UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO

IN SCIENZE VETERINARIE E DELL'ALLEVAMENTO

DIPARTIMENTO

DI MEDICINA VETERINARIA

CICLO XXX

Flow cytometry for the diagnosis and the characterization of canine lymphoproliferative tumors

PhD student: Marzia Cozzi

Tutor: prof. Stefano Comazzi

Coordinatore : prof. Fulvio Gandolfi

A.A. 2016-2017

INDEX

RIASSUNTO

La citofluorimetria è una tecnica in continuo sviluppo e, ad oggi, ricopre un ruolo fondamentale nella diagnosi e nella classificazione dei tumori ematopoietici in medicina umana.

Diversi studi affermano che le informazioni ottenute dall'analisi citofluorimetrica, oltre a definire l'immunofenotipo delle cellule tumorale, quindi l'origine della neoplasia, possono aiutare a prevederne il comportamento biologico.

Questa tecnica è così diventata un metodo semplice e oggettivo per caratterizzare i tumori ematopoietici, dalla quale ottenere anche preziose informazioni prognostiche (Swerdlow et al., 2016).

In medicina veterinaria, la citofluorimetria è sempre più utilizzata per la diagnosi, la stadiazione ed il monitoraggio dei tumori ematopoietici, ma nonostante i progressi nella generazione e nella validazione di anticorpi per l'utilizzo nel cane, la caratterizzazione di tali neoplasie rimane una sfida, soprattutto per alcuni sottotipi di neoplasie meno conosciuti (Wilkerson et al., 2005; Comazzi and Gelain, 2011).

L'obiettivo di questo progetto di dottorato è di descrivere alcuni sottotipi meno frequenti di linfomi e leucemie del cane tramite l'applicazione della citofluorimetria, di definirne il comportamento biologico e di indagare se vi siano delle variabili di valore prognostico tra tutti i fattori analizzati.

A questo scopo, verranno illustrati quattro studi; il primo, è un lavoro retrospettivo che ha la finalità di valutare fattori preanalitici che possono influire sull'utilità diagnostica della citofluorimetria in campioni di aspirato linfonodale. Nel lavoro sono stati inclusi 987 casi selezionati nel periodo 2009-2015, giunti con sospetto di patologia linfoproliferativa e con presenza di un campione di aspirato linfonodale. Le variabili analizzate sono relative all'animale (razza, sesso, età), relative all'operatore (anno, stagione, metodo di spedizione al laboratorio, veterinario referente) e relative al campione (tipo di materiale, concentrazione cellulare, presenza di vetrini citologici, presenza di artefatti). Dei fattori considerati, la cellularità del campione e la presenza di cellule morte sono quelli che hanno maggiormente influenzato la possibilità di ottenere una diagnosi. La citofluorimetria è comunque risultata conclusiva nella quasi totalità dei campioni, caratterizzati da buona qualità ed adeguate condizioni di campionamento.

Il secondo studio, focalizzato sul T-zone lymphoma (TZL), un sottotipo peculiare di linfoma canino con caratteristico comportamento indolente, ha come scopo quello di caratterizzare questa entità tumorale dal punto di vista clinico e patologico. La prima fase del lavoro è di tipo retrospettivo, col fine di descrivere presentazione clinica e outcome di 51 casi selezionati tra il 2009 e il 2014. La seconda fase dello studio ha lo scopo di chiarire l'origine del peculiare immunofenotipo T CD45 negativo (Martini et al., 2013; Seelig et al., 2014); nello specifico, è stata confermata l'assenza della proteina di superficie tramite due diverse tecniche (citofluorimetria ed immunoistochimica) ed è stato valutato se fossero presenti il trascritto e il gene codificante la proteina. Dai risultati è emerso che questo tipo di linfoma ha comportamento indolente e con lunghi tempi di sopravvivenza, nonostante sia spesso diagnosticato al V stadio della patologia. Inoltre, è stato constatato che l'origine dell'aberranza fenotipica è probabilmente attribuibile a fattori trascrizionali, vista l'assenza di trascritto e la presenza del corrispondente tratto genomico.

Il terzo studio ha invece come scopo la descrizione del comportamento biologico del linfoma marginale di tipo nodale (nMZL). Nonostante in letteratura sia classificato come linfoma indolente, alcune pubblicazioni riportano casi con un comportamento piuttosto aggressivo (Flood-Knapik et al., 2012; Valli et al., 2013; Aresu et al., 2015; Marconato et al., 2015). Sono state raccolte informazioni cliniche di 35 casi di nMZL retrospettivamente selezionati, con stadiazione completa e terapie standardizzate. E' così emerso che nella nostra casista questo linfoma aveva comportamento tutt'altro che indolente, con un coinvolgimento generalizzato e con tempi di sopravvivenza brevi, quasi sovrapponibile al linfoma diffuso B a grandi cellule (DLBCL). Grazie ai risultati di questo lavoro, si potrebbero aprire discussioni sull'approccio terapeutico più corretto.

L'ultimo studio ha invece lo scopo di proporre nuovi anticorpi per la diagnosi e la stratificazione dei pazienti affetti da leucemia linfocitica cronica a cellule B (CLL-B), un sottotipo di leucemia meno frequente nel cane ma dall'elevato interesse comparativo. L'espressione dei markers ZAP70 e CD38 in medicina umana è strettamente relazionata all'andamento della malattia, con sopravvivenze molto inferiori nei pazienti con espressione oltre certe soglie (Rossi et al., 2010; Sulda et al., 2012). Nel presente progetto, questi markers sono stati valutati per la prima volta in citofluorimetria su 37

casi di leucemia cronica B di cane, insieme a CD25 e ki67. Sono state raccolti i follow-up dei casi selezionati e l'analisi della sopravvivenza ha infine rivelato che ZAP70 è un potenziale marker di prognosi, fornendo basi per ulteriori studi prognostici, con casistica più ampia e terapia standardizzate.

I risultati del mio progetto di dottorato confermano che la citofluorimetria è una buona tecnica per la diagnosi e lo studio degli aspetti clinico-patologici dei tumori linfoproliferativi del cane. Inoltre, la descrizione di questi tumori ha fornito informazioni utili a completarne il profilo biologico, gettando anche le basi per futuri approfondimenti in merito ai risultati ottenuti.

ABSTRACT

Flow cytometry (FC) is a diagnostic technique in continuous development and, to date, it plays a fundamental role in human medicine for the diagnosis and the classification of hematopoietic tumors.

