Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples

Journal:	*Journal of Feline Medicine and Surgery*
Manuscript ID	JFMS-17-0025.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Martini, Valeria; Department of Veterinary Medicine Bernardi, Serena; Department of Veterinary Medicine Marelli, Priscilla; Department of Veterinary Medicine Cozzi, Marzia; Department of Veterinary Medicine Comazzi, Stefano; Department of Veterinary Medicine
Keywords:	feline, flow cytometry, lymphoma, sample quality, preanalytical variability, needle size
Abstract:	Objective Flow cytometry (FC) is becoming increasingly popular among veterinary oncologists for the diagnosis of lymphoma or leukemia. It is accurate, fast, and minimally invasive. Several studies on FC have been carried out in canine oncology and applied with great results, whereas there is limited knowledge and use of this technique in feline patients. This is mainly due to the high prevalence of intra-abdominal lymphomas in this species and the associated discomfort in the diagnostic procedures needed to collect the sample. The purpose of the present study is to investigate whether any pre-analytical factor might affect the quality of suspected feline lymphoma samples for FC analysis. Methods 97 consecutive samples of suspected feline lymphoma were retrospectively selected from the authors' institution FC database. The referring veterinarians were contacted and interviewed about several different variables, including signalment, appearance of the lesion, features of the sampling procedure and the experience of veterinarians performing the sampling. Statistical analyses were performed to assess the possible influence of these variables on the cellularity of the samples and the likelihood of it being finally processed for FC. Results Sample cellularity is a major factor in the likelihood of the sample being processed. Moreover, sample cellularity was significantly influenced by the needle size, with 21G needles providing the highest cellularity. Notably, the sample cellularity and the likelihood of being processed did not vary between peripheral and intra-abdominal lesions. Approximately half of the cats required pharmacological restraint. Side effects were reported in one case only (transient swelling after peripheral lymph node sampling). Conclusion and relevance FC can be safely applied to cases of suspected feline lymphomas, including intra-abdominal lesions. 21G needle should be preferred for sampling. This study provides the basis for the increased use of this minimally invasive, fast and cost-effective technique in feline medicine.

Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples

Martini V[1], Bernardi S[1], Marelli P[1], Cozzi M[1], Comazzi S[1].

[1] Department of Veterinary Medicine, University of Milan, Milan, Italy

Corresponding author:

VALERIA MARTINI, DVM, PhD

Department of Veterinary Medicine

University of Milan

Via Celoria 10 20133 Milan, Italy

Phone: +39 0250318153

valeria.martini@unimi.it

Keywords: feline, flow cytometry, lymphoma, sample quality, preanalytical variability, needle size

Abstract

Objective Flow cytometry (FC) is becoming increasingly popular among veterinary oncologists for the diagnosis of lymphoma or leukemia. It is accurate, fast, and minimally invasive. Several studies on FC have been carried out in canine oncology and applied with great results, whereas there is limited knowledge and use of this technique in feline patients. This is mainly due to the high prevalence of intra-abdominal lymphomas in this species and the associated discomfort in the diagnostic procedures needed to collect the sample. The purpose of the present study is to investigate whether any pre-analytical factor might affect the quality of suspected feline lymphoma samples for FC analysis.

Methods 97 consecutive samples of suspected feline lymphoma were retrospectively selected from the authors' institution FC database. The referring veterinarians were contacted and interviewed about several different variables, including signalment, appearance of the lesion, features of the sampling procedure and the experience of veterinarians performing the sampling. Statistical analyses were performed to assess the possible influence of these variables on the cellularity of the samples and the likelihood of it being finally processed for FC.

Results Sample cellularity is a major factor in the likelihood of the sample being processed. Moreover, sample cellularity was significantly influenced by the needle size,

with 21G needles providing the highest cellularity. Notably, the sample cellularity and the likelihood of being processed did not vary between peripheral and intra-abdominal lesions. Approximately half of the cats required pharmacological restraint. Side effects were reported in one case only (transient swelling after peripheral lymph node sampling).

Conclusion and relevance FC can be safely applied to cases of suspected feline lymphomas, including intra-abdominal lesions. 21G needle should be preferred for sampling. This study provides the basis for the increased use of this minimally invasive, fast and cost-effective technique in feline medicine.

Introduction

Lymphoproliferative malignancies are fairly common in dogs and cats.[1] In the canine species, the diagnosis of lymphoma and leukaemia is nowadays easy, fast and minimally invasive thanks to useful tools like flow cytometry (FC).[2] FC is widely used in canine oncology, not only for the determination of neoplastic cell lineage, but also because of the increasing number of available leucocyte markers, which strengthens the chance to recognize different lymphoma subtypes.[3,4] Moreover, in dogs FC allows to assess the stage of the tumour or the minimal residual disease (MRD) after treatment.[5,6]

In cats, the prevalence of lymphoma is believed to be high: in the 1970s and 1980s one third of feline neoplasms was estimated to be of hematopoietic origin, either lymphoid or myeloid, in association with a high prevalence of feline leukaemia virus (FeLV) infections. Since late 1980s the situation has changed: the prevalence of FeLV+ cats and associated forms of hematopoietic tumours decreased thanks to efficient diagnostic tests, vaccination and infected cats isolation, whereas the number of not-FeLV-related forms of lymphoid tumours increased (mostly alimentary and cutaneous forms).[7]

Cytology of suspected feline lymphomas is often heterogeneous and thus generally not conclusive for neoplasia. Histopathology and immunohistochemistry remain the gold standard for the diagnosis and immunophenotyping of feline lymphoma,[8] but these techniques are invasive, as they require a biopsy specimen, and time-expensive, as some days are needed for results.

