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NEOPLASTIC DISEASE

Short Title: Flow Cytometric Characterization in Canine Lymphoma

Flow Cytometric Characterization of S-phase Fraction and Ploidy in Lymph Node Aspirates from Dogs with Lymphoma

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Summary

Canine lymphoma is a multifaceted disease encompassing numerous entities with different prognosis. Objective assessment of the proliferation rate is of importance from the pathological and clinical perspectives. Different methods have been described in the literature to assess proliferation rate, including evaluation of Ki67 expression in fresh lymph node (LN) aspirates measured by flow cytometry (FC). This test has a high accuracy in discriminating between low- and high-grade lymphomas, and provides prognostic information among high-grade B-cell lymphomas. DNA content analysis is less expensive and suitable for well-preserved samples. We describe DNA-content analysis using LN aspirates from 112 dogs with lymphoma. S-phase fraction (SPF) accurately discriminated between low- and high-grade lymphomas, with 3.15% being the best discriminating cut-off value. SPF values strongly correlated with Ki67 expression as assessed by FC. Survival analyses were restricted to 33 dogs with high-grade B-cell lymphoma receiving standardized multi-agent chemotherapy, but no significant result was obtained for SPF. We also describe a subset of aneuploid cases and their respective follow-up. We conclude that DNA content analysis may be combined with morphological examination of LN aspirates to improve the objectivity in lymphoma subtype classification in dogs. Further studies are needed to assess the possible prognostic role of SPF and ploidy status within specific lymphoma subtypes in dogs.

Keywords: dog; lymphoma; proliferation rate; DNA content

Introduction

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

The DNA content of cells varies with progression in the cell cycle: cells in the G0 and G1 phases maintain two complete sets of chromosome (i.e. diploid cells, 2n); during the S-phase, the amount of DNA increases progressively until duplication; cells in the G2 and M phases are tetraploid (i.e. 4n). Therefore, cell distribution within the phases of the cell cycle can be revealed by analysing the DNA content of a cellular population. This is generally achieved via flow cytometry (FC), using fluorescent DNA dyes (Ormerod *et al.*, 1998; Wilkerson 2012; Darzynkiewicz *et al.*, 2017) and particularly by assessing the fraction of cells in S-phase (SPF). As replicating DNA is more prone to damage by carcinogens, the S-phase is a crucial stage 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 the urinary bladder, mast cell tumours, melanomas, mammary tumours, osteosarcomas and lymphomas (Bolon *et al.*, 1990; Fox *et al.*, 1990; Ayl *et al.*, 1992; Hellmen *et al.*, 1993; Teske *et al.*, 1993; Clemo *et al.*, 1994). In particular, Teske *et al.* (1993) investigated the DNA content and ploidy status in canine lymphoma, but failed to detect any correlation with

lymphoma subtype or phenotype, or with survival. Canine lymphoma is a heterogeneous disease and encompasses numerous entities with different clinical presentation, behaviour and follow up (Valli *et al.*, 2013; Aresu *et al.*, 2015). The study by Teske *et al.* dates back to 1993 and was based on the Working Formulation (WF) classification scheme. From then on, other classification schemes were introduced for canine lymphoma. Based on a recent review, the WF classification appears to be of poor clinical and prognostic value, different to both the World Health Organization (WHO) histopathological scheme and the updated Kiel cytological scheme (Sayag *et al.*, 2018). Still, morphological evaluation alone remains operator dependent, and the inclusion of other objective data can contribute to improvement in the intra- and interobserver reproducibility (Teske and van Heerde, 1996).

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

The aim of the present study was to assess the diagnostic and prognostic value of DNA content analysis in canine lymphoma. In particular, we describe: (1) the SPF in different lymphoma subtypes; (2) the correlation between SPF and another marker of proliferation (Ki67); (3) the prevalence of aneuploidy and the follow up of aneuploid cases;

and (4) the possible prognostic role of SPF in dogs with high-grade B-cell lymphoma undergoing a standardized treatment regime.