Several studies state that the information obtained from the FC analysis in addition to defining the immunophenotype of the tumor cells, hence the origin of the neoplasia, can help to predict its biological behavior (Swerdlow et al., 2016).

FC has thus become a simple and objective method to characterize hematopoietic tumors in order to obtain also valuable prognostic information.

In veterinary medicine, FC is increasingly adopted for the diagnosis, staging, and monitoring of hematopoietic tumors, but despite advances in the generation and validation of antibodies for the use in dogs, the characterization of such neoplasms remains challenging (Wilkerson et al., 2005; Comazzi and Gelain, 2011).

The aim of this doctoral project is to describe some less frequent subtypes of lymphomas and leukemia of the dog via FC, in order to define its biological behavior and to investigate whether there is any variable of prognostic value among all the factors analyzed.

For this purpose, four studies will be illustrated; the first is a retrospective work, aiming to evaluate pre-analytical factors that may affect the diagnostic utility of FC in samples of lymph node aspirates. The work included 987 cases selected in the period 2009-2015, in which a lymph node aspirate was sent to our laboratory with suspect of lymphoproliferative disease. In order to define any possible bias affecting the outcome

of the FC diagnosis, the variables analyzed were related to the animal (breed, sex, age), related to the operator (year, season, method of delivery to the laboratory, referring veterinarian) and related to the sample (type of material, cell concentration, presence of cytological slides, presence of artifacts). Of the factors considered, the sample cellularity and the presence of dead cells were the ones that most influenced the possibility of obtaining an adequate diagnosis. FC was, however, conclusive in almost all the samples, that were characterized by good quality and adequate sampling conditions.

The study focused on TZL, a peculiar canine lymphoma with an indolent behavior, aimed to characterize this entity from a clinical and pathological point of view. The first phase of the work was retrospective, with the aim of describing clinical presentation and outcome of 51 cases selected between 2009 and 2014. The second phase of the study was aimed at clarifying the origin of the peculiar CD45 negative T-immunophenotype (Martini et al., 2013; Seelig et al., 2014); specifically, we confirmed the absence of the surface protein by means of two different techniques (flow cytometry and immunohistochemistry) and verified whether the transcript and the gene encoding the protein were present. The results confirmed that this type of lymphoma has indolent behavior with long survival times, despite being often diagnosed at the V stage of the disease. Furthermore, we can note that the origin of the phenotypic aberration is probably attributable to transcription factors, given the absence of transcription associated with the presence of the corresponding genomic tract.

The objective of the third study was focused on the description of the biological behavior of nodal-type marginal lymphoma (nMZL). Although in literature it is classified as indolent lymphoma, some publications reported cases with a rather aggressive behavior (Flood-Knapik et al., 2012; Valli et al., 2013; Aresu et al., 2015; Marconato et al., 2015). Clinical information was collected from 35 retrospectively selected nMZL cases, with complete staging and standardized therapies. In our cohort, this lymphoma did not show and indolent behavior, with a generalized involvement and with short survival times, almost overlapping with the high-grade diffuse B-cell lymphoma (DLBCL). Thanks to the results of this work, discussions could be opened on the correct therapeutic approach.

The latest study aimed to evaluate new antigens for the diagnosis and stratification of patients with CLL-B. The expression of the ZAP70 and CD38 markers in human medicine is closely related to the progression of the disease, with much lower survivals in patients with expression over specific thresholds (Rossi et al., 2010; Sulda et al., 2012). In the present project, these markers were evaluated for the first time in 37 blood samples of dogs with chronic B-cell leukemia, together with CD25 and ki67. Clinical data of the cases were obtained and survival analysis finally revealed that ZAP70 is a potential prognostic marker, providing bases for further studies with larger case studies and standardized therapy.

The results of my doctoral project confirm that FC is a good technique for the study of the clinical-pathological aspects of lymphoproliferative tumors of dogs, and provides useful information to complete the biological profile of these tumors, also laying the foundations for future investigations on the usefulness of the proposed new markers.

1. Introduction

When a canine patient presents with lymphadenopathy, the list of differential diagnosis suggested by clinicians and clinical pathologists generally includes lymphoproliferative disorders. Lymphoproliferative disorders often present with a diagnostic dilemma: is this a neoplastic process?

Immunophenotyping by Flow Cytometry (FC), to date, represents an important step for distinguishing the origin of cells, thus helping in differentiating non-neoplastic from neoplastic cells.

Over the last decade, FC evolved from a promising new technology to an integral component of research in the life sciences, particularly immunology, and a common element of diagnostic and prognostic investigations in human oncology, specifically, hemato-oncology (Craig and Foon 2008; Wood et al., 2008; Stewart and Stewart, 2011).

In Veterinary Medicine, FC is a service provided by an increasing number of laboratories, most of which are in veterinary schools. In veterinary research, measurement of cell fluorescence may be employed for immunophenotyping, ploidy analysis, functional cell assays, or determination of transfection efficiency with genes encoding fluorescent proteins. However, one of the most important applications is the diagnosis, classification, staging, and monitoring of hematologic neoplasms (Comazzi and Gelain 2011; Wilkerson 2012; Comazzi et al., 2017, Marconato et al., 2013; Aresu et al., 2014; Riondato et al., 2017).

In both humans and canines, lymphomas and leukemias demonstrate heterogeneity in regards to presentation, behavior, genetic characteristics, and patient outcome, offering a multitude of challenges to better characterize the disease (Valli and MacEwen, 2010; Khanna et al. 2006; Marconato et al., 2013, Richards and Suter, 2015). Indeed, the area of major promise is the identification of molecular markers, that may predict the response to therapy and tumor relapse, thereby offering clinically useful information.

Over the last years, several researchers have attempted to stratify canine patients into prognostic groups within the application of different techniques. It is known that many flow cytometric features, such as cell size and expression of specific antigens, can be important prognostic factors for certain types of lymphoproliferative tumors (Comazzi et al., 2011; Seelig et al., 2014). Richards et al. (2015) used immunohistochemistry and

the gene expression profile (GEP) on canine DLBCLs for the identification of germinal center and post-germinal center subtypes, including different survival times, partially reflecting human DLBCL. Another group used GEP on 35 canine lymphoma samples to define three major genomic groups: (i) low-grade T-cell lymphoma, (ii) high-grade T-cell lymphoma and (iii) B-cell lymphoma, associated with different clinical outcomes (Frantz et al., 2012).

Also the DNA methilome of canine DLBCLS has been investigated by genome-wide CpG microarray, revealing a common signatures of tumorigenesis and defined epigenetic prognostic subtypes (Ferraresso et al., 2017).