FC could overcome these limits, although only a single study has been published so far on this topic.[9] In spite of the high prevalence of lymphoma in cats, the application of FC for the diagnosis and characterization of this tumour in the feline species is still limited and considered challenging for several possible reasons.[10] Firstly, because of the high prevalence of intra-abdominal forms, which are anecdotally reported to yield poor quality samples because of sampling difficulties. Secondly, the availability of species-specific monoclonal antibodies (mAbs) binding to feline leukocyte differentiation antigens (LDA) is restricted for FC application and only a few studies have been conducted for evaluation of cross reactivity with mAbs of other species.[11,12]

The general thought concerning the difficulty of obtaining good quality samples from abdominal lesions in cats is unsubstantiated, and has never been documented (nor contradicted) in the scientific literature. Going the long way round, to the authors' knowledge, there are no published studies concerning the quality of samples for FC analysis of feline lymphomas.

The aim of the present study was to evaluate if and which specific pre-analytical factor could affect the quality of feline lymphoma samples for FC and, as a consequence, the likelihood of being processed. Factors taken into account concerned both sampling and processing procedures. This might be an important assessment in order to provide useful indications for a good quality sampling for FC in cats with suspected lymphomas.

The use of FC may improve the diagnosis and classification of feline lymphomas, raising it to the levels achieved in the dog. However, the assessment of the diagnostic accuracy of FC for feline lymphomas does not fall within the aims of the present study, as it has already been described in the literature.[9]

Materials and methods

The database of the FC service of the authors' Institution from January 2009 to February 2016 was interrogated and feline cases were extracted. Inclusion criteria were: 1) cases sent to the laboratory for suspected lymphoma; 2) request for FC immunophenotyping of the primary lesion, including effusions. Cases were excluded from the study if only peripheral blood and/or bone marrow samples had been sent to the laboratory.

For each case, data concerning the subject, the lesion, the sampling technique, the ancillary tests performed as well as data concerning the clinician who collected the sample were requested from the referring veterinarian, if not provided at the time of sample submission (Table 1). Since no objective parameters exist to assess and quantify the veterinarians' clinical experience, this was evaluated with two parameters set for this study. These latter were both the timespan between the veterinarians license acquisition and the sample collection (years of expertize) and the presence of any post-

graduate specialization, irrespective of the type of specialization and its specific field of application (whether clinical or not). If samples from multiple sites were available for a single animal, the data were recorded for each sample independently. Additional data were retrieved from the FC database (Table 1).

All FC data were reviewed by a single operator (VM) who was aware of the cytological diagnosis (when available) but was blinded to the previous FC report and to all other tests performed on the lesion.

Flow cytometry

FC was performed on tissue aspirates collected in a liquid medium (either saline solution or RPMI 1640) or on effusions collected in EDTA-tubes with an adaptation of a previously published procedure.[9]

Prior to labelling, all samples were counted via an automated haematology analyser (Sysmex XT-2000iV, Sysmex, Kobe, Japan) to assess cellularity. Also, a visual inspection of the sample was made by the operator, to assess both the total volume of the sample and the presence of artifacts such as gross hemocontamination, clots, necrotic material, or any other abnormality. Based on all these parameters, the operator dealing with each sample decided whether to admit it to FC or not, based on his/her subjective opinion and experience.

The antibody panel varied among samples: indeed, for samples processed between January 2009 and December 2010, a single-color approach was used with the inclusion of a FITC-conjugated secondary antibody (rabbit anti-mouse IgG-FITC, polyclonal, Serotec), whereas a multi-colour approach was used for samples processed between January 2011 and February 2016 (CD5-FITC/CD21-PE/CD18-AlexaFluor647; CD4-FITC/CD8-PE/CD18-AlexaFluor647). Antibody clones and source are listed in Table 2.

All samples were acquired with a FACScalibur flow cytometer (Becton Dickinson, San Josè, CA, USA) and analysed with a specific software (CellQuest, Becton Dickinson).

Statistical analysis

Statistical analyses were performed to assess whether the cellularity of FC samples and the likelihood of samples to be finally processed for FC were influenced by any pre-analytical variable (Table 1).

To this aim, continuous variables were arbitrarily categorized as follows: age of cat (≤1 or >1 year); years of experience of the vet (i.e. timespan between veterinarian license acquisition and sample collection, <15 or ≥15 years). The lesions were classified into five groups based on their site: peripheral lymph nodes (pLN), abdominal lymph nodes or masses (aLN), thoracic lymph nodes or masses (tLN), effusions (grouped altogether irrespective of their primary location, either thoracic or abdominal), and any other site.

Contingency tables were prepared for each of the investigated variables, and the Pearson χ^2 test was performed to assess their possible association with the likelihood of samples to be processed for FC.

