Flow cytometric characterization of S-phase fraction and ploidy in lymph node aspirates from dogs with lymphoma

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16 **Abstract**

17 Canine lymphoma is a multifaceted disease encompassing numerous entities with different
18 prognosis. Objective assessment of the proliferation rate is of great importance from both
19 pathological and clinical point of view. Different methods have been described in the literature to
20 assess it, including evaluation of Ki67 expression on fresh lymph node (LN) aspirates by flow
21 cytometry (FC). This test has a high accuracy in discriminating low- and high-grade lymphomas,
22 and provides prognostic information among high-grade B-cell lymphomas. DNA content analysis is
23 less expensive and suitable for preserved samples. We describe DNA-content analysis on LN
24 aspirates from 112 dogs with lymphoma. Based on our results, S-phase fraction (SPF) accurately
25 discriminates between low and high-grade lymphomas, being 3.15% the best discriminating cut-off.
26 SPF values strongly correlate with Ki67 expression assessed by FC. Survival analyses were
27 restricted to 33 dogs with high-grade B-cell lymphoma receiving a standardized multi-agent
28 chemotherapy, but no significant result for SPF was obtained. We also describe a subset of
29 aneuploid cases and their respective follow-up. We conclude that DNA content analysis may be
30 combined with morphological examination of LN aspirates to improve the objectivity in lymphoma
31 subtype classification in dogs. Further studies are needed to assess possible prognostic role of both
32 SPF and ploidy status within specific lymphoma subtypes in the canine population.

33

34 **Keywords**

35 Proliferation rate, DNA content, Ki67, aneuploidy

36

Introduction

The definition of neoplasia has evolved during the past decades, as new concepts were taken into account, such as sustenance of the proliferative signalling, evasion of growth suppression, resistance to cell death, replicative immortality and induction of angiogenesis (Hanahan and Weinberg, 2000; Vincent, 1987; Willis, 1952). However, uncontrolled proliferation continues to be considered a hallmark of cancer cells (Fouad and Aanei, 2017; Golias et al., 2004; Hanahan and Weinberg, 2000, 2011; Kaufman et al., 2007). Indeed, dysregulation of the cell cycle is a major contributor to the pathogenesis of neoplasms (Dictor et al., 1999; Wiman and Zhivotovsky, 2017).

The DNA content of the cells varies with progression in the cell cycle: cells in G0 and G1 phases maintain two complete sets of chromosome (diploid cells, 2n); during the S-phase, the DNA amount progressively increases until duplication; cells in G2 and M phases are tetraploid (4n). Thus, cell distribution within the phases of the cell cycle can be revealed analysing the DNA content of the cellular population. This is generally achieved via flow cytometry (FC), using fluorescent DNA dyes (Darzynkiewicz et al., 2017; Ormerod et al., 1998; Wilkerson 2012) and particularly assessing the fraction of cells in S-phase (SPF). As replicating DNA is more prone to carcinogen attacks, S-phase is a crucial step of the cell cycle in the process of neoplastic transformation and development (Kaufman et al., 2007).

In dogs, DNA content and ploidy have been described for transitional cell carcinomas of urinary bladder, mast cell tumours, melanomas, mammary tumours, osteosarcomas and lymphomas (Ayl et al., 1992; Bolon et al., 1990; Clemo et al., 1994; Fox et al., 1990; Hellmen et al., 1993; Teske et al., 1993).

In particular, Teske and colleagues investigated the DNA content and ploidy status in canine lymphoma, but failed to detect any correlation with lymphoma subtype or phenotype, and with survival (Teske et al., 1993). Canine lymphoma is a heterogeneous disease and encompasses numerous entities with different clinical presentation, behaviour and follow up (Aresu et al., 2015;

62 Valli et al 2013). The study by Teske and colleagues dates back to 1993 and was based on the
63 Working Formulation (WF) classification scheme. From then on, other classification schemes have
64 been introduced for canine lymphoma. Based on a recent review, the WF classification appears to
65 be of poor clinical and prognostic interest, differently from both the World Health Organization
66 (WHO) histopathological scheme and the updated Kiel cytological scheme (Sayag et al., 2018).
67 Still, morphological evaluation alone remains operator-dependent, and the inclusion of other
68 objective data can contribute to improve the intra- and inter-observer reproducibility (Teske and van
69 Heerde, 1996).