Materials and Methods

Case Selection and Classification

The FC database of the Veterinary Teaching Hospital of the University of Turin, Italy, was investigated retrospectively for cases submitted between January 2011 and September 2014. All consecutive cases fulfilling the following inclusion criteria were included in this study: (1) a final diagnosis of nodal lymphoma based on clinical presentation, complete blood count and 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 the LN. Dogs treated with glucocorticoids 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 the written informed consent of the owners. Therefore, specific formal approval of the Institutional 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 h of sampling as described previously (Gelain *et al.*, 2008). Different combinations of the following monoclonal antibodies were used for this purpose: 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 from Serotec (Oxford, UK) except for anti-CD34 (Becton Dickinson; San Josè, California, USA). Ki67 expression was determined as described previously (Poggi *et al.*, 2015). All samples were acquired with a BD Accuri C6 flow cytometer (Becton Dickinson) and analysed with the specific software CFlow Plus (Becton Dickinson).

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

DNA Content Analysis

LN aspirates were fixed in 70% ethanol and stored at –20°C for a minimum of 2 h, until analysis for DNA content. Immediately prior to processing, all samples were washed twice in 1× phosphate buffered saline (PBS) and the supernatant was discarded. Five-hundred microlitres of a staining solution containing propidium iodide (50 μg/ml) and ribonuclease (RNase, 0.2mg/ml) were added to the cell pellet (1×10^6 cells/tube), incubated for 15 min and then acquired with the BD Accuri C6 flow cytometer.

Nucleated cells from the peripheral blood of a healthy dog, obtained by red blood cell (RBC) lysis with a solution containing 8% ammonium chloride, were fixed in 70% ethanol and stored at –20°C for processing alongside each neoplastic LN sample, to serve as a normal diploid control.

Analyses were performed with dedicated software (Multicycle for Windows, in FCS Express). Data were represented by a histogram with the fluorescence intensity on the X

axis, and the number of cells on Y axis. The SPF was calculated as the area under the curve between the G0/G1 and G2/M peaks.

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

Statistical Analysis

All statistical analyses were performed with specific software (SPSS v21.0, SPSS Inc., Chicago, Illinois, USA) and significance was set at $P < 0.05$ for all tests. Many cytological subtypes were poorly represented or absent in this case series. Therefore, for statistical purposes, cases were re-classified based on phenotype and cytological malignancy grade as: 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. 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 receiver 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.

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 proven to be of prognostic value

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-week 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.

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

Univariate Cox's proportional hazard regression analysis was performed, to assess possible influence on DFS and LSS of the following variables: breed (pure or mixed), sex (male or female), age (< or ≥8 years), stage (I to V), substage (a or b), Ki67 expression (≤20%, between 20 and 40%, >40%), SPF (≤5.90%, between 5.90 and 11.72%, >11.72%), obtaining CR (yes or no). Variables with *P*-value ≤0.30 were then included in a backward elimination multivariate analysis. In addition, Kaplan Meier curves were drawn and compared with log-rank test to assess possible variations in median DFS and LSS according to the aforementioned variables.

Results

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

Concerning lymphoma subtype, 41 (36.6%) cases were centroblastic polymorphic B cell, 16 (14.3%) centroblastic monomorphic B cell, 10 (8.9%) small clear T cell, nine (8.0%) immunoblastic B cell, nine (8.0%) pleomorphic mixed small and large T cell, six (5.4%) macronucleolated medium-sized B cell, six (5.4%) pleomorphic large T cell, four (3.6%) lymphoblastic B cell, four (3.6%) lymphoblastic T cell, three (2.7%) pleomorphic small T cell and one (0.9%) each of the following: plasmacytoid B cell, plasmacytoid T cell, prolymphocytic B cell and prolymphocytic T cell. Therefore, 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 seven (6.2) a low-grade B-cell lymphoma.

