

## **Flow-cytometric detection of phenotypic aberrancies in canine small clear cell lymphoma**

V. Martini<sup>1</sup>, A. Poggi<sup>2</sup>, F. Riondato<sup>2</sup>, M. E. Gelain<sup>3</sup>, L. Aresu<sup>3</sup> and S. Comazzi<sup>1</sup>

1 Department of Veterinary Sciences and Public Health, Faculty of Veterinary Medicine, University of Milan, Milan, Italy

2 Department of Veterinary Science, University of Turin, Turin, Italy

3 Department of Comparative Biomedicine and Food Science, Faculty of Veterinary Medicine, University of Padua, Padua, Italy

### **Corresponding address:**

V. Martini, DVM Department of Veterinary Sciences and Public Health Faculty of Veterinary Medicine

University of Milan, via Celoria 10

20133 Milan, Italy e-mail:

valeria.martini@unimi.it

**Abstract**

Histopathology and immunohistochemistry are mandatory to solve the differential between canine low-grade lymphoma and reactive hyperplasia. However, clinicians and owners often show reluctance toward these invasive tests. However, molecular biology techniques are still not sensitive and specific enough to be regarded as a reliable tool for final diagnosis. In humans, flow cytometry (FC) allows a definitive diagnosis of T-cell lymphoma based on high prevalence of antigen aberrancies. We describe here the immunophenotype of 26 cases of suspect canine small-clear cell lymphoma, determined by multi-colour FC. All cases showed antigen aberrancies and therefore neoplasia was always confirmed. As a consequence, we argue that the combined use of cytology and FC allows solving the differential diagnosis between small clear cell lymphoma and non-neoplastic reactive conditions when histopathology is not available. Further studies are needed to establish if any aberrancy can be considered indicative of specific histotypes.

**Keywords**

aberrancies, diagnosis, flow cytometry, lymphoma, small clear cell

## Introduction

Canine peripheral T-cell lymphoma of small cell type is an indolent lymphoma involving peripheral lymph nodes. The diagnosis traditionally relies on morphological criteria. Cytologically, a pattern with highly prevalent population of small lymphoid cells with clear cytoplasm and frequent 'hand-mirror' shape (small-clear cell appearance) is considered suggestive, although not conclusive, of T-zone lymphoma.<sup>1– 3</sup> However, a reactive paracortical hyperplasia cannot be completely ruled out based on cytological evaluation alone and histopathology should always be performed in order to confirm the tentative diagnosis of neoplasia.

Histologically, T-zone lymphoma shows neoplastic cells expanding the paracortex and medullary cords without effacing the nodal architecture. The neoplastic population is composed by small lymphocytes with sharp, shallow nuclear indentations, unapparent nucleoli and a moderate volume of pale cytoplasm.<sup>4</sup> Mitoses are rare. Different studies have identified the importance of immunohistochemistry to confirm diagnosis and define immunophenotype in T-zone lymphomas.<sup>2,4– 7</sup>

The main issue in veterinary medicine, as far as histology and immunohistochemistry are concerned, is the surgical removal of at least one lymph node, which is usually poorly accepted by the owners due to the increase of the costs and the need for anaesthesia. On the basis of a recent survey, indeed, only 28% of responding participants recommended lymph node histopathology among staging procedures, although only high-grade lymphomas were considered in the survey.<sup>8</sup>

A faster and less invasive method to distinguish reactive from neoplastic cases is the PCR for Antigen Receptor Rearrangement (PARR): the development of one or two prominent bands is considered suggestive of clonality. Several attempts have been made to set up this technique. Unfortunately, optimal sensitivity and specificity have never been reached.<sup>4,9– 14</sup> PARR results in different histotypes were reported in one study: among eight T-zone lymphomas, only five had a clonal rearrangement.<sup>4</sup> Therefore, neoplasia cannot be ruled out based on the negative PARR result and the interpretation of this test should be always associated with histopathology and immunohistochemistry.

