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CLINICAL PATHOLOGICAL FEATURES OF CANINE HEMATOPOIETIC NEOPLASMS ASSESSED VIA FLOW CYTOMETRY

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ABSTRACT

Flow cytometry (FC) is an advanced diagnostic technique widely used in human medicine to confirm, classify and stage hematopoietic neoplasms (HNs): it supplies an objective evaluation of the size and internal complexity of the cells and identifies the antigenic pattern expressed by each cell, which is specific for cellular lineage and maturative stage. FC is presently spreading also in veterinary medicine and is mostly used to confirm the diagnosis and determine the immunophenotype of canine HNs.

Aim of this study was to apply FC to the study of specific clinical pathological features of canine HNs. In particular, we focused on three points:

1) staging of diffuse large B-cell lymphoma. We validated a FC technique to assess peripheral blood and bone marrow infiltration by neoplastic cells and proved its prognostic value.
2) antigen aberrancies. We investigated the prognostic role of specific antigen aberrancies in canine lymphomas and found the dogs with CD4+CD8+ T-cell lymphomas had a poorer prognosis compared to other T-cell aberrant lymphomas. In addition, we highlighted an important diagnostic role for antigen aberrancies in canine small clear cell lymphoma.
3) CD44 expression. We proved that CD44, which has a role in the pathogenesis and dissemination of many human and canine neoplasms, is expressed at different degrees on neoplastic cells from different canine HNs, with acute leukemias showing the highest degree of expression.

Overall, our results suggest that FC can be a useful tool not only to confirm the diagnosis and assess the immunophenotype of canine HNs, but also to assess clinical pathological aspects and additional prognostic parameters specific for the neoplastic subtype identified.

Keywords: dog, hematopoietic neoplasms, flow cytometry, staging, aberrancies, CD44

1- INTRODUCTION

Hematopoietic neoplasms, which are some of the most represented neoplasms in dogs, are a heterogeneous group of diseases that originate from the clonal expansion of different hematopoietic cells (either lymphoid or myeloid) in lymphoid tissues, in the bone marrow or less commonly in other non-lymphoid tissues. On the basis of the lineage and stage of maturation of the neoplastic cells, and of the site where neoplasia first arises, hematopoietic neoplasms can be subdivided into acute and chronic leukemias, of different lineages, and lymphomas.

The dog is presently given some attention as a possible animal model for human non-Hodgkin lymphomas (NHL). Indeed, the classification of canine NHL has been compared to the human one and many similarities between the two species have been highlighted (Fournel-Fleury et al, 1997).

Compared to traditional animal models, dogs show some advantages, including:

- dogs play a useful tool as sentinel hosts for cancer, possibly leading to early detection of carcinogens hazards in the environment. In recent years, their quality of life has improved, particularly thanks to improvement in veterinary cares and nutrition, resulting in a longer survival and an increased probability of experiencing spontaneous diseases of old age, including cancer. Dogs share the same environment with humans and are therefore exposed to pollutants and carcinogens, but they have a shorter lifespan compared to humans. As a consequence, the latency period between exposure to potential carcinogens and cancer development is shorter (Van der Schalie *et al*, 1999; Backer *et al*, 2001)
- dog is an immune-competent host developing spontaneous diseases. In addition to the clinical, clinical pathological and histological correspondence, also genomic instability is similar between canine and human NHL (Thomas

et al, 2001; Thomas *et al*, 2011). The correlation between genetic factors and development and progression of canine NHL is more significant because the confounding effect of human genetic heterogeneity is not present in purebred dogs. Although cytogenetic studies in canine hematopoietic neoplasms are only at an embryonic stage compared to those published in human medicine, some abnormalities shared by the two species have been reported so far (Hahn *et al*, 1994; Thomas *et al*, 2003; Pelham *et al*, 2003; Breen and Modiano, 2008; Gaurnier-Hausser *et al*, 2011)

- dog can represent a great model for preclinical stage of anticancer drug development. This is a highly expensive and time-consuming process, a critical part of which is the demonstration of the antitumor efficacy in a relevant tumor model *in vivo*. Because of the lack of gold standard treatments, pet owner may have the opportunity to enroll their pets in new clinical trials being potentially of benefit in terms of improved quality of life and prolonged survival. In addition, the regulation for the development of new drugs in pets is not as strict as for humans and owners' informed consent is often enough for recruitment of dogs. Additionally, in dogs, cancer progresses faster than in humans and clinical trials are completed earlier, allowing assessing toxicity and efficacy data in a shorter time (Marconato *et al*, 2013).

Unfortunately, however, many gaps still have to be filled in the study of canine hematopoietic neoplasms, concerning pathogenesis, diagnosis, prognosis and therapeutic protocols specific for each subtype.

2- FLOW CYTOMETRY

Flow cytometry (FC) is a technique for qualitative and quantitative assessment of multiple parameters of individual cells or particles in complex cells suspensions. Presently, it is commonly used in human oncology for confirmation of diagnosis and assessment of immunophenotype in lymphoid and non-lymphoid malignancies (Wood *et al*, 2007; Barrena *et al*, 2011).

FC is nowadays spreading in veterinary medicine, as well, and is mostly used for the diagnosis and classification of canine hematopoietic neoplasms (Comazzi and Gelain, 2011; Reggeti and Bienzle, 2011; Joetzke *et al*, 2012).

2.1- TECHNICAL ASPECTS

Flow cytometers capture light scatter properties and light emitted by individual cells labeled with fluorescent antibodies. Cells or particles are submerged in a fluid medium and pass one-by-one through a cell where are interrogated by light, either from a lamp or from one or more lasers of defined wavelengths; light scatters and emission signals are then captured by specific detectors.

Specimens for FC have to be sampled as a fluid (peripheral blood, bone marrow, effusions) or to be transformed into cell suspensions by means of different techniques. The cell suspension is ejected into the center of a sheet fluid, usually a buffered saline solution, which achieves a laminar flow under pressure and will carry the cells in a conduits system through the cytometer. By means of mechanical tools, cells are placed in the core of the laminar flow and narrowed to a single line in order to make them passing into the analysis point and being hit by the light stream one-by-one. The optical signals created by the interaction between the light stream and the cell are captured by photo detectors and converted into electrical signals:

- a photo detector is located at 0.5° in the path of the light stream and captures only the light diffracted at low angles (forward scatter, FSC). The light captured by this photodiode is proportional to the cross-sectional area of the cell and to the refractive index of the membrane: FSC is therefore considered an index of the cell volume;
- a photo detector is located at 90° and captures the light scattered by the cells at high-angle (side scatter, SSC). The light captured by this photodiode is proportional to the internal complexity and the surface texture of the cell: SSC is therefore considered an index of the cellular complexity;
- additional photo detectors are equipped with filters that capture specific wavelengths. These signals are usually relatively weak and require photomultiplier tubes to generate a useful signal.

Electric signals generated by conversion of optical signals can be directly captured on a linear scale, as typical for FSC and SSC, or amplified on linear or logarithmic scale, as typical for fluorescence channels.

A consensus on labeling techniques has never been reached and procedures may largely vary from laboratory to laboratory. However, authors agree that pre-analytic quality of the sample strongly influence FC analysis, particularly when cellularity and cell viability are concerned. Indeed, some authors suggest to assess the cellularity of the sample via an automated blood cell counter prior to immunolabeling and to adjust the concentration to approximately 10,000 cells/μl (Comazzi and Gelain, 2011). To preserve cell integrity and antigenic expression, samples for FC should be fresh: labeling of cells within 24 hours from sampling is essential for effective immunophenotyping (Jalla *et al*, 2004). Aspirate from lymph nodes can be collected in PBS if FC is performed within a few hours. Otherwise, tissue culture media such as RPMI 1640 should be preferred, possibly

supplemented with protein sources such as fetal bovine serum or plasma (Gibson *et al*, 2004).

If FC is performed to evaluate nucleated cells populations, RBC need to be removed before analysis. Indeed, although the size and light scatter of RBC differ from those of leukocytes, they outnumber leukocytes by about 1000-fold in the blood. Many different techniques can be used to eliminate RBC; however, osmotic lysis with water, ammonium chloride solutions or other buffers is more likely than density gradient methods to preserve leukocytes in their native state (Nguyen *et al*, 2007). RBC lysis is mandatory for bone marrow specimens' analysis, too. On the contrary, it could be not necessary for slightly blood contaminated samples such as lymph node aspirates, effusions or cerebrospinal fluid (Duque *et al*, 2002; Gibson *et al*, 2004; Gelain *et al*, 2008).

Once the pre-analytic quality of sample has been verified and RBC have been eliminated, immunolabeling with monoclonal antibodies can be performed. As a first step, cells are usually incubated or washed with a buffer containing serum to block non specific antibody binding to the cell surface: this step is particularly relevant when indirect antibody staining protocols are adopted. Next, cells are divided into aliquots for negative and positive controls and for staining with antibodies of interest. Negative controls may be unstained cells, cells incubated with just fluorochrome-linked secondary antibody or cells incubated with fluorescent antibodies directed to epitopes not expressed by the population of interest. Positive controls are cells incubated with a fluorescent antibody directed to an epitope which is known to be expressed by the population of interest. Which negative and/or positive control use is highly dependent from the cytometric assay and the preference of the operator (Hurley, 2010).

Antibody incubations are generally performed at 4° for 15 to 30 minutes. The optimal concentration of each antibody has to be determined by titration and if antibody combinations are applied, these need to be optimized in concert. Antibodies can be

directly conjugated to fluorochromes (direct staining) or may be used in a 2-step procedure (indirect staining), which includes sequential incubation with a primary antibody, removal of unbound antibody, and incubation with a secondary fluorochrome-conjugated antibody. Application of a direct staining procedure reduces incubation steps, time requirement and potential for non-specific antibody binding (Stewart and Stewart, 2010).

Intracellular staining of antigens can be performed, as well, but it is a more laborious procedure than cell surface antigen detection and artifacts are more common (Sachdeva and Asthana, 2007). Indeed, cells have to be fixed and permeabilized to allow antibodies entry the cytoplasm and additional negative and/or positive controls are needed. Once again, procedures vary among laboratories, but as a rule only directly conjugated antibodies are suitable for intracellular staining.

After incubation with antibodies, cells can be immediately acquired at the flow cytometer or preserved with formaldehyde- or alcohol-based fixatives for delayed analysis. Unfortunately, fixed cells have different light scatter properties and reduced fluorescence emissions, thus immediate acquisition should be preferred (Shapiro, 2003). Data analysis system may vary among laboratories but 10,000 cells are usually acquired for each tube. For each cell, the flow cytometer records a numeric value for FSC, SSC and for each fluorescence channel. Data can thereafter be visualized by dot, density or contour plots or histograms (fig 2.1). Selecting regions of cells with distinct features (gating) allows restricting analysis to specific population of cells. For example, fluorescence could be assessed either on the whole acquired sample, or on the lymphoid population alone.

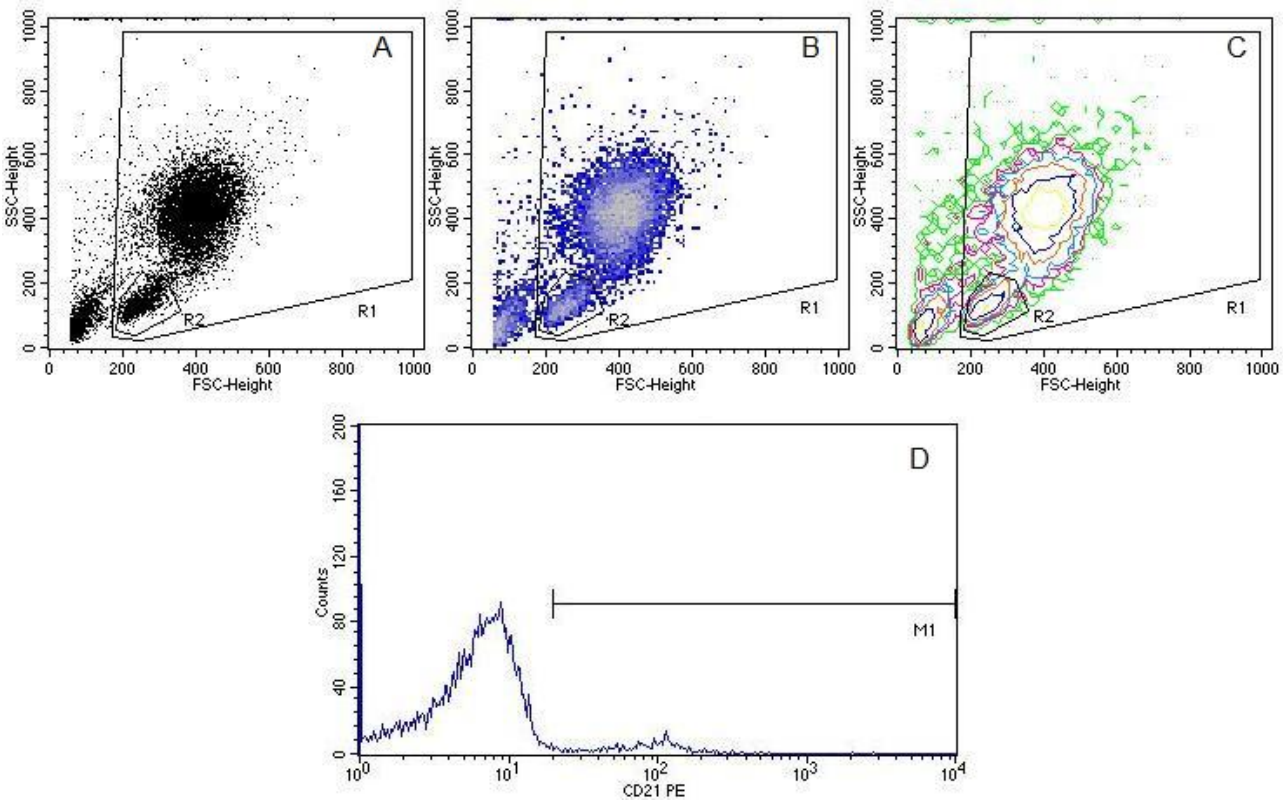

Figure 2.1: representation of flow cytometric data obtained from peripheral blood sample of an healthy dog. **A, B, C**: forward VS side-scatter; all acquired events are shown; a first gate (R1) was set to exclude debris; a second gate (R2) was set including only small-sized cells with low cellular complexity (lymphocytes). **A**: dot plot: every dot represents a single cell. **B**: density plot: color shades represent frequency of events; the paler is the color, the higher is frequency of events. **C**: contour plot: lines of different colors surround areas with different frequency of events. **D**: histogram; only cells included in R1 are shown according to the degree of fluorescence after staining with anti-CD21 antibody: only a few cells (B-lymphocytes) stained positive (M1)

2.2- APPLICATIONS IN COMPANION ANIMALS ONCOLOGY

In veterinary oncology, FC is most often applied to companion animals' hematopathology. Immunophenotyping of hematopoietic cells is based on the use of a panel of antibodies directed to cell surface or cytoplasmic antigens (clusters of differentiation, CD) to characterize cells that appear microscopically similar. Indeed, each

cell lineage and maturative stage within the same lineage are characterized by a distinct antigenic pattern.

A great interest is nowadays paid to canine hematopoietic neoplasms, as they are one of the most diffuse oncologic diseases in the dog. In particular, although it is quite difficult to estimate the real annual incidence in the absence of a tumor registry, canine lymphomas comprise approximately 7% to 24% of all canine neoplasms, and 83% of all canine hematopoietic neoplasms. On the other hand, frequency of the different forms of leukemias is still not clear, even if lymphoid origin seems more common than the myeloid one (Vail *et al*, 2012).

Immunophenotyping is usually predicated on clinicopathological evidence of putative neoplastic cells either on cytological preparation from solid tissues (suspect lymphoma) or on peripheral blood or bone marrow smears (leukemias): indeed, FC can be performed to assist the classification of these diseases but it should never replace diagnosis based on cytological and/or histological examination.

In human medicine, an antigen is considered expressed if >20% of the gated population stains positive for it (Zeppa *et al*, 2004); unfortunately, no similar guidelines exist in veterinary medicine. As a rule, if a high proportion of the putative neoplastic cells shares the same immunophenotype, this is considered a persuasive evidence for neoplasia, but does not confirm clonality. Molecular analysis of gene receptor rearrangement should be performed to demonstrated clonality in lymphoproliferative diseases (Burnett *et al*, 2003).

2.2.1- CANINE LYMPHOMA

Phenotype is one of the most important prognostic factors in canine lymphomas (Ponce *et al*, 2004; Vail *et al*, 2012). Although morphological appearance can be considered suggestive of immunophenotype (Fournel-Fleury *et al*, 1997), it should be

always confirmed by additional tests, such as immunocytochemistry, immunohistochemistry or flow cytometry. FC is nowadays routinely performed on lymph node aspirates for the diagnosis, classification and immunophenotyping of canine lymphomas.

