized by an indolent disease course.[1,2]

42 Defining the immunophenotype is also reported to be important in predicting prognosis.[3-7] In fact

43 multicentric T-cell forms, when compared with B-cell forms, seem to be associated with similar

44 initial response rates, but have significantly lower response durability, even following an

45 appropriate chemotherapy protocol.[2]

46 Moreover, although several prognostic factors that independently influence the response rate and

47 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.

72

Materials and methods

73

74

Case selection

75

Dogs with multicentric high grade B-cell lymphoma diagnosed at the Veterinary Teaching

76

Hospital of the University of Turin between April 2011 and September 2014 were considered.

77

The diagnosis was based on clinical presentation (lymph node enlargement), cytological

78

examination of lymph nodes and flow cytometric analysis.

79

Inclusion criteria for the study were: cytological diagnosis of high grade lymphoma according to

80

the updated Kiel classification,[27,28] presence of flow cytometric B-cell immunophenotype, flow

81

cytometric Ki67 determination, treatment with a modified version of the University of

82

Wisconsin-Madison chemotherapy protocol (UW-25)[29] and the availability of follow up data.

83

Dogs previously treated with corticosteroid or chemotherapy agents were excluded. For each

84

included dog, signalment data (breed, sex and age), when available, were retrieved and clinical

85

stage (I-V) and substage (a or b) were assigned according to the World Health Organization

86

(WHO) system.[30] In particular, stage V was assigned when the neoplastic population, detected by

87

flow cytometry, was ≥ 3% in peripheral blood and/or bone marrow.

88

89

Flow cytometric immunophenotyping and Ki67 determination

90

At time of initial staging, flow cytometric immunophenotyping was performed on lymph node

91

fine-needle aspirate biopsies (FNABs), peripheral blood samples and/or bone marrow aspirates

92

within 24 h from collection as previously reported.[31] The following panel of monoclonal

93

antibodies (mAbs) was used: CD45-Alexa647 (pan-leucocyte marker; clone YKIX716.1, AbD

94

Serotec, Oxford, UK), CD3-FITC (T-cells marker; clone CA17.2A12, AbD Serotec), CD5-FITC

95

96　(T-cells marker; clone YKIX322.3, AbD Serotec), CD4-Alexa647 (T-helper marker; clone

97　YKIX302.9, AbD Serotec), CD8-PE (T-cytotoxic/suppressor marker; clone YCATE55.9, AbD

98　Serotec), CD21-PE (B-cells marker; clone CA21D6, AbD Serotec), CD79b-FITC (B-cells

99　marker; clone AT107-2, AbD Serotec) and CD34-PE (precursor cells marker; clone 1H6,

100　Pharmingen, Becton Dickinson, San Jose, CA, USA).

101　The proliferative activity was determined using the same lymph node FNABs utilized for

102　immunophenotyping. Cells were labelled with anti-Ki67-FITC monoclonal antibody (clone MIB-

103　1, DAKO, Glostrup, DK) using a fixation and permeabilization method with methanol, as

104　described previously.[26] A minimum of 10,000 events were acquired both for immunophenotype

105　and Ki67 determination on BD Accuri C6 flow cytometer (Becton Dickinson). Data were

106　analyzed using CFlow Plus software (Becton Dickinson). A gate of analysis was depicted on

107　forward (FSC) versus side scatter (SSC) plot to exclude debris and background. The proliferative

108　activity was expressed as the percentage of Ki67 positive cells (Ki67%) calculated on SSC versus

109　fluorescence intensity plot.

110

111　*Cytological evaluation*

112　Smears obtained by FNABs of enlarged lymph nodes were air-dried, fixed and stained with May-

113　Grünwald-Giemsa. Each case was classified according to the updated Kiel classification[27,28] and

114　allocated to a specific grade of malignancy and cytological subtype.

115

116　*Follow up*

117　Information pertaining to the achievement of remission, occurrence of relapse, survival at the end

118　of first chemotherapy protocol, lymphoma specific survival (LSS), relapse-free interval (RFI),

119　date and cause of death was collected.