Shapiro-Wilk test was performed to assess whether the FC samples cellularity was normally distributed. Then, Student t-test, Mann-Whitney test, ANOVA or Kruskal-Wallis test were performed to compare the mean sample cellularity among different categories, based on the data distribution (normal or not) and the number of groups (2 or more). The Kruskal-Wallis test was also performed to compare the mean cellularity among samples with different FC diagnosis (conclusive for lymphoma, negative for lymphoma, not conclusive): this analysis was restricted to samples finally processed for FC. When significant results were obtained, appropriate post-hoc tests were performed based on data distribution and homoscedasticity assessment (Mann-Whitney, Bonferroni or Dunnett test).

Initially, all samples were included in the analyses. Thereafter, samples were investigated according to the five lesion site groups.

All analyses were performed with SPSS v20.0 for Windows. Significance was set at $P \leq 0.05$ for all tests.

Results

105 consecutive suspect lymphoma samples were retrospectively extracted from the FC service's database of DIVETLAB (Department of Veterinary Medicine, University of Milan). Eight were then excluded from the study for different causes: three were delivered to the laboratory 48 hours after sampling, three were sampled after the administration of chemotherapy. Lastly, in two cases the sampling was repeated twice because the first one had a low cellular concentration and had not been processed: in these two cases, only the first (poorly cellular) sample was included in the study. Thus, 97 samples were finally included in the present study, from 86 different feline patients: 73 (75.3%) out of these samples were analysed for FC, while the remaining 24 (24.7%) were discarded by the operator. Thirty-one (32%) samples were collected before 2011 and were analysed with a single-color approach while 66 (68%) samples were collected subsequently and analysed with a multi-colour approach. The proportion of samples finally processed for FC did not vary with year of sampling, nor with the FC approach used.

Total nucleated cell count (TNCC) was reported for 91 samples, with a mean of 12.96 ± 21.19 x 10^3 cells/µL (median: 3.11 x 10^3 cells/µL; minimum-maximum: 0.01-89.88 x 10^3 cells/µL). In particular, it was significantly higher in samples eventually processed for FC (mean: 14.78 ± 22.12 x 10^3cells/µL; median: 4.09 x 10^3 cells/µL; minimum-maximum: 0.16-89.88 x 10^3 cells/µL) than in discarded samples (mean 7.26 ± 17.20 x 10^3cells/µL; median: 0.54 x 10^3 cells/µL; minimum-maximum: 0.01-58.02 x 10^3

cells/µL; P=0.000). None of the remaining variables significantly influenced the likelihood of samples of being finally processed for FC.

In turn, TNCC was significantly influenced by the size of the needle and by the presence of post-graduate specialization of the veterinarian performing the sampling. The 21 G needle gave the most cellular samples, with a statistically significant difference from the other needles (P=0.045). Size of the needles with relative average cellular concentration are listed in Table 3. Of 37 samples for which this information was available, 33 (89.2%) were collected by veterinarians with post-graduate qualifications: veterinarians with post graduate qualifications collected samples with an average cellular concentration of 9.42 ± 19.86 $x10^3$ cells/µL (median 1.71 $x10^3$ cells/µL; minimum-maximum 0.01-87.54 $x10^3$ cells/µL), whereas other first opinion veterinarians collected samples with an average cellular concentration of 33.16 ± 29.5 $x10^3$ cells/µL (median 34.01 $x10^3$ cells/µL; minimum-maximum 5.35-59.26 $x10^3$ cells/µL). The difference was statistically significant (p=0.027).

None of the other variables (including lesion site and size) gave significant results. Raw results are listed below.

Breed was known for 75 cats: 64 (85.3%) domestic shorthair (DSH), 4 (5.3%) Maine Coon, 2 (2.7%) British shorthair, 2 (2.7%) Chartreux, 2 (2.7%) Persian and 1 (1.3%) Norwegian Forest. Sex was known for 81 cats: 18 (22.2%) were intact females, 21 (25.9%) were neutered females, 9 (11.1%) were intact males and 33 (40.7%) were

neutered males. Age was known for 85 cats, who aged from 5 months to 16 years, with a median age of 8 years. FIV/FeLV status was known only for 16 patients: 7 (43.8%) were negative for both, 7 (43.8%) were FeLV+ and 2 (12.5%) were FIV+.

The site of the lesion was known for all 97 samples: 24 (24.7%) pLN, 21 (21.6%) aLN, 21 (21.6%) tLN, 17 (17.5%) effusions and 14 (14.4%) other sites, including skin, kidney, spleen, liver and urine. Lesion's size was known for 49 samples: 13 (26.5%) were beneath 2 cm, 22 (44.9%) were between 2 and 5 cm and 14 (28.6%) measured more than 5 cm.

Concerning cytology, 67 samples were received with a cytological report: of these, 30 (44.8%) were conclusive for lymphoma, for 16 (23.9%) lymphoma was suspected with different confidence levels (diagnostic but not conclusive), for 9 (13.4%) lymphoma was excluded, 1 (1.5%) was diagnostic for thymoma (for a total of 10 lymphoma-negative samples) and 11 (16.4%) were considered non-diagnostic because of poor cellular concentration, high hemodilution or poor quality of the preparation.