70 The combined use of cytology, immunophenotype and FC assessment of Ki67 expression has been
71 recently proposed as a reliable tool for the classification of canine lymphomas (Poggi et al., 2015).
72 Ki67 FC quantification has also a prognostic value in dogs with high-grade B-cell lymphomas
73 (Poggi et al., 2017). Unfortunately, this technique has been applied only on fresh samples (within
74 24 hours from sampling) in the dog (Poggi et al., 2015). In addition, changes >20% in Ki67
75 expression are reported in human blood stored for 72-96 hours before analysis, even if collected in
76 tubes containing a cell preservative commonly used for FC analysis (Sun et al., 2016). On the
77 contrary, preserved or archive material is suitable for DNA content analysis (Ormerod et al., 1998).
78 In addition, this technique is less expensive, requiring only fluorescent DNA dyes, whereas
79 monoclonal antibodies and permeabilizing solutions are needed to assess Ki67 expression via FC
80 (Kim and Sederstrom, 2015).

81 Aim of the present study was to assess the diagnostic and prognostic role of DNA content analysis
82 in canine lymphomas. In particular, we describe: 1) the S-phase fraction (SPF) in different
83 lymphoma subtypes; 2) the correlation of SPF with other proliferation rate markers (Ki67); 3) the
84 prevalence of aneuploidy and the follow up of aneuploid cases; and 4) the possible prognostic role
85 of SPF among dogs with high-grade B-cell lymphoma undergoing a standardized treatment
86 regimen.

Materials and methods

Case selection and classification

The FC database of the Veterinary Teaching Hospital of the University of Turin was retrospectively investigated from January 2011 to September 2014. All consecutive cases fulfilling the following inclusion criteria were extrapolated: 1) a final diagnosis of nodal lymphoma based on clinical presentation, complete blood count, cytological and FC examination of an enlarged peripheral lymph node (LN), 2) availability of a LN cytological sample for review, and 3) availability of FC immunophenotype and DNA content analysis on LN. Dogs treated with corticosteroids or chemotherapy agents prior to FC analysis were excluded as well as samples of poor quality for DNA content analysis: Background Aggregates and Debris (BAD) >20% and/or G0/G1 peak Coefficient of Variation (CV) >8% (Ormerod et al., 1998).

All dogs were privately owned and sampled for diagnostic purposes with a written informed consent of the owners. Thus, a specific formal approval of the Institution Committee for Animal Care of the University of Turin was not required.

Cytological samples were reviewed by a single operator and cases were classified according to the updated Kiel classification scheme (Fournel-Fleury et al., 1997). LN aspirates collected into tubes containing RPMI-1640 or saline solution were processed for FC immunophenotyping within 24 hours from sampling as previously described (Gelain et al., 2008). Different combinations of the following monoclonal antibodies were used for this aim: CD45 (clone YKIX716.13), CD3 (clone CA17.2A12), CD5 (clone YKIX322.3), CD4 (clone YKIX302.9), CD8 (clone YCATE55.9), CD21 (clone CA2.1D6), CD79b (clone AT107-2), CD34 (clone 1H6). All antibodies were provided by Serotec (Oxford, UK) except CD34 (BD Becton Dickinson; San Josè, CA, USA). Ki67 expression was determined as previously described (Poggi et al., 2015). All samples were acquired with a BD

111 Accuri C6 (BD Becton Dickinson) and analysed with the specific software CFlow Plus (BD Becton
112 Dickinson).

113 For high-grade B-cell lymphoma and aneuploid tumours cases, the referring veterinarians were
114 contacted to retrieve signalment data (including breed, sex and age at diagnosis), as well as clinical
115 stage and substage according to the WHO staging system (Owen, 1980), and follow up data.

116 *DNA content analysis*

117 LN aspirates were fixed in 70% ethanol and stored at -20°C for a minimum of 2 hours, until
118 analysis for DNA content. Immediately prior to processing, all samples were washed twice in PBS
119 1x and the supernatant was finally discarded. 500µl of a staining solution containing propidium
120 iodide (50 µg/ml) and Ribonuclease (RNase, 0.2mg/ml) were added to the cell pellet ($1x10^6$
121 cells/tube), incubated for 15 minutes, and then acquired with the BD Accuri C6 flow cytometer.

122 Nucleated cells from peripheral blood of a healthy dog, obtained by RBC lysis with a solution
123 containing 8% ammonium chloride, fixed in 70% ethanol and stored at -20°C were processed along
124 with each neoplastic LN sample, to serve as a normal diploid control.