Again, IGHV mutational status, which is the major prognostic factor in human CLL, has been recently explored in canine CLL by sequencing the IGHV-D-J rearrangements, disclosing that Boxer breed has prevalently an unmutated IGVH status, thus opening for further detailed studies (Rout et al., 2018).

A number of different methods can be used for the characterization of hematopoietic tumors, however, molecular biology techniques are still not sensitive and specific enough to be regarded as a reliable tool for final diagnosis.

In spite of the different techniques available, FC remains a cost-effective, minimally invasive, sensitive and rapid tool to generate results from a cell population. It gives the opportuniti to obtain a better range of information, thanks to the examination of multiple parameters, including cell surface proteins, intracellular proteins, size, and complexity of individual cells (Nguyen 2007). The main edge of FC is that the expression of several antigens can be assessed simultaneously on any given cell population, in contrast with the limited number of markers that can be used in immunostaining on tissue sections or cytological smears.

The availability of a wide range of specific antibodies and different fluorochromes have improved the ability of FC to characterize cell lineages and to recognize phenotypic aberrancies in many neoplasms. Consequently, FC has acquired a prominent position in the current World Health Organization (WHO) classification of hematological malignancies in humans. The final goal of the human's WHO classification is helping to identify homogeneous groups of well-define entities and to facilitate the oncologist to include the patient in specific risk groups. In dogs WHO classification is currently based on the principles initially defined in the Revised European-American Classification of

Lymphoid Neoplasms (REAL) (Harris et al., 2004), endorsing a multiparametric approach to diagnosis and outlines the morphologic, immunophenotypic, molecular and clinical features characteristic of each disease entity; it is not static and continues to be refined (Swerdlow et al., 2016).

The past decade has also seen refinement of the criteria for classification and grading of canine lymphoma, with widespread adoption of the human's WHO classification: the classification tend to reflect the analogous in people, grouping entities by cytology, histology, and immunophenotype, while clinical, pathological, and genetic/molecular data are not yet well established (Valli et al., 2011). However, the range of monoclonal antibodies for a veterinary use is still restricted to allow standardization of panels for specific diseases subtypes (Williams et al., 2008). Thus, the characterization of tumor entities according to phenotypic markers is still a challenge.

Nevertheless, canine tumors represent valuable tools for studying interesting aspects of human cancer: the canine model has some advantageous characteristics compared to mice, since neoplasia arises and grows in immune-competent animals, thus behaving similarly to what happens in affected humans. In addition, privately owned dogs share the same environment of humans and are exposed to the same risk factors that contribute to the development of many neoplasms, including lymphoma and leukemia. Careful attention to anatomic, morphologic, molecular, and clinical features of these diseases can accelerate discovery and improve the efficiency of translation, ultimately benefiting dogs and humans, and address unmet medical needs (Hayes et al., 1995; Reif et al., 1995; Khanna et al., 2006; Rowell et al., 2011; Marconato et al., 2013).

1.1 PRINCIPLES OF FLOW CYTOMETRY

The basic principle of FC is that a cells population is defined by its morphological features and the presence/absence of specific antigens (generally called Cluster of Differentiation or CD), recognized by monoclonal antibodies labelled with fluorochromes and detected by the lasers.

Technical aspects

The flow cytometer is an instrument that defines characteristics of cells or particles in a fluid stream as they pass through a light source (laser).

The main components of a flow cytometer consist of a fluidic, optic and electronic system, and a computer for the visualization of data (fig 1).

The fluidic directs a liquid stream containing particles through the focused light source. Fluorescence results from the excitation of fluorochromes: the absorption of light by a population of these molecules raises their energy level to a brief excited state. As they decay from this excited state, they emit fluorescent light. The excitation laser focuses the light source on the cells/particles, where collection optics (filters and mirrors) directs the light scatter of the cells/particle or various fluorescent signals of excited fluorochromes to an electronic network.

The electronic network detects the light signals as it passes through the light beam and then converts the signals to a digital readout that is proportional to light intensity. The computer records the digital signals from the electronic detectors, allowing the operator to analyze the data and place it in one of several outputs (eg, histograms, dot plots, contour plots, density plots).

When the laser beam interrogates the cell, two types of light scatter occurs: forward-angle scatter (FSC) and side-angle scatter (SSC) (Fig. 2-A). Forward angle scatter is the light that has diffracted around the cell and is proportional to cell size.

Side-angle scatter is 90-degree angle scatter and SSC is proportional to the internal complexity (granularity or nuclear lobularity) and surface texture of cells, as readily illustrated by higher SSC resulting from granulocytes than lymphocytes (Fig. 2-B).

The unique light scatter properties of cells is used to distinguish subpopulations of cells based on their size and internal complexity. Fluorescent light from fluorochrome excitation is captured at the same angle as side-angle scatter.

Commonly used fluorochromes are listed in Figure 3. Such an example, fluorescein isothiocyanate (FITC) and phycoerythrin (PE), absorb light at wavelengths of 488 nm and emit light at approximately 500 and 575 nm, respectively, which can be individually captured through the use of appropriate bandpass filters.

Flow cytometers may have a variety of laser configurations that dictate the types of fluorochromes that can be excited. One widely used flow cytometer is FACSCalibur from Becton Dickinson, characterized by two-laser (argon and red diode) and four-color detector system in which dyes in the green, orange, and red emission spectra are excited by the argon laser and detected by three photomultiplier tubes (PMT) referred to as FL1, FL2, and FL3 (see Fig. 1), whereas a red diode laser excites far red dyes that are detected by the FL4 PMT (see Fig. 1).

Choice of antibodies

The availability of antibody clones conjugated with different fluorochromes allows the simultaneous evaluation of several cellular antigens. This multi-tube antibody panel, also defined as "multicolor approach", is extensively adopted for flow cytometric analysis, giving the more relevant performance than the use of individual markers.

One of the advantages of a multicolor approach is that the higher number of fluorochromes is used for simultaneous analysis, the lower is the number of tubes in the antibody panel and the lower is the total number of cells required for the entire FC study; reagents and cells are thus utilized more efficiently. The analysis with three-four color is currently the standard for human medicine, and is generally the best available for Veterinary Medicine in a clinical setting (Wood et al., 2007; Comazzi et al., 2017).

Anyway, a careful selection of unique combinations of markers is based on their degree of specificity for the identification of a given cell lineage, maturation stage and aberrant phenotypes. However, a number of basic principles should be followed for the construction of the antibody panel:

- Highly expressed antigens should be coupled with dim fluorochromes and dimly expressed antigens should be coupled with bright fluorochromes. This principle ensures reasonable sensitivity for each antigen and avoids compensation problems due to excessively bright fluorescence.