120   Responses were classified as follows: complete remission (CR), which indicated ~~100%~~ reduction

121   to normal size ~~in the size~~ of all measurable lymph nodes; partial remission (PR), which indicated

122   more than 50% but less than 100% reduction of all measurable lesions and stable disease (SD),

123   which indicated less than 50% reduction or no change in the size of all measurable lesions.

124   Relapse was defined as clinical reappearance and cytological evidence of lymphoma in any

125   anatomic site in dogs having experienced CR. RFI was defined as the time in days from when a

126   dog achieved CR until relapse. LSS was defined as the interval in days between the date on

127   which chemotherapy was started and the date of death due to lymphoma related causes.

128

129   *Statistical analysis*

130   LSS and RFI for all dogs were estimated using the Kaplan-Meier product limit method.

131   Contingency tables were prepared for each of following variables: Ki67% (low, intermediate,

132   high), breed (purebred or crossbred), sex (male or female), age ($<$ or $\geq$ 10 years), stage (I-IV or

133   V), substage (a or b), and CR (yes or no). Pearson's χ^2 with z-test for column proportion

134   comparisons and Bonferroni adjustment for multiple comparisons were calculated to test the

135   association between each variable and the achievement of CR and survival at the end of first

136   chemotherapy protocol (UW-25). Dogs that died from causes other than lymphoma and dogs that

137   had not yet completed the protocol and did not meet the event (CR or death) were excluded from

138   the contingency tables. Ki67% cut-off values were defined rounding the thresholds of 25th and

139   75th percentiles to 20% and 40%, respectively, and thus generating the following groups

140   ~~arbitrarily identified on the basis of the data distribution as follows~~: low if Ki67 $\leq$ 20%,

141   intermediate if Ki67 between 20.1% and 40%, and high if Ki67>40%.

142   To evaluate the prognostic significance of each variable, univariate logistic regression for LSS

143   and RFI was first used and variables with P<0.3 were then included in a multivariate Cox

144   proportional hazards model progression analysis with a backward step selection. Kaplan-Meier

145   curves were drawn for Ki67% groups and compared by log-rank test to assess the survival

146   analysis. Dogs that were alive at the end of the study, lost to follow-up or dead due to causes

147   other than lymphoma were censored for survival analysis. Differences were considered

148   significant with P<0.05. Statistical analyses were performed using SPSS software (IBM SPSS

149   Statistics, IBM Corporation, Chicago, IL, USA).

150

151

152   **Results**

153   *Lymphoma cases*

154   Forty cases met the inclusion criteria and were enrolled in the study. Data about the identification

155   of breed were reported for 39 cases. There were 26 (66.7%) purebred dogs (three Labrador

156   retrievers, two German shepherds, two Dobermans, two Bloodhounds, two Pit bull terriers and

157   one each of Italian Mastiff, Great Dane, Poodle, Dachshund, Beagle, Bernese mountain dog,

158   Cavalier King Charles Spaniel, Golden retriever, Jack Russell, Rottweiler, White Swiss shepherd,

159   Cocker Spaniel, English Bulldog, Lagotto Romagnolo, American Staffordshire terrier) and 13

160   (33.3%) crossbred dogs. Sixteen dogs (42.1%) were males (1 castrated) and 22 (57.9%) were

161   females (9 spayed), while in 2 cases the sex was unknown. The age was only reported for 37 dogs

162   and the median age was 9 years (range, 4-15 years). The included lymphomas were cytologically

163   classified as follows: 8 (20%) centroblastic monomorphic, 24 (60%) centroblastic polymorphic

164   predominantly large cell, 5 (12.5%) immunoblastic, 2 (5%) lymphoblastic and 1 (2.5%)

165   plasmacytoid. At time of diagnosis, 27 dogs (67.5%) were in stage IV (10 substage a, 16 substage

166   b and 1 unknown) and 13 (32.5%) in stage V (all substage b).

167

168   *Response to treatment*

169   CR was achieved in 25 (62.5%) dogs. Twelve out of these 25 (48%) relapsed (median RFI=180

170   days; range 28-530 days), 10 (40%) were still in CR at the end of the study (median follow up

171   period=321 days; range 60-1005 days) and 3 (12%) died of causes unrelated to lymphoma after

172   34, 210 and 240 days from the beginning of chemotherapy, with lymphoma remaining in CR.