S-phase Fraction

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

SPF variation among lymphoma subgroups was 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 curves identified a high accuracy of SPF in discriminating between low- and high-grade lymphomas (area under the curve [AUC] = 0.996), with 3.15% being the best cut-off value (sensitivity 97.8%, specificity 100.0%) for identification of 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%; range 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. Therefore, 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 seven (6.2%) were aneuploid. These included three (42.9%) high-grade B-cell lymphomas (two immunoblastic and one centroblastic monomorphic), three (42.9%) high-grade T-cell lymphomas (two pleomorphic mixed small and large and one lymphoblastic) and one (14.3%) low-grade T-cell lymphoma (pleomorphic small cells).

The mean DI of aneuploid cases was 1.20 ± 0.07 (median 1.18; range 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 six different pure breeds. Three (42.9%) were females (one neutered) and four (57.1%) were males (one neutered). Mean age at diagnosis was 10.8 ± 2.3 years (median 11 years; range 7–14 years), but age was not reported in one case.

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

Survival Analyses

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

Breed was known in 32 cases, including 21 (65.6%) pure-breed dogs of 17 different breeds and 11 (34.4%) mixed-breed. Sex was known in 32 cases, with 16 (50%) entire males and 16 (50%) females (seven neutered). 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: three (10.3%) were classified as having stage IV disease and 26 (89.7%) as stage V. Eight (24.2%) dogs were in substage a and 25 (75.8%) were in substage b. Ki67 expression

was tested in 29 dogs: it was ≤20% in five (17.2%) dogs, between 20% and 40% in 16 (55.2%) and >40% in eight (27.6%) animals. SPF was ≤5.90% in six (18.2%) dogs out of 33, between 5.90% and 11.72% in 19 (57.6%) dogs and >11.72% in eight (24.2%) dogs. All dogs except one were diploid and 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 one 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 the 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 the log-rank test ($P = 0.042$); the *P*-value for univariate analysis was 0.063.

Ten (30.3%) dogs were censored for LSS analysis: seven were still alive at data analysis closure with a median follow-up of 528 days (range 28–872 days), while three died of lymphoma-unrelated causes after 34, 45 and 210 days, respectively. Overall median LSS was 365 days (range 15–1,086 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 34–1,086 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 between 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 28–1,086 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 (Bolon *et al.*, 1990; Fox *et al.*, 1990; Ayl *et al.*, 1992; Hellmen *et al.*, 1993; Teske *et al.*, 1993; Clemo *et al.*, 1994). In the present study, we describe the variation in SPF between different lymphoma subtypes in dogs, and its diagnostic and prognostic implications. We also describe a subset of aneuploid cases.

Identification of the grade of malignancy 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 (Ponce *et al.*, 2004; Valli *et al.*, 2013; Aresu *et al.*, 2015). Indeed, assessment of the mitotic index is required by all morphological classification schemes (either cytological or histopathological) (Greenle *et al.*, 1990; Teske *et al.*, 1994; Fournel-Fleury *et al.*, 1997). Morphological assessment is somewhat operator-dependent, and a slight level of intra- and interobserver disagreement may occur, even among the most experienced and specialized pathologists (Valli *et al.*, 2011). Therefore, tools that estimate the proliferation rate in histopathological sections of canine lymphomas more objectively were introduced decades ago, such as argyrophilic nucleolar organiser region (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). Therefore, strategies to quantify proliferation rate have been adapted to lymph node aspirates: AgNOR count can be performed with a silver stain on cytological smears (Bauer *et al.*, 2007) and Ki67 expression can be quantified either by immunolabelling 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 the face of a minimally invasive sampling procedure, is one of the major advantages of FC, and partially explains its wide use in veterinary oncology (Comazzi and Gelain, 2011; Burkhard and Bienzle, 2015). FC has been used also to assess Ki67 expression in canine lymphomas, therefore 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 advantages of FC (i.e. minimal invasiveness, rapidity and 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 must be excluded from DNA content analysis due to poor sample quality: we had to discard about 10% of the samples in the present study due to BAD >20% or CV >8%.