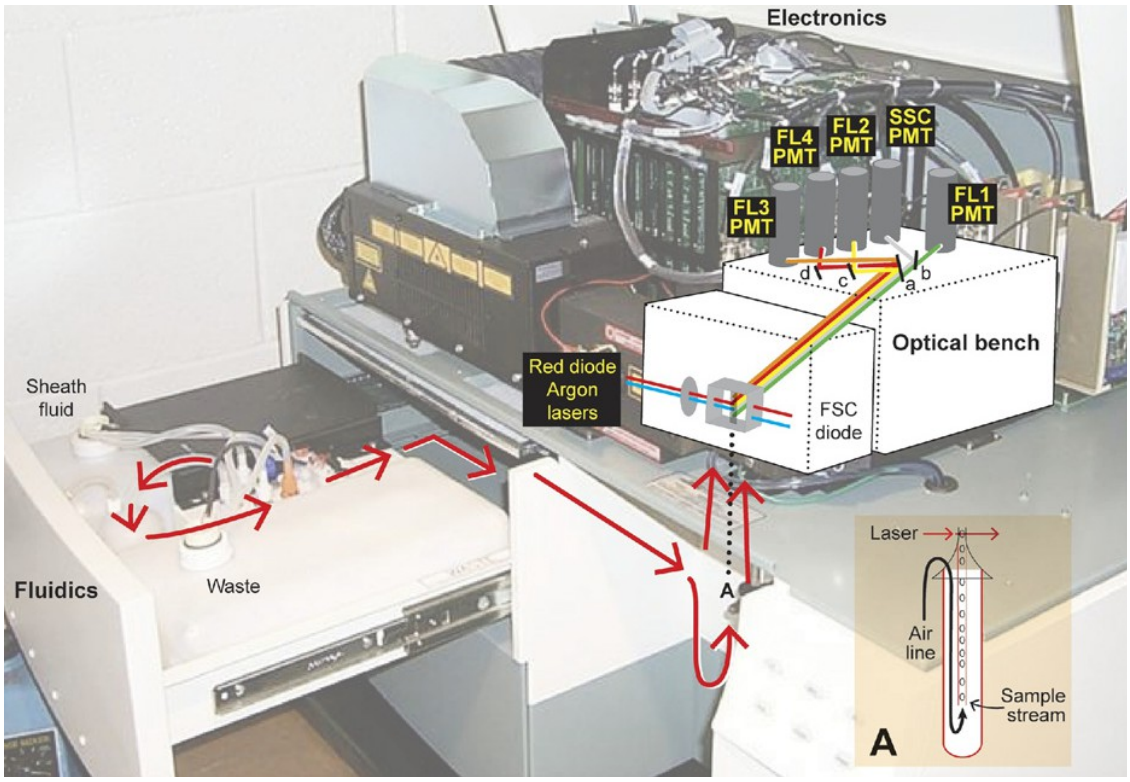

Figure 1: Essential components of the bench top flow cytometer (FACSCalibur). Fluidics (sheath fluid and waste resevoir), optical bench including excitation optics (lasers) and collection optics (a, b, c, and d), and the electronic network including photo diode for FSC and detectors (SSC, FL1, FL2, FL3, and FL4 PMTs) for side angle scatter and 4-fluorescent wavelengths. Red arrows represent the pressurized flow of the sheath fluid to the flow chamber (gray box where lasers interegate cells). (A) Inset of the sample tube (Wilkerson 2012).

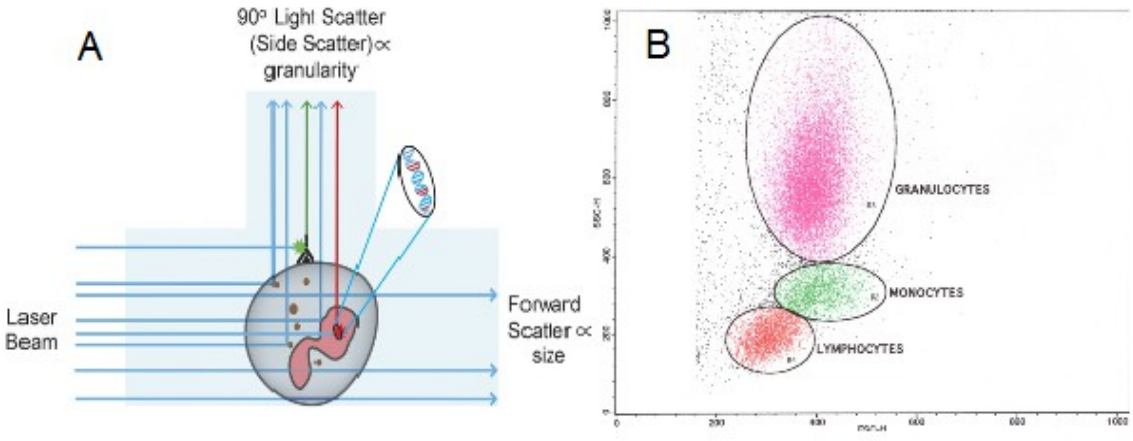

Figure 2: (A) When a laser beam interrogates a cell. (B) Scatter plot (SSC-H vs FSC-H) of peripheral blood leukocytes from a healthy dog. Lymphocytes (Region 4, red gate) have the lowest forward and side scatter indicating their small size and low complexity, whereas monocytes are intermediate (R2 or green gate), and granulocytes are most complex (R3 or pink gate) (Wilkerson 2012).

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm
DyLight 405		400	420
Alexa Fluor 405		401	421
Pacific Blue		410	455
Alexa Fluor 488		495	519
FITC		490	525
DyLight 550		562	576
PE*		490; 565	578
APC		650	661
Alexa Fluor 647		650	665
DyLight 650		654	673
PerCP		490	675
Alexa Fluor 700	Infrared	702	723

Figure 3: Commonly used fluorochromes and respective wavelengths. PE= phycoerythrin, APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

- At least one common reagent should be present in each of the tubes used for the evaluation of a particular lineage to allow correlation or tracking of populations between tubes. For exemple, CD45 in combination with side scatter allows the identification of basic hematopoietic populations (lymphocytes, monocytes, neutrophils, myeloid and lymphoid blasts) and is commonly used to allow tracking of these populations between tubes in a panel.
- Antigens for subpopulation identification within a particular lineage should be combined with a lineage associated antigen. This ensures the identification of subpopulations within the appropriate lineal context and aids in the recognition of nonspecific patterns of antibody binding. i.e. the identification of CD4 and CD8 population should be combined with a T cell common antigen as CD5.
- Antigens that are differentially expressed with maturation within a particular lineage should be combined to recognize maturational stage of cells. This is often best accomplished by combining antigens preferentially expressed early in maturation with those expressed later in differentiation. i.e. for B cell lineage, CD21 should be

combined with CD79a for the differentiation between early precursors (CD21- CD79a+) from naive mature stage (CD21+ CD79a+).