The method of sampling for FC was known for 66 samples: 16 (24.2%) were made by blind aspiration, 41 (62.1%) ultrasound-guided, 7 (10.6%) computed tomography-guided, 1 (1.5%) was obtained by surgical access and 1 (1.5%) by urethral catheterization. Patient waking condition was known for 53 samples: 24 (45.3%) were awake, 17 (32.1%) needed mild sedation, 12 (22.6%) needed general anaesthesia.

Of the 44 patients for which this information was available, side effects of sampling were reported in one case only: this cat showed a transient mild swelling in the sampling region (submandibular lymph node).

Transport medium was known for 58 aspirates: 6 (10.3%) were collected in saline solution and 52 (89.7%) were collected in culture medium (RPMI or DMEM). All effusions were collected in EDTA tubes.

Concerning the experience of the clinician performing the sampling, the timespan between graduation and sample collection was < 15 years for 25 (46.3%) samples and > 15 years for 29 (53.7%) samples.

As a second step, the statistical analyses were performed including samples according to the five different lesion sites (pLN, aLN, tLN, effusions, and other sites). Results are reported in the supplementary materials (Tables S1 to S10). Significant differences were noted only within the tLN group: clinicians without postgraduate qualifications collected samples with a higher cellularity (P=0.036).

Finally, cellular concentration was evaluated according to the FC diagnosis. TNCC was not recorded for 4 samples. The remaining 69 processed samples were divided in three categories: positive for lymphoma, negative for lymphoma and non-diagnostic. TNCC significantly varied among the three groups (P=0.022; Table 4): in particular, non-

diagnostic samples were less cellular than lymphoma and non-lymphoma samples (P=0.009 and P=0.040, respectively). The difference in TNCC according to FC diagnosis remained significant also within the pLNs and effusions groups (P=0.029 for both): the significant difference was between lymphoma and non-diagnostic samples for both groups (P=0.016 for pLNs and P=0.036 for effusions). TNCC values are shown in Table 5 and 6.

Discussion

FC is widely used in human medicine and its use in veterinary medicine has been increasing in the last years, especially for canine lymphoproliferative diseases. In the canine species, this diagnostic tool turned out to be very helpful for a rapid and non-invasive lymphoma diagnosis.[2] Moreover, some studies have been published in the last years about the prognostic value of the flow cytometric immunophenotype in this species.[5,13,14]

Nevertheless, in the feline species, FC is not commonly used; to the authors' knowledge, it was never described in the last decade until last year, when Guzera *et al* published the first scientific study about the application of FC in the diagnosis of feline lymphoma, highlighting the diagnostic accuracy of this technique.[9]

Being most of feline lymphomas localized in the intra-abdominal region, reaching the primary lesion with a needle might be uncomfortable for the clinician, and mild or general anaesthesia may be necessary. Based on this general thought, FC is usually not included in the diagnostic workup for suspected feline lymphomas. The lack of confidence in this technique is confirmed by the fact that feline samples represent only about 2% of the samples included in the authors' FC database in the last seven years (2009-2016).

The study published by Guzera *et al*[9] and the present study somehow deny this common belief, because a high number of samples in both studies were likely to be processed and to be diagnostic. In the present study, 75% of samples were finally processed for FC; of these, only 20% were non-diagnostic and they had a lower cellular concentration compared to the diagnostic samples. In our laboratory, samples are usually admitted to be processed for FC only if suitable to be analysed with the whole antibody panel, irrespective of the FC approach used (single- VS multi-colour). The percentage of processed samples in the study by Guzera and colleagues[9] was slightly higher, but only a limited antibody panel was applied to a subset of samples, which may explain this discrepancy between the two studies. Summarizing the results of the two studies, we could state that 75-85% of feline samples is suitable for FC analysis. Samples with a low cellular concentration could still be investigated through a more limited panel of antibodies, although they are less likely to be of diagnostic usefulness.

Based on our results, cellular concentration is a key-point in the discrimination between samples suitable or unsuitable for FC. Still, some poorly cellular samples were admitted to processing and, *vice versa*, some highly cellular samples were not. The choice whether to process or not the samples was left to the operator dealing with the sample, and was likely based also on other features, resulting from the visual inspection of the sample, together with the TNCC. Unfortunately, these data were not reported in the FC records and their influence on TNCC and on the likelihood of samples of being processed could not be evaluated in the present study. Still, gross inspection of the specimen is recommended, before admission to processing for FC.

In our case series, similar numbers of pLN, aLN and tLN were present, in spite of the higher prevalence of alimentary lymphoma reported in cats.[15,16] One possible explanation for this discrepancy is the presence of many non-lymphoma cases. Another possible reason is that clinicians prefer not to sample hardly reachable lesions such as gastro-intestinal lesions, spleen, liver or aLN, due to a supposed poor quality of the sample. Still, our data support the application of FC even for intra-abdominal lesions, as these had the same likelihood of being finally processed for FC than the aspirates taken from peripheral lymph nodes (which are expected to be more comfortably reached).

Although sedation or anaesthesia of the cat may be of aid to the clinician during specimen collection, these did not ensure to catch higher quality samples. Thus, they are not essential and the choice whether to use them or not should only be based on the

297 cat's character. The possible occurrence of side effects might worry the operator as
298 well. However, for the cases included in the present study, no side effects have been
299 reported following intra-thoracic/abdominal fine needle aspiration (FNA), but just one
300 patient had a transient swelling after FNA of a pLN.