Based on our results, SPF discriminates accurately between high- and low-grade lymphomas in dogs. This is in contrast with the results obtained by Teske *et al.* (1993), who failed to detect any association between SPF and lymphoma malignancy. The difference in the samples used between the two studies (i.e. frozen tissue versus fresh nodal 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 updated Kiel scheme. Most of the cases in the study by Teske *et al.* were allocated to the intermediate grade, which is not covered in the Kiel classification used in the present study. Furthermore, Teske *et al.* did not stratify cases according to the neoplastic cell phenotype when analysing SPF data. In line with Teske *et al.* (1993), we found no prognostic significance for SPF. Different reasons may account for this lack of significant results in the two studies. Teske *et al.* included many different lymphoma subtypes in the survival analysis, 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 were 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 has been reported previously 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 of developing 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 the present case series, but if both parameters allow a reliable discrimination between high- and low- grade lymphomas, survival analyses gave significant results only for the latter. The difference in the information provided by the two techniques is probably due to the fact that all proliferating cells label positively for Ki67, while 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 for discriminating between 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, 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 and it can easily be run beside the immunophenotyping analysis. Moreover, the Ki67 protocol does not involve the use of hazardous reagents such as propidium iodide. SPF remains an excellent second option if the analysis must be delayed.

Contrasting results have been obtained also in human medicine concerning the clinical usefulness of DNA content analysis for non-Hodgkin's lymphomas: some studies claim a prognostic value for SPF and/or ploidy status (Rehn *et al.*, 1990; Joensuu *et al.*, 1991; Lackowska *et al.*, 1999; Pinto *et al.*, 2003), while other authors do not support 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 the specific subtypes of 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 the present case series (6.2%) was lower compared with that previously reported for 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 where two distinct G0/G1 peaks were identifiable, while Teske *et al.* 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 a high G0/G1 peak CV in our case series, while aneuploid cases with higher DI are more easily detected, irrespective of the CV.

Teske *et al.* (1993) found no prognostic relevance for ploidy status. We did not attempt a prognostic evaluation because of the low number of aneuploid cases. Interestingly, however, a complete follow up was available for three aneuploid T-cell lymphoma cases: the two high-grade cases died of lymphoma after 20 and 70 days, respectively, and the only low-grade case died of lymphoma after 111 days. This is in line with what has already been reported for dogs with high-grade T-cell lymphomas (Aresu *et al.*, 2015), while dogs with low-grade T-cell lymphoma commonly have a longer survival (Valli *et al.*, 2013; Martini *et al.*, 2016). 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 is required to clarify the potential diagnostic role of aneuploidy in dogs with lymphoma. Based on a recent study, numerical chromosomal aberrations found in the tumours of dogs with lymphoma are often present also in the peripheral blood. Therefore, the authors of that study hypothesized 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 speculative. 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 in assessing the grade of malignancy in LN aspirates from dogs with lymphoma. These data can be combined with morphological examination to improve objectivity in 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 Statement

The authors declare no conflict of interest with respect to the publication of this manuscript.

References

Aresu L, Martini V, Rossi F, Vignoli M, Sampaolo M *et al.* (2015) Canine indolent and aggressive lymphoma: clinical spectrum with histologic correlation. *Veterinary and Comparative Oncology*, **13**, 348-362.

Ayl RD, Couto CG, Hammer AS, Weisbrode S, Ericson JG *et al.* (1992) Correlation of DNA ploidy to tumor histologic grade, clinical variables, and survival in dogs with mast cell tumours. *Veterinary Pathology*, **29**, 386-390.

Bauer KD, Bagwell B, Giaretti W, Melamed M, Zarbo RJ *et al.* (1993) Consensus review of the clinical utility of DNA flow cytometry in colorectal cancer. *Cytometry*, **14**, 486-491.

Bauer NB, Zervos D, Moritz A (2007) Argyrophilic nucleolar organizing regions and Ki67 equally reflect proliferation in fine needle aspirates of normal, hyperplastic, inflamed, and neoplastic canine lymph nodes (*n* = 101). *Journal of Veterinary Internal Medicine*, **21**, 928-935.