- Antigens differently expressed between closely related lineages may be used to allow improved lineage distinction. i.e. CD11b in combination with HLA should be combined for distinguish neutrophils (HLA -) from monocytes (HLA +).

The antibody panel to be used in multicolor combinations should be designed to answer one or multiple relevant clinical questions. Usually, a priori decision is made regarding the cell lineages and antigens to be evaluated, that is based on the type of specimen and other available information, such as the medical indication on the requisition, clinical history, morphologic findings, results of other laboratory testing or prior flow cytometric testing. Therefore, a different strategy may be required in each situation, as the target cell population may not be known in advance or might have been defined previously.

In the former situation, is generally preferable a rapid screening step based on a limited number of antibodies directed at the identification of all relevant cell subsets in the specimen. Based on the results, a second antibody panel is performed with optimal markers for definitive diagnosis, classification, and prognosis. This approach is generally more suitable in terms of efficiency and cost reduction, providing a rapid screen for hematologic neoplasms of clinical specimens (Wood et al., 2006).

The antibody panels for screening and the disease classification usually fit in a diagnostic algorithm that may vary among laboratories. A consensus panel for the diagnostic approach in lymphoproliferative cancers has never been proposed in Veterinary Medicine, although the European Canine Lymphoma Network (ECLN) is working with the aim to provide guidelines (Comazzi et al., 2017).

Sample preparation and processing

Fresh specimens for FC fall into two broad categories: liquid samples (peripheral blood, bone marrow, body fluids) and solid tissues (lymph nodes, spleen, and extranodal sites). Cells must be in liquid suspension for flow cytometric assay. Thus, peripheral blood cells or cells in suspension are well suited for the analysis, while solid tissue or adherent cell lines required processing into single cell suspension.

At the time of collection, blood, bone marrow and body cavity fluids may be collected in EDTA tubes, while tissue aspirates should be suspended in a tube containing a

preservation medium that may vary depending on the diagnostic laboratory (usually RPMI 1640 added with Na azide) (Liu et al., 2001).

The time between withdrawal and delivery to the laboratory, and the environmental conditions during transport are critical factors affecting the viability of the cells in the sample. Despite 48 hours from collection is generally considered the maximum storage time for retaining adequate viability, samples should be shipped in a refrigerated packaging and analyzed within 24 hours to preserve cell integrity and antigen expression (McCoy 2010; McKenna et al., 2009; Nguyen et al., 2007). This expedient is even more adopted when working with neoplastic cells: samples from tumors have a high cell turnover and cells are more prone to damage before 48 h (Jalla et al., 2004). Apart from the present work no specific information on canine samples regarding pre-analytical variables possibly affecting the quality of FC results are currently available, but , a recent study conducted on feline lymph nodes samples shows that major artifacts influencing the likelihood of being processed was poor cellularity (Martini et al., 2017). For this reason, adequate sample quality and sampling conditions are essential factors to obtain conclusive results from FC. The optimization of sample preparation steps is crucial to obtain good results from FC analysis mainly in samples shipped to a reference lab like in veterinary medicine. One of the goals of the present research is to provide the basis to create recommendations on how pre-processing the sample from canine neoplastic lymph nodes.

Herein, the steps for labeling and processing samples for FC analysis will be briefly described in order to identify possible source of bias that may influence the results of the analysis.

The first step when receiving a sample is to evaluate the cellularity of the samplesince a low cell yields limits the number of markers that can be analyzed.

Although red blood cells (RBC) have different size and light scatter characteristics then white blood cells (WBC), it could be useful to remove them before analysis, to obtain a good distinction of leukocytes population at the scatters. Red cells result in a large number of non-staining cells acquired that will make difficult the acquisition of events (cells), in particular when the abnormal population is poorly represented (Paietta 2003). However, for lymph node samples the erythrocytes lysis could be unnecessary, unless gross hemodiluition. RBC lysis is usually made with osmotic damage using water

and ammonium chloride. This method is preferable then the use of density gradient methods, because preserves leukocytes integrity (Nguyen 2007).

Then, a washing and centrifugation step is necessary to separate leukocytes from RBC debris, dead cells and other contaminants.

Once obtained a cell pellet of a sufficient number of leukocytes, cells are aliquoted into polystyrene tubes for the antibody stains. The number and the type of tubes is dictated regarding the cell lineages and antigens to be evaluated. Positive, negative and isotypic controls preparation depends on the panel of markers used. These controls are useful to discriminate between right signals and erroneous signals that may result from autofluorescence of cells, lack of antibody titration, non-specific antibody binding, interactions of different fluorochromes, lack of instrument optimization, and many other factors (Hurley 2010).

A blocking reagent is applied prior to adding the specific antibodies to capture un-specific binding sites on cells. For practical and economics reasons, the block usually consist in serum from bovine or equine, despite a serum of the same species is ideal.

Then, antibody stains are performed: antibodies are added to tubes at the correct working dilutions and incubations are generally performed at 4°C for 20-30'.

Antibodies may be conjugated directly with fluorochromes (direct staining) or might be un-conjugated. In the second case, a two-steps procedure in needed (indirect staining), consisting in sequential application of a primary antibody, removal of unbound antibody, and application of a secondary fluorescent antibody.

Intracellular stains instead, are directed to cytokine or cytoplasmic antigens. It is a more laborious protocol then the surface antigen detection and more prone to artifacts, because cells have to be fixed and permeabilized, to allow the antibodies enter the membrane. The fixation step with formaldehyde may alter epitopes and increase non-specific bindings. Also in this case, additional negative and positive controls are essential.

Once cells are labeled with fluorescent antibodies the samples are ready for the acquisition at the flow cytometer. The practice suggest to acquire at least 5000 or 10,000 FC events to have a good representation of cells in the plots (Shapiro 2004).

Data analysis

Multiparameter analysis and the possible selection of region of cells (gating) are some of the most important benefits of flow cytometry.