125 Analyses were performed with a dedicated software (Multicycle for Windows, in FCS Express).
126 Data were represented by a histogram with the fluorescence intensity on X-axis, and the number of
127 cells on Y-axis. The SPF was calculated as the area under the curve between G0/G1 and G2/M
128 peak.

129 Cases were considered aneuploid if two G0/G1 peaks were identified. When a single peak was
130 present or the diploid peak was not clearly recognizable, an aliquot of the control tube was added.
131 The DNA index (DI) was calculated as the ratio between the mean channel number of the G0/G1
132 extra-peak and the mean channel number of the G0/G1 diploid peak.

133 *Statistical analysis*

All statistical analyses were performed with a specific software (SPSS v21.0, SPSS Inc, Chicago, USA) and significance was set at $p \leq 0.05$ for all tests.

Many cytological subtypes were poorly represented or absent in our case series. Thus, for statistical purposes, cases were re-classified based on phenotype and cytological malignancy grade in: high-grade B-cell, high-grade T-cell, low-grade B-cell and low-grade T-cell subtypes.

A Shapiro-Wilk test was performed to assess whether SPF data were normally distributed within groups. A Levene's test was performed to test the homoscedasticity assumption. Thereafter, possible SPF variations among lymphoma subtypes were assessed with a Brown-Forsythe ANOVA test. Post-hoc analyses were performed with a Dunnett test.

A Receiving Operator Curve (ROC) was drawn to select the SPF cut-off best discriminating between low- and high-grade lymphomas, based on the best compromise between sensitivity and specificity.

The Pearson's correlation coefficient between SPF and Ki67 expression was calculated. The equation of the regression line was used to identify the SPF values corresponding to 20% and 40% Ki67 expression, respectively, as these values were proved to be of prognostic impact in dogs with high-grade B cell lymphoma (Poggi et al., 2017). These SPF cut-offs were then used for survival analyses.

Survival analyses were restricted to dogs with high-grade B-cell lymphoma treated with a 25-weeks Winsconsin-Madison chemotherapy protocol (UW-25) (Garrett et al., 2002), in order to reduce the bias linked to inclusion of different lymphoma subtypes and treatment regimens.

Follow up data were also recorded for aneuploid cases, although survival analyses were not attempted because of the low number of cases and the wide spectrum of lymphoma subtypes and treatment regimens adopted.

157 According to the official guidelines (Vail et al., 2010), complete response (CR) was defined as
158 disappearance of all evidence of disease in target lesions, disease-free survival (DFS) as time from
159 documentation of CR and relapse, and lymphoma-specific survival (LSS) as time from initiation of
160 treatment and death for lymphoma. Dogs lost to follow up, still in CR at data analysis closure, or
161 dead for lymphoma-unrelated causes before lymphoma relapse were censored for DFS analyses.
162 Dogs lost to follow up, still alive at data analysis closure, or dead for lymphoma-unrelated causes
163 were censored for LSS analysis.

164 Univariate Cox's proportional hazard regression analysis was performed, to assess possible
165 influence on DFS and LSS of the following variables: breed (pure or mixed), sex (male or female),
166 age (< or ≥8 years), stage (I to V), substage (a or b), Ki67 expression (≤20%, between 20% and
167 40%, >40%), SPF (≤5.90%, between 5.90% and 11.72%, >11.72%), obtainment of CR (yes or not).
168 Variables with P-value ≤0.30 were then included in a backward elimination multivariate analysis. In
169 addition, Kaplan Meier curves were drawn and compared with log-rank test to assess possible
170 variations in median DFS and LSS according to the aforementioned variables.

171 **Results**

172 Overall, DNA content analysis was performed in 124 cases. Among them, 12 (9.7%) were of poor
173 quality (BAD >20% and/or G0/G1 peak CV >8%). Thus, 112 cases were finally included in the
174 study.