The first report of FC immunophenotyping of lymph node aspirates in a large number of dogs with lymphoma goes back up to 2001, when Culmsee and colleagues described the FC phenotype of 30 canine lymphoma cases. All analyzed samples had sufficient quality and cellularity for FC analysis and diagnosis of lymphoma was always confirmed. B-cell phenotype was more common than T-cell phenotype. Since then, other studies have been published describing FC features of canine lymphoma subtypes (Sozmen *et al*, 2005; Wilkerson *et* al, 2005; Gelain *et al*, 2008). In particular, Gelain and colleagues investigated whether FC could add more objective data about morphology of lymphoma cells to conventional cytology (Gelain *et al*, 2008). Authors described the FC properties of lymph node aspirates from 50 dogs with lymphoma. Each lymphoma case had been classified according to the Kiel updated classification based on lymph node cytological preparations. No statistical difference in FC properties among lymphoma subtypes and no correlation between cellular size in cytology and FC was detected. Interestingly, authors identified unusual phenotypes, either qualitative or quantitative, in 64% of the lymphoma cases, particularly when small-cell lymphoma were considered. Authors concluded that FC, when used in conjunction with cytology, is a useful technique to refine classification of canine lymphomas, since it ads objective morphological information on a higher number of cells and allows identification of the phenotype, thus confirming neoplasia in ambiguous cases as low-grade, small cell lymphomas.

As highlighted by Gelain and colleagues, FC allows a quantitative evaluation of the expression of single antigens on the neoplastic cell surface. Whether unusual or aberrant phenotypes are associated to different prognosis has still to be defined. Recently, one

study investigated the influence on survival of Class II Major Histocompatibility Complex (MHCII) or CD34 expression by neoplastic cells in canine B-cell lymphomas (Rao *et al*, 2011). Based on FC results, expression of CD34 appeared as a dichotomous variable (that is, cells were easily divided into positive and negative), while MHCII exhibited a range of expression and cases had to be arbitrarily divided into two groups based on the median fluorescence intensity, with low and high MHCII expression, respectively. Based on the results of this study, MHCII had a negative association with mortality and relapse (low-MHCII group had shorter disease free interval and survival); on the contrary, CD34 was not associated with prognosis. In addition, cases in which neoplastic cells had a very large size (high FSC) also had a poorer outcome. Taken together, these results suggest that FC can be useful not only in confirming diagnosis and immunophenotype of lymphomas, but also in evaluating distinct variable associated with prognosis.

Although it is still not clearly described in literature, FC is often used to stage canine lymphoma. WHO clinical staging system recognizes five different stages of lymphoma based on organ invasion and two substages related to clinical signs (Vail *et al*, 2012), but techniques used for stage assessment largely vary among studies.

A recent study wondered if aspirates from non-lymphoid tissues in dogs are feasible for FC analysis (Joetzke *et al*, 2012). Authors examined by FC peripheral blood and bone marrow samples and aspirates from lymph nodes, spleen and liver from dogs with lymphoma and from healthy dogs. Only 12 out of 245 samples were not suitable for FC analysis due to a low cellularity: among them, 9 were liver aspirates, 1 a spleen aspirate and 2 bone marrow samples. Authors conclude that since sample from all different tissues included in the study were suitable for FC, this technique may aid in the detection of extra nodal lymphoma involvement in dogs. However, all samples were stored at 4° C and processed within 12 hours from collection and only three antibodies were tested (CD45, CD3 and CD21); thus, further studies should be performed to confirm suitability for FC of

aspirates from extra nodal tissues after a longer storage and for staining with a wider panel of antibodies.

The detection of peripheral blood and bone marrow infiltration has traditionally relied on cytological evaluation (Ponce *et al*, 2004; Flory *et al*, 2007; Flory *et al*, 2011). However, such an approach lacks specificity since the neoplastic cells, particularly those in low grade lymphomas, are difficult to distinguish from normal and reactive lymphocytes. Morphological assessment may also lack sensitivity and diagnostic accuracy, since slight marrow infiltration is difficult to observe and inter-observer variation can occur (Comazzi and Gelain, 2011). FC has been suggested in human medicine as a useful ancillary technique to be used in conjunction with histopathology to increase sensitivity and specificity and detecting bone marrow infiltration by lymphoma cells (Hanson *et al*, 1999; Talaulikar *et al*, 2009). Bone marrow histopathology is not routinely performed in veterinary medicine, mostly because of clinicians' reluctance, but FC has been used in some studies to quantify bone marrow infiltration (Grindem *et* al, 1998; Marconato *et al*, 2008; Marconato *et al*, 2011). However, use of FC for canine lymphoma staging is still far from being standardized.

In conclusion, today FC is largely used on lymph node aspirates to confirm and classify canine lymphomas. Recent studies are aimed to extend the application of FC to the staging of lymphomas by detecting neoplastic cells in non-lymphoid tissues.

2.2.2- CANINE LEUKEMIAS

The main applications of FC for canine leukemias are the confirmation of neoplasia, the discrimination between acute or chronic leukemias or leukemic lymphomas, the identification of the cell lineage involved and the identification of prognostic features. Until recently, the classification of canine leukemias was dependent upon cell morphology and, in some instances, the use of cytochemical staining. However, these techniques have

been superseded in human medicine by immunophenotyping by FC, which is now considered the gold standard for classification of leukemias (Jennings and Foon, 1997).

As a rule, clinical presentation is an important aid in solving the differential among leukemic lymphomas, chronic leukemias and acute leukemias. Indeed, dogs with leukemic lymphoma usually present with generalized painless lymphadenopathy (or with any other primary lesion in case of extra nodal lymphoma); other clinical signs are variable and depend on the extent and location of the neoplasm; blood and/or bone marrow infiltrations can occur, but they only rarely cause peripheral cytopenias. Dogs with chronic leukemias are usually asymptomatic, even if owners can report lethargy and decreased appetite; lymph nodes are usually not or minimally enlarged; in most cases, severe leukocytosis is found during routine hematologic checks; concomitant mild cytopenias can occasionally be found; at blood smear evaluation, neoplastic cells appear as normal mature cells (in most cases lymphocytes, only rarely myeloid cells). Dogs with acute leukemias have an acute onset of aspecific clinical signs such as lethargy, inappetence and weight loss, persistent fever, pallor and petechiae; moderate lymphadenopathy is common but not always present; blood cell count reveals a variable leukocytosis combined with severe non regenerative anemia and thrombocytopenia; at blood smear evaluation, neoplastic cells have an immature aspect and recognizing cellular lineage is usually difficult or impossible by morphological evaluation alone (Young and Vail, 2012).

Clinical presentation and routine blood analysis including smear evaluation, although strongly suggestive of the final diagnosis, are not conclusive and do not allow identifying the lineage involved: thus, further tests have to be performed. From the time FC is available on large scale in veterinary medicine, it has been used to get a final diagnosis in many case reports and in a few studies including a large number of dogs.

Most case reports refer of acute myeloid leukemias. Clinical presentation and morphological description of neoplastic cells in these cases are usually suggestive of

acute leukemia and sometimes myeloid origin can be supposed based on cytological evaluation alone. However, only FC immunophenotyping with a large antibody panel allows to make a definitive diagnosis and to identify the specific cellular lineage involved (Ameri *et* al, 2010; Comazzi *et* al, 2010; Tomiyasu *et al*, 2011; Valentini *et* al, 2011; Figueiredo *et al*, 2012).

In 2008, Williams and colleagues described the immunophenotype of circulating lymphoid cells in 202 dogs with lymphoproliferative disease characterized by lymphocytosis and investigated its prognostic relevance. Authors were able to identify four main phenotypic groups: CD8+ T-cells, CD21+ B-cells, CD5+CD4-CD8- aberrant T-cells and CD34+ undifferentiated cells. Median survival was compared among phenotypic groups, and CD34 expression appeared to predict a poorer prognosis, while no difference was found among the remaining three phenotypic groups. CD34 is considered a marker of acute leukemias (Vernau and Moore, 1999). Dogs included in the study by Williams and colleagues had peripheral lymphocytosis with apparently mature lymphocytes: the presence of cases with CD34+ cells and poor outcome suggests that morphologic description alone can be insufficient to discriminate acute and chronic leukemias. Additional findings from the study of Williams and colleagues include: 1) among cases with CD8+ T-cell lymphocytosis, a higher lymphocytes count was associated to poorer prognosis; 2) among cases with CD21+ B-cell lymphocytosis, large cell size was associated to poorer outcome and to the presence of lymphadenopathy at diagnosis. Unfortunately, authors did not distinguish between primary leukemias and leukemic lymphomas, thus their results could have been biased by the inclusion of dogs with different diseases.

Similar inclusion criteria were applied in a study by Tasca and colleagues in 2009. In this study, immunophenotype of 210 dogs with hematologic neoplasia was described. Based on morphology and FC, authors subdivided cases in acute lymphoid leukemias

(ALL), acute myeloid leukemias (AML), chronic leukemias (CLL) and leukemic high-grade lymphomas (HGL). Authors reported that most cases of ALL and HGL were of B-cell phenotype whereas most cases of CLL were of T-cell phenotype and that cytopenias (anemia, thrombocytopenia and neutropenia) were more common and more severe in ALL and AML cases compared to CLL and HGL; in addition, thrombocytopenia was more common in T-cell than in B-cell HGL. Authors concluded that a standard CBC can be useful in suggesting the type of hematopoietic neoplasm. Unfortunately, the phenotypic pattern of each subgroup is not well defined in the study. Indeed, cases were diagnosed as AML based only on positive staining for CD34 and CD45 and negative staining for two lymphoid markers (CD3 and CD79): these cases would have better been classified as undifferentiated acute leukemias, since no myeloid marker was tested confirming the lineage of origin. In addition, authors declared they differentiated ALL from CLL and HGL based on the expression of CD34; however, CD34+ lymphomas (Rao *et al*, 2011) and CD34- acute leukemias (Ameri *et* al, 2010; Comazzi *et* al, 2010; Tomiyasu *et* al, 2011; Valentini *et* al, 2011; Mylonakis *et al*, 2012) have been reported, thus this marker should not be considered conclusive by itself to solve the differential.

Clinical pathological and epidemiological features of dogs with confirmed leukemia were assessed via a prospective study by Adam and colleagues, in 2009. Lymphoma cases were not included in the study. Cases were subdivided into three groups based on the final diagnosis (ALL, AML, CLL). Differently from the study of Tasca and colleagues, CD34 was considered suggestive of acute leukemias, but some cases were classified as ALL or AML based on morphological evaluation of the blood smear, irrespective of the results of CD34 staining; in addition, negative staining for lymphoid markers was not considered conclusive for AML, but cells from these cases had to stain positive for at least one myeloid marker. Based on the results of Adam and colleagues, dogs with ALL had more cytopenias and more severe neutropenia and thrombocytopenia compared to dogs

with CLL, whereas the severity and prevalence of cytopenias was not different between ALL and AML cases. Total white blood cell count was not different among the three groups. Percentage and absolute number of atypical cells detected via smear evaluation was higher in ALL than in AML cases. The advent of immunophenotyping has facilitated more accurate leukemias classification than even before and has led to question the knowledge based upon the less firm foundations of the diagnosis in older studies. Still, results from Adam and colleagues support previous statements that acute leukemias give rise to more profound cytopenias than chronic ones. However, the main advantage of FC appears to be the clear identification of the lineage of neoplastic cells in acute leukemias, more than the confirmation of acute leukemia itself. Further prospective studies should be performed to assess the prognostic relevance of different phenotypes in acute leukemias.

On the contrary, confirming the diagnosis of chronic leukemias is mainly based on exclusion of any possible cause of leukocytosis. Indeed, cells keep the morphological appearance of normal mature cells and are easily identified at smear evaluation. Immunophenotyping by FC can aid in distinguishing non-neoplastic from neoplastic mature lymphocytosis determining the phenotypic diversity of circulating cells (Avery and Avery, 2007).

FC has been used in a recent study to define the immunophenotype of CLL in dogs and its prognostic influence (Comazzi *et al*, 2011). Forty-three dogs were included in the study, and subdivided into three groups based on FC phenotype: B-CLL (CD21+), T-CLL (CD3+CD8+) and atypical-CLL (any other phenotype, including CD3-CD8+, CD3+CD4-CD8-, CD3+CD4+CD8+, CD3+CD21+). Among all clinical pathological variables considered, only immunophenotype was associated with survival: dogs with T-CLL had the longest survival and a 3-fold higher and 19-fold higher probability of surviving than dogs with B-CLL and atypical-CLL, respectively. In addition, among B-CLL cases old dogs survived longer than younger dogs and among T-CLL cases anemic dogs had a shorter

survival than dogs without anemia. Although preliminary and obtained via a retrospective study where treatment was not standardized, these results suggest that immunophenotype is of great importance for dogs with CLL. Phenotypic groups in this study were created evaluating the contemporary expression of different antigens on the surface of neoplastic cells, a characteristic easily evaluable only via FC.

In conclusion, application of FC to canine leukemias is presently aimed at confirming diagnosis and cellular lineage. In addition, it brings prognostic information when used in CLL cases: whether this can be applied to acute leukemias as well has still to be determined.

2.2.3- RNA-DNA CONTENT ANALYSIS

In addition to detecting proteins with fluorescent antibodies, FC can be used to analyze nucleic acid content and to characterize DNA or RNA synthesis, cell cycle stages, apoptosis, necrosis and individual chromosomes. In oncology, DNA analysis can serve as an adjunct to estimate the proliferating tumor fraction or aneudiploid states, which may relate to prognosis of tumors.

Nucleic acid dyes significantly fluoresce only when bound to their target. A range of nucleic acid stains is available, with different emission wavelength, cell membrane penetration and nucleotide specificity. The most widely used nucleic acid stain is propidium iodide which is unable to penetrate into viable cells allowing exclusion of non-viable cells from analysis and distinction of necrosis from early apoptosis (Smolewski *et al*, 2002; Schmid *et al*, 2007).

Cells of an organism have a consistent amount of nuclear DNA unless they undergo meiosis, mitosis or apoptosis; cells populations containing nuclei with increased or decreased DNA content (aneuploid) are commonly observed in human tumors and, in many instances, reflect malignant properties of the tumor. For in vitro assessment of the

cell cycle, it is often desirable to quantify the proportion of cells in diploid stages (G0/G1), in the S-phase (with an intermediate amount of DNA) and in the G2/M phases, where DNA synthesis has been completed to the tetraploid stage (Huang *et al*, 2005).

Tumor DNA or RNA content analysis is not routinely applied in veterinary clinical oncology. However, it has been used in some research studies. In theory, a high proportion of cells containing asymmetric DNA content (aneuploid cells) should reflect gross genetic abnormalities of a tumor, and an increased proportion of cells synthesizing DNA should reflect rapid tumor growth (Hall, 2004). Accordingly, aneuploid cells have been identified by FC in canine mammary tumors (Rutteman *et* al, 1988), prostatic carcinoma (Madewell *et* al, 1991), plasma cell tumors (Frazier *et al*, 1993), transitional cells carcinomas (Clemo *et al*, 1994) and hemangiopericytoma (Kang *et al*, 2006). DNA ploidy was also analyzed by FC in canine lymphomas (Teske *et al*, 1993). In this study, aneuploidy was detected in 20 out of 94 lymphoma cases and the mean S-phase fraction was significantly higher in lymphomas than in non-neoplastic controls. No difference was found in DNA ploidy and S-phase fraction between T- and B-cell lymphomas or among different lymphoma histotypes. Finally, neither DNA ploidy nor S-phase fraction were correlated to survival. Authors concluded that the frequency of DNA aneuploidy in canine lymphomas is similar to that described in human medicine, whereas the absence of correlation between DNA ploidy or S-phase fraction and histomorphology or prognosis is in contrast with findings in human medicine.

Other studies found aneuploidy in neoplastic cells, but none of them could find a significant correlation with prognosis (Clemo *et al*, 1994; Kang *et al*, 2006). Many reasons for the limited prognostic significance of aneuploidy are possible, including: admixture of multiple non-neoplastic cells (including multinucleated polyploid cells) in analysis, tumor heterogeneity or tumor necrosis.

2.2.4- FELINE SAMPLES

Hematopoietic neoplasms are quite common in the cat, too; however, only one report has been published about use of FC in feline lymphomas (Roccabianca *et al*, 2006) and the clinical relevance of immunophenotype, which is one of the most important prognostic factors in canine lymphoma, has never been described in the cat. Only few reports have been published about use of FC to confirm diagnosis and immunophenotype of feline leukemias, as well (Tebb *et* al, 2004; Gelain *et al*, 2006; Sharifi *et al*, 2007; Campbell *et al*, 2012).