Data interpretation is based on a graphical visualization of FC output, assessing the complex patterns formed by the shape and relative position of the cell clusters on various graphics.

Data could be represented on one-parameter display (histograms), two-parameter display (cytogram, density, or contour plots) or three-parameter display (cloud plots) based on the operator's choice (Fig. 4).

The cytograms (dot plots), are useful in determining correlation between parameters and for determining gating strategies, as any other form of display. Typically, data are initially displayed in 2-dimensional plots of FSC versus SSC (also known as morphological plot)(Fig. 5), before fluorescence analysis. In blood samples, this configuration separates lymphocytes, monocytes, and granulocytes into relatively distinct clouds. Then, dot plots to examine cell fluorescence of a gated population may be set to display FSC or SSC versus fluorescence or, alternately, fluorescence in one channel versus fluorescence in another channel. Which parameters are evaluated on dot plots depends largely on the nature of the antigen being examined and the preference of the operator. Regarding gating strategies, most operators generally prefer to use an elliptical window then rectangles or parallelograms to select cells. Gates could be done on the bases of morphological or fluorescence features (Shapiro 2004). Based on the specific needs, it is possible to analyze only lymphocytes population or to gate on a putative neoplastic population with peculiar morphological features. Again, analysis restricted to CD45+ cells may be useful to exclude non-leukocytes if RBC lyses are not performed (Comazzi and Gelain 2011).

A quadrant statistics is applied to get information from cells: four rectangular gating regions are created on the two-dimensional space of plots and information about cells lying in quadrants could be obtained. The operator can move the boundaries of the rectangular gating to include in the quadrants the cells of interest; data on the percentage of a given population and the fluorescence intensity of markers are achieved.

Finally, information regarding the expression of a given antigen may be reported as percentage of positivity (or negativity), as ‘‘dim or low,’’ ‘‘bright or high,’’ or ‘‘partially

expressed' according the fluorescence intensity (Wood et al., 2007). This is the most common form of data analysis performed to two-parameter distribution (Shapiro 2004).

The issue of how reporting FC for canine lymphoma has been debated in the context of the European Canine Lymphoma Network and some guidelines for providing information of clinical usefulness will be edited (Comazzi et al., 2016).

However, the diagnostic interpretation of FC takes into account the other clinical and laboratory data, such as the hemogram findings and the cytologic/morphologic features. A correlation between the FC findings and the available morphologic data should be performed in all cases (Mathiot et al., 2006; Comazzi and Gelain, 2011; Ensani et al., 2012). Knowledge of the pertinent clinical history, especially the type of therapy (e.g. corticosteroids), is also useful to further refine FC diagnostic interpretation.

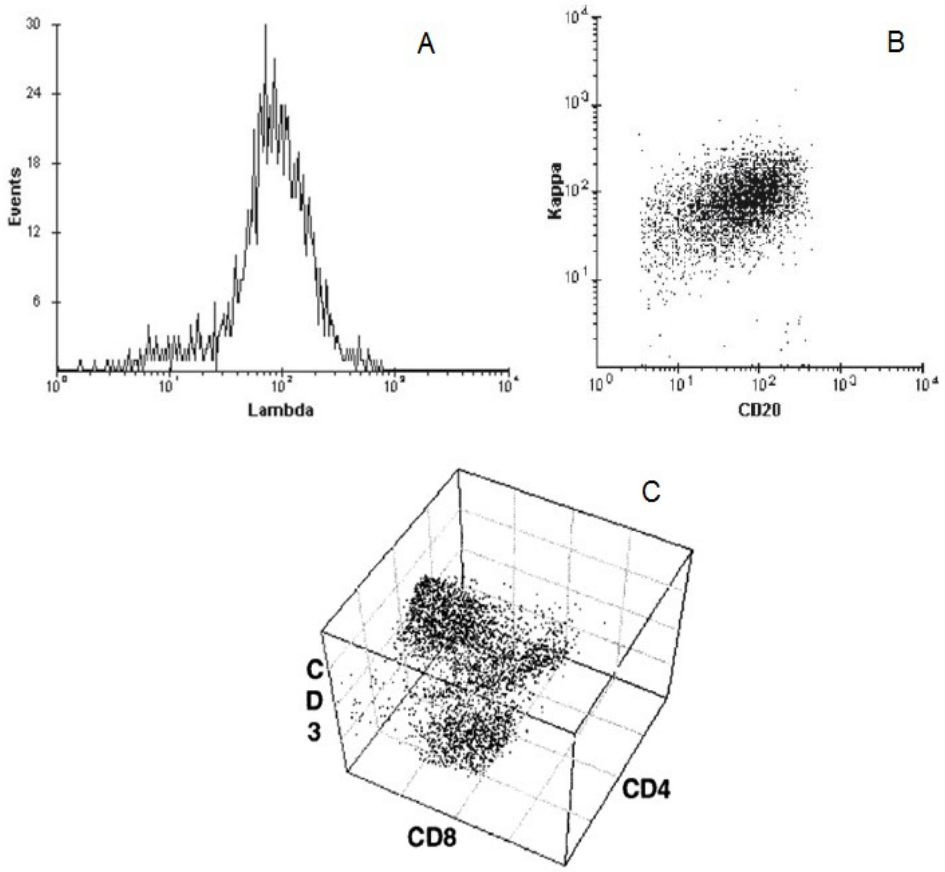

Figure 4: Different types of data rappresentation of a FC output- A: histogram. B: cytogram. C: cloud plot (Shapiro 2004)