175 Concerning lymphoma subtype, 41 (36.6%) cases were centroblastic polymorphic B-cell, 16
176 (14.3%) centroblastic monomorphic B-cell, 10 (8.9%) small clear T-cell, 9 (8.0%) immunoblastic
177 B-cell, 9 (8.0%) pleomorphic mixed small and large T-cell, 6 (5.4%) macronucleolated medium-
178 sized B-cell, 6 (5.4%) pleomorphic large T-cell, 4 (3.6%) lymphoblastic B-cell, 4 (3.6%)
179 lymphoblastic T-cell, 3 (2.7%) pleomorphic small T-cell, and 1 (0.9%) each of the following:
180 plasmacytoid B-cell, plasmacytoid T-cell, prolymphocytic B-cell and prolymphocytic T-cell. Thus,

71 (63.4%) dogs had a high-grade B-cell lymphoma, 20 (17.9%) had a high-grade T-cell lymphoma, 14 (12.5%) a low-grade T-cell lymphoma, and 7 (6.2) a low-grade B-cell lymphoma.

S-phase fraction

Overall mean SPF was 9.36±7.81% (median 8.50%; min-max 0.2-46.4%). In particular, high-grade B-cell lymphomas had a mean SPF of 11.35±8.11% (median 9.30%; min-max 1.3-46.4%), high-grade T-cell lymphomas a mean SPF of 10.95±4.60% (median 10.70%; min-max 3.4-21.5%), low-grade T-cell lymphomas a mean SPF of 1.08±0.94% (median 0.85%; min-max 0.2-3.1%) and low-grade B-cell lymphomas a mean SPF of 1.30±0.77% (median 1.30%; min-max 0.6-2.8%). SPF values for specific cytological subtypes are listed in table 1.

SPF variation among lymphoma subgroups was statistically significant ($p<0.001$). In particular, SPF was higher in high-grade B-cell than in low-grade B- and T-cell lymphomas and higher in high-grade T-cell than in low-grade B- and T-cell lymphomas ($p<0.001$ for all comparisons). Differences between high-grade B- and T-cell lymphomas and between low-grade B- and T-cell lymphomas were not significant ($p>0.05$) (Fig.1).

ROC curve identified a high accuracy of SPF in discriminating between low- and high-grade lymphomas (area under the curve AUC=0.996), being 3.15% the best cut-off (sensitivity 97.8%, specificity 100.0%) to identify high-grade lymphomas.

Correlation between SPF and Ki67

Ki67 expression was assessed in 100 cases, with a mean overall value of 31.66±18.69% (median 30.00%; min-max 1.00-71.00%). A strong correlation between Ki67 expression and SPF was detected ($p<0.001$, $r=0.753$). The equation was: SPF=0.075+0.291*Ki67. Thus, the SPF cut-offs corresponding to 20% and 40% Ki67 expression were 5.90% and 11.72%, respectively. These cut-offs were then used in the survival analyses.

Ploidy

Among the 112 cases included, 105 (93.8%) were diploid and 7 (6.2%) aneuploid. These included 3 (42.9%) high-grade B-cell lymphomas (2 immunoblastic and 1 centroblastic monomorphic), 3 (42.9%) high-grade T-cell lymphomas (2 pleomorphic mixed small and large and 1 lymphoblastic) and 1 (14.3%) low-grade T-cell lymphoma (pleomorphic small cells).

Mean DI of aneuploid cases was 1.20±0.07 (median 1.18; min-max 1.14-1.36). Five (71.4%) cases had DI<1.20 and were subclassified as near-diploid (Bauer et al 1993). A lymphoblastic T-cell lymphoma had a DI=1.21, and a centroblastic monomorphic B-cell lymphoma had a DI=1.36.

Breed was not reported in one case; the other dogs represented 6 different pure breeds. Three (42.9%) were females (1 spayed) and 4 (57.1%) males (1 neutered). Mean age at diagnosis was 10.8±2.3 years (median 11 years; min-max 7-14 years) but was not reported in one case.

Follow up data were available for the 5 near-diploid cases. One dog with high-grade B-cell lymphoma was treated with corticosteroid alone and was still alive after 58 days from the diagnosis; the other one entered the UW-25 chemotherapy protocol but died for lymphoma-unrelated causes after 45 days from the diagnosis. One dog with high-grade T-cell lymphoma was treated with single-agent chemotherapy and died for lymphoma on day 20; the other one entered the UW-25 chemotherapy protocol, had a DFS of 56 days and died for lymphoma on day 70. The dog with low-grade T-cell lymphoma received chlorambucile and prednisone, did not achieve CR and died for lymphoma on day 111.

Survival analyses

Follow up data were available for 51 dogs with high-grade B-cell lymphoma. Among them, 8 (15.7%) received prednisone alone, 6 (11.8%) no treatment, and 4 (7.8%) single-agent chemotherapy. Thus, survival analyses were restricted to 33 dogs that were treated with the UW-25 chemotherapy protocol.