301 Among all the factors we evaluated, only two of them affected significantly the cellular
302 concentration of samples: the size of the needle and the post-graduation qualification of
303 the clinician.

304 The results show that 21G needle, a medium size needle, is related with a higher cellular
305 concentration of the sample. On one hand, smaller needles could damage the cells, that
306 are more fragile for their neoplastic origin, and necrotic or clotted material or
307 connective tissue could plug the needle. On the other hand, larger needles could be more
308 traumatic on the tissue, producing bleeding and thus contamination of the sample with
309 too much blood and other surrounding tissues (necrosis, connective, fat). Also, the small
310 number of samples collected with different needle sizes may have influenced the
311 statistical results. Still, following these results, the advice is to use 21G needle to have
312 good quality samples.

313 The fact that less cellular samples came from theoretically more qualified veterinarians
314 is surprising: the most probable explanation for this result is a statistical artefact due to
315 few samples coming from operators without post-graduate qualification (4). Most of

these clinicians regularly send canine samples to our FC service, so they have a steady practical experience in sampling for this purpose. Otherwise, new inexperienced operators may have asked to the FC service's staff for instructions about sampling, transport, medium and sample storage prior to sampling. However, this result is of questionable value and should be better addressed by future studies.

One of the most important result from the present study is that the likelihood of processing the sample and the cellular concentration are not affected by size and site of the lesion, unlike what has been thought until now. This makes FC appropriate for application in the feline species, even if lesions are intra-abdominal or thoracic.

In our case series, less than a half of cytological preparations was conclusive for lymphoma: despite this poor diagnostic value of cytology alone for the diagnosis of feline lymphomas, this test remains a mandatory first step in the diagnostic workup. In addition, a subset of samples in the present study were sent to our laboratory with a suspect of lymphoma even in face of a negative cytological report. Although the referring veterinarians have provided no clear explanation for this phenomenon, it may be considered a proof of the scarce confidence of clinicians toward negative cytological reports, when a strong suspicion of neoplasia is present based on clinical signs and imaging. The diagnostic performances of cytology alone and cytology plus FC is still to be elucidated in a clinical setup.

This is only the second paper published concerning FC as a diagnostic tool for feline lymphoma patients. Clinicians are only slightly familiar with this technique and there is the need to enhance their confidence, based on its promising large spread in the human and canine species. Thus, we support the contemporary sampling for FC and histopathology/immunohistochemistry in cases of suspected feline lymphomas: this would provide a rapid report (within 24 hours) from FC and a subsequent confirmation and more detailed classification from histology/immunohistochemistry.

The retrospective formulation of the present study is its main limitation: information collected were often incomplete and there was no standard sampling procedure. Confirmation will be necessary in the future, through a prospective sample collection and a complete submission of the case. Another main limitation of the present study is the lack of a confirmatory test, as histology was available only for few cases (data not shown) and PARR for none. This prevented us from assessing the diagnostic accuracy of FC for feline lymphomas; anyway, Guzera et al. already evaluated it in their study, though on a narrow sample.[9]

Conclusions

The results of the present study show how FC can be used for immunophenotyping in feline lymphomas, regardless of the site and the size of the lesion sampled. The use of

21G needles may enhance the probability to catch highly cellular samples. This is a pilot study aimed at making FC more widely known in the feline medicine world, and future studies are necessary to make this tool as useful as it currently is in dogs, from both a diagnostic and a prognostic point of view.

Supplementary material

Results obtained from the analyses within lesion-site groups are available as Supplementary material.

Funding

The authors received no financial support for the research, authorship and/or publication of this article.

Conflict of interest

The authors declared no potential conflicts of interest with respect to research, authorship and/or publication of this article.

References

1. Vail DM, Pinkerton ME and Young KM. Hematopoietic tumors. In: SJ Withrow, DM Vail and RL Page (eds) *Withrow and MacEwen's Small Animal Clinical Oncology.* St. Louis, MO: Elsevier Health Sciences, 2013, pp. 608-637
2. Comazzi S and Gelain ME. Use of flow cytometric immunophenotyping to refine the cytological diagnosis of canine lymphoma. *Vet J* 2011; 188(2): 149-155
3. Seelig DM, Avery P, Webb T et al. Canine T-zone lymphoma: Unique immunophenotypic features, outcome, and population characteristics. *J Vet Intern Med* 2014; v28: 878–886
4. Martini V, Poggi A, Riondato F et al. Flow cytometric detection of phenotypic aberrancies in canine small clear cell lymphoma. *Vet Comp Oncol* 2015; 13: 281–287
5. Marconato L, Martini V, Aresu L et al. Assessment of bone marrow infiltration diagnosed by flow cytometry in canine large B cell lymphoma: Prognostic significance and proposal of a cut-off value. *Vet J* 2013; 197: 776–781
6. Aresu L, Aricò A, Ferraresso S, et al. Minimal residual disease detection by flow cytometry and PARR in lymph node, peripheral blood and bone marrow, following treatment of dogs with diffuse large B-cell lymphoma. *Vet J* 2014; 200: 318–324