Flow cytometry (FC) is a fast technique that requires a minimally invasive sampling and is becoming diffusely available in veterinary medicine. Moreover, many antibodies have been validated for canine samples processing, particularly as far as T-lymphocytes subclasses are concerned. Therefore, nowadays FC is often used for immunophenotyping of canine lymphomas.<sup>15,16</sup>

Immunocytochemistry on lymph node aspirate smears can be a useful tool to determine B- or T-phenotype of canine lymphomas. Furthermore, procedures for automated immunostaining have recently been described.<sup>17</sup> This technique requires a minimally invasive sampling and as a consequence could be preferred to histopathology by owners and clinicians. Unfortunately, it is by far less suitable than FC for assessment of co-expression of different antigens by the same cellular population and therefore it does not allow recognition of antigen aberrancies.

The aims of this study are to assess the usefulness of flow cytometry for confirming a cytological suspect of nodal small clear cell lymphoma in the dog, and to describe the antigen pattern of this particular neoplasm through a retrospective overview of the samples sent to the Authors' institutions' Flow cytometric Services for immunophenotyping.

## Materials and methods

### Case selection

Laboratory databases of the Authors' Institutions from January 2010 to January 2013 were interrogated and cases with a suspect of nodal small clear cell lymphoma were selected. Cytological preparations of lymph node fine needle aspiration were revised. Inclusion criteria were high prevalence of small-sized cells with round to slightly irregular nuclei and extended, pale cytoplasm; low mitotic index (0–1 mitosis/five fields 500×). These features are considered suggestive of low-grade T-cell lymphomas with small-clear cell appearance and presumable T-zone histotype.<sup>2,3,6</sup>

In addition, cases with a final diagnosis of reactive process based on lymph node cytology, flow cytometry and when needed histopathology were included in the study as negative controls.

### Flow cytometry

Flow cytometric immunophenotyping of lymph node samples collected into RPMI (Sigma Aldrich, St. Louis, MO, USA) was performed as previously described.<sup>18</sup> A panel of antibodies including CD45 (pan-leukocyte), CD3 and CD5 (T-cells), CD4 (T-helper cells and neutrophils), CD8 (T-cytotoxic cells), CD21 (mature B-cells) and CD79a (B-cells) was used. Cells were acquired either with a FACScalibur or with a BD Accuri C6 (Becton Dickinson, San Jose, CA, USA) and analysed using the specific software CellQuest Pro or CFlow Plus (Becton Dickinson).

A multi-colour flow cytometric analysis was performed, thus allowing the evaluation of contemporary expression of different antigens in the same cellular population. Aberrancies were defined as diminished or absent expression of pan-leukocyte antigens, co-expression of antigens from different cellular lineage and co-expression or loss of both CD4 and CD8 by T-cells.

A diagnosis of lymphoma was made if almost all cells in the sample showed the same antigenic pattern or if any aberrant antigen expression was present even in a smaller percentage of cells.

## Results

From January 2010 to January 2013, 447 canine lymph node samples with suspected lymphoid neoplasm were processed in the authors' laboratories and recorded in their databases. Cytological preparations were available for 259 (57.9%) cases. Among them, 26 (10.0%) showed a small-clear cell morphology and were included in this study. In addition, eight cases with a final diagnosis of reactive process were included and considered as negative controls.

On the basis of flow cytometric immunophenotyping, a lymphoid population showing aberrant antigen expression was identified in all samples with a suspect of small-clear cell lymphoma, thus allowing a definitive diagnosis of neoplasia.

Neoplastic cells stained negative for CD45 in 25 of 26 cases (96.2%). CD3 and CD5 had been tested in 25 and 21 samples and stained positive in 18 (72%) and 19 (90.5%) cases, respectively. Thirteen cases (50%) stained positive either for CD4 or CD8, three (11.5%) were positive for both, nine (34.6%) were double negative; in one case, two distinct CD45-negative populations were identified staining positive for CD4 and CD8, respectively. Twenty of 26 (76.9%) samples stained positive for CD21 and 3 of 20 (15%) for CD79a.

Results of immunophenotyping for each case are shown in Table 1. Flow cytometric scattergrams from one representative case are pictured in Figure 1.