Some important differences make feline samples less suitable for FC analysis compared to canine samples:

- differently from the dog, the most common clinical presentation of feline lymphoma is a low-grade small cell lymphoma involving the gastrointestinal tract (Vail, 2012) which is likely more difficult to be sampled than peripheral lymph nodes (that are most commonly involved in canine lymphomas). Cytological evaluation of fine needle aspirate of the primary lesion if often not sufficient to distinguish among lymphoma (particularly low-grade lymphoma), benign hyperplastic or reactive lymphoid conditions. Histopathological assessment is required in these cases, as only architectural abnormalities and invasiveness can bring to a definitive diagnosis of neoplasia. PARR analysis can help refining diagnosis in equivocal cases, but its application has been described as being approximately only 80% sensitive for the diagnosis of feline lymphoma, whereas specificity has not been defined (Avery, 2009).
- besides biological differences between canine and feline hematopoietic neoplasms, the most important limiting factor for the application of FC to feline samples is the reduced number of monoclonal antibodies available:

this prevents the assessment of precise phenotype and of antigen aberrancies. Studies describing the phenotype of feline hematopoietic neoplasms and its potential prognostic value are still lacking. Publication of such data in the future would perhaps improve the production and validation of monoclonal antibodies reacting with feline antigens, and consequently the application of FC to the study of feline hematopoietic neoplasms.

3- AIM OF THE STUDY

Aim of the present study was to evaluate some clinical-pathological features of canine hematopoietic neoplasms via flow cytometry (FC). Peculiar characteristics and advantages of FC will be applied to different specific aims, described one-by-one here below.

3.1- CANINE LARGE B-CELL LYMPHOMA STAGING

Lymphoid neoplastic cells are well characterized by FC, and both cell size and immunophenotype can be determined, thus identifying a specific fingerprint of the neoplastic cells for each case. Once this has been made, other tissues from the same dog can be investigated by FC to detect and quantify the infiltration by the same neoplastic cells. This is particularly useful in lymphomas in which other tissues, such as peripheral blood (PB) and bone marrow (BM), may be investigated for the presence of the same neoplastic cells identified in the lymph node. This detection is particularly easy and specific when neoplastic cells have characteristics that clearly distinguishes them from normal or reactive lymphocytes, such as large size or aberrant phenotype.

In this section of the present study, the attention will be paid to large B-cell lymphoma (LBCL), the most common lymphoma subtype in the dog. In this particular subtype, neoplastic cells are easily distinguishable from normal or reactive lymphocytes thanks to their large size. A FC method to quantify PB and BM infiltration will be validated and compared to cytological evaluation, and its prognostic value will be assessed via a prospective study.

Based on WHO clinical staging system, canine lymphoma is classified as stage V when PB or BM infiltration (or any other non-lymphoid tissue) occurs (Vail *et al*, 2012). However, methods of evaluation are not standardized and the prognostic influence is still

not clear: as a consequence, BM evaluation is considered not mandatory under official guidelines unless peripheral blood cytopenias are present (Vail *et al*, 2010). The link between PB cytopenias and BM infiltration will be investigated in this section, too.

3.2- PHENOTYPIC ABERRANCIES IN CANINE LYMPHOMAS

One of the main peculiarities of FC is the possibility to assess the contemporary expression of different antigens on the same cellular population, and the subsequent identification of antigen aberrancies. In human medicine some specific aberrancies are related to different prognosis in specific neoplasms (Jennings and Foon, 1997; Lai *et* al, 2000; Gorczyca *et* al, 2002; Mitrovic *et al*, 2009), but no data are at the moment available in dogs. The detection of aberrant patterns, however, is useful to confirm clonal expansion and to accurately check tissue infiltration (Comazzi and Gelain, 2011).

First aim of this section of the study was to assess the prevalence and the prognostic value of specific phenotypic aberrancies detected by FC in canine lymphomas, irrespective of the subtype, via a retrospective study.

Differently from other lymphoma subtypes, cytological evaluation of canine small clear cell lymphoma (a T-cell lymphoma) cannot be considered conclusive for neoplasia, and further tests have to be performed. In humans, aberrancies are quite common in T-cell lymphomas and are considered an excellent diagnostic tool (Jamal *et al*, 2001; Arun *et al*, 2010). In this section of the study, as a second aim, we will retrospectively describe the prevalence of aberrancies in canine small clear cell lymphoma and the usefulness of FC to confirm the diagnosis of neoplasia when histopathology, which is the gold standard technique, is not available or permitted by clinicians or owners.

3.3- EXPRESSION OF CD44 IN BLOOD FROM DOGS WITH HEMATOPOIETIC NEOPLASMS

FC allows a semi-quantitative evaluation of the expression of molecules on the cell surface. Indeed, the more is a molecule expressed by the cells, the higher will be the mean fluorescence intensity (MFI) of the antibody specific for that molecule, when compared to the MFI of unstained cells.

This property of FC will be used in the last section of the study to assess the degree of expression of CD44 in PB sample from dogs with hematopoietic neoplasms.

CD44 is being studied in human medicine because of its pathogenetic and potential therapeutic role in many neoplasms. It is the main receptor for the hyaluronic acid and different isoforms are expressed by the different cells of the organism (Mackay *et al*, 1994; Ponta *et al*, 1998). It is involved in many cellular processes, such as proliferation, migration, survival and apoptosis (Lesley *et al*, 1993) and could therefore be involved in the proliferation and spread in the organism of neoplastic cells. In particular, the degree of expression of CD44 has been correlated to a worse prognosis in many hematopoietic neoplasms in humans (Hertweck *et al*, 2011). Some authors opened the way for the development of new drugs targeting CD44, by demonstrating that the administration of anti-CD44 antibodies can induce cellular differentiation in AML-M0 cell lines (Charrad *et al*, 2002).

Although it has been studied in many different reactive and neoplastic conditions in the dog (Moore *et* al, 1996; Sanchez *et* al, 2004; Serra e*t al*, 2004; Lin *et al*, 2009; Madrazo *et* al, 2009; Paltian *et al*, 2009; Sabattini and Bettini, 2009; Cogliati *et al*, 2010), no study is at the moment available about CD44 expression in canine hematopoietic neoplasms. In the last section of present study we focused on the expression of CD44 in hematopoietic neoplasms with particular regard for peripheral blood, due to the possible role played by CD44 in the systemic spread of the neoplastic cells.

4- MATERIALS AND METHODS

Samples included in the present study were collected and sent by the Oncology Service of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy; Dr Damiano Stefanello), by the Centro Oncologico Veterinario (Sasso Marconi, Bologna, Italy; Dr Laura Marconato), or by private vets for diagnostic purposes or routine hematologic analysis to the Laboratory of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy) or of the Department of Veterinary Science (University of Turin, Turin, Italy; Dr Fulvio Riondato).

Flow cytometry (FC) was performed on lymph node (LN), peripheral blood (PB) and/or bone marrow (BM) samples. LN samples were collected into 1 ml of RPMI, PB and BM samples in EDTA tubes. All samples were stored at 4°C and processed within 24 hours from collection. When not provided by the referring veterinarian, PB and BM smears were prepared and stained with *May-Grunwald-Giemsa*.

Before processing for FC, all samples were analyzed via an automated hematology analyser (Sysmex XT-2000iV; Sysmex, Kobe, Japan) to assess cellularity.

BM samples were not admitted to the FC analysis if subjectively considered excessively hemodiluted by smear evaluation (considering the presence of spicules, megakaryocytes and immature cells) and/or by automated count (comparing the number of RBC and nucleated cells in PB and BM samples from the same dog).

PB and BM samples underwent a pre-labeling lysis using a buffer containing 8% ammonium chloride to eliminate erythrocytes. This was usually not necessary for LN samples as blood contamination was never severe and did not interfere with FC analysis.
Sample processing for FC analysis was performed as previously described (Gelain *et al*, 2008).

All antibodies utilized for FC were provided by Serotec (Oxford, UK) except for anti-CD34 that was provided by BD Pharmingen (San Diego, CA, USA), and had been

previously titered to define the correct working dilution. Antibody panel applied to each sample included: anti-CD3-FITC (clone CA17.2A12), anti-CD5-PE (clone YKIX322.3), anti-CD4-FITC (clone YKIX302.9), anti-CD8-PE (clone YCATE55.9), anti-CD21-PE (clone CA21D6), anti-CD79a-PE (clone HM57), and anti-CD34-PE (clone 1H6). In order to properly select cells via a tricolor approach, anti-CD45-FITC or –APC (clone YKIX716.13) was included in each tube.

Samples were finally acquired via a FACScalibur or a BD Accuri C6 flow cytometer (Becton Dickinson, San Josè, CA, USA) and data were analyzed via specific software CellQuest or CFlow Plus (Becton Dickinson, San Josè, CA, USA).

All statistical analysis were performed using SPSS 19.0 for Windows and results were considered significant when $p \leq 0.05$.

Materials and methods for specific aims are described here below.

4.1- CANINE LARGE B-CELL LYMPHOMA STAGING

FC was utilized to quantify PB and BM infiltration by neoplastic cells in dogs with large B-cell lymphoma (LBCL). In a first scatter gram based on forward- (FSC) and side-scatter (SSC), a gate (R1) was set to exclude debris. In a second scatter gram based on SSC and CD45-fluorescence, a gate (R2) was set including only CD45-positive cells. Finally, only cells included in both R1 and R2 were visualized and a FSC versus CD21-fluorescence scatter gram was drawn. PB and BM infiltration were defined as the percentage of large (FSC-H channel higher than 400) CD21-positive cells (large B-cells) out of total CD45-positive cells (fig 4.1).

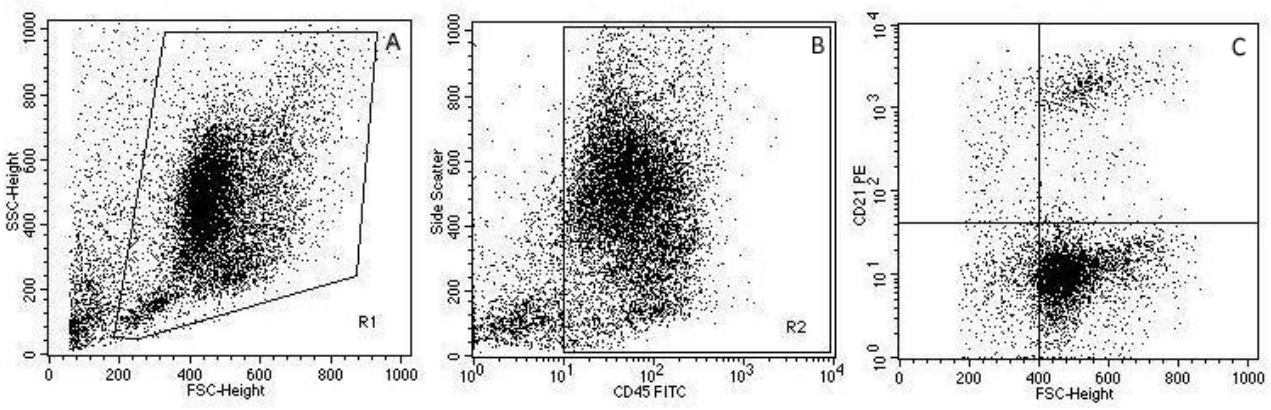

Fig 4.1: bone marrow flow cytometric scatter grams from a dog with large B-cell lymphoma and a moderate (9.6%) bone marrow infiltration by large B-cells. **A**: all acquired events are shown; a gate (R1) was set to exclude debris based on the forward and side scatter properties. **B**: all acquired events are shown; a gate (R2) was set to include only CD45-positive cells (all leukocytes). **C**: only cells belonging to both R1 and R2 are shown; the discriminator was set on the basis of the fluorescence of cells incubated with isotypic control, large B-cells are in the upper right quadrant.

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4.1.1- DIAGNOSTIC PERFORMANCES

To establish the diagnostic performances of this FC method, samples from dogs with a final diagnosis of LBCL based on LN cytology, FC and PARR analysis were prospectively enrolled.

PARR analysis was regarded as a gold standard to identify PB and BM infiltrated samples, and only PARR-positive PB and/or BM samples were included in the study. Additionally, samples from healthy dogs or dogs with diseases other than LBCL were enrolled as negative controls.

A ROC curve was drawn to establish the percentage of large B-cells that best distinguishes between infiltrated (PARR-positive) and control samples (from healthy dogs or dogs with diseases other than LBCL). PB and BM samples were analyzed separately.

4.1.2- COMPARISON BETWEEN FC AND CYTOLOGY

In order to assess if FC and cytology are interchangeable and give the same results about PB and BM infiltration by LBCL, 60 consecutive cases of canine LBCL were retrospectively extracted from the database of the Laboratory. Additional inclusion criteria were: availability of a CBC by an automated analyser (Sysmex XT-2000iV; Sysmex, Kobe, Japan), availability of PB and BM smears stained with *May-Grunwald-Giemsa*, and availability of FC data about LN, PB and BM.

Cytological infiltration was calculated as the percentage of atypical cells among 100 nucleated cells on PB smears, and as the percentage of lymphoblasts among 500 nucleated cells on BM smears.

Cases were subdivided into four groups based on BM infiltration evaluated by FC and cytology, respectively. When FC results were used for classification, group A had <5% infiltration, group B from 5 to 10%, group C from 10 to 20%, group D >20%. When cytological results were used for classification, group 1 had <5% infiltration, group 2 from 5 to 10%, group 3 from 10 to 20%, group 4 >20%.

Cohen's Kappa value was calculated to assess the agreement between the two techniques.

4.1.3- BM INFILTRATION AND PB ABNORMALITIES

The same cases included in section 4.1.2 were analyzed to check if BM is infiltrated by LBCL only in dogs with PB abnormalities, as supposed by official guidelines (Vail *et al*, 2010) but never proved.

PB abnormalities were defined as any hematological value out of internal laboratory reference range and/or the presence of morphologically unclassifiable cells at smear evaluation.

Mann-Whitney test was performed to analyze the difference in BM infiltration between dogs with and without anemia, thrombocytopenia, leukocytosis, lymphocytosis or atypical cells, and between dogs with or without any hematological abnormality. Generalized Linear Models were used to evaluate the correlation between BM infiltration and hemoglobin concentration, platelet, leukocytes, lymphocytes, atypical cells number and PB infiltration.

4.1.4- PROGNOSTIC VALUE

Prognostic value of PB and BM infiltration was investigated via a prospective study. To this aim, dogs admitted at the Centro Oncologico Veterinario (Sasso Marconi, Bologna, Italy; Dr Laura Marconato) and diagnosed with LBCL on the basis of lymph node cytology, FC and when possible histopathology were prospectively enrolled.

Based on a complete staging workup (consisting of history, physical evaluation, CBC and biochemical profile, cytological evaluation of liver and spleen, diagnostic imaging of thorax and abdomen, cytological and FC analysis of PB and BM) dogs were assigned to a clinical stage according to the WHO staging system (Vail *et al*, 2012) and treated with the same CHOP-based chemotherapy protocol.

Generalized Linear Models were performed to check the independence of PB and BM infiltrations from the following variables, which have shown some prognostic value in the literature (Marconato *et al*, 2011): breed (pure- or mixed breed), gender (male or female), and age (< or ≥10 years), weight (< or ≥10 kg), substage (a or b), anemia (present or absent), high LDH activity (present or absent) and pretreatment with corticosteroids (present or absent).

Univariate and multivariate Cox's proportional hazard regression analysis were performed to determine any possible relationship between PB or BM infiltration and TTP or LSS. When a significant association was detected, all cases were subdivided into two

groups based on arbitrary cutoff value of 1%, 3%, 5%, 10%, 15% and 20%. Kaplan-Meier curves were drawn for each cutoff and compared by Log-rank test. Finally, hazard ratios (HRs) were calculated for each cutoff using a Cox's proportional hazard regression analysis.

4.1.5- LINK BETWEEN PB AND BM INFILTRATION DEGREE

To establish if PB infiltration can predict BM infiltration, some consecutive cases with a final diagnosis of LBCL were retrospectively extracted from the FC database of the Laboratory of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy). For each case PB and BM infiltration by large B-cells were recorded.

Different degrees of BM infiltration were arbitrarily selected and ROC curves were drawn to identify PB infiltration cutoff values suitable to safely identify BM samples with at least 1%, 3%, 5%, 10% and 20% large B-cells, respectively. Sensitivity and specificity for each selected PB infiltration cutoff were recorded.

4.2- PHENOTYPIC ABERRANCIES IN CANINE LYMPHOMAS

4.2.1- PROGNOSTIC VALUE

Prevalence and prognostic role of specific aberrancies in canine lymphomas was evaluated by a retrospective study. The FC database of the Laboratory of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy) was interrogated over a five-year period. All cases with a final diagnosis of lymphoma were extracted and antigenic pattern for each case was recorded.