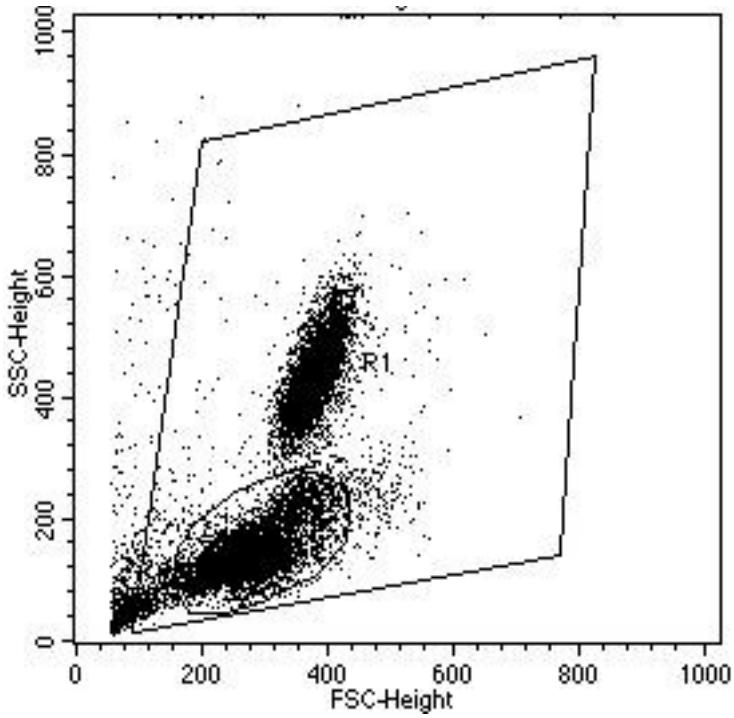

Figure 5: morphological plot of a blood sample

1.2 FLOW CYTOMETRY APPLICATION IN THE DIAGNOSIS OF LYMPHOPROLIFERATIVE DISEASES

Canine lymphoma/leukemia are malignant disorders derived from a clonal proliferation of lymphoid cells. The utility of FC is multifold and has been firmly established for lineage assignment, analysis of cellular maturation and heterogeneity within the malignant cell population, as well as detection of minimal residual disease and monitoring of disease. In some groups of lymphoid neoplasms, FC study also provides prognostic information (Williams et al., 2008; Gelain et al., 2008; Comazzi et al., 2011; Marconato et al., 2011; Marconato et al., 2013; Martini et al., 2013; Rao et al., 2015; Mizutani et al., 2016; Riondato et al., 2016).

Below are described the main aspects of canine lymphoma and leukemia in the dog and the possible FC application for immunophenotypic assessment.

Lymphoma

Lymphoma represents the most frequent hematopoietic cancer in dogs, making up 83% of all hematopoietic tumors and showing significant overlap with the human disease (Marconato 2011; Richard and Suter 2015). Similarly to human non-Hodgkin's lymphoma (h-NHL), canine lymphoma (c-NHL) is a heterogeneous group of lymphoid malignancies that have different subtypes and biological behaviors.

Several information, starting from the signalment of the patient, have been found to be important factors for the definition of disease entities.

Lymphoma affects primarily middle-aged to older dogs and many studies have reported variable occurrence of lymphoma by canine breed, showing that certain dog breeds have a statistically significant increased risk of this tumor, suggesting a hereditary compound of the disease (Modiano et al., 2005; Villamil et al., 2009). Approximately 85% of all lymphomas in Boxers is comprised of T-cell, with >50% of these malignancies being CD3+ CD4+ in origin (Lurie et al., 2004; Lurie et al., 2008; Ponce et al., 2010). In contrast, Basset hounds and Cocker spaniels almost always develop B-cell lymphomas, while Golden retrievers has been described with a high incidence of both B-cell and T-cell lymphoma overall (19%) and T-zone lymphoma (TZL, 40%), with differences in different geographical areas in United States (Modiano et al., 2005; Seelig et al., 2014); the high prevalence of Golden retriever seems confirmed in Japan but the same conclusions have not been reached by an European study (Pastor et al., 2009).

Regarding clinical signs, most animals are asymptomatic at the time of presentation, but sometimes there is a history of unspecific signs as weight loss, lethargy, anorexia, and febrile episodes. A common lymphoma-related hematologic abnormality is anemia, followed by thrombocytopenia seen in 30% to 50% of cases, while lymphocytosis is uncommon and occurs in approximately 20% of affected dogs (Madewell and Feldman, 1980; Jain 1986). Anemia has been also reported as a negative prognostic factor influencing survival and response to therapy (Abbo et al., 2007)

The most important clinical finding at presentation is the enlargement of lymph nodes: it is reported that 80% of dogs develops a multicentric form characterized by superficial lymphadenopathy (Vail and Young, 2013).

When a dog presents to the veterinarian with lymphadenomegaly, the microscopic evaluation of lymph node fine needle aspirate is still the first approach, mainly because is minimally invasive and cost effective. Despite a classification of lymphoma subtypes and different prognosis have been reported for a number of well described cytological entities (Ponce et al., 2004), this morphological diagnosis does not provide sufficient information to the clinicians for the management of the patient. Thus, a cytological evaluation is usually followed by ancillary techniques such as immunohistochemistry, molecular biology and FC (Dong et al., 2001; Comazzi and Gelain, 2011).

Although histopathologic evaluation of architectural patterns have been historically considered as the gold standard for lymphoma classification, using WHO scheme, the advent of FC in addition of cytology allowed to putatively diagnose and classify most canine lymphoma subtypes that have a characteristic cytomorphology and immunophenotype (Gelain 2008; Comazzi and Gelain, 2011; Martini et al., 2013; Seelig et al., 2014). Specific studies on the diagnostic performances of different techniques in defining different lymphoma subtypes are still lacking in dogs, but according to a survey conducted in US, histopathology (Regan et al., 2011) is required just on a small percentage of dogs with lymphoma prior chemotherapy.

When the WHO scheme was applied to the dog, it was found that the vast majority of lymphoma cases consisted of only five histological subtypes: diffuse large B-cell lymphoma (54%), marginal zone (B-cell) lymphoma (4%), peripheral T-cell lymphoma not otherwise specified (16%), nodal T-zone lymphoma (14%) and T lymphoblastic lymphoma (5%) (Valli et al., 2011). Apart from WHO, among the other classification schemes available for canine lymphoma, just the updated-Kiel classification appears of some clinical usefulness according to a recent paper (Sayag et al., 2018). This scheme is mainly based on morphologic appearance of neoplastic cells and tissue architecture is just minimally considered, that's why updated-Kiel classification is generally preferred for the use in cytology. Some papers have attempted a correlation between WHO and updated-Kiel (Ponce et al 2010) (fig. 6) .

Studies evaluating lymphoma in dogs by FC have generally confirmed the predominance of one type of B-cell lymphoma and identified a variety of T-cell phenotypes. Nevertheless, a quantitative and a qualitative evaluation of antigen expression by FC resulted to be useful to identifying aberrant subtypes and further

subclassify lymphomas into clinically relevant subcategories (Wilkerson et al., 2005; Gelain et al., 2008).

Anyway, the knowledge of features typical of an healthy lymph node are fundamental for the recognition of neoplastic conditions. Generally, polyclonality and the absence of phenotypic aberrancies characterize a benign sample. It is important to be aware, however, that the absence of abnormalities by FC analysis does not necessarily exclude the presence of a lymphoma; sampling errors or selective loss of neoplastic cells during preparation, or other tumors that may not be recovered in the cell suspension, could result in a "false picture" dot plot (Nguyen 2007).