On the contrary, when reactive controls were considered, all cells in each sample stained positive for CD45. Flow cytometry revealed a non-committed lymphoid population composed by CD4+ T-cells (mean 35.9%, min– max 19.0 – 59.3%), CD8+ T-cells (mean 12.9%, min– max 8.0 – 21.2%) and B-cells (mean 43.5%, min– max 23.0 – 53.0%). Furthermore, all T-cells stained positive for both CD3 and CD5 and either CD4 or CD8; B-cells stained positive for both CD21 and CD79a.

## Discussion

The aim of this work was to describe the combined use of cytology and FC to differentiate canine low-grade lymphoma and non-neoplastic reactive hyperplasia. In this study, we reviewed flow-cytometric immunophenotype of 26 suspect small clear cell lymphomas and found a prevalence of 100% for antigen aberrancies, thus allowing a definitive diagnosis of neoplasia. Eight cases with reactive hyperplasia served as negative controls.

Cytology plays a crucial role in the diagnosis of canine lymphoma<sup>19</sup>: in fact, the cytological pattern and the mitotic index easily allow to characterize low-grade and high-grade lymphomas. However, while the presence of a single population of immature or large cells at microscopic evaluation can be considered definitive of neoplasia, the distinction between low-grade lymphoma and reactive hyperplasia is a crucial point for which detection of clonality and/or the evaluation of architectural pattern is often required. Unfortunately, from a clinical point of view, this remains a challenging issue as histopathology and immunohistochemistry require an invasive approach and molecular biology tests for clonality (PARR) are far from being accurate enough. Therefore, the use of a rapid and quite cheap test to distinguish between low-grade lymphomas and reactive processes could be of great interest for oncologists.

Sampling procedures for FC are non-invasive: fine-needle aspiration is required and general anaesthesia is usually not needed. This technique has been used for diagnosis and staging of canine large-cell lymphomas<sup>15,20</sup> because large-sized cells are easily recognizable on morphological scattergrams. In addition, FC allows the assessment of the simultaneous expression of different antigens on the same cellular population as well as a quantitative evaluation of the expression of each antigen.<sup>18</sup> These features allowed to identify different aberrancies in canine neoplastic lymphoid cells.<sup>18,21,22</sup>

Similar to neoplastic samples included in this study, cytological preparations from reactive lymph nodes showed a highly prevalent population of small lymphoid cells with a moderate number of large blast cells, interdigitating cells and a variable amount of plasma cells. Therefore, although useful to discriminate between reactive hyperplasia and low grade lymphoma, cytology alone is generally not definitive: this is why excision, followed by histopathology and immunohistochemistry, is generally mandatory. In this study, flow cytometry revealed a non-committed population of cells without any aberrancy in reactive lymph nodes. On the other hand, aberrancies were always identified in small-clear cell lymphoma cases.

In particular, CD45 expression was lost in all small clear cell lymphoma cases but one. CD45 is a pan-leukocyte marker in the dog, with different degree of expression in the leukocytes subclasses.<sup>23</sup> None of the reactive samples included in this study as negative controls had a CD45-negative lymphoid population. Therefore, the absence of

CD45 expression could be considered a hallmark of neoplasia. A decreased or absent expression has been previously reported in canine T-cell neoplasms.<sup>18,24</sup>

Interestingly, CD21 expression was quite frequent in our case series. Despite the positivity for this antigen, all cases could be considered of T-cell phenotype, because of the small clear cell appearance, which was the inclusion criteria for this study and is itself suggestive, although not conclusive, of a T-zone lymphoma,<sup>1–3</sup> and because of the contemporary expression of at least one T-cell marker. T- and B-cell populations were clearly separate in reactive lymph nodes and co-expression of markers from different lineages was never found. Furthermore, CD21-positivity has already been described in canine T-cell neoplasms in the past.<sup>21</sup> Finally, in a recent study GEP analysis identified a group of genes, including CD21-gene, expressed at higher levels in T-zone lymphomas than in high-grade T-cell lymphomas.<sup>25</sup>