Aberrancies were defined as diminished or absent expression of pan-leukocyte antigens or antigen specific of the involved cellular lineage, co-expression of antigens from different cellular lineages, co-expression or lost of both CD4 and CD8 by T-cells, and expression of CD34 in cases in which acute leukemia had been excluded.

For aberrant lymphoma cases, referring vets were interrogated and therapy, time-to-progression (TTP), lymphoma-specific-survival (LSS) and cause of death were recorded. Kaplan-Meier curves were drawn and compared by Log-rank test to assess the influence of specific aberrancies on TTP and LSS.

4.2.2- DIAGNOSIS OF SMALL CLEAR CELL LYMPHOMA

To establish the diagnostic role of antigen aberrancies in canine lymphomas, only small clear cell lymphoma cases were taken into account, as this morphological appearance is considered suggestive, although not conclusive, of T-zone histopathological pattern and therefore T-cell phenotype (Fournel-Fleury *et al*, 1997; Fournel-Fleury *et al*, 2002; Ponce *et al*, 2004).

Laboratory databases of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy) and of the Department of Veterinary Science (University of Turin, Turin, Italy; Dr Fulvio Riondato) were interrogated. Cases with a suspect of nodal small clear cell lymphoma were selected and cytological preparations of the lymph node 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 mitoses/five fields 500x). In addition, cases with a final diagnosis of reactive hyperplasia based on lymph node cytology, FC and when needed histopathology were included as negative controls.

The phenotype of each lymphoma case was recorded, and prevalence of specific aberrancies was calculated. Aberrancies were defined as diminished or absent expression of pan-leukocyte antigens or antigens of the involved cellular lineage, co-expression of antigens from different cellular lineages and co-expression or lost of both CD4 and CD8 by T-cells.

4.3- EXPRESSION OF CD44 IN BLOOD FROM DOGS WITH HEMATOPOIETIC NEOPLASMS

In the last section of this study, the degree of expression of CD44 by the lymphoid population in PB samples from dogs with lymphoma or leukemia was evaluated. To this aim, some antibodies were added to the previously described panel: anti-CD44-FITC (clone IM7), anti-CD14-PE (clone TUK4), and anti-CD11b-PEcy5 (clone m1/70), anti-MPO-FITC (clone 2C7). These were all provided by Serotec (Oxford, UK) except anti-CD44 that was provided by BD Pharmingen (San Diego, CA, USA) and anti-CD11b that was provided by eBioscience (San Diego, CA, USA).

Besides neoplastic samples, PB samples from healthy dogs and dogs with non-neoplastic diseases were included as controls.

Diagnosis of acute or chronic leukemia was made on the basis of clinical signs, CBC, smear evaluation and FC immunophenotype. Positive staining for CD34 was considered suggestive but not conclusive for acute leukemia; when differential with leukemic lymphoma could not be solved, cases were not included in the study.

Acute leukemias were classified as lymphoid (ALL) if stained positive for any lymphoid antigen (CD3, CD5, CD4, CD8, CD79a, CD21) and negative for all myeloid antigens (CD14, CD11b, MPO), as myeloid (AML) if stained positive for any myeloid marker and negative for all lymphoid markers, and as unclassified (AUL) if stained negative for all lymphoid and myeloid antigens.

Chronic leukemias were classified as T-CLL if stained positive for T-cell markers (CD3, CD5, CD4 or CD8) and negative for B-cell markers (CD21 and CD79a), as B-CLL if stained positive for B-cell markers and negative for T-cell markers, as atypical-CLL if any other phenotype was detected (Comazzi *et al*, 2011).

For each sample, the population of interest was gated based on morphological properties using a FSC versus SSC scatter gram where only CD45-positive cells were

shown. CD44 mean fluorescence intensity (MFI) was calculated as the ratio of MFI of stained cells/MFI of unstained cells.

Kruskal-Wallis test was performed to compare CD44 MFI among different disease groups (acute leukemias, chronic leukemias, lymphomas, reactive controls, healthy controls) and among different phenotypes within the same disease group. Mann-Whitney test was performed to compare CD44 MFI between T- and B-lymphocytes in healthy controls, and between leukemic and non-leukemic lymphomas within B- and T-cell lymphomas.

5- RESULTS

5.1- CANINE LARGE B-CELL LYMPHOMA STAGING

5.1.1- DIAGNOSTIC PERFORMANCES

Overall, thirty-one consecutive cases were prospectively enrolled to determine the diagnostic performances of FC to quantify PB and BM infiltration in canine LBCL. Among them, 15 dogs had LBCL and 16 were negative controls, including 3 healthy dogs, 4 dogs with Leishmaniasis, 2 dogs with Evans' syndrome, 2 dogs with T-cell lymphoma, 1 dog with disseminated histiocytic sarcoma, 1 dog with cutaneous mast cell tumor and 3 dogs with reactive lymph node hyperplasia of unknown origin.

PB samples were available for 29 dogs: 14 (48.3%) dogs with LBCL and 15 (51.7%) negative controls. Overall mean percentage of large B-cells in PB samples was 6.47±16.72% (median 1.77%; min-max: 0.15-83.60%). Among lymphoma cases, mean percentage of large B-cells was 12.0±23.18% (median 2.25%; min-max: 0.32-83.60%). Among negative controls, mean percentage of large B-cells was 1.31±1.17% (median 0.85%; min-max: 0.15-4.29%). In four negative control samples (3 healthy dogs and 1 dog with cutaneous mast cell tumor), large CD21-positive cells also had a high cellular complexity (high SSC). When these cases were excluded from analysis, mean percentage of large B-cells in negative controls was 0.77±0.54% (median 0.72%, min-max: 0.15-1.81%).

When ROC curve was drawn including all control samples, an AUC of 0.812 was found, and 1.56% was identified as the best cut-off to distinguish between positive and negative samples, having an 85.7% sensitivity and a 73.3% specificity. When ROC curve was drawn excluding the four control sample with large CD21-positive cells with high cellular complexity, the AUC increased to 0.912 and the best cut-off identified was 1.50%, having an 85.7% sensitivity and a 90.9% specificity.

BM samples were available for 20 dogs: 11 (55%) dogs with LBCL and 9 (45%) negative controls. Overall mean percentage of large B-cells was 11.17±20.45% (median 1.04%; min-max: 0.12-73.6%). Among lymphoma cases, mean percentage of large B-cells was 20.00±24.57% (median 12.58%; min-max: 0.83-73.6%); among negative controls, mean percentage of large B-cells was 0.39±0.26% (median 0.33%; min-max: 0.12-1.02%).

When ROC curve was drawn, an AUC of 0.990 was found, and 1.04% was identified as the best cut-off, having a 90.9% sensitivity and a 100% specificity.

5.1.2- COMPARISON BETWEEN FC AND CYTOLOGY

Sixty consecutive cases were extracted from the database of the Laboratory to compare FC and cytological results about PB and BM infiltration by LBCL.

At PB smear evaluation, atypical cells were found only in 5 (16.7%) cases, accounting for 1% of all nucleated cells in one case, 4% in two cases, 15% and 72% in one case each. Atypical cells found were usually medium to large sized, with high nucleus/cytoplasm ratio, slightly basophilic scant to moderate cytoplasm, round or irregular nucleus with finely reticular chromatin and occasional prominent nucleoli. In the five cases with atypical cells, FC infiltration was 0.13%, 1.80%, 5.4%, 1.96% and 80.41%, respectively. Due to the reduced number of cases with atypical cells, agreement tests between FC and cytology were not performed.

In fourteen cases, BM smear quality was subjectively considered not sufficient for evaluation, mostly due to bad preparation or preservation. Among the 46 remaining cases, cytological infiltration was <5% in 22 (47.8%) cases, between 5 and 10% in 11 (23.9%), between 10 and 20% in 6 (13%) and >20% in 7 (15.2%). Although all BM samples had sufficient quality for FC evaluation, analysis was restricted to the 46 for which also cytological evaluation was possible: among them, FC infiltration was <5% in 31 (67.4%)

cases, between 5 and 10% in 3 (6.5%), between 10 and 20% in 4 (8.7%) and >20% in 8 (17.4%). Combined results from cytology and FC are shown in table 5.1.

Cohen's kappa value for BM samples was 0.51.

		Cytology				
		<5%	5-10%	10-20%	>20%	Total
Flow Cytometry	<5%	19	8	3	1	31
	5-10%	2	1	0	0	3
	10-20%	0	2	2	0	4
	>20%	1	0	1	6	8
	Total	22	11	6	7	46

Table 5.1: distribution of 46 dogs with LBCL based on FC and cytological BM infiltration

5.1.3- BM INFILTRATION AND PB ABNORMALITIES

The same cases included in section 5.1.2 were analyzed to assess if there is a link between BM infiltration and peripheral cytopenias.

Among 60 dogs included, thirty (50%) had at least one PB abnormality, including: moderate anemia (17 cases, 56.7%), thrombocytopenia (15 cases, 50%), leukocytosis (12 cases, 40%), lymphocytosis (11 cases, 36.7%), presence of atypical cells (5 cases, 16.7%). Fourteen (46.7%) cases had multiple abnormalities. No dog had lymphopenia or granulocytic abnormalities.

Among cases with hematological abnormalities, PB infiltration was <5% in 18 (60%) cases, between 5% and 10% in 3 (10%), between 10% and 20% in 1 (3.3%) and >20% in 8 (26.7%); BM infiltration was <5% in 16 (53.3%) cases, between 5% and 10% in 1 (3.3%), between 10% and 20% in 5 (16.7%) and >20% in 8 (26.7%).

Thirty dogs did not have any hematological abnormality. Among them, PB infiltration was <5% in 20 (66.7%) cases, between 5% and 10% in 7 (23.3%), between 10% and 20% in 3 (10%) and >20% in no one case; BM infiltration was <5% in 21 (70%) cases, between 5 and 10% in 5 (16.7%), between 10% and 20% in 3 (10%) and >20% in 1 (3.3%) case.

Median PB infiltration among all cases was 2.82% (min-max: 0.00-80.41%). Median BM infiltration among all cases was 3.05% (min-max 0.03-74.50%). PB and BM infiltration for each case are shown in fig 5.1.

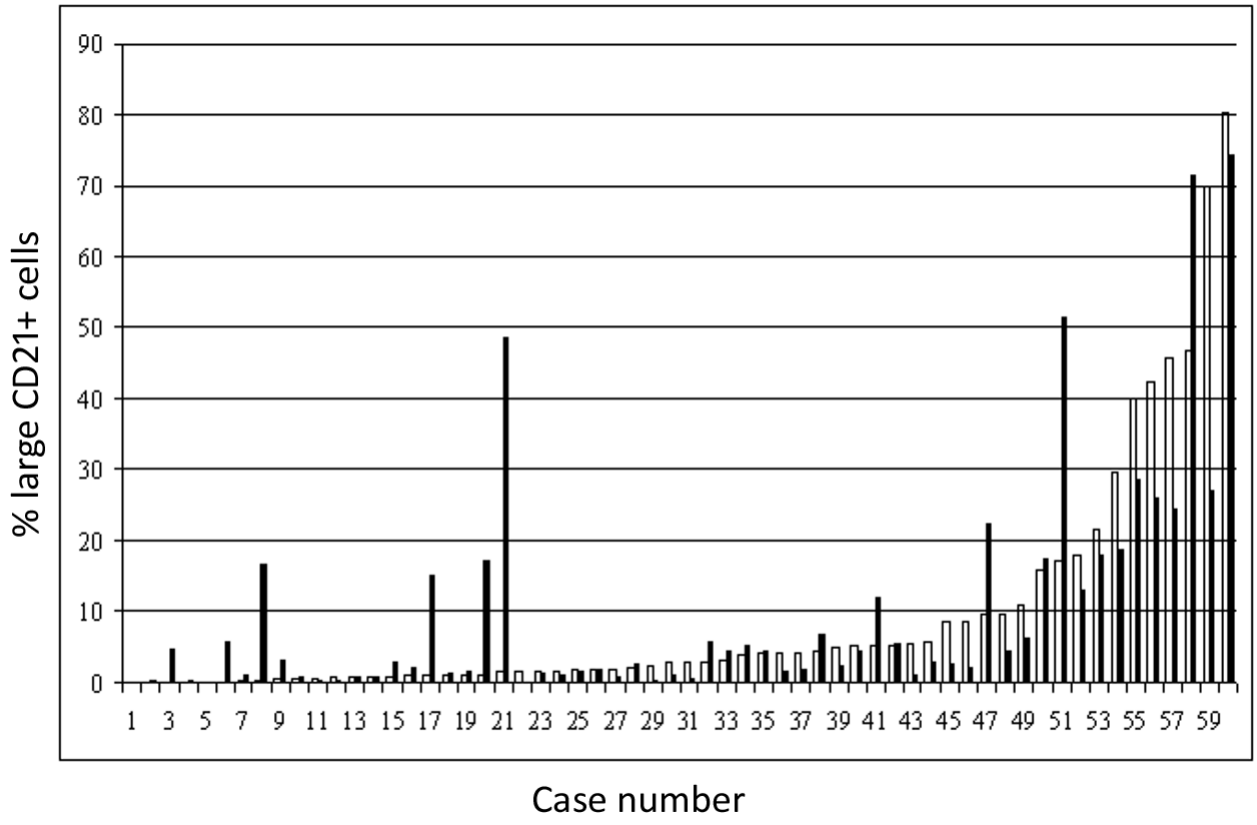

Fig 5.1: flow cytometric blood (white columns) and bone marrow (black columns) infiltration by large B-cells in 60 dogs with large B-cell lymphoma. A significant correlation was found between blood and bone marrow infiltration degree (p=0.022).

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On the basis of Mann-Whitney test, BM infiltration was significantly higher in dogs with thrombocytopenia (p=0.002), leukocytosis (p=0.032) and lymphocytosis (p=0.000). On the other hand, there was no significant difference in BM infiltration between dogs with or without anemia or atypical cells and between dogs with or without any hematological abnormality (tab 5.2).

Peripheral blood abnormality	Number of cases (%)	BM infiltration (%)	
		Median	min-max
Low hemoglobin concentration	17 (28.3)	3.03	0.3-74.5
Normal hemoglobin concentration	43 (71.7)	3.07	0.03-48.62
Low platelet count	15 (25)	18	0.35-74.5
Normal platelet count	45 (75)	2.49	0.03-27
High leukocytes number	12 (20)	21.55	0.34-74.5
Normal leukocytes number	48 (80)	2.58	0.03-51.42
High lymphocytes number	11 (18.3)	24.43	1.2-74.5
Normal lymphocytes number	49 (81.7)	2.2	0.03-51.42
Presence of atypical cells	5 (8.3)	1.35	0.3-74.5
Absence of atypical cells	55 (91.7)	3.25	0.03-71.26
Any abnormality	30 (50)	3.97	0.13-74.5
No abnormalities	30 (50)	2.58	0.03-22.4

Table 5.2: bone marrow infiltration in different groups of dogs with large B-cell lymphoma, based on the presence of abnormalities at complete blood cell count or at blood smear evaluation

Generalized Linear Models showed a significant correlation between BM infiltration and platelet count (p=0.012) and PB infiltration (p=0.022): BM infiltration was negatively correlated to platelet count and positively correlated to PB infiltration. Interestingly, however, in some cases involvement was noticeably higher in bone marrow than in peripheral blood (fig 5.1). No significant correlation was found between BM infiltration and hemoglobin concentration, leukocytes, lymphocytes and atypical cells number.

5.1.4- PROGNOSTIC VALUE

Forty-six dogs with LBCL were prospectively enrolled at the Centro Oncologico Veterinario (Sasso Marconi, Bologna, Italy; Dr Laura Marconato) to evaluate the prognostic value of BM infiltration quantified by FC. There were 33 (71.7%) purebred and 13 (28.3%) mixed breed dogs. Twenty-four (52.2%) dogs were males and 22 (47.8%) were females. Median age was 8 years (min-max: 3-15 years). Median weight was 28.1 kg (min-max: 2.6-69 kg). Seventeen (36.9%) dogs received low-dose prednisone for a median of 12 days (min-max: 4-18 days) before being referred but in all cases it had been discontinued for at least 3 days before admission at the Centro Oncologico Veterinario. Thirty-one (67.4%) dogs were asymptomatic and 15 (32.6%) showed clinical signs at admission. Sixteen (34.8%) dogs had a moderate anemia and 30 (65.2%) had high LDH activity.

All dogs were treated with the same CHOP-based chemotherapy protocol. Thirty-eight (82.6%) dogs obtained lasting complete remission (CR) within 24 hours of the start of treatment; 1 (2.2%) dog obtained CR within 24 hours but progressed 24 days later; 2 (4.3%) went into partial remission (PR) but one progressed after 19 days; 5 (10.9%) dogs experienced progressive disease (PD). Among dogs that achieved lasting CR, 31 (81.6%) relapsed during the study period, 5 (13.2%) were still in CR at data analysis closure, 2 (5.3%) died for causes unrelated to their lymphoma after 20 and 112 days respectively,

still in CR for lymphoma. The dog with lasting PR relapsed after 34 days. Overall median time-to-progression (TTP) was 87 days (min-max: 0-1211 days).