Bolon B, Calderwood Mays MB, Hall BJ (1990) Characteristics of canine melanomas and comparison with histology and DNA ploidy to their biologic behaviour. *Veterinary Pathology*, **27**, 96-102.

Burkhard MJ, Bienzle D (2015) Making sense of lymphoma diagnostics in small animal patients. *Clinics in Laboratory Medicine*, **35**, 591-607.

Clemo FA, DeNicola DB, Carlton WW, Morrison WB, Walker E (1994) Flow cytometric DNA ploidy analysis in canine transitional cell carcinoma of urinary bladders. *Veterinary Pathology*, **31**, 207-215.

Comazzi S, Gelain ME (2011) Use of flow cytometric immunophenotyping to refine the cytological diagnosis of canine lymphoma. *Veterinary Journal*, **188**, 149-155.

Darzynkiewicz Z, Huang X, Zhao H (2017) Analysis of cellular DNA content by flow cytometry. *Current Protocols in Cytometry*, **82**, 7.5.1-7.5.20.

Devitt JJ, Maranon DJ, Ehrhart EJ, Bachand AM, Lana SE *et al.* (2009) Correlations between numerical chromosomal aberrations in the tumor and peripheral blood in canine lymphoma. *Cytogenetic and Genome Research*, **124**, 12-18.

Dictor M, Ehinger M, Mertens F, Akervall J, Wennerberg J (1999) Abnormal cell cycle regulation in malignancy. *American Journal of Clinical Pathology*, **112**, S40-S52.

Fouad YA, Aanei C (2017) Revisiting the hallmarks of cancer. *American Journal of Cancer Research*, **7**, 1016-1036.

Fournel-Fleury C, Magnol JP, Bricaire P, Marchal T, Chabanne L *et al.* (1997) Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. *Journal of Comparative Pathology*, **117**, 35-59.

Fox MH, Armstrong LW, Withrow SJ, Powers BE, LaRue SM (1990) Comparison of DNA aneuploidy of primary and metastatic spontaneous canine osteosarcomas. *Cancer Research*, **50**, 6176-6178.

Garrett LD, Thamm DH, Chun R, Dudley R, Vail DM (2002) Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. *Journal of Veterinary Internal Medicine*, **16**, 704-709.

Gelain ME, Mazzilli M, Riondato F, Marconato L, Comazzi S (2008) Aberrant phenotypes and quantitative antigen expression in different subtypes of canine lymphoma by flow cytometry. *Veterinary Immunology and Immunopathology*, **12**, 179-188.

Golias CH, Charalabopoulos A, Charalabopoulos K (2004) Cell proliferation and cell cycle control: a mini review. *International Journal of Clinical Practice*, **58**, 1134-1141.

Greenlee PG, Filippa DA, Quimby FW, Patnail AK, Calvano SE *et al.* (1990). Lymphomas in dogs. A morphologic, immunologic, and clinical study. *Cancer*, **66**, 480-490.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell*, **100**, 57-70.

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell*, **144**, 646-674.

Hellmen E, Bergstrom R, Holmberg L, Spangberg IB, Hansson K *et al.* (1993) Prognostic factors in canine mammary tumours: a multivariate study of 202 consecutive cases. *Veterinary Pathology*, **30**, 20-27.

Joensuu H, Klemi PJ, Söderström KO (1991). Comparison of S-phase fraction, working formulation, and Kiel classification in non-Hodgkin's lymphoma. *Cancer*, **68**, 1564-1571.

Kaufman DG, Cordeiro-Stone M, Brylawski BP, Cohen SM, Chastain PD (2007) Early S phase DNA replication: a search for targets of carcinogenesis. *Advances in Enzyme Regulation*, **47**, 127-138.

Kim KH, Sederstrom JM (2015) Assaying cell cycle status using flow cytometry. *Current Protocols in Molecular Biology*, **111**, 28.6.1-28.6.11.