7. Louwerens M, London CA, Pedersen NC et al. Feline lymphoma in the post-feline leukaemia virus era. *J Vet Intern Med* 2005; 19(3): 329-335
8. Moore PF, Rodriguez-Bertos A and Kass PH. Feline gastrointestinal lymphoma: mucosal architecture, immunophenotype, and molecular clonality. *Vet pathol* 2012; 49(4): 658-668
9. Guzera M, Cian F, Leo C et al. The use of flow cytometry for immunophenotyping lymphoproliferative disorders in cats: a retrospective study of 19 cases. *Vet Comp Oncol* 2016; 14(1): 40-51
10. Burkhard MJ, Bienzle D. Making sense of lymphoma diagnostics in small animal patients. *Vet clin north am small anim pract* 2013; 43(6): 1331-47
11. Brodersen R, Bijlsma F, Gori K et al. Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against various leucocyte surface antigens of human and 11 animal species. *Vet Immunol Immunopathol* 1998; 64(1): 1-13
12. Meister RK, Taglinger K, Haverson K et al. Progress in the discovery and definition of monoclonal antibodies for use in feline research. *Vet Immunol Immunopathol* 2007; 119(1-2): 38-46
13. Comazzi S, Gelain ME, Martini V et al. Immunophenotype predicts survival time in dogs with chronic lymphocytic leukaemia. *J Vet Intern Med* 2011; 25: 100–106

14. Rao S, Lana S, Eickhoff J et al. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *J Vet Intern Med* 2011; 25: 1097–1105

15. Richter K. Feline gastrointestinal lymphoma. *Vet clin north am small anim pract.* 2003; 33(5): 1083-1098

16. Patterson-Kane JC, Kugler BP, Francis K. The possible prognostic significance of immunophenotype in feline alimentary lymphoma: a pilot study. *J comp pathol* 2004; 130(2-3): 220-222

420 **Table 1**: Pre-analytical data asked to the referring veterinarians or reported in the
421 laboratory record for 97 samples of suspected feline lymphoma, sent to the laboratory
422 for flow cytometric immunophenotyping.

Feature group	**Specific variables**
Animal	*Breed*
	Sex (male, neutered male, female, spayed female)
	Age (years)
	FIV/FeLV status (positive or negative)
	Presenting complaint
	Clinical findings
Sampling procedure	*Sample catching* (blind aspiration, ultrasound-guided, computed tomography-guided, surgical approach, any other)
	*Pharmacological restraint (*none, sedation, general anaesthesia)
	Sampling technique (fine needle capillary biopsy, fine needle aspiration, any other)
	Needle size (G)
	Occurrence of side effects (yes or not)
Lesion	*Site* (peripheral lymph node, thoracic mass, intra-abdominal mass,

	effusion, any other)
	Size (≤2 cm, between 2 and 5 cm, ≥5 cm)
	Cytological diagnosis
	Histopathological diagnosis
	Any other test performed
Clinician who collected the sample	*Timespan between degree in Veterinary Medicine and sample collection* (years)
	Post-degree specialization, including European/American College, master, PhD (yes or not)
Flow cytometry	*Year of analysis*
	Tube of sample collection (culture medium, saline solution, EDTA, any other)
	Cell concentration ($x10^3/\mu l$)
	Sample processed (yes or not)
	Flow cytometric approach (single-color or multi-colour)

423 Italics: variable included in the statistical analyses

424

425 **Table 2** Antibodies used for flow cytometric analysis of suspected feline lymphoma
426 samples

antibody	**specificity**	**clone**	**Source**
CD5	T cells	FE1.1B11	Lab/UCDavis, Davis, CA
CD4	T helper cells	vpg39	Serotec, Oxford, UK
CD8	T cytotoxic cells	FE1.10E9	Serotec
CD21-PE	B cells	CA2.1D6	Serotec
CD5-FITC	T cells	f43	SouthernBiotech, Birmingham, AL, USA
CD4-FITC	T helper cells	3-4F4	SouthernBiotech
CD8-PE	T cytotoxic cells	fCD8	SouthernBiotech
CD18-AlexaFluor647	All leukocytes	CA1.4E9	Serotec

427

428

429 **Table 3** cellular concentration of 52 samples of suspected feline lymphoma sent to the
430 laboratory for flow cytometric immunophenotyping, according to the size of the needle
431 used for sampling. The mean cellular concentration significantly varied with needle size
432 (P=0.045). [a,b] significant difference at post-hoc analysis.

Needle size (G) [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
18 [6]	12.67 ± 22.92	3.7	0.03	59.26
20 [1]	21.03			
21 [4][a,b]	49.61 ± 36.72	51.90	4.75	89.88
22 [30][a]	9.49 ± 20.61	2.00	0.01	87.54
23 [8][b]	5.05 ± 8.32	1.83	0.63	21.99
25 [2]	20.19 ± 0.02	20.19	20.17	20.20
27 [1]	19.14			

433

434

435 **Table 4** cellular concentration of 69 samples of suspected feline lymphoma sent to the
436 laboratory for flow cytometric immunophenotyping, according to the flow cytometric
437 diagnosis. The mean cellular concentration significantly varied among the three
438 diagnostic groups (P=0.022). [a,b] significant difference at post-hoc analysis