Six (13%) dogs were still alive and in CR at the end of study, 2 (4.3%) died for causes unrelated to their lymphoma, 38 (82.6%) died for PD. Overall lymphoma-specific survival (LSS) for all dogs was 188 days (min-max 9-1403 days).

Median PB infiltration was 3.0% (min-max: 0.2-45%); median BM infiltration was 3.8% (min-max: 0.2-47.4%). PB infiltration was directly correlated with BM infiltration (p=0.000). PB infiltration was significantly higher in dogs <10 years old (p=0.007), in females (p=0.009), in symptomatic dogs (p=0.049) and in dogs with high LDH activity (p=0.006), whereas it was not associated with breed, weight, anemia or pretreatment with corticosteroids. BM infiltration was significantly higher in symptomatic dogs (p=0.000) and in dogs with high LDH activity (p=0.002), whereas it was not associated with breed, sex, age, weight, anemia or pretreatment with corticosteroids.

On the basis of univariate Cox's proportional hazard regression, TTP was influenced by BM infiltration (p=0.001), substage (p=0.002), LDH activity (p=0.028) and anemia (p=0.044), whereas LSS was influenced by BM infiltration (p=0.000), substage (p=0.000) and anemia (p=0.034). In the multivariate analysis, only substage was associated with TTP (p=0.002), whereas substage and anemia were associated with LSS (p=0.000 and p=0.008, respectively).

Kaplan-Meier curves were drawn and compared by Log-rank test for BM infiltration groups, with arbitrary cutoffs of 1%, 3%, 5%, 10%, 15% and 20% (tab 5.3 and 5.4, fig 5.2 and 5.3).

A cutoff of 1% gave the lowest Log-rank test's p-value for TTP (p=0.000) and one of the lowest for LSS (p=0.003). Median TTP and LSS were 208 and 344 days for dogs with BM infiltration <1%, and 64 and 164 days for dogs with BM infiltration ≥1%, respectively. Dogs with BM infiltration ≥1% had a 6.7 times (95% CI: 2.2-20.4) higher

probability of recurrence and 3.4 times (95% CI: 1.4-8.1) higher probability of death compared to dogs with BM infiltration <1%.

Using a cutoff of 3% (close to the median value in our case series), median TTP and LSS were 149 and 322 days for dogs with BM infiltration <3%, and 69 and 155 days for dogs with BM infiltration ≥3%, respectively (p=0.004 for TTP, p=0.001 for LSS). Dogs with BM infiltration ≥3% had a 3.3 times (95% CI: 1.4-7.6) probability of recurrence and a 3.6 times (95% CI: 1.6-8.0) higher probability of death compared to dogs with BM infiltration <3%.

BM infiltration (%)	Progressed dogs	Censored dogs	Median TTP (days)	Log-rank test p-value	Hazard ratio
<1	9	2	208	0.000	
≥1	30	5	64		6.70
<3	15	3	149	0.004	
≥3	24	4	69		3.25
<5	22	5	118	0.002	
≥5	17	2	43		2.99
<10	30	6	95	0.033	
≥10	9	1	43		2.30
<15	31	6	95	0.018	
≥15	8	1	43		2.60
<20	34	7	95	0.006	
≥20	5	0	34		3.62

Table 5.3: Time-to-progression (TTP) in 46 dogs with large B-cell lymphoma

BM infiltration (%)	Dogs dead for lymphoma	Censored dogs	Median LSS (days)	Log-rank test p-value	Hazard ratio
<1	9	2	344	0.003	
≥1	29	6	164		3.42
<3	14	4	322	0.001	
≥3	24	4	155		3.59
<5	21	6	201	0.007	
≥5	17	2	133		2.94
<10	29	7	200	0.075	
≥10	9	1	164		2.00
<15	30	7	200	0.033	
≥15	8	1	164		2.36
<20	33	8	200	0.002	
≥20	5	0	171		4.28

Table 5.4: Lymphoma-specific-survival (LSS) in 46 dogs with large B-cell lymphoma

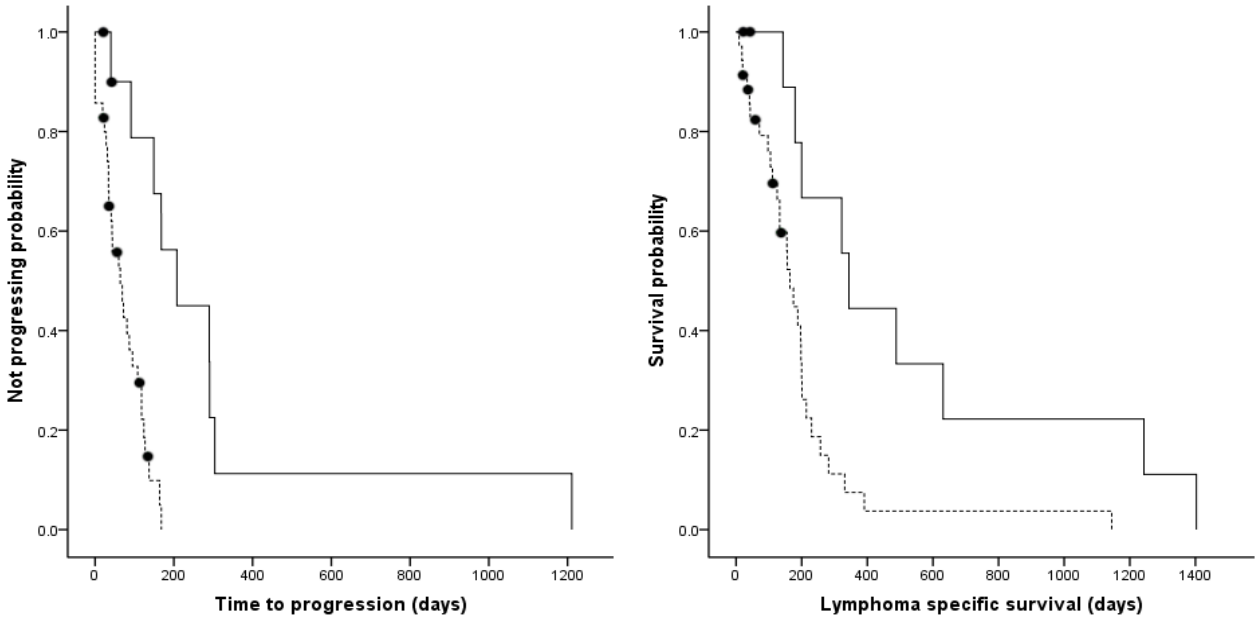

Fig 5.2: Kaplan-Meier curves representing time-to-progression and lymphoma-specific survival of 46 dogs with large B-cell lymphoma with <1% (continuous line) and ≥1% (dotted line) bone marrow flow cytometric infiltration. • = censored data.

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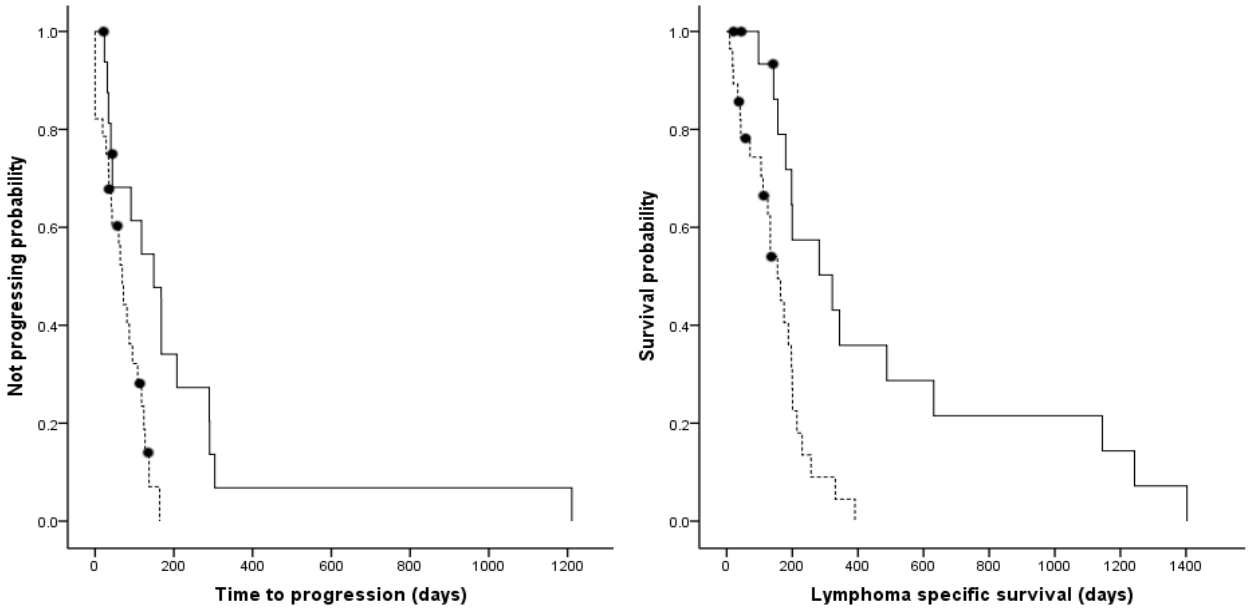

Fig 5.2: Kaplan-Meier curves representing time-to-progression and lymphoma-specific survival of 46 dogs with large B-cell lymphoma with <3% (continuous line) and ≥3% (dotted line) bone marrow flow cytometric infiltration. • = censored data.

Published in Veterinary and Comparative Oncology, doi:10.1111/vco.12024

5.1.5- LINK BETWEEN PB AND BM INFILTRATION DEGREE

Samples from other forty-six dogs with LBCL were analyzed to establish if PB infiltration degree can predict BM infiltration degree. Among them, mean PB infiltration was 9.65±16.48% (median 2.67%, min-max 0.00-80.41%) and mean BM infiltration was 10.59±17.93% (median 2.51%, min-max 0.03-74.5%). In particular, BM infiltration was ≥1% in 35 (76.1%) dogs, ≥3% in 21 (45.7%), ≥5% in 15 (32.6%), ≥10% in 12 (26.1%), ≥20% in 8 (17.4%).

Results from different ROC curves are shown in Table 5.5. Using a 1% cutoff to define positive BM samples, the best PB infiltration cutoff was 3.05%, which discriminated positive and negative BM samples with a 60% sensitivity and a 100% specificity. However, the best results were obtained when a 10% cutoff was used to define positive BM samples: the selected PB infiltration cutoff was 13.41%, which discriminated positive and negative BM samples with a 75% sensitivity and a 100% specificity.

BM cutoff (%)	Number (%) of BM samples		PB cutoff (%)	Sensitivity (%)	Specificity (%)
	Negative	Positive			
1	11 (23.9)	35 (76.1)	3.05	60.0	100
3	25 (54.3)	21 (45.7)	9.15	52.4	100
5	31 (67.4)	15 (32.6)	9.17	73.3	100
10	34 (73.9)	12 (26.1)	13.41	75.0	100
20	38 (82.6)	8 (17.4)	34.73	62.5	100

Table 5.5: data from ROC curves drawn to select the PB infiltration cutoff most suitable to predict BM infiltration status.

5.2- PHENOTYPIC ABERRANCIES IN CANINE LYMPHOMAS

5.2.1- PROGNOSTIC VALUE

371 consecutive cases with a final diagnosis of lymphoma were retrospectively extracted from the FC database of the Laboratory of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy) in a five-year period, from 2006 to 2011. Among them, 260 (70.1%) were classified as B-cell, 110 (29.6%) as T-cell and 1 (0.3%) as bi-phenotypic lymphoma in which the lineage of origin could not be defined since only a T-cell and a B-cell markers were expressed by the neoplastic population.

In 56 (15.1%) cases, at least one phenotypic aberrancy was detected. Cytological preparation from lymph nodes was available only for 23 (41.1%) aberrant cases (8 B-cell and 15 T-cell lymphomas).

Aberrancies were more common among T- than B-cell lymphoma cases (35.5% and 5.8%, respectively). Prevalence of each aberrancy is shown in table 5.6. In particular, the most common aberrancy among T-cell lymphomas was a diminished or absent expression of CD45 (more than 18% of cases), whereas the most common aberrancy in B-cell lymphomas was the expression of CD34 (3.5% of cases).

Follow-up data were available for 38 (67.9%) aberrant lymphoma cases (7 B-cell, 30 T-cell and 1 biphenotypic lymphomas). Unfortunately, therapeutic protocol was not standardized among cases: 26 (68.4%) dogs received chemotherapy with different drugs and protocols (mostly CHOP-based multidrug chemotherapy), 5 (13.2%) received only corticosteroids, and 7 (18.4%) were not treated.

Thirty (79%) dogs progressed during the study period, whereas 8 (21%) were still in remission at data analysis closure. Overall median TTP was 150 (min-max: 0-450 days) and 60 (min-max: 0-375 days) days for B-cell and T-cell aberrant lymphomas, respectively.

Twenty-eight (73.7%) dogs died for progressive disease during the study period, whereas 10 (26.3%) were still alive at data analysis closure. Median LSS was 180 (min-max: 30-450 days) and 150 (min-max: 7-1020 days) days for B-cell and T-cell aberrant lymphomas, respectively.

The dog with biphenotypic lymphoma was treated with CHOP-based chemotherapy, progressed within 11 days and died for progressive disease 73 days after diagnosis.

Phenotypic aberrancy	Cases (%) among all B-cell lymphomas	Cases (%) among all T-cell lymphomas
Diminished or absent CD45	3 (1.2)	20 (18.2)
Expression of CD34	9 (3.5)	1 (0.9)
Diminished or absent CD5		8 (7.3)
Diminished or absent CD3		3 (2.7)
Loss of both CD4 and CD8		9 (8.2)
Co-expression of CD4 and CD8		6 (5.5)
Expression of CD21		9 (8.2)
Expression of CD79a		1 (0.9)
Diminished or absent CD21	1 (0.4)	
Diminished or absent CD79a	1 (0.4)	
Expression of CD5	2 (0.8)	
Expression of CD3	2 (0.8)	

Table 5.6: Prevalence of phenotypic aberrancies among 259 and 110 dogs with B- and T-cell lymphoma, respectively

Differences in TTP and LSS between B-cell and T-cell aberrant lymphomas were not statistically significant.

Survival analyses were performed only for aberrancies represented in at least 4 cases. Only CD4-CD8 double-positive cases showed a significantly shorter LSS, but not TTP, when compared to all other T-cell aberrant lymphomas (p=0.014, fig 5.4): median LSS was 50 and 180 days, respectively.

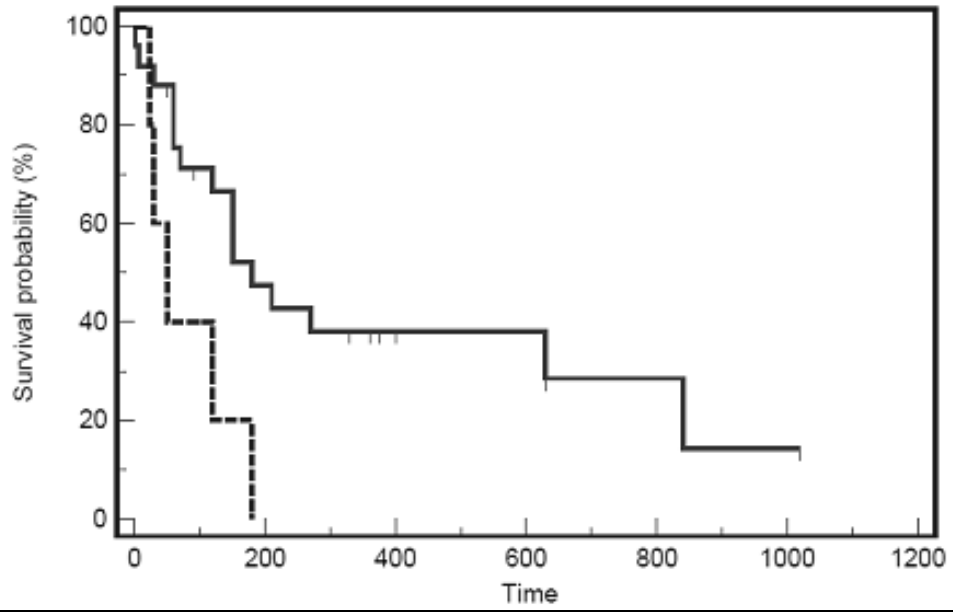

Fig 5.4: Kaplan-Meier curves representing lymphoma-specific survival in dogs with CD4+CD8+ T-cell lymphoma (dotted line) and all other aberrant T-cell lymphomas (continuous line). Difference was statistically significant (p=0.014)

Although not statistically significant, diminished or absent CD45 expression by T-cell lymphomas seemed linked to a longer LSS when compared to other T-cell aberrant lymphomas (median LSS was 211 and 74 days, respectively). Interestingly, this aberrancy was detected in all small clear cell lymphomas (4 out of 4 for which lymph node cytological preparation was available) but in one case only out of 11 high-grade T-cell lymphomas.