173   Relapses were treated with a second UW-25 or with other rescue protocols (DMAC, L-

174   asparaginase + lomustine), depending on when the relapse occurred and owner compliance. At

175   the end of the study 8 out of 12 relapsed dogs (66.7%) were dead because of PD (median LSS =

176   390 days; range 150-866 days), 3 (25%) were in PR (follow up period of 515, 800 and 1108

177   days) and 1 (8.3%) was in SD (follow up period=295 days).

178   Among the 15 dogs that did not achieve CR, 11 (73.3%) died because of PD (median LSS=42

179   days; range 15-1100 days), 3 (20%) were in PR at the end of the study (follow up period of 28,

180   157 and 653 days) and 1 (6.7%) died of causes unrelated to lymphoma after 45 days.

181   Estimated median RFI and LSS for all dogs were 414 days (95% CI range 228-600 days) and 442

182   days (95% CI range 236-648 days), respectively.

183

184   *Proliferative activity*

185   The mean Ki67% was 33.8% (SD=14.2%) and the median was 30.7% (range 10-67%). Six cases

186   presented low Ki67% (≤20%), 24 were in the intermediate group (20.1-40%) and 10 were in the

187   high group (>40%).

188

189   *Survival at the end of chemotherapy protocol and achievement of CR*

190   Survival at the end of chemotherapy protocol was significantly associated with the achievement

191   of CR (P=0.001). In fact, 91.7% of the dogs that achieved CR were alive compared with 33.3%

192   of dogs that did not reach CR (Table 1). Ki67% showed near-significant association with both

193   survival (P=0.063) and achievement of CR (P=0.075) at the end of chemotherapy protocol. In

194   fact, percentages of both survival and CR were higher for dogs with intermediate Ki67% (85.7%

195   and 81%, respectively) compared with dogs with low (50% and 33%) and high Ki67% (50% and

196   60%) (Table 1).

197

198   *Prognostic factors for LSS and RFI*

199   Ki67% (P=0.007) and achievement of CR (P=0.001) significantly influenced LSS on univariate

200   analysis and were confirmed to be independent prognostic factors for LSS (P=0.009 and P=0.001,

201   respectively) in the multivariate analysis (Table 2). None of the variables significantly influenced

202   RFI in the univariate analysis and none were of prognostic significance for RFI in the

203   multivariate analysis (Table 2).

204   The Kaplan-Meier analysis showed that dogs with intermediate Ki67% had significantly longer

205   LSS (median=866 days) than dogs with low (median=42 days; P<0.001) and high Ki67%

206   (median=173 days; P=0.038) (Fig. 1). Intermediate Ki67% was a significant predictor also for

207   one year and two years survival (P=0.001 and P=0.004 vs low and high Ki67%, respectively, at

208   both time points) (Fig. 1).

209   Dogs with intermediate Ki67% reported also longer RFI (median=428 days) than dogs with low

210   (median=159 days; P=0.014) and high Ki67% (median=100 days; P=0.126), although the

211   difference with the high Ki67% group did not reach statistical significance (Fig. 2).

212

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

237 determination could account for these discrepancies. In fact, in the previous studies,[24,25] both high

238 and low grade lymphomas and both B and T-cell immunophenotypes were included. Moreover,

239 Ki67 detection was carried out through immunohistochemistry on bioptic specimens, while we

240 used flow cytometric analysis of FNABs. Furthermore, the different selection of the cut-off

241 values to define groups may have influenced the results. In our work we used an approach similar

242 to that of Phillips et al.[24], using median and 75th percentile to differentiate two prognostic groups

243 and we get near significant results with the latter (data not shown). Observing that the longest

244 survival times were associated with intermediate Ki67% values, we assessed the prognostic

245 significance of Ki67% dividing cases in three different groups using quartiles. Furthermore, we

246 rounded the quartile cut-off values to 20% and 40% in order to simplify the use in clinical

247 practice. Unfortunately, a direct comparison of our cut-off values with those assessed by Phillips

248 et al is not possible because they did not reported the actual percentages that define quartiles in