Diagnosis [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [29][a]	23.45 ± 29.6	10.00	1.10	89.88
Non-Lymphoma [25][b]	10.73 ± 12.6	4.75	0.63	43.59
Non-Diagnostic [15][a,b]	4.76 ± 7.5	2.48	0.16	26.89

439

440

441 **Table 5** cellular concentration of 21 feline peripheral lymph node aspirates sent to the
442 laboratory for flow cytometric immunophenotyping for suspected lymphoma, according
443 to the flow cytometric diagnosis. The mean cellular concentration significantly varied
444 among the three diagnostic groups (P=0.029). [a] significant difference at post-hoc
445 analysis

Diagnosis [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [5][a]	45.96 ± 38.05	21.99	13.65	87.54
Non-Lymphoma [11]	13.32 ± 13.77	7.3	1.68	43.59
Non-Diagnostic [5][a]	4.78 ± 7.52	2.61	0.16	18.02

446

447

448 **Table 6** cellular concentration of 11 feline effusions sent to the laboratory for flow
449 cytometric immunophenotyping for suspected lymphoma, according to the flow
450 cytometric diagnosis. The mean cellular concentration significantly varied among the
451 three diagnostic groups (P=0.029). [a] significant difference at post-hoc analysis

452

Diagnosis [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [5][a]	57.56 ± 26.24	66.04	21.03	89.88
Non-Lymphoma [3]	12.50 ± 14.86	4.75	3.11	29.63
Non-Diagnostic [3][a]	2.55 ± 2.30	2.34	0.36	4.94

453

454

Table S1 24 peripheral lymph nodes samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	5	2
2-5 cm	5	1
Sample catching		
Blind aspiration	12	3
ultrasound-guided	1	0
Pharmacological restraint		
None	8	2
Mild sedation	1	1
Needle size		
18 G	2	0
22 G	6	3
23 G	1	0
25 G	1	0
27 G	1	0
Sampling technique		
Fine needle aspiration	8	3
Fine needle capillary biopsy	7	0
Transport tube		
Saline solution	2	1
Culture medium	11	2
Years since graduation		
<15	9	1
≥15	5	1
Post-degree qualifications		
No	0	0
Yes	10	2

Table S2 cellular concentration of 24 peripheral lymph nodes samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	11.36±16.30	1.68	0.01	43.59
2-5 cm	20.47±33.50	5.71	2.29	87.54
Sample catching				
Blind aspiration	21.20±29.35	8.81	0.01	87.54
ultrasound-guided	0.17			
Pharmacological restraint				
None	21.27±26.87	16.40	0.22	87.54
Mild sedation	3.20±4.51	3.20	0.01	6.39
Needle size				
18 G	4.66±2.45	4.66	2.92	6.39
22 G	11.75±28.55	2.29	0.01	87.54
23 G	21.99			
25 G	20.17			
27 G	19.14			
Sampling technique				
Fine needle aspiration	13.95±26.09	2.42	0.01	87.54
Fine needle capillary biopsy	13.63±14.82	8.81	0.17	43.59
Transport tube				
Saline solution	2.95±3.16	2.29	0.17	6.39
Culture medium	23.28±31.14	13.65	0.01	87.54
Years since graduation				
<15	15.30±26.53	4.34	0.16	87.54
≥15	6.46±8.15	2.77	0.01	21.99
Post-degree qualifications				
No	-			
Yes	12.70±24.70	2.36	0.01	87.54

Table S3 21 abdominal lymph nodes samples from cats with suspected lymphoma, processed or not for flow cytometry, according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	4	1
>5 cm	3	2
Sample catching		
Blind aspiration	0	0
Ultrasound-guided	9	5
Pharmacological restraint		
None	2	2
Mild sedation	5	1
General anaesthesia	1	0
Needle size		
18 G	1	0
22 G	6	1
23 G	2	1
Sampling technique		
Fine needle aspiration	8	3
Fine needle capillary biopsy	4	0
Transport tube		
Saline solution	0	0
Culture medium	10	2
Years since graduation		
<15	3	2
≥15	6	1
Post-degree qualifications		
No	1	0
Yes	5	3

Table S4 cellular concentration of 21 abdominal lymph nodes samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	1.58			
2-5 cm	1.90±1.61	1.38	0.30	4.40
>5 cm	3.63±4.15	2.37	0.07	10.03
Sample catching				
Blind aspiration	-			
Ultrasound-guided	2.01±2.76	1.16	0.03	10.03
Pharmacological restraint				
None	2.05±2.26	1.26	0.34	5.35
Mild sedation	3.64±3.71	2.48	0.63	10.03
General anaesthesia	0.3			
Needle size				
18 G	3.39			
22 G	7.69±3.31	7.69	5.35	10.03
23 G	1.56±1.31	1.56	0.63	2.48
Sampling technique				
Fine needle aspiration	2.67±3.41	1.34	0.30	11.58
Fine needle capillary biopsy	4.61±4.04	3.51	1.38	10.03
Transport tube				
Saline solution	-			
Culture medium	2.75±2.97	1.63	0.30	10.03
Years since graduation				
<15	4.50±4.17	3.51	0.93	10.03
≥15	1.44±1.16	1.38	0.3	3.39
Post-degree qualifications				
No	5.35			
Yes	2.32±3.45	1.38	0.3	10.03