Finally, CD34-positive B-cell lymphomas had a shorter LSS when compared with other B-cell aberrant lymphomas (median LSS was 140 and 270 days, respectively), although differences were not statistically significant.

5.2.2- DIAGNOSIS OF SMALL CLEAR CELL LYMPHOMA

Cases of suspect nodal small clear cell lymphoma were extracted from the databases of the Laboratories. After review of the cytological preparations from lymph nodes, 26 cases fulfilled the inclusion criteria and were therefore included in this section of the study. Besides pathological samples, 8 cases with a final diagnosis of reactive process were included as negative controls.

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

Neoplastic cells stained negative for CD45 in 25/26 (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, 3 (11.5%) were positive for both, 9 (34.6%) were double negative, and in one case (3.8%) 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%) stained positive for CD79a. Results of immunophenotyping for each case are shown in table 5.7. Flow cytometric scatter grams from one representative case are pictured in fig 5.5.

When reactive controls were considered, all cells in each sample stained positive for CD45. FC revealed a mixed lymphoid population composed by CD4-positive T-cells (mean 35.9%, min-max 19.0-59.3%), CD8-positive 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.

Case nr	CD45	CD3	CD5	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	-	+	+	+	+	-	-

Table 5.7: flow cytometric immunophenotype of the neoplastic population identified in lymph node aspirates from 26 dogs with suspect small clear cell lymphoma

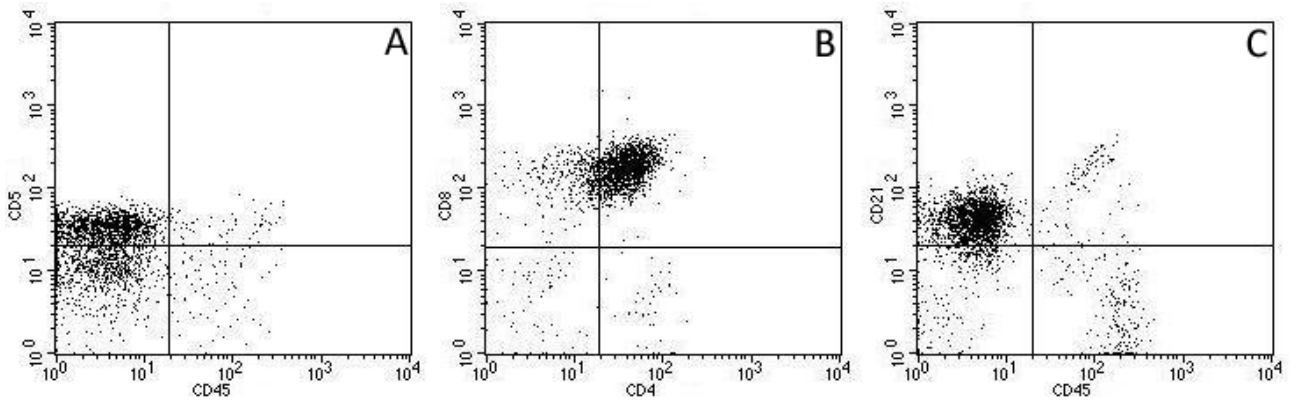

Fig 5.5: flow cytometric scatter grams of the lymph node of one dog with suspect small clear cell lymphoma, showing aberrant antigen pattern. Most cells are CD45-negative (**A** and **C**), CD5-positive (**A**), CD4 and CD8 double positive (**B**) and CD21-positive (**C**).

5.3- EXPRESSION OF CD44 IN BLOOD FROM DOGS WITH HEMATOPOIETIC NEOPLASMS

Overall, 145 PB samples were included in this section of the study: 13 (9%) from healthy dogs, 7 (4.8%) from dogs with non-neoplastic diseases, 20 (13.8%) from dogs with acute leukemias (10 ALL, 6 AML, 4 AUL), 21 from dogs with chronic leukemias (10 T-CLL, 8 B-CLL, 3 atypical-CLL), 84 (57.9%) from dogs with lymphoma (59 B-cell, 25 T-cell). In 4 lymphoma cases, it was not possible to define the clinical stage because of the lack of BM samples; among the remaining 80 lymphoma cases, 54 (67.5%) were classified as stage III (41 B-cell, 13 T-cell) and 26 (32.5%) as stage V (16 B-cell, 10 T-cell).

Results from CD44 expression (CD44 MFI/unstained cells MFI) analysis are shown in table 5.8.

Mean CD44 expression was significantly different among the disease groups (p=0.000). In particular, CD44 expression was significantly lower in healthy dogs than in dogs with acute leukemias, chronic leukemias, stage III and stage V lymphomas (p=0.000); in dogs with non-neoplastic diseases than in dogs with acute (p=0.001) and chronic leukemias (p=0.003); in dogs with stage III and stage V lymphomas than in dogs with acute leukemias (p=0.000); in dogs with chronic leukemias than in dogs with acute leukemias (p=0.002). Finally, acute leukemias showed the highest CD44 expression, and it was significantly higher than in healthy dogs (p=0.000), in dogs with non-neoplastic diseases (p=0.001), in stage III and V lymphomas (p=0.000) and in chronic leukemias (p=0.002).

Mean CD44 expression was not different among the three phenotypes of acute leukemias, among the three phenotypes of chronic leukemias (although a progressive decrease was found in T-CLL, B-CLL and atypical-CLL), between B- and T-cell lymphomas, between stage III and V B-cell lymphomas, nor between stage III and V T-cell lymphomas.

Disease group	Number of cases	CD44 expression		
		Median	Mean	standard deviation
Healthy	13	26.69	35.07	27.89
Granulocytes	3	307.76	265.55	73.28
Monocytes	3	204.40	191.83	61.38
T-lymphocytes	3	20.54	18.55	5.21
B-lymphocytes	3	18.44	16.68	4.13
Non-neoplastic diseases	7	141.85	184.93	150.83
Acute leukemias	20	429.03	400.35	167.39
ALL	10	407.15	362.91	178.66
AML	6	459.67	450.15	195.52
AUL	4	411.75	419.25	88.80
Chronic leukemias	21	235.28	240.44	131.44
T-CLL	10	261.97	291.06	157.65
B-CLL	8	173.45	206.31	75.32
Atypical-CLL	3	99.08	162.75	120.63
Lymphomas	84	133.28	156.13	104.03
B-cell	59	141.85	88.51	148.15
Stage III	41	147.16	150.06	85.38
Stage V	16	110.67	119.03	69.69
T-cell	25	132.22	174.96	134.04
Stage III	13	132.22	172.02	103.68
Stage V	10	187.24	209.71	164.04

Table 5.8: CD44 expression by the neoplastic population of dogs with hematopoietic neoplasms and by the lymphoid population of healthy and reactive controls

6- DISCUSSION

Hematopoietic neoplasms (HNs) are some of the most common oncologic diseases in dogs. However, pathogenetic and clinical pathological aspects of canine HNs are still far from being completely understood.

Flow cytometry (FC) has been largely used for the diagnosis, characterization and staging of human HNs. This technique is spreading also in veterinary medicine and it is increasingly used for the diagnosis and characterization of canine HNs.

The present study demonstrated that FC can be a useful technique not only for the diagnosis and immunophenotyping of canine HNs, but also to study pathogenetic and clinical-pathological aspects with clinical relevance. Indeed, we demonstrated that:

- ✓ FC allows the quantification of PB and BM infiltration in canine LBCL with great diagnostic performances. BM infiltration can be present even in the absence of peripheral cytopenias, influences both TTP and LSS, and can be predicted by PB infiltration with an optimal specificity, but an unsatisfactory sensitivity;
- ✓ FC allows the identification of phenotypic aberrancies in canine lymphomas. Some specific aberrancies can give prognostic information to the clinician. In addition, aberrancies have a 100% prevalence in canine small clear cell lymphoma and are therefore of great diagnostic usefulness;
- ✓ FC allows a semi-quantitative evaluation of the expression of molecules on the cell surface. Thanks to this peculiarity, we demonstrated that CD44 is expressed at higher degrees by neoplastic cells in canine HNs compared to the lymphoid population of healthy and reactive controls. Although this result has no clinical relevance by itself, it deepens the knowledge of the pathogenesis of canine HNs and opens the way to the development of new drugs targeting CD44, which are presently under study in human medicine.

Among canine HNs, lymphoma is the most represented, since it accounts for more than 80% of all HNs by itself (Vail *et al*, 2012). It is no more regarded as a single disease, but as an heterogeneous group of diseases with different prognosis. One study, indeed, demonstrated that morphological appearance in conjunction with immunophenotype give important prognostic information: B-cell lymphomas (which are more commonly high-grade lymphomas) bear a better prognosis than high-grade T-cell lymphomas, whereas small-clear cell T-zone lymphomas are usually characterized by an indolent course and a better prognosis (Ponce *et al*, 2004). The biological basis for the identification of these three major groups was recently given by Frantz and colleagues, in 2013: they confirmed by gene expression profiling (GEP) that canine lymphomas can be subdivided into three major molecular subgroups with prognostic significance (high-grade T-cell lymphomas, low grade T-cell lymphomas and B-cell lymphomas). Low-grade B-cell lymphomas appeared as a subgroup within the B-cell lymphoma group (Frantz *et al*, 2013). In addition, a recent retrospective study investigated survival of dogs with different lymphoma histotypes, stressing the need for a precise pathological diagnosis leading to tailored chemotherapeutic protocols (Valli *et al*, 2013). As a consequence, in the authors' opinion, diagnostic and staging procedures, as well as therapeutic protocols, should be differentiated and adapted to the specific properties of each lymphoma subtype.

6.1- CANINE LARGE B-CELL LYMPHOMA STAGING

The first section of this study was focused only on canine large B-cell lymphoma (LBCL). LBCL is the most common lymphoma subtype in the dog (Teske *et* al, 1994; Ponce *et* al, 2010; Valli *et al*, 2011). Different cytological entities are included in the "LBCL" definition, among which the most common is the high-grade centroblastic polymorphic lymphoma, which is composed by a mixture of small blastic cells, medium-sized macronucleated cells, centroblasts and immunoblasts; usually, this morphological subtype

presents with a diffuse histological pattern (Ponce *et al*, 2004). LBCL is easily recognizable by FC, too: indeed, neoplastic cells in the lymph node are large sized (FSC-H higher than 400) and stain positive for B-cell markers (CD21 and CD79a) (Gelain *et al*, 2008).

In the present study, we looked for the infiltration by cells with these characteristics in PB and BM from dogs with LBCL, validated this method of quantifying infiltration and highlighted its prognostic value. Some points have to be remarked about this method:

- ✓ It was not possible to completely confirm that these cells, when found in PB and BM samples, were neoplastic. However, to the authors' opinion, their detection in very low percentages in control samples, including samples from dogs with severe systemic conditions such as Leishmaniasis, suggests that they are likely neoplastic cells. Interestingly, in some control dogs, a higher percentage of these cells were found in PB (never exceeding 5%) showing an elevated cellular complexity, thus resembling more a non-specific antibody binding to granulocytes than true large B-cells. Sensitivity and specificity of FC in detecting PB infiltration increased by excluding these cases from analysis. Therefore, careful analysis of all parameters (including SSC) recorded by FC should always be done in order to avoid mistakes. In addition, quantification of large B-cells in the PB and BM should never be performed without a definitive diagnosis of LBCL and a confirmation of the neoplastic cells' size and phenotype. Furthermore, the presence of large B-cells in the PB or BM should not be considered conclusive of lymphoma.
- ✓ Based on our results, a flow cytometric percentage >1.04% of large B-cells in the BM from dogs with LBCL may be considered conclusive for infiltration (100% specificity). Unfortunately, sensitivity for this cutoff was slightly lower. This could indicate that minimal infiltration could not be detected via FC but

can still be identified by PARR analysis. The prognostic value of this minimal infiltration has still to be understood, and prospective studies with the combined use of these two techniques should be performed.

- ✓ We compared results obtained by FC with those obtained by cytology in the quantification of PB and BM infiltration. Unfortunately, among 60 cases analyzed, atypical cells suggesting PB infiltration could be identified only in 5 cases, in which FC infiltration varied from negative to >80%. Agreement analysis could not be performed on these samples; however, it can be derived that the presence of atypical cells at PB smear evaluation should not be considered conclusive for infiltration, maybe because of the atypical appearance of reactive lymphocytes, and that high degrees of PB infiltration should not be excluded based on the absence of atypical cells.
- ✓ Our results revealed only a moderate agreement between cytology and FC in the evaluation of BM samples. This could be in part due to the different sensitivity and specificity of the two techniques, which are based on different methods (morphological VS immunological) and analyze a different number of cells (500 VS 10,000). In addition, it is important to underline that we expressed FC infiltration as a percentage out of CD45-positive cells instead of total nucleated cells, since data in human medicine (Allan *et* al, 2008) and some preliminary results in the dog (Novacco *et al*, manuscript in preparation) showed a disruption of some RBC precursors and immature RBC by the RBC lysis solution used during the sample processing for FC. RBC precursors and immature RBC express CD45 only at a minimal degree, thus even the portion not disrupted by the lysing procedure was not included in the gates set for FC analysis. On the other hand, when infiltration is quantified by cytological evaluation, it is expressed as percentage of all

nucleated cells, including RBC precursors and immature RBC. These aspects could bias and reduce the agreement between the two techniques, particularly when the myeloid-to-erythroid ratio in the BM is abnormal due to erythroid abnormalities. However, in our case series, no sample with evident alteration of the erythroid lineage was present. Still, we strongly agree that cytological evaluation has to be always performed in conjunction with FC, in order to validate FC results and to assess alterations of the other hematopoietic lineages.

- ✓ The percentage of BM infiltration obtained by FC could be influenced by hemodilution and/or by granulocytic hypo- or hyperplasia. Gross hemodilution could influence cytological assessment of BM infiltration, as well. Therefore, when cytological evaluation or FC is used for BM infiltration assessment, samples should be screened for gross hemodilution to avoid misjudgment. Samples with gross hemodilution were not included in the present study. However, a mild hemodilution can minimally influence percentage of neoplastic cells due to the different concentration of nucleated cells in PB and BM. Histopathology would be the only diagnostic tool not influenced by blood contamination. Granulocytic abnormalities could influence not only FC, but also other techniques such as conventional cytology and histopathology. On the contrary, PARR is not affected by the granulocytes presence, thus representing a more reliable test for cases with granulocytic abnormalities, even if the prognostic value of PARR-positive bone marrow sample is still not clear (Lana et al, 2006a). None of the cases included in the present study had granulocytic hyperplasia or hypoplasia.
- ✓ The FC method we applied to quantify PB and BM infiltration in LBCL cannot be used for other lymphoma subtypes. Indeed, when large T-cell lymphomas

are concerned, quantifying all T-cells (CD3+ or CD5+) in PB and/or BM would bring to an overestimation of the neoplastic cells number, as they would be included different classes of T-lymphocytes (T-helper CD4+ and T-cytotoxic CD8+). Thus, CD4+ or CD8+ cells should be quantified based on the immunophenotype identified in the lymph node. Unfortunately, in the dog CD4 is expressed also by neutrophils and this makes it very hard to safely identify neoplastic cells in CD4+ cases. CD8+ lymphoma cases, on the contrary, do not stumble upon this inconvenience, but in the authors' experience this phenotype is uncommon in the dog. However, use of FC to stage this lymphoma subtype should be deepened. On the other hand, as far as small-cell lymphomas are concerned, it would be impossible to distinguish between neoplastic and normal or reactive lymphocytes in PB and BM samples. This problem could be climbed by the presence of aberrant antigen expression which represents a specific fingerprint of each lymphoma case allowing the identification of the same population in PB and BM from these dogs (Comazzi and Gelain, 2011).