Breed was known for 32 cases, including 21 (65.6%) pure-breed dogs of 17 different breeds and 11 (34.4%) mixed-breed. Sex was known for 32 cases, with 16 (50%) intact males and 16 (50%) females (7 spayed). Among 29 dogs whose age was known, 13 (44.8%) were <8 years old and 16 (55.2%) were ≥8 years old. Twenty-nine dogs underwent full staging: 3 (10.3%) were classified as stage IV disease and 26 (89.7%) as stage V. Eight (24.2%) dogs were in substage a and 25 (75.8%) in substage b. Ki67 expression had been tested in 29 dogs: it was ≤20% in 5 (17.2%) dogs, between 20% and 40% in 16 (55.2%), and >40% in 8 (27.6%). SPF was ≤5.90% in 6 (18.2%) dogs out of 33, between 5.90% and 11.72% in 19 (57.6%) dogs and >11.72% in 8 (24.2%) dogs. All dogs but one were diploid: the aneuploid case had a DI of 1.18. Nineteen (59.4%) dogs achieved CR and 13 (40.6%) did not; this information was not available for 1 dog.

None of the 19 dogs that achieved CR was censored for DFS analysis. Overall median DFS was 235 days (range 14-747 days). Among the investigated variables, significant results were obtained only for sex and Ki67 expression. In particular, median DFS was 349 days (range 159-747 days) for female dogs and 102 days (range 14-508 days) for male dogs: significant results were obtained with univariate and multivariate Cox's analysis (p=0.020 and p=0.022, respectively) as well as with log-rank test (p=0.014). Median DFS was 159 days (range 159-200 days) for dogs with low Ki67 expression, 329 days (range 14-747 days) for dogs with intermediate Ki67 expression, and 75 days (range 70-349 days) for dogs with high Ki67 expression significant results were obtained with multivariate Cox's analysis (p=0.039) and with log-rank test (p=0.042); p-value for univariate analysis was 0.063.

Ten (30.3%) dogs were censored for LSS analysis: 7 were still alive at data analysis closure with a median follow-up of 528 days (range 28-872 days), whereas 3 died for lymphoma-unrelated causes after 34, 45 and 210 days, respectively. Overall median LSS was 365 days (range 15-1086 days). Among the investigated variables, significant results were obtained only by the achievement of CR (p<0.001 for univariate Cox's analysis and log-rank test, and p=0.001 for multivariate analysis):

median LSS was 531 days (range 34-1086 days) for dogs that achieved CR and 45 days (range 15-320 days) for dogs that did not. A difference in the median LSS was also noted among the three Ki67 expression groups, although it did not reach statistical significance (p=0.099 for univariate Cox's analysis and p=0.081 for log-rank test): median LSS was 240 days (range 34-365 days) for dogs with low Ki67 expression, 728 days (range 28-1086 days) for dogs with intermediate Ki67 expression, and 150 days (range 15-872 days) for dogs with high Ki67 expression.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Discussion

DNA content analysis has been used in veterinary medicine to characterize the proliferative activity of different neoplasms (Ayl et al., 1992; Bolon et al., 1990; Clemo et al., 1994; Fox et al., 1990; Hellmen et al., 1993; Teske et al., 1993). In the present study, we describe the variation in SPF among different lymphoma subtypes in dogs, and its diagnostic and prognostic implications. We also describe a subset of aneuploid cases.

Identification of the malignancy grade is a crucial step in the diagnostic workup for dogs with lymphoma because of its prognostic impact: high-grade lymphomas have a more aggressive clinical behaviour than low-grade indolent lymphomas (Aresu et al., 2015; Ponce et al., 2004; Valli et al., 2013). Indeed, assessment of the mitotic index is required by all morphological classification schemes (either cytological or histopathological) (Fournel-Fleury et al., 1997; Greenle et al., 1990; Teske et al., 1994). Morphological assessment is somewhat operator-dependent, and a slight level of intra- and inter-observer disagreement may occur, even among the most experienced and specialized pathologists (Valli et al., 2011). Thus, tools that more objectively estimate the proliferation rate in histopathological sections of canine lymphomas have been introduced decades ago, such as AgNOR count and Ki67 expression (Kiupel et al., 1998, 1999; Phillips et al., 2000). Unfortunately, many veterinary oncologists do not include histopathological examination in their

diagnostic workup for canine lymphoma (Regan et al., 2013). Thus, strategies to quantify proliferation rate have been adapted to lymph node aspirates: AgNOR count can be performed with silver stain on cytological smears (Bauer et al., 2007), and Ki67 expression can be quantified either by immunostaining of cytological smears (Bauer et al., 2007) or by FC (Poggi et al., 2015).