249 their caseload. However this comparison, although interesting, would presumably be limited by

250 the different method used to measure Ki67 expression. In this regard, Kiupel et al.[25] did not get

251 significant results despite the application of thresholds similar to ours (Ki67≤20%; 21-40%; 41-

252 60%; >60%)

253 Contradictory results on prognostic significance of Ki67 have also been reported in human non-

254 Hodgkin's lymphoma, due to the heterogeneity of the different subtypes of this disease.[19] Many

255 studies have assessed Ki67 in aggressive diffuse large B-cell lymphomas, with a wide range of

256 expression.[21] In accordance with our results, Jerkeman et al.[32] found that patients with either low

257 (<60%) or high Ki67 (>90%) expression demonstrated a trend toward overall lower survival than

258 patients with moderate expression (60-90%). Moreover, a low proliferation index was associated

259 with a low level of failure-free survival compared with moderate or high indexes. This behavior

260 is likely because lymphomas with a low proliferation rate exhibit resistance to cycle specific

261 cytotoxic chemotherapy, given that the majority of cells are resting in the G0 phase of the cell

262 cycle. Conversely, in cases with high proliferation rates, treatment failure may be caused through

263 regrowth or by the increasing the likelihood of further mutations.

264 In addition to the proliferative activity, we also found that achievement of CR was an

265 independent prognostic factor for LSS, as reported in previous studies, where obtaining CR led to

266 prolonged survival for dogs with aggressive lymphoma.[7,33,34] Stage and substage did not show

267 prognostic significance for LSS or RFI, in contrast with some authors,[8,35,36] but in accordance

268 with others.[37,38] These differences may be due to the inclusion of different types of lymphoma,

269 different therapeutic strategies and different methods and cut-offs used to stage the disease.

270 Major limits of this work are its retrospective nature and the limited number of cases. Prospective

271 studies considering a larger number of lymphomas are required to confirm the clinical usefulness

272 of a Ki67-based stratification of patients.

273 In conclusion flow cytometric determination of Ki67 was found to be an independent predictor

274 for LSS in treated high grade B-cell lymphomas; intermediate values were associated with the

275 best prognosis. We previously demonstrated that this determination is useful in discriminating

276 between low and high grade lymphomas. Thus, we suggest the introduction of Ki67 in the routine

277 panel of labeling to add diagnostic and prognostic value to the flow cytometric analysis.

278

282

283 **References**

284   1. Teske E. Canine malignant lymphoma: a review and comparison with human non-
285   Hondgkin's lymphomas. *Veterinary Quarterly* 1994; **16**: 209–219.

286   2. Vail DM, Pinkerton ME and Young KM. Canine lymphoma and lymphoid leukemia. In:
287   *Withrow and MacEwen's Small Animal Clinical Oncology*. 5th edn., St Louis, Saunders
288   Elseviers, 2012; 608–638.

289   3. Greenlee PG, Filippa DA, Quimby FW, Patnaik AK, Calvano SE, Matus, *et al*.
290   Lymphomas in dogs. A morphologic, immunologic and clinical study. *Cancer* 1990; **66**:
291   480–490.

292   4. Teske E, van Heerde P, Rutteman GR, Kurzman ID, Moore PF and MacEwen EG.
293   Prognostic factors of treatment of malignant lymphoma in dogs. *Journal of the American*
294   *Veterinary Medical Association* 1994; **205**: 1722–1728.

295   5. Ruslander DA, Gebhard DH, Tompkins MB, Grindem CB and Page RL.
296   Immunophenotypic characterization of canine lymphoproliferative disorders. *In Vivo*
297   1997; **11**: 169–172.

298   6. Dobson JM, Blackwood LB, McInnes EF, Bostock DE, Nicholls P, Hoather TM, *et al.*
299   Prognostic variables in canine multicentric lymphosarcoma. *Journal of Small Animal*
300   *Practice* 2001; **42**: 377–384.

301   7. Simon D, Moreno SN, Hirschberger J, Moritz A, Kohn B, Neumann S, *et al*. Efficacy of a
302   continuous, multiagent chemotherapeutic protocol versus a short-term single-agent
303   protocol in dogs with lymphoma. *Journal of the American Veterinary Medical*
304   *Association* 2008; **232**: 879–885.