BM assessment is considered not mandatory in staging dogs with lymphoma under official guidelines, unless peripheral cytopenias are present, even if this relationship has never been demonstrated. Another important concern of official guidelines is that prognostic value of BM infiltration is still controversial, and it does not drive any clinical and therapeutic decision (Vail *et al*, 2010). The present study offers some preliminary answers to these points:

- ✓ Based on our results, BM infiltration is higher in dogs with thrombocytopenia, leukocytosis or lymphocytosis, and is negatively correlated to platelet count and positively to PB infiltration. Hence, even if a high BM infiltration can be

expected in dogs with thrombocytopenia, leukocytosis or lymphocytosis, its degree cannot be directly predicted by the number of leukocytes or lymphocytes: a heavy BM infiltration can be found both in cases with a slight and a strong lymphocytosis. On the contrary, the lower is the platelet count, the higher is BM infiltration. No difference in BM infiltration was found, instead, between dogs with and without any haematological abnormality. Our results suggest that BM status should be assessed even in dogs without PB abnormalities, as it is not possible to exclude a high BM infiltration based only on PB analysis. Some abnormalities such as thrombocytopenia and lymphocytosis are likely to be suggestive of a higher BM infiltration. It should be underlined, however, that only few cases in our study had a high BM infiltration, which could perhaps result in secondary myelophthisis and consequent cytopenias. Therefore, the potential of some blood abnormalities, such as anemia, to predict BM status could have been underestimated and should be further investigated including a larger number of cases with a high BM infiltration.

- ✓ Our results highlight a significant correlation between degree of BM infiltration by FC and biological behaviour. Notably, BM infiltration was not completely independent from other variables, since it lost significance in multivariate analysis and was correlated to substage, which also influenced prognosis: this association might reflect wider disease spread. In addition, it should be underlined that not statistically significant correlation with prognosis at multivariate analysis does not exclude influence on prognosis, since this analysis is strongly influenced by the number and type of variables included and by the number of samples analyzed. Some previous studies reported no association between stage and prognosis (Garrett *et al*, 2002;

Morrison-Collister *et al*, 2003; Flory *et al*, 2007), while other reported a shorter survival for dogs in stage V compared to dogs in stage III-IV (Lana *et al*, 2006a; Marconato *et al*, 2011). These differences may be due to the inclusion of different lymphoma entities, differing therapeutic strategies and to the varying methods and cut-offs used to assess infiltration: therefore, we evaluated the diagnostic value of BM infiltration in canine LBCL accurately defining the inclusion criteria in order to avoid bias that could interfere with results. Indeed, only LBCL cases were enrolled, and all dogs underwent the same protocol for full staging and were treated by means of the same chemotherapeutic regimen.

- ✓ The identification of a BM infiltration cut-off that can be used to define subgroups of dogs with different prognosis might be clinically useful. Based on our results, a 1% BM infiltration cut-off is the best to discriminate between two prognostic groups. However, this cut-off is very close to the limit of detection of FC, and we found large B-cells in healthy dogs and in dogs with diseases other than B-cell lymphoma at a percentage close to 1%. As a consequence, the value of 3% seems to be a more feasible putative cut-off to discriminate two prognostic groups, since it showed the lowest log-rank p-value for LSS and one of the lowest for TTP, and did not overlap with the lower limit of detection of FC. In conclusion, we suggest the use of a large B-cells cut-off of 1% to discriminate between positive and negative BM samples, and of 3% to discriminate between two prognostic groups.

Based on a recent survey (Regan *et* al, 2012), almost all clinicians require a complete blood cell count during canine high-grade lymphoma staging, but only a minority require BM aspirate to be performed. A challenging issue is whether PB infiltration could

be used in substitution or to predict BM infiltration, even if it did not show a prognostic value by itself in our case series. Results from the present study suggest that PB infiltration can predict BM infiltration with an optimal specificity (100%), but only a moderate sensitivity (ranging from 52.4% to 75.0%). Therefore, the presence of PB infiltration could be considered suggestive of BM infiltration, which is a negative prognostic factor; on the contrary, an absent or slight PB infiltration is not enough to exclude BM infiltration.

Unfortunately, BM histopathology and immunohistochemistry were not performed on any of the samples included in the present study. In human medicine, these are considered the gold standard technique to assess BM infiltration in lymphoma cases and to characterize the pattern of infiltration. On the contrary, only a single study has been published in veterinary medicine demonstrating that trephine biopsy is more sensitive in detecting infiltration compared to BM aspirate (Raskin and Krehbiel, 1988). This paucity could be due to a reduced clinicians' and owners' compliance, because of the lack of proved clinical benefits in spite of an invasive and expensive procedure. On the other hand, BM aspirate is easier to collect and cytology is much cheaper and faster than histopathology, even though sensitivity and specificity are lower: therefore, morphological evaluation via light microscopy is the most commonly used technique for BM infiltration assessment in the dog (Ponce *et al*, 2004; Flory *et al*, 2007; Abbo and Lucroy, 2007; Flory *et al*, 2011). FC shares the advantages of cytology, since sampling is made by BM aspirate and diagnosis is available within one day from sampling. In addition, the present study demonstrated a prognostic value for BM infiltration quantified by FC, at least for canine LBCL. As a consequence, including this procedure in the staging work-up would not strongly increase costs, but would bring to a more precise prognosis. In addition, it should be underlined that studies in human medicine suggest adding FC to histopathology

in order to increase sensitivity and specificity in the detection of BM infiltration (Hanson *et al*, 1999; Talaulikar *et al*, 2009).

6.2- PHENOTYPIC ABERRANCIES IN CANINE LYMPHOMAS

A great advantage of FC is the possibility to evaluate the contemporary expression of different antigens on the same cellular population and detect phenotypic aberrancies. Phenotypic aberrancies are largely documented in human lymphomas (Schmidt *et al*, 1999; Jamal *et al*, 2001; Arun *et al*, 2010), are considered a great diagnostic tool particularly for peripheral T-cell lymphomas (Jamal *et al*, 2001) and, in addition, some distinct aberrancies have shown a prognostic role (Jennings and Foon, 1997; Lai *et* al, 2000; Gorczyca *et* al, 2002; Mitrovic *et al*, 2009). Aberrant antigen expression has been reported in canine hematopoietic neoplasms, as well (Wilkerson *et* al, 2005; Gelain *et al*, 2008; Rao *et al*, 2011). However, very little is known about their prognostic role. One study investigated the prognostic role of CD34 expression in canine B-cell lymphomas, but failed in detecting any association with disease outcome (Rao *et al*, 2011). Another study investigated the influence of phenotypes on survival time in dogs with chronic lymphocytic leukemia, and found that cases with atypical phenotype had the worst prognosis; however, this group included mostly cases with NK-like phenotype, which cannot be considered aberrant (Comazzi *et al*, 2011).

Our results highlight that phenotypic aberrancies are not uncommon in canine lymphomas, as they accounted for around 15% of cases referred to our laboratory in a five-year period. In particular, phenotypic aberrancies were more common in T-cell than in B-cell lymphomas, as already reported (Gelain *et al*, 2008). This could be due to the different number of antigens tested for each lineage. Indeed, only two B-cell antigens were tested in the present study (CD21 and CD79a), while T-cells were identified by means of

four different antibodies (CD3, CD5, CD4, CD8). Including a wider number of antibodies could unmask aberrancies in a larger number of cases which are presently classified as non-aberrant. Another possible explanation for the different prevalence of aberrancies between T- and B-cell lymphomas is that canine B-cell lymphomas are in the vast majority represented by diffuse large B-cell lymphoma (DLBCL), which could be less prone to show phenotypic aberrancies compared to other lymphoma subtypes. Unfortunately, cytological preparation from lymph nodes was available only for a few cases in our series, and histopathology had hardly ever been performed, which prevented us from evaluating the prevalence of aberrancies among different lymphoma subtypes.

The prognostic information about phenotypic aberrancies obtained in the present study could be strongly biased by the fact that treatment was not standardized among cases. In addition, the lack of morphological data entailed the classification of cases with the same antigen aberrancy in a single group, irrespective of the grade of the malignancy, which is an important prognostic factor particularly among T-cell lymphomas (Ponce *et al*, 2004). Further studies should be performed, including a larger number of cases with complete and standardized diagnostic and staging workup and treatment protocol, in order to validate our results. Unfortunately, this would require a multicentric study and several years of case enrollment to gain a significant number of aberrant cases. Still, results from the present study suggest a possible prognostic role for distinct aberrancies in canine lymphomas:

- ✓ A significant influence on survival was detected only when comparing CD4+/CD8+ T-cell to other aberrant T-cell lymphoma cases. To the authors' knowledge, no data is available in the literature about the prognostic influence of this phenotype in canine lymphomas; however, one study hypothesized that this

phenotypic pattern is more frequent in lymphoblastic lymphomas, which is associated to a more aggressive clinical behavior (Fournel-Fleury *et al*, 2002).

- ✓ Among B-cell lymphomas, the most common aberrancy was the expression of CD34. This molecule is a membrane glycoprotein present on the cell surface of both lymphoid and myeloid precursors and its expression is usually considered suggestive of acute leukemia (Vernau and Moore, 1999). Acute leukemia cases were not included in this section of the present study, nor were included cases in which the differential between leukemic lymphoma and leukemia could not be solved. CD34 expression has already been reported in canine lymphoma cases (Wilkerson *et al*, 2005; Rao *et al*, 2011). Based on our results and on those obtained by Rao and colleagues, however, positivity to CD34 in canine B-cell lymphomas has no influence on prognosis.
- ✓ Among T-cell lymphomas, on the contrary, CD45 diminished or absent expression was the most common aberrancy and seemed to bear a better prognosis compared to other aberrant T-cell lymphomas, even if the difference was not statistically significant. After evaluation of the small number of cytological preparations available, this phenotype resulted more common in small clear cell lymphomas, which are associated to a better prognosis (Ponce *et al*, 2004). Furthermore, all small clear cell lymphomas included in this section stained negative for CD45. As a consequence, in a second step we investigated whether phenotypic aberrancies can have a diagnostic role in canine small clear cell lymphomas.

Furthermore, we also highlighted a diagnostic role for aberrancies in canine small clear cell lymphomas. This is an indolent lymphoma involving peripheral lymph nodes whose 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 (Fournel-Fleury *et al*, 1997; Fournel-Fleury *et al*, 2002; Ponce *et al*, 2004). However, while the presence of a single population of immature or large cells and numerous mitoses at cytological evaluation can be considered conclusive for neoplasia, the distinction between low-grade lymphomas and reactive hyperplasia is still challenging and requires further tests for confirmation. Histologically, T-zone lymphomas show 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; mitoses are rare (Valli *et al*, 2006). Different studies have proved the importance of immunohistochemistry to confirm the diagnosis and define the immunophenotype of T-zone lymphomas (Fournel-Fleury *et al*, 2002; Valli *et al*, 2006; Valli *et al*, 2011; Flood-Knapik *et al*, 2012). Unfortunately, surgical removal of a lymph node for histopathological evaluation is poorly accepted by the owners due to the increase of costs and the need for anesthesia. On the basis of a recent survey, indeed, only 28% of the responding participants recommended lymph node histopathology among staging procedures, although only high-grade lymphomas were considered in the survey (Regan *et al*, 2012).

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 (Vernau and Moore, 1999; Burnett *et* al, 2003; Valli *et al*, 2006; Yagihara *et al*, 2007; Chaubert *et al*, 2010; Keller and Moore, 2012a; Keller and Moore, 2012b). PARR results in different histotypes were reported in one study: among eight T-zone lymphomas,

only five had a clonal rearrangement (Valli *et al*, 2006). Therefore, neoplasia cannot be ruled out based on negative PARR results and the interpretation of this test should be always associated with histopathology and immunohistochemistry. Immunocytochemistry on lymph node aspirate smears can be a useful tool to determine B- or T-cell phenotype and procedures for automated staining have recently been described in veterinary literature (Aulbach *et* al, 2010). This technique requires a minimally invasive sampling and as a consequence could be preferred to histopathology by clinicians and owners; unfortunately, it is not suitable for assessment of co-expression of different antigens by the same cellular population and therefore it does not allow recognition of phenotypic aberrancies, which 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 (Jamal *et al*, 2001; Arun *et al*, 2010).

Although aberrancies have been described in human and canine B-cell lymphomas, as well (Inaba *et al*, 2001; Gelain *et al*, 2008; Wang *et al*, 2009; Rao *et al*, 2011), we selected only small clear cell lymphomas for this section of the study, not all low-grade lymphomas, because T-zone histotype (and therefore T-cell phenotype) can be expected in these cases. We reviewed FC immunophenotype of 26 cases of suspect nodal small clear cell lymphoma 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: cytological preparation from lymph nodes in reactive cases, similar to the neoplastic ones, 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 was not sufficient to definitively discriminate between reactive controls and small clear cell lymphomas. On the contrary, FC revealed a mixed population of small lymphoid cells without any aberrancy in reactive cases, whereas

aberrancies were always identified in small clear cell lymphoma cases. Therefore, differently from cytology, FC allowed a definitive discrimination between reactive and neoplastic cases.

The most common aberrancies found in our case series were:

- ✓ Loss of the expression of CD45, which was found in all small clear cell lymphoma cases but one. On the other hand, none of the reactive samples had a CD45-negative lymphoid population. CD45 is a pan-leukocyte marker in the dog, with different degrees of expression in the leukocytes subclasses (Comazzi *et al*, 2006). Therefore, the absence of CD45 expression could be considered a hallmark of neoplasia. A decreased or absent CD45 expression has been previously reported in canine T-cell neoplasms (Gelain *et al*, 2008; Williams *et al*, 2008).
- ✓ CD21 expression, which 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 section of the study and is itself suggestive, although not conclusive, of a T-zone lymphoma (Fournel-Fleury *et al*, 1997; Fournel-Fleury *et al*, 2002; Ponce *et al*, 2004) and because of the contemporary expression of at least one T-cell marker. T- and B-cell populations were clearly distinct in reactive samples and co-expression of markers from different lineages was never found. CD21-positivity has already been described in canine T-cell neoplasms in the past (Wilkerson *et al*, 2005). Furthermore, in a recent study GEP analysis identified a group of genes, including CD21-gene, expressed at higher levels in T-zone than in high-grade T-cell lymphomas (Frantz *et al*, 2012).
- ✓ Co-expression or loss of both CD4 and CD8, which 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 FC on mediastinal masses aspirates drives the differential diagnosis between thymoma and thymic lymphoma (Lana *et al*, 2006b). In the present 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 aberrant and suggestive of lymphoma. Some cases of CD4+CD8+ double positive or double negative canine lymphoid neoplasms have been signaled in literature. In particular, these phenotypes were found in some cases of unclassifiable high-grade T-cell lymphomas (Fournel-Fleury *et al*, 2002) and in a subgroup of dogs with chronic lymphocytic leukemia harboring a poor prognosis (Comazzi *et al*, 2011). Finally, results from the present study show a shorter lymphoma-specific-survival for CD4+CD8+ double positive cases compared to other aberrant T-cell lymphomas.

Unfortunately, histopathology and immunohistochemistry were available only for a few cases: this circumstance could be considered a proof of the reluctance of clinicians and owners toward these invasive tests. However, the few cases for which histopathology and immunohistochemistry were available had a final diagnosis of T-zone lymphoma, thus supporting what already reported in the literature (Fournel-Fleury *et al*, 1997; Fournel-Fleury *et al*, 2002; Ponce *et al*, 2004). In addition, the lack of histopathological data prevented the comparison between different lymphoma subtypes and the evaluation of possible relationship between specific FC phenotypes or aberrancies and histotypes. Further studies should be performed on a larger number of cases comparing cytological, FC 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, results from this section of the study support the use of multi-color FC for the diagnosis of canine lymphomas since specific aberrancies can bear prognostic information; in addition, FC represents a useful tool to confirm neoplasia and exclude reactive hyperplasia when a small clear cell lymphoma is suspected and histopathology is not available or permitted.

6.3- EXPRESSION OF CD44 IN BLOOD FROM DOGS WITH HEMATOPOIETIC NEOPLASMS

In the last section of the present study, we used FC to evaluate the degree of expression of CD44 on blood cells from dogs with HNs and negative controls and found decreasing expressions in acute leukemias, chronic leukemias, lymphomas, reactive controls and healthy dogs.

CD44 is a class I trans-membrane glycoprotein expressed ubiquitously on the surface of all cells of the organism (Mackay *et al*, 1994). The sequence of the CD44-gene has been defined in many species, including humans and dogs, is highly conserved and is composed by constant and variable exons (Screaton *et al*, 1992). Constant exons are expressed in all isoforms, whereas the expression of different combination of variable exons gives rise to several CD44 variables or isoforms (CD44v). In particular, the expression of constant but not variable exons causes the production of the so called “CD44 standard” (CD44s) (Ponta *et al*, 1998). Normal hematopoietic cells express almost only CD44s (Mackay et al*,* 1994). CD44 is the main receptor for hyaluronic acid, but can interact also with other components of the extra cellular matrix (ECM), including fibronectin, collagen type I and type IV, laminin and osteopontin; binding to its ligands

promotes the connection to the cytoskeleton and initiates some intracellular signaling cascades that ultimately influence cellular shape, adhesion, migration, proliferation, survival, apoptosis and differentiation (Hertweck *et al*, 2011). CD44 has been largely studied in human oncology since its interaction with ECM could be one of the pathogenetic mechanisms of metastasis formation and tumor spread, as it can promote both extravasation of cells and their arrest in distant sites. CD44 expression has been studied in some canine neoplasms, as well (Moore *et al*, 1996; Serra *et al*, 2004; Madrazo *et al*, 2009; Paltian *et al*, 2009; Sabattini and Bettini, 2009; Cogliati *et al*, 2010); however no study is available to date regarding CD44 expression in canine HNs.