Kiupel M, Bostock D, Bergmann V (1998) The prognostic significance of AgNOR counts and PCNA-positive cell counts in canine malignant lymphomas. *Journal of Comparative Pathology*, **119**, 407-418.

Kiupel M, Teske E, Bostock D (1999) Prognostic factors for treated canine malignant lymphoma. *Veterinary Pathology*, **36**, 292-300.

Lackowska B, Gruchala A, Niezabitowski A, Radkowski A, Skołyszewski J *et al.* (1999) Prognostic and predictive evaluation of DNA content, S-phase fraction and immunophenotyping in non-Hodgkin's lymphomas in adults: a prospective study. *Polish Journal of Pathology*, **50**, 23-29.

Lackowska B, Gruchala A, Jaszcz-Gruchala A, Rolski J, Zemełka T *et al.* (2012) Diagnostic, predictive and prognostic verification of DNA flow cytometric measurements performed at diagnosis for non-Hodgkin's lymphoma adult patients. *Polish Journal of Pathology*, **1**, 18-24.

Martini V, Marconato L, Poggi A, Riondato F, Aresu L *et al.* (2016) Canine small clear cell/T-zone lymphoma: clinical presentation and outcome in a retrospective case series. *Veterinary and Comparative Oncology*, **14 (Suppl. 1)**, 117-126.

Ormerod MG, Tribukait B, Giaretti W (1998) Consensus report of the task force on standardization of DNA flow cytometry in clinical pathology. *Analytical Cellular Pathology*, **17**, 103-110.

Owen L (1980) *TNM Classification of Tumours in Domestic Animals*. World Health Organization, Geneva, pp. 46-47.

Phillips BS, Kass PH, Naydan DK, Winthrop MD, Griffey SM *et al.* (2000) Apoptotic and proliferation indexes in canine lymphoma. *Journal of Veterinary Diagnostic Investigation,* **12**, 111-117.

Pinto AE, Cabeçadas J, Nóbrega SD, Mendonça E (2003) Flow cytometric S-phase fraction as a complementary biological parameter for the cytological grading of non-Hodgkin's lymphoma. *Diagnostic Cytopathology*, **29**, 194-199.

Poggi A, Miniscalco B, Morello E, Comazzi S, Gelain ME *et al.* (2015) Flow cytometric evaluation of Ki67 for the determination of malignancy grade in canine lymphoma. *Veterinary and Comparative Oncology*, **13**, 475-480.

Poggi A, Miniscalco B, Morello E, Gattino F, Delaude A *et al.* (2017) Prognostic significance of Ki67 evaluated by flow cytometry in dogs with large B-cell lymphoma. *Veterinary and Comparative Oncology*, **15**, 431-440.

Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V *et al.* (2004) Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. *Veterinary Journal*, **167**, 158-166.

Regan RC, Kaplan MS, Bailey DB (2013). Diagnostic evaluation and treatment recommendations for dogs with substage-a high grade multicentric lymphoma: results of a survey of veterinarians. *Veterinary and Comparative Oncology*, **11**, 287-295.

Rehn S, Glimelius B, Strang P, Sundström C, Tribukait B (1990). Prognostic significance of flow cytometry studies in B-cell non-Hodgkin lymphoma. *Hematological Oncology*, **8**, 1-12.

Sansregret L, Swanton C (2017) The role of aneuploidy in cancer evolution. *Cold Spring Harbor Perspectives in Medicine*, **7**, a028373.

Sayag D, Fournel-Fleury C, Ponce F (2018) Prognostic significance of morphotypes in canine lymphomas: a systematic review of literature. *Veterinary and Comparative Oncology*, **16**, 12-19.

Sokolowska J, Micun J, Malicka E, Lechowski R (2012) Proliferation activity in canine lymphomas. *Polish Journal of Veterinary Sciences*, **15**, 727-734.