305   8. Marconato L, Stefanello D, Valenti P, Bonfanti U, Comazzi S, Roccabianca P, *et al.*
306   Predictors of long-term survival in dogs with high-grade multicentric lymphoma. *Journal*
307   *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.

332   18. Miller TP, Grogan TM, Dahlberg S, Spier CM, Braziel RM, Banks PM, *et al.* Prognostic
333   significance of the Ki-67 associated proliferative antigen in aggressive non-Hodgkin's
334   lymphomas: a prospective Southwest Oncology Group trial. *Blood* 1994; **83**: 1460–1466.

335   19. Broyde A, Boycov O, Strenov Y, Okon E, Shpilberg O and Bairey O. Role and prognostic
336   significance of the Ki-67 index in non-Hodgkin's lymphoma. *American Journal of*
337   *Hematology* 2009; **84**: 338–343.

338   20. Palestro G, Pich A, Chiusa L, Geuna M, Ponti R, Kerim S, *et al.* Biological heterogeneity
339   of diffuse mixed small and large cell non-Hodgkin's lymphomas assessed by DNA flow
340   cytometry and Ki67. *Leukemia and Lymphoma* 1995; **19**: 467–472.

341   21. Bairey O. Role and prognostic significance of the Ki-67 index in non Hodgkin's
342   lymphoma. *European Journal of Clinical & Medical Oncology* 2010; **2**: 113– 119.

343   22. Fournel-Fleury C, Magnol JP, Chabanne L, Ghernati I, Marchal T, Bonnefond C, *et al.*
344   Growth fractions in canine non-Hodgkin's lymphomas as determined in situ by the
345   expression of the Ki-67 antigen. *Journal of Comparative Pathology* 1997; **117**: 61–72.

346   23. Patruno R, Zizzo N, Zito AF, Catalano V, Valerio P, Pellecchia V, *et al.* Microvascular
347   density and endothelial area correlate with Ki-67 proliferative rate in the canine non-
348   Hodgkin's lymphoma spontaneous model. *Leukemia and Lymphoma* 2006; **47**: 1138–
349   1143.

350   24. Phillips BS, Kass PH, Naydan DK, Winthrop MD, Griffey SM and Madewell BR.
351   Apoptotic and proliferation indexes in canine lymphoma. J*ournal of Veterinary*
352   *Diagnostic Investigation* 2000; **12**: 111–117.

353   25. Kiupel M, Teske E and Bostock D. Prognostic factors for treated canine malignant
354   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.

402

403

404

405

406

407

408

409

410

411

412

413

414

415

416 **Table 1.** Association between different variables and survival or achievement of complete

417 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	11a	4a		3a	12b	
		73.3%	26.7%		20.0%	80.0%	
	Tot	24	11		12	23	
Age	<10years	14a	5a		6a	13a	
		73.7%	26.3%	0.471	31.6%	68.4%	0.600
	≥10years	10a	5a		5a	10a	
		66.7%	33.3%		33.3%	66.7%	
	Tot	24	10		11	23	
Stage	Stage I-IV	19a	6a		7a	18a	
		76.0%	24.0%	0.235	28.0%	72.0%	0.320
	Stage V	7a	5a		5a	7a	
		58.3%	41.7%		41.7%	58.3%	
	Tot	26	11		12	25	
Substage	a	7a	1a		3a	6a	
		87.5%	12.5%	0.210	33.3%	66.7%	0.665
	b	18a	10a		9a	18a	
		64.3%	35.7%		33.3%	66.7%	
	Tot	16	10		12	24	
Ki67%	≤20%	3a	3a		4a	2a	
		50.0%	50.0%		66.7%	33.3%	
	20.1-40%	18a	3b		4a	17a	
		85.7%	14.3%	0.063	19.0%	81.0%	0.075
	>40%	5a	5a		4a	6a	
		50.0%	50.0%		40.0%	60.0%	
	Tot	26	11		12	25	
Complete	No	4a	8b	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

438

439

440

441

442

443

444

445

446

447

448

449

450

451

452

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)