Based on our results, CD44 is differently expressed in canine hematopoietic neoplasms:

- ✓ Acute leukemias showed the highest CD44 MFI. Among canine HNs, acute leukemias have the fastest progression and the poorest prognosis (Vail *et al*, 2012). This aggressive behavior could be partly due to the high expression of CD44 on the cell surface, which could facilitate tissue invasion and organic failure. CD44 MFI was not different among the three phenotypic groups of acute leukemias (ALL, AML, AUL): therefore, it seems linked more to an early stop of maturation than to the cellular lineage involved.
- ✓ In CLL, CD44 MFI resulted significantly higher than in healthy dogs and in dogs with non-neoplastic diseases, but lower than in acute leukemias; on the opposite, there was no statistically significant difference between CLL and lymphomas, irrespective of lymphoma stage. Among CLL, CD44 MFI progressively decreased in T-CLL, B-CLL and atypical-CLL, even if differences were not significant. Based on the analysis performed on a small number of healthy dogs, CD44 MFI was higher in T- than in B-lymphocytes:

as a consequence, the higher CD44 MFI in T-CLL compared to B-CLL could be due to the higher percentage of T-cells, independently from their neoplastic transformation. Interestingly, atypical-CLL had a lower CD44 MFI: this could be attributed to a stop of maturation at earlier stages, since CD44 shows a characteristic pattern of expression during lymphocytes maturation, with two waves of down-regulation separated by phases of higher expression (Vaskova *et al*, 2008). A lower maturation of neoplastic cells could explain the more aggressive behavior of atypical-CLL compared to T- and B-CLL (Comazzi *et al*, 2011).

- ✓ In lymphoma cases, CD44 MFI was significantly higher than in healthy controls and lower than in acute leukemias, whereas no significant difference was found with CLL and with dogs with non-neoplastic diseases. No significant difference was found between B- and T-phenotypes or between stage III and V lymphomas. However, CD44 MFI was slightly higher in stage III than in stage V B-cell lymphomas and in stage V than in stage III T-cell lymphomas: this could be due to the different percentages of B- and T-cells, which seem to express CD44 at different degrees when analyzed in healthy dogs. Finally, the lack of significant difference between dogs with non-neoplastic diseases and stage III lymphomas could be due to an increased expression of CD44 on the cell surface of non-neoplastic lymphocytes in PB from dogs with lymphoma, which could reflect an immune response of the organism against the neoplasm.

In the present study, CD44 MFI was evaluated only on peripheral blood samples, not on lymph nodes: this could have influenced the data about CD44 MFI in lymphoma cases. Indeed, in these cases, the lymphoid population analyzed was heterogeneous,

composed by a mixture of normal lymphocytes and neoplastic cells, whose percentages varied from case to case, based on the PB infiltration degree. Lymph node samples had been regularly analyzed by FC in order to confirm the diagnosis and immunophenotype of lymphoma, but data about CD44 expression were not included in the present study since it was not possible to compare them with data from normal or reactive lymph nodes. Studies including control lymph node samples are required to evaluate the pathogenetic role of CD44 in canine lymphomas, and possibly the prognostic role of different degree of expression within the same lymphoma subtype.

To assess CD44 expression, we used the anti-CD44 antibody clone IM7 (BD Pharmingen, San Diego, CA, USA). It binds to an epitope of the constant region of CD44: therefore, distinction among CD44s and single CD44 variants is not possible. Normal hematopoietic cells express almost exclusively CD44s (Mackay *et al*, 1994), but some variants can be over-expressed during neoplastic transformation, especially CD44v6 (Hertweck *et al*, 2011). As a consequence, the higher degrees of CD44 expression detected in the present study could be due to 1) an increase in CD44s expression; 2) an expression of CD44 variants that physiologically are not expressed or expressed at very low degrees on the surface of hematopoietic cells; 3) a combination of these two situations. Antibodies that specifically bind to each CD44v should be employed to untangle this bundle.

In conclusion, results from the last section of the present study show a different degree of expression of CD44 among canine HNs, thus supporting its possible pathogenetic role. Genetic or epigenetic abnormalities underlying the higher expression of this molecule should be investigated. Finally, our results support the research and clinical

trials of new drugs targeting CD44, as is presently happening in human medicine (Charrad *et al*, 2002).

7- CONCLUSIONS

Altogether, results from the present study show that FC is a great tool to study some clinical pathological aspects of canine HNs. Furthermore, it can be applied in a clinical set up to confirm diagnosis and immunophenotype, and to evaluate additional prognostic parameters based on the neoplastic subtype identified (BM infiltration in LBCL, specific aberrancies in T-cell lymphomas).

However, besides its advantages, FC also has some important limitations:

- ✓ as cytology, FC is a great tool to analyze cellular morphology, but does not allow to assess the architectural pattern of neoplasms;
- ✓ samples for FC have to be analyzed within a few hours from sampling. Presently, new products are being validated which preserve cells for some days and therefore facilitate delivery to reference laboratory, but FC analysis of archive material is still not possible;
- ✓ flow cytometers and monoclonal antibodies have high costs and specifically trained personnel is necessary: as a consequence, FC is usually performed only in reference laboratories, and delivering of samples could increase costs and time to diagnosis.

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9- APPENDIX

During my PhD period, I've been involved in other studies, not directly bound to my main research project. They will be shortly described here below.

Flow cytometric immunophenotyping of acute erythroid leukemia (AML-M6) in a dog

Martini V, Stefanello D, Ghisleni G, Novacco M, Aresu L, Proverbio D, Comazzi S.

Veterinary Clinical Pathology, submitted

A male, mixed breed, 10-years-old dog was presented with lasting severe pancytopenia unresponsive to treatment with different antibiotics and low-dose prednisone. Myelodisplastic syndrome (MDS) was diagnosed based on bone marrow cytology. The dog was treated with whole blood transfusion and high-dose prednisone, but anemia and thrombocytopenia progressed. Within five days, a few unclassifiable immature cells appeared on the blood smear. Bone marrow cytology was repeated. More than 60% of the cells were rubriblasts or prorubricytes with prominent dyserythropoiesis; among non-erythroid cells, more than 40% were blasts. Immunocytochemistry and electron microscopy confirmed erythroid origin of the predominant cells. These cells were disrupted by RBC lyses buffer, thus flow cytometry for immunophenotyping was performed also on mononucleated cells isolated by gradient separation. The final diagnosis was acute erythroid leukemia (AML-M6). Despite therapy with high-dose prednisone, clinical conditions got worse and the dog was euthanatized after 38 days from admission. AML-M6 is a rare disease in humans. Pancytopenia is usually present, while circulating blasts are less common. Recent studies suggest that acute erythroid leukemia, acute myeloid leukemia with MDS-related changes and MDS with erythroid hyperplasia are biologically related diseases arbitrarily separated into different entities even in the 2008 revised WHO classification scheme. Although not rare in cats, only two cases of spontaneous AML-M6 have been reported in dogs. Clinical-pathological features overlapped those described in

humans. Evolution of MDS into AML-M6 could be supposed in this case, supporting recent findings in humans. Poor prognosis of AML-M6 was confirmed in all species.

Canine indolent and aggressive lymphoma: clinical spectrum with histologic correlation

Aresu L, **Martini V**, Rossi F, Vignoli M, Sampaolo M, Aricò A, Laganga P, Pierini A, Frayssinet P, Mantovani R, Marconato L.

Veterinary and Comparative Oncology, epub ahead of print

Sixty-three dogs with newly diagnosed lymphoma underwent complete staging and received the same chemotherapy. Diffuse large B-cell lymphoma was the leading histotype (44.4%), followed by peripheral T-cell lymphoma (20.6%). Indolent lymphomas accounted for 30.2% of cases. Most dogs with aggressive B-cell lymphoma had stage IV disease. Dogs with indolent and aggressive T-cell lymphoma had more often stage V disease and were symptomatic. Liver and bone marrow were predominantly involved in B-cell and T-cell lymphoma, respectively. The clinical stage was significantly related to substage, sex and total lactic dehydrogenase (LDH) level. Aggressive B-cell lymphomas were more likely to achieve remission. Median survival was 55 days for aggressive and indolent T-cell lymphoma, 200 and 256 for indolent and aggressive B-cell lymphoma, respectively. The prognosis of advanced indolent lymphoma does not appear to be appreciably different from that of aggressive disease. Familiarity with the various histotypes is critical to make the correct diagnosis and drive therapy.

Evaluation of tyrosine-kinase receptor c-KIT (c-KIT) mutations, mRNA and protein expression in canine leukemia: might c-Kit represent a therapeutic target?

Giantin M, Aresu L, Aricò A, Gelain ME, Riondato F, **Martini V**, Comazzi S, Dacasto M.

Veterinary Immunology and Immunopathology 2013;152:325-332

The tyrosine-kinase receptor c-KIT (c-KIT) plays an important role in proliferation, survival and differentiation of progenitor cells in normal hematopoietic cells. In human hematological malignancies, c-KIT is mostly expressed by progenitor cell neoplasia and seldom by those involving mature cells. Tyrosine-kinase inhibitors (TKIs) are actually licensed for the first- and second-line treatment of human hematologic disorders. Aim of the present study was to evaluate c-KIT mRNA and protein expression and complementary DNA (cDNA) mutations in canine leukemia. Eleven acute lymphoblastic leukemia (ALL) and acute undifferentiated leukemia (AUL) and 12 chronic lymphocytic leukemia (CLL) were enrolled in this study. The amounts of c-KIT mRNA and protein were determined, in peripheral blood samples, by using quantitative real time RT-PCR, flow cytometry and immunocytochemistry, respectively. The presence of mutations on c-KIT exons 8-11 and 17 were investigated by cDNA sequencing. Higher amounts of c-KIT mRNA were found in ALL/AUL compared to CLL, and this latter showed a lower pattern of gene expression. Transcriptional data were confirmed at the protein level. No significant gain-of-function mutations were ever observed in both ALL/AUL and CLL. Among canine hematological malignancies, ALL/AUL typically show a very aggressive biological behavior, partly being attributable to the lack of efficacious therapeutic options. The high level of c-KIT expression found in canine ALL/AUL might represent the rationale for using TKIs in future clinical trials.

Reticulocyte enumeration errors with Sysmex XT-2000iV in blood from leukemic dogs

Gelain ME, Paltrinieri S, **Martini V**, Tvedten H

European Society for Veterinary Clinical Pathology (ESVCP) 13th Annual Congress. Veterinary Clinical Pathology 2012;41:E1-E61

The Sysmex XT-2000iV hematology analyzer enumerates reticulocytes using a fluorescent dye that stains nucleic acids. It subdivides reticulocytes based on staining into low-, medium-, and high-fluorescence reticulocytes (LFR, MFR, HFR). The immature reticulocyte fraction (IRF) includes MFR and HFR, which increase in dogs with regenerative anemia. In humans, an increased IRF due to blast cell fragments in leukemic patients has been reported, and increased HFR% without polychromasia in a leukemic dog has been described. Aim of this study was to investigate potential errors in reticulocyte analysis in blood from leukemic dogs compared with non-anemic controls. Full reticulocytes and other automated hematology variables were determined in blood from 35 leukemic dogs (acute lymphoid leukemia: 8 cases; acute undifferentiated leukemia: 10 cases; chronic lymphocytic leukemia: 17 cases) and 40 healthy dogs. The Sysmex RET and RET-EXT scatter grams were analyzed to investigate the presence of an abnormal reticulocyte distribution. The percentage of reticulocytes was higher in leukemic dogs than in controls. In 29/35 samples the IRF was above the reference interval whereas the LFR% was lower; 20 of these dogs were also anemic. In 13 cases increased IRF was due to increased HFR% whereas in 16 dogs both HFR% and MFR% increased. In 18 cases an atypical cloud of high-fluorescence events was detected in the scatter grams. An inappropriate HFR% may be found in blood from leukemic dogs, likely due to dead cells, cell fragments, or altered staining of leukemic cells rather than to a true increase in reticulocyte production. This error can be visually detected in the scatter grams. Therefore,

Sysmex users should check for errors in the IRF% and other reticulocyte variables in leukemic dogs.

Leukemic small cell lymphoma or chronic lymphocytic leukemia in a horse

Cian F, Tyner G, **Martini V**, Comazzi S, Archer J.

Veterinary Clinical Pathology, epub ahead of print

A 16-year-old, Irish Draf mare was admitted to the referring veterinarian for annual health check. A mild generalized lymphadenomegaly was noted. Rectal palpation and transrectal ultrasonographic examination revealed prominent mesenteric lymph nodes. A transcutaneous abdominal ultrasonographic evaluation was unremarkable. A CBC revealed a marked leukocytosis (63.06 x $10^3/\mu l$) and lymphocytosis (58.2 x $10^3/\mu l$) due to increased numbers of small lymphocytes. No evidence of anemia or thrombocytopenia was found and neutrophil counts were low-normal. Cytologic examination of fine-needle aspirates of multiple lymph nodes and a bone marrow aspirate revealed the presence of a monomorphic population of small lymphocytes similar to those observed in the peripheral blood, suggesting a leukemic small cell lymphoma (SCL) or chronic lymphocytic leukemia (CLL). As the lymphadenomegaly and peripheral blood lymphocytosis were present simultaneously, the distinction between these 2 conditions was not possible. Immunophenotyping by immunocytochemistry and flow cytometry of the lymphoid cells in peripheral blood determined a T-cell phenotype. As the horse was clinically stable, no treatment was initiated, but regular examinations were undertaken. A CBC repeated 120 days after the diagnosis showed a marked lymphocytosis (157.6 x $10^3/\mu l$) with no evidence of anemia or other cytopenias. The horse was euthanized 194 days after the initial diagnosis. Histopathology and immunohistochemistry of submandibular lymph nodes and bone marrow confirmed the diagnosis of leukemia SCL or CLL, and a T-cell phenotype. SCL and CLL are rare in horses; previous immunohistochemical studies determined that

the T-cell phenotype is predominant. To the authors' knowledge, this is the first report of the combined use of immunocytochemistry and flow cytometry in a horse with leukemic SCL or CLL.

CD4+/CD8+ ratio in atopic dogs before and after treatment with Ciclosporin A: preliminary results

Cornegliani L, Comazzi S, Beccati M, **Martini V**, Vercelli A.

Veterinaria 2012;3:31-37

Ciclosporin is generally used in veterinary dermatology for the treatment of canine atopic dermatitis (CAD), due to its selectively ability to block production of inflammatory cytokine. CD4+/CD8+ ratio is considered useful to evaluate immune system for the indirect correlation with specific lymphocytic immune response (Th1/Th2 ratio). The aim of this prospective study was to evaluate CD4+/CD8+ ratio in atopic dogs before and after treatment with ciclosporin A (CsA). Eight dogs with CAD were included and flow cytometry was performed on day 0 (V0), day 30 (V30) and 90 (V90) from beginning CsA therapy. CADESI (Canine Atopic Dermatitis Extend Severity Index) and VAS (Visual Analogical Scale) were assessed at the same time. Healthy dogs were used as control group for CD4+/CD8+ ratio evaluation. Statistical analysis was performed with SPSS17 software: ANOVA for repeated measures was used for CADESI and CD4+/CD8+ ratio, Friendman test for VAS difference analysis. P-value <0.05 was considered statistically significant. Results showed a p-value=0.001 for CADESI and VAS, a p-value=0.694 for CD4+/CD8+ ratio. Based on our results, CsA statistically decreases CADESI and VAS index improving clinical condition of affected dogs, but do not modify CD4+/CD8+ ratio

PUBLICATIONS ON NATIONAL AND INTERNATIONAL PEER-REVIEW JOURNALS

1. Cornegliani L, Comazzi S, Beccati M, **Martini V**, Vercelli A. Valutazione del rapporto CD4-CD8 nei cani affetti da dermatite atopica prima e dopo terapia con ciclosporina A: risultati preliminari. *Veterinaria* 2012;3:31-37
2. Giantin M, Aresu L, Aricò A, Gelain ME, Riondato F, **Martini V**, Comazzi S, Dacasto M. Evaluation of tyrosine-kinase receptor c-KIT (c-KIT) mutations, mRNA and protein expression in canine leukemia: might c-KIT represent a therapeutic target? *Vet Immunol Immunopath* Doi: 10.1016/j.vetimm.2013.01.003 [epub ahead of print]
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