Sun Y, Yang K, Bridal T, Ehrhardt AG (2016) Robust Ki67 detection in human blood by flow cytometry for clinical studies. *Bioanalysis*, **8**, 2399-2413.

Teske E, Rutteman GR, Kuipers-Dijshoorn NJ, van Dierendonck JH, van Heerde P *et al.* (1993) DNA ploidy and cell kinetic characteristics in canine non-Hodgkin's lymphoma. *Experimental Hematology*, **21**, 579-584.

Teske E, van Heerde P (1996) Diagnostic value and reproducibility of fine-needle aspiration cytology in canine malignant lymphoma. *Veterinary Quarterly*, **18**, 112-115.

Teske E, Wisman P, Moore PF, van Heerde P (1994) Histologic classification and immunophenotyping of canine non-Hodgkin's lymphomas: unexpected high frequency of T-cell lymphomas with B cell morphology. *Experimental Hematology*, **22**, 1179-1187.

Valli VE, San Myint M, Barthel A, Bienzle D, Caswell J *et al.* (2011) Classification of canine malignant lymphomas according to the World Health Organization criteria. *Veterinary Pathology*, **48**, 198-211.

Valli VE, Kass PH, San Myint M, Scott F (2013) Canine lymphomas: association of classification type, disease stage, tumor subtype, mitotic rate and treatment with survival. *Veterinary Pathology*, **50**, 738-748.

Villamil JA, Henry CJ, Hahn AW, Bryan JN, Tyler JW *et al.* (2009) Hormonal and sex impact on the epidemiology of canine lymphoma. *Journal of Cancer Epidemiology*, **2009**, 591753.

Vincent MD (1987) The definition of a neoplasm. *Medical Hypotheses*, **24**, 151-160.

Willis RA (1952) *The Spread of Tumors in the Human Body*. Butterworth & Co, London.

Wilkerson MJ (2012) Principles and applications of flow cytometry and cell sorting in companion animal medicine. *Veterinary Clinics of North America: Small Animal Practice*, **42**, 53-71.

Wiman KG, Zhivotovsky B (2017) Understanding cell cycle and cell death regulation provides novel weapons against human diseases. *Journal of Internal Medicine*, **281**, 483-495.

Winter JN, Andersen J, Variakojis D, Gordon LI, Fisher RI *et al.* (1996) Prognostic implications of ploidy and proliferative activity in the diffuse, aggressive non-Hodgkin's lymphomas. *Blood*, **88**, 3919-3925.

Fig.1. Distribution of S-phase fraction values within the four subtypes of lymphoma considered in the study (high grade B-cell, low grade B-cell, high grade T-cell, and low grade T-cell).