Until now, DNA content in canine lymphoma has been analysed in one study only, on frozen tissue samples (Teske et al., 1993). In the present study, we used LN aspirates suspended in a liquid medium. This makes the examination more feasible for routine clinical practice, as surgical approach is not required. The opportunity of analysing different parameters on thousands of cells simultaneously, in face of a minimally invasive sampling procedure, is one of the major advantages of FC, and partially explains its wide spread in veterinary oncology (Burkhard and Bienzle, 2015; Comazzi and Gelain, 2011). FC has been used also to assess Ki67 expression in canine lymphomas, thus allowing a rapid and objective evaluation of the proliferative activity in a large number of cells (Poggi et al., 2015). Unfortunately, storage of the samples for few days can affect the results of this analysis (Sun et al., 2016). The technique presented in the present study fills this gap, as it combines the pros of FC (minimal invasiveness, rapidity, number of cells analysed) with the possible use of stored material from LN aspirates. Still, the analysis of Ki67 expression should be preferred whenever possible, as a relevant percentage of samples have to be excluded from DNA content analysis due to poor sample quality: we had to discard about 10% of the samples due to BAD>20% or CV>8%.

Based on our results, SPF accurately discriminates between high- and low-grade lymphomas in dogs. This is in contrast with the results obtained by Teske and colleagues (1993), who failed to detect any association between SPF and lymphoma malignancy. The difference in the samples used between the two studies (frozen tissues vs fresh ~~nodal~~LN aspirates) may partially explain this discrepancy, as well as the different classification schemes applied. In particular, the WF scheme includes three grades while only the low and high grade are described in the ~~Kiupel~~updated Kiel

scheme. Most of the cases in the study by Teske and colleagues were allocated exactly in the intermediate grade, which is not considered ~~not provided for~~ in the Kiel classification used in our study. Furthermore, Teske and colleagues did not stratify cases according to the neoplastic cells phenotype when analysing SPF data.

~~Furthermore, Teske and colleagues determined the B- vs T-cell lineage but they did not considered it for the classification. Cell lineage was detected only in a part of the tumors and a very different antibody panel was used.~~

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In line with Teske and colleagues (1993), we found no prognostic significance for SPF. Different reasons may account for this lack of significant results in the two studies. Teske and colleagues included in the survival analysis many different lymphoma subtypes, irrespective of the phenotype and the malignancy grade. In order to avoid this bias, we restricted our analysis to high-grade B-cell lymphoma cases that underwent a standardized chemotherapy protocol. Unfortunately, only 33 dogs corresponded to these inclusion criteria, and about one third of them was censored for LSS analysis. Therefore, our results may be affected by a low statistical power and caution should be used when considering them. This may explain the lack of significant results for LSS among Ki67 expression groups, as the median values are in line with what previously reported by our research group (Poggi et al., 2017). This is also true for the significant results obtained for sex, which may be over-estimated: female dogs have a lower risk to develop lymphoma (Villamil et al., 2009) but to date a prognostic impact of male versus female sex has never been reported in dogs with lymphoma. Further studies are warranted, including a larger standardized case series.

SPF was strongly correlated with Ki67 expression in our cases series. ~~but if b~~Both parameters allow a reliable discrimination between high- and low- grade lymphomas, but survival analyses gave significant results only for the latter. The difference in the information provided by the two techniques may account for this: all proliferating cells stain positive for Ki67, whereas only the subset of cells in the S-phase of the cell cycle are counted with DNA content analysis. In addition,

the SPF cut-offs we selected may be unsuitable to discriminate different prognostic groups, and other values may work better.

Even though both analyses (Ki67 and DNA content) can be run in a routine panel for canine lymphoma diagnosis, ~~in our experience~~ Ki67 determination is currently the first choice because of the limitations of SPF and ploidy as prognostic markers. Furthermore, the protocol for Ki67 determination is common to all intracytoplasmic labelling (it can easily be run beside the immunophenotyping analysis) and does not use hazardous reagents such as propidium iodide. SPF remains an excellent second option in the case the analysis must be delayed.