A co-expression or loss of both CD4 and CD8 was detected in about 50% of the lymphoma cases, but in none of the reactive controls. Co-expression of CD4 and CD8 is a phenotype suggestive of thymocytes: therefore, the percentage of lymphoid cells with this phenotype identified by flow cytometry on mediastinal masses aspirates drives the differential diagnosis between thymoma and thymic lymphoma.<sup>26</sup> In this study, only lymph node samples were included, thus excluding a possible thymic origin of the CD4 – CD8 double positive cells: therefore this phenotype was considered as aberrant and suggestive of lymphoma. Some cases of CD4 – CD8 double positive or double negative canine lymphoid neoplasms have been signalled in literature. In particular, these phenotypes were found in some cases of unclassifiable high-grade T-cell lymphomas<sup>2</sup> and in a subgroup of dogs with chronic lymphocytic leukaemia harbouring a poor prognosis.<sup>27</sup>

Aberrancies are one of the most important features in human peripheral T-cell lymphomas and are considered a hallmark of clonality and neoplasia allowing flow cytometric definitive diagnosis of lymphoma.<sup>28,29</sup> Aberrancies have been described in human and canine B-cell lymphomas as well.<sup>18,22,30,31</sup> However, we selected only small clear cell lymphomas for this study, not all low-grade lymphomas, because T-zone histotype (and therefore T-cell phenotype) can be expected in these cases. In fact, only a few antibodies are available for characterizing canine B-lymphocytes: consequently, a reduced number of B-cell antigens can be tested and many aberrancies would remain undetected. Validation of new anti-canine or cross-reacting antibodies in the future may permit the identification of aberrancies in canine B-cell neoplasms too.

The main pitfall of this study is the lack of histopathology and immunohistochemistry for a high number of the cases: this circumstance could be considered a proof of the reluctance of clinicians and owners towards invasive tests. However, the few cases of suspect small clear cell lymphoma for which histopathology was available had a final diagnosis of T-zone lymphoma, thus supporting what already reported in literature.<sup>1–3</sup>

The lack of histopathological data unfortunately prevented the comparison between different lymphoma subtypes and the evaluation of possible relationship between specific flow cytometric phenotypes or aberrancies and histotypes. Therefore, further studies should be performed on a larger number of cases comparing cytological, flow cytometric and histopathological data, in order to assess if specific aberrancies are pathognomonic of T-zone lymphomas and could therefore be considered for the definitive diagnosis of this histological subtype. In conclusion, this study has documented the high prevalence of antigen aberrancies in canine small clear cell lymphoma, which were not found in reactive controls, and highlighted their diagnostic role. We support the use of multi-colour flow cytometry when a small clear cell

appearance is found at cytological evaluation, as it represents a useful tool to confirm neoplasia and exclude reactive hyperplasia in those cases in which histopathology is not available or permitted.

### **Acknowledgements**

The authors wish to thank all private vets who submitted their clinical samples to our institutes for routine examination. Supported by Progetti di Ricerca di Interesse Nazionale (PRIN 2008), Ministry of Education, University and Research, Italy.