Table S5 21 thoracic lymph node/masses samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	6	0
>5 cm	4	3
Sample catching		
ultrasound-guided	11	3
computed tomography-guided	2	2
Pharmacological restraint		
None	4	1
Mild sedation	6	0
General anaesthesia	2	3
Needle size		
18 G	1	0
22 G	6	2
23 G	3	0
25 G	1	0
Sampling technique		
Fine needle aspiration	10	2
Fine needle capillary biopsy	4	2
Transport tube		
Saline solution	1	0
Culture medium	11	3
Years since graduation		
<15	5	1
≥15	4	1
Post-degree qualifications		
No	2	1
Yes	5	1

Table S6 cellular concentration of 21 thoracic lymph node/masses samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	1.99			
2-5 cm	18.99±23.50	7.88	3.16	59.26
>5 cm	18.46±24.58	5.86	0.01	58.02
Sample catching				
ultrasound-guided	12.64±21.12	1.99	0.61	59.26
computed tomography-guided	15.94±21.14	7.88	0.01	39.93
Pharmacological restraint				
None	13.86±24.71	3.16	1.71	58.02
Mild sedation	18.51±24.04	10.00	1.51	59.26
General anaesthesia	10.41±19.68	0.85	0.01	39.93
Needle size				
18 G	59.26			
22 G	10.00±19.65	2.44	0.01	58.02
23 G	1.75±0.34	1.75	1.51	1.99
25 G	20.2			
Sampling technique				
Fine needle aspiration	10.61±19.80	1.59	0.01	59.26
Fine needle capillary biopsy	16.70±24.44	1.99	1.58	58.02
Transport tube				
Saline solution	1.99			
Culture medium	10.37±18.96	1.65	0.01	59.26
Years since graduation				
<15	12.41±22.62	2.44	1.58	58.02
≥15	13.56±25.59	1.99	0.61	59.26
Post-degree qualifications				
No	42.43±28.09	58.02	10.00	59.26
Yes	2.30±1.50	1.71	0.61	4.44

Table S7 17 effusion samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Sample catching		
Blind aspiration	1	0
ultrasound-guided	3	3
computed tomography-guided	3	0
Pharmacological restraint		
None	1	1
Mild sedation	1	1
General anaesthesia	4	0
Needle size		
20 G	1	0
21 G	3	1
22 G	2	1
Sampling technique		
Fine needle aspiration	5	0
Fine needle capillary biopsy	1	1
Years since graduation		
<15	2	0
≥15	3	2
Post-degree qualifications		
No	0	0
Yes	4	0

Table S8 cellular concentration of 17 effusion samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Sample catching				
Blind aspiration	89.88			
ultrasound-guided	31.95±28.56	37.75	0.47	66.04
computed tomography-guided	12.50±14.86	4.75	3.11	29.63
Pharmacological restraint				
None	70.22±27.81	70.22	50.55	89.88
Mild sedation	21.35±23.20	21.35	4.94	37.75
General anaesthesia	12.50±14.86	4.75	3.11	29.63
Needle size				
20 G	21.03			
21 G	49.61±36.72	51.90	4.75	89.88
22 G	27.75±32.25	27.75	4.94	50.55
Sampling technique				
Fine needle aspiration	19.97±26.76	4.94	3.11	66.04
Fine needle capillary biopsy	50.55			
Years since graduation				
<15	4.94			
≥15	53.05±26.41	50.55	21.03	89.88
Post-degree qualifications				
No	-			
Yes	30.67±31.67	21.03	4.94	66.04

Table S9 14 different samples (skin, kidney, spleen, liver, urine) from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	3	0
2-5 cm	1	3
Sample catching		
ultrasound-guided	3	3
Surgical access	0	1
Catheterisation	1	0
Pharmacological restraint		
None	2	1
Mild sedation	1	0
General anaesthesia	2	0
Needle size		
18 G	0	2
22 G	2	1
23 G	1	0
Sampling technique		
Fine needle aspiration	5	4
Fine needle capillary biopsy	2	0
Transport tube		
Saline solution	1	0
Culture medium	3	3
Years since graduation		
<15	1	1
≥15	4	2
Post-degree qualifications		
No	0	0
Yes	2	1

Table S10 cellular concentration of 14 different samples (skin, kidney, spleen, liver, urine) from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	2.49±1.15	2.49	1.67	3.30
2-5 cm	4.78±6.65	2.34	0.03	14.40
Sample catching				
ultrasound-guided	0.74±0.64	0.66	0.02	1.67
Surgical access	4.01			
Catheterisation	14.40			
Pharmacological restraint				
None	1.45±1.68	1.02	0.02	3.30
Mild sedation	1.67			
General anaesthesia	14.40			
Needle size				
18 G	2.02±2.81	2.02	0.03	4.01
22 G	1.66±2.32	1.66	0.02	3.30
23 G	1.67			
Sampling technique				
Fine needle aspiration	4.80±8.45	1.67	0.02	26.89
Fine needle capillary biopsy	3.30			
Transport tube				
Saline solution	3.30			
Culture medium	3.51±6.13	1.45	0.02	14.40
Years since graduation				
<15	0.02			
≥15	4.07±5.27	2.49	0.03	14.40
Post-degree qualifications				
No	-			
Yes	0.52±0.71	0.52	0.02	1.02