Contrasting results have been obtained also in human medicine about the clinical usefulness of DNA content analysis for non-Hodgkin's lymphomas: some studies claim for a prognostic value of SPF and/or ploidy status (Joensuu et al., 1991; Lackowska et al., 1999; Pinto et al., 2003; Rehn et al., 1990) whereas other authors downsize this hypothesis (Winter et al., 1996). A more recent study performed on a large case series of human non-Hodgkin's lymphomas revealed a prognostic impact for SPF (but not ploidy status) only within specific subtypes (namely, B-cell small lymphocytic lymphoma, diffuse large B-cell lymphoma and anaplastic large cell lymphoma) (Lackowska et al., 2012): the different inclusion criteria used may therefore account for the different results obtained in human medicine. Such a detailed study is still lacking for canine lymphoma.

The prevalence of aneuploidy in our case series (6.2%) was lower compared to the one previously reported in dogs with lymphoma (21.3%) (Teske et al., 1993). This is likely due to the different criteria used to define aneuploidy. Indeed, we only selected cases were two distinct G0/G1 peaks were identifiable, whereas Teske and colleagues also included cases with marked asymmetry and high CV in the G0/G1 peak. This difference particularly affects the possibility to correctly classify near-diploid cases: samples with DI only slightly >1 may have gone unnoticed in cases with high

G0/G1 peak CV in our case series, whereas aneuploid cases with higher DI are more easily detected, irrespective of the CV.

Teske and colleagues (1993) found no prognostic relevance for ploidy status. We did not attempt such an analysis because of the low number of aneuploid cases. Interestingly, however, a complete follow up was available for 3 aneuploid T-cell lymphoma cases: the 2 high-grade cases died for lymphoma after 20 and 70 days, respectively, and the only low-grade case died for lymphoma after 111 days. This is in line with what already reported for dogs with high-grade T-cell lymphomas (Aresu et al., 2015), whereas dogs with low-grade T-cell lymphoma commonly have a longer survival (Martini et al 2016; Valli et al., 2013). Further studies including a large cohort of aneuploid cases with a complete follow up are needed to assess whether ploidy status may affect survival within specific lymphoma subtypes.

Future investigation should also be aimed at clarify potential diagnostic role of aneuploidy in dogs with lymphoma. Based on a recent study, indeed, numerical chromosomal aberrations found in the tumour of dogs with lymphoma are often present also in the peripheral blood. Therefore, the authors hypothesize the use of peripheral blood as a matrix for cytogenetic analysis to monitor the status of the disease during treatment (Devitt et al., 2009). Gross numerical chromosomal aberrations may lead to aneuploidy (Sansregret and Swanton, 2017). If aneuploidy is detected in peripheral blood from dogs with lymphoma, it may serve as a case-specific tumour fingerprint to assess the minimal residual disease after treatment. To the authors' knowledge, however, DNA content in the peripheral blood from dogs with lymphoma has never been investigated, and these considerations remain purely speculative.

~~Even though both analyses can be run in a routine panel for canine lymphoma diagnosis, in our experience Ki67 determination is currently the first choice because of the limitations of SPF and ploidy as prognostic markers. Furthermore, the protocol for Ki67 determination is common to all intracytoplasmic labelling (it can easily be run beside the immunophenotyping analysis) and does~~

~~not use hazardous reagents such as propidium iodide. SPF remains an excellent second option in the case the analysis must be delayed~~

The retrospective nature of the present study represents its major limitation, as well as the small number of aneuploid cases included: these two factors prevented us from performing large-scale survival analyses and perhaps from identifying a prognostic role for the parameters investigated. In addition, only lymphoma samples were included in the present study, and the power of SPF to discriminate between neoplastic and non-neoplastic LNs is still to be elucidated.

In conclusion, DNA content analysis can be of aid to assess the malignancy grade on LN aspirates from dogs with lymphoma. These data can be combined to the morphological examination to improve the objectivity in the subtype definition. Identification of the prognostic role of both SPF and ploidy status requires further studies on a large number of cases.

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Conflict of interest

The authors declare no conflict of interest.

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524

525 **Fig.1** Distribution of S-phase fraction values within the four subtypes of lymphoma considered in

526 the study (high grade B-cell, low grade B-cell, high grade T-cell, and low grade T-cell)