## References

1. Fournel-Fleury C, Magnol JP, Bricaire P, Marchal T, Chabanne L, Delverdier A, Bryon PA and Felman P. Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. *Journal of Comparative Pathology* 1997; 117: 35 – 59.
2. Fournel-Fleury C, Ponce F, Felman P, Blavier A, Bonnefont C, Chabanne L, Marchal T, Cadore JL, Goy-Thollot I, Ledieu D, Ghernati I and Magnol JP. Canine T-cell Lymphomas: a morphological, immunological and clinical study of 46 new cases. *Veterinary Pathology* 2002; 39: 92 – 109.
3. Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K and Fournel-Fleury C. Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. *The Veterinary Journal* 2004; 167: 158 – 166.
4. Valli V, Vernau W, Lorimier LD, Graham P and Moore PF. Canine indolent nodular lymphoma. *Veterinary Pathology* 2006; 43: 241 – 256.
5. Valli V. Veterinary pathologists achieve 80% agreement in application of WHO diagnoses to canine lymphoma. *Cancer Therapy* 2008; 6: 221 – 226.
6. Valli V, San Myint M, Barthel A, Bienzle D, Caswell J, Colbatzky F, Durham A, Ehrhart EJ, Johnson Y, Jones C, Kiupel M, Labelle P, Lester S, Miller M, Moore P, Moroff S, Roccabianca P, Ramos-Vara J, Ross A, Scase T, Tvedten H and Vernau W. Classification of canine malignant lymphomas according to the World Health Organisation Criteria. *Veterinary Pathology* 2011; 48: 198 – 211.
7. Flood-Knapik KE, Durham AC, Gregor TP, Sanchez MD, Durney ME, and Sorenmo KU. Clinical, histopathological and immunohistochemical characterization of canine indolent lymphomas. *Veterinary and Comparative Oncology* 2012; doi:10.1111/j.1476-5829.2011.00317.x.
8. Regan RC, Kaplan MS, and Bailey DB. Diagnostic evaluation and treatment recommendations for dogs with substage-a high-grade multicentric lymphoma: results of a survey of veterinarians. *Veterinary and Comparative Oncology* 2012; doi:10.1111/j.1476-5829.2012.00318.x.
9. Vernau W and Moore PF. An immunophenotypic study of canine leukemias and preliminary assessment of clonality by polymerase chain reaction. *Veterinary Immunology and Immunopathology* 1999; 69: 145 – 164.
10. Burnett RC, Vernau W, Modiano JF, Olver CS, Moore PF and Avery AC. Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. *Veterinary Pathology* 2003; 40: 32 – 41.
11. Yagihara H, Tamura K, Isotani M, Ono K, Washizu T and Bonkobara M. Genomic organization of the T-cell receptor gamma gene and PCR detection of its clonal rearrangement in canine T-cell lymphoma/leukaemia. *Veterinary Immunology and Immunopathology* 2007; 115: 375 – 382.
12. Chaubert P, Baur Chaubert AS, Sattler U, Forster U, Bornand V, Suter M and Welle M. Improved polymerase chain reaction-based method to detect early-stage epitheliotropic T-cell lymphoma (mycosis fungoides) in formalin-fixed, paraffin-embedded skin biopsy specimens of the dog. *Journal of Veterinary Diagnostic Investigations* 2010; 22: 20 – 29.



13. Keller SM and Moore PF. Rearrangement patterns of the canine TCR gamma locus in a distinct group of T cell lymphomas. *Veterinary Immunology and Immunopathology* 2012; 145: 350 – 361.
14. Keller SM and Moore PF. A novel clonality assay for the assessment of canine T cell proliferations. *Veterinary Immunology and Immunopathology* 2012; 145: 410 – 419.
15. Comazzi S and Gelain ME. Use of flow cytometric immunophenotyping to refine the cytological diagnosis of canine lymphoma. *The Veterinary Journal* 2011; 188: 149 – 155.
16. Joetzke AE, Eberle N, Nolte I, Mischke R and Simon D. Flow cytometric evaluation of peripheral blood and bone marrow and fine-needle aspirate samples from multiple sites in dogs with multicentric lymphoma. *American Journal of Veterinary Research* 2012; 73: 884 – 893.
17. Aulbach AD, Swenson CL and Kiupel M. Optimized processing of fine-needle lymph node biopsies for automated immunostaining. *Journal of Veterinary Diagnostic Investigation* 2010; 22: 383 – 388.
18. Gelain ME, Mazzilli M, Riondato F, Marconato L and Comazzi S. Aberrant phenotypes and quantitative antigen expression in different subtypes of canine lymphoma by flow cytometry. *Veterinary Immunology and Immunopathology* 2008; 121: 179 – 188.
19. Marconato L. The staging and treatment of multicentric high-grade lymphoma in dogs: a review of recent developments and future prospects. *The Veterinary Journal* 2011; 188: 34 – 38.
20. Martini V, Melzi E, Comazzi S, and Gelain ME. Peripheral blood abnormalities and bone marrow infiltration in canine large B cell lymphoma: is there a link? *Veterinary and Comparative Oncology* 2013; doi: 10.1111/vco.12024.
21. Wilkerson MJ, Dolce K, Koopman T, Shuman W, Chun R, Garrett L, Barber L and Avery A. Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules. *Veterinary Immunology and Immunopathology* 2005; 106: 176 – 196.
22. Rao S, Lana S, Eickhoff J, Marcus E, Avery PR, Morley PS and Avery AC. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *Journal of Veterinary Internal Medicine* 2011; 25: 1097 – 1105.
23. Comazzi S, Gelain ME, Spagnolo V, Riondato F, Guglielmino R and Sartorelli P. Flow cytometric patterns in blood from dogs with non-neoplastic and neoplastic hematologic diseases using double labelling for CD18 and CD45. *Veterinary Clinical Pathology* 2006; 35: 47 – 54.
24. Williams MJ, Avery AC, Lana SE, Hillers KR, Bachand AM and Avery PR. Canine lymphoproliferative disease characterized by lymphocytosis: immunophenotypic markers of prognosis. *Journal of Veterinary Internal Medicine* 2008; 22: 596 – 601.
25. Franz AM, Sarver AL, Ito D, Phang TL, Karimpour-Fard A, Scott MC, Valli VEO, Lindblad-Toh K, Burgess KE, Husbands BD, Henson MS, Borgatti A, Kisseberth WC, Hunter LE, Breen M, O'Brien TD, and Modiano JF. Molecular profiling reveals prognostically significant subtypes of canine lymphomas. *Veterinary Pathology* 2012; doi: 10.1177/0300985812465325.
26. Lana S, Plaza S, Hampe K, Burnett R and Avery AC. Diagnosis of mediastinal masses in dogs by flow cytometry. *Journal of Veterinary Internal Medicine* 2006; 20: 1161 – 1165.