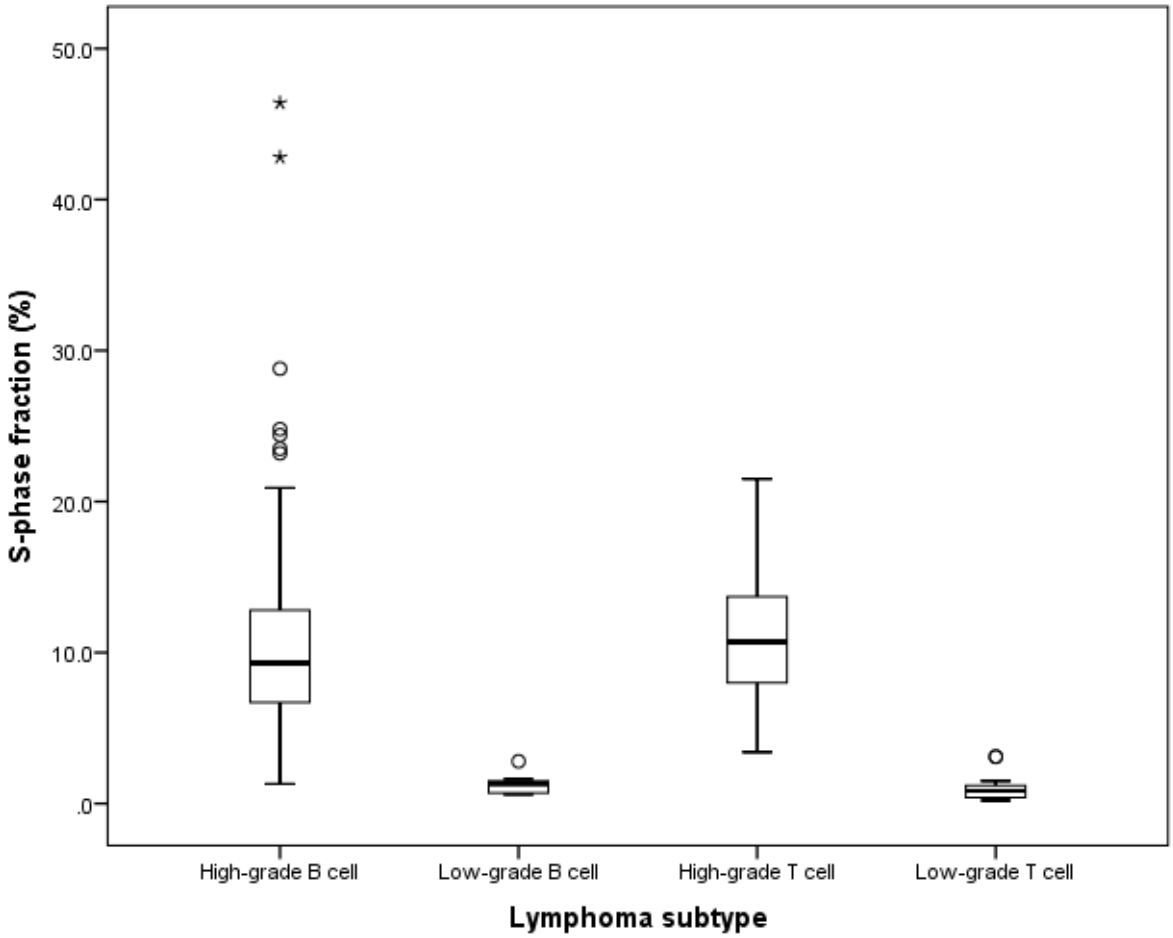

Table 1

S-phase fraction (SPF) in 112 cases of canine nodal lymphoma

Lymphoma subtype	*Number of cases*	*SPF (%) Mean ± SD (median; range)*
High-grade B cell	71	11.35 ± 8.11 (9.30; 1.3–46.4)
Centroblastic polymorphic	41	11.45 ± 8.17 (9.30; 4.0–46.4)
Centroblastic monomorphic	16	13.90 ± 9.23 (11.55; 4.4–42.8)
Immunoblastic	9	6.30 ± 3.57 (6.30; 1.3–12.6)
Lymphoblastic	4	13.43 ± 6.86 (11.60; 7.3–23.2)
Plasmacytoid	1	3.2
High-grade T cell	20	10.95 ± 4.60 (10.70; 3.4–21.5)
Pleomorphic mixed small and large	9	9.61 ± 4.28 (9.50; 3.4–16.6)
Pleomorphic large	6	12.95 ± 3.14 (13.55; 9.0–17.8)
Lymphoblastic	4	10.83 ± 7.42 (8.65; 4.5–21.5)
Plasmacytoid	1	11.4
Low-grade T cell	14	1.08 ± 0.94 (0.85; 0.2–3.1)
Small clear	10	0.62 ± 0.33 (0.60; 0.2–1.2)
Pleomorphic small	3	2.57 ± 0.92 (3.1; 1.5–3.1)
Prolymphocytic	1	1.2
Low-grade B cell	7	1.30 ± 0.77 (1.30; 0.6–2.8)
Macronucleolated medium-sized	6	1.05 ± 0.43 (1.00; 0.6–1.6)
Prolymphocytic	1	2.8

Cases classified according to the updated Kiel classification scheme (Fournel-Fleury *et al.*, 1997)