In a normal lymph nodes, the majority of cells express CD45 (around 90%), with a degree of expression (fluorescence intensity) that vary among lymphocyte population (Comazzi et al., 2006). T-lymphocytes expressing CD3, CD4, or CD8 represented about 60 %, 40%, or 20% of the total cells, whereas B-lymphocytes expressing either CD21 or surface IgM represented 30% or 15%, respectively. CD34 is not expressed on mature lymphocytes, CD14 (monocytes) positive cells are few (4%) while granulocytes (CD11b) are rare (2-3%) and attributable to neutrophils or eosinophils (Wilkerson 2005; Rutgen 2015).

Small lymphocytes could be distinguished from large lymphocytes by forward light scatter. According FSC channel, both small and large lymphocytes are present in B and T cell populations: usually small cells fall between 200 and 400 FSC, while medium to large cells fall over 400 FSC; the size parameters may vary among flow cytometer according the calibration of the instrument (Wilkerson et al., 2005; Rutgen et al., 2015).

When interpreting the staining patterns of tumor cell preparations, a cutoff value of 60% of the cells labeling for a specific cell marker is usually used (Wilkerson et al., 2005). This guideline is similar to the criteria used for cytological assessment in which a diagnosis of lymphoma can be confidently made when >50% of the cells have immature features (Cowell et al., 1999).

However, this cut-off value is very high and may provide false negative results in early stage lymphomas or in neoplastic lymph nodes with a mixed population of cells, such as T-cell rich B-cell lymphoma, or with a high percentage of residual resident lymphoid cells, such as in follicular lymphomas.

B-cell-origin lymphomas in the dog are characterized by the expression of the markers CD21, CD79a, CD20, IgM, MHC class II (Wilkerson 2005; Gelain et al., 2008). Some case do not express CD21 but expressed CD79a and IgM, likely representing an arrest at an immature stage of B-cell maturation: CD79a expression precedes immunoglobulin heavy-chain gene rearrangement during B-cell development and is expressed in lymphoma/leukemias arrested at the pre-B-cell stage (Chu and Arber, 2001) (Fig. 7).

Cell size observed by morphological evaluation is usually confirmed by the FSC scatter. Centroblastic B-cell lymphoma and pleomorphic large B-cell lymphoma are the most common subtypes and show higher mean FSC. B-macronucleolated medium size cells usually show an higher size at FC then on slides, maybe due to the fact that cells on slides rapidly undergo dehydratation and rigidity (Gelain et al., 2008). Low-grade lymphomas and lymphoblastic lymphoma are characterized by low FSC, thus gating on neoplastic populations is quite difficult on scatter cytograms alone; therefore, the detection of a prevalent immunophenotype and quantitative antigen expression may be of particular value to confirm the neoplastic origin (Comazzi et al., 2006; Comazzi and Gelain, 2011).

Cell size is also reported to be partially related to the behaviour of the disease. A recent study on B-cell lymphomas disclosed that patients with large tumor cells were 2.8 times more likely to die compared with those with small tumor cells (Rao et al., 2011); this result could be explained by the fact that large cells might correspond to a more immature cell type that has a more aggressive neoplastic behavior.

Among the T-cell lymphomas, small clear cells, pleomorphic large and mixed cells, large granular lymphocytic cells and lymphoblastic are cytologic subtypes most represented in the dog (Gelain et al., 2008; Ponce et al., 2010).

T-cell lymphomas express a combination of CD3, CD5, CD4, and CD8 markers, with CD3+CD4+CD8- the most frequent subtype (Fig. 7). CD3+CD4+CD8+ phenotype profile is instead indicative of an early thymocyte differentiation stage. One study reported how FC of mediastinal mass aspirates may provide a definitive diagnosis of thymoma or ymphoma: samples containing ≥ 10% lymphocytes coexpressing CD4 and CD8 was reported to correlate with a histologic diagnosis of thymoma, whereas thymic lymphomas expressed either CD4/CD8 or B-cell antigens, demonstrating that FC is a useful tool for discriminating mediastinal masses (Lana et al ., 2006).

Updated Kiel Malignant Lymphoma Classification	No. Cases	% Cases	World Health Organization Malignant Lymphoma Classification
B-cell neoplasms	388	64	
Low-grade malignancy	81	13	Peripheral B-cell neoplasms
Small B-cell lymphoma	12		
Small lymphocytic	2	<1	B-cell chronic lymphocytic leukemia / small lymphocytic lymphoma? *OR* low-grade small B-cell lymphoma not otherwise specified?
Prolymphocytic	1	<1	
Lymphoplasmacytic	9	1	Lymphoplasmacytic lymphoma
Marginal zone	66	11	Nodal marginal zone lymphoma Extranodal marginal zone lymphoma Splenic marginal zone lymphoma
Centroblasto-centrocytic	3	<1	Follicular lymphoma grade I/II
High-grade malignancy	307	51	
Centroblastic monomorphic	5		
Follicular subtype	1	<1	Follicular lymphoma grade III
Diffuse subtype	4	<1	Diffuse large B-cell lymphoma
Centroblastic polymorphic	234	38	Diffuse large B-cell lymphoma
Immunoblastic	47	8	Diffuse large B-cell lymphoma
Anaplastic/mediastinal	1	<1	Mediastinal B-cell lymphoma (diffuse large B-cell lymphoma)
Burkitt type	10	2	Burkitt lymphoma
Plasmacytoid	6	1	No World Health Organization correlate
Small cell not otherwise specified	4	<1	Mantle cell lymphoma?
T-cell neoplasms	215	35	
Precursor T-cell lymphoma: Lymphoblastic	17	3	Precursor T-cell lymphoblastic lymphoma/leukemia
Mature T-cell lymphoma			Peripheral T-cell and natural killer–cell neoplasms
Low-grade malignancy	26	13	
Prolymphocytic	1	<1	T-cell chronic lymphocytic leukemia / prolymphocytic leukemia
Pleomorphic small cell	5	<1	Peripheral T-cell lymphomas, unspecified
Small clear cell (T-zone)	20	3	Peripheral T-cell lymphomas, unspecified
High-grade malignancy	100	22	
Pleomorphic mixed	39	6	Peripheral T-cell lymphomas, unspecified
Pleomorphic large cell	13	2	Peripheral T-cell lymphomas, unspecified
Immunoblastic	7	2	Peripheral T-cell lymphomas, unspecified
Plasmacytoid	