27. Comazzi S, Gelain ME, Martini V, Riondato F, Miniscalco B, Marconato L, Stefanello D and Mortarino M. Immunophenotype predicts survival time in dogs with chronic lymphocytic leukemia. *Journal of Veterinary Internal Medicine* 2011; 25: 100 – 106.
28. Jamal S, Picker LJ, Aquino DB, McKenna RW, Brian Dawson D and Kroft SH. Immunophenotypic analysis of peripheral T-cell neoplasms. A multiparameter flow cytometric approach. *American Journal of Clinical Pathology* 2001; 116: 512 – 526.
29. Arun I, Wulu JA, Janik JE, Jasper GA, Yuan CM, Venzon D and Stetler-Stevenson M. Visual inspection versus quantitative flow cytometry to detect aberrant CD2 expression in malignant T cells. *Cytometry. Part B, Clinical Cytometry* 2010; 78: 169 – 175.
30. Inaba T, Shimazaki C, Sumikuma T and Nakagawa M. T-cell associated antigen positive B-cell lymphoma. *Leukemia & Lymphoma* 2001; 42: 1161 – 1171.
31. Wang J, Chen C, Lau S, Raghavan RI, Rowsell EH, Said J, Weiss JM and Huang Q. CD3-positive large B-cell lymphoma. *The American Journal of Surgical Pathology* 2009; 33: 505 – 512.

**Table 1.** Flow cytometric immunophenotype of the neoplastic population identified in lymph node aspirates from 26 dogs with suspect small clear cell lymphoma

no.	CD45	CD3	CD5	Case CD4	CD8	CD21	CD79a
1	-	+	+	-	-	+	+
2	-	+	+	-	-	+	+
3	-	-	nd	-	+	+	-
4	-	-	+	+	-	-	nd
5	-	+	+	-	-	-	nd
6	+	-	+	-	+	-	-
7	-	+	+	-	+	-	nd
8	-	+	+	-	+	+	-
9	-	-	+	-	-	+	-
10	-	-	+	+	+	+	-
11	-	-	+	-	-	+	nd
12	-	-	-	-	+	+	-
13	-	+	+	-	+	+	-
14	-	+	+	-	+	+	nd
15	-	+	nd	-	+	+	-
16	-	+	+	-	-	+	-
17	-	-	+	+ (33%)	+ (60%)	+	nd
18	-	+	-	-	-	+	-
19	-	+	+	+	+	+	-
20	-	+	-	+	-	+	-
21	-	nd	+	-	+	+	-
22	-	+	nd	-	-	+	-
23	-	+	+	-	+	-	-
24	-	+	nd	-	-	+	+
25	-	+	nd	-	+	+	-
26	-	+	+	+	+	-	-

nd, not determined.

**Figure 1.** Flow cytometric scattergrams of dog number 10, representative of a typical aberrant antigen pattern. Most cells are CD45-negative (A) and (C), CD5-positive (A), CD4-CD8 double positive (B) and CD21-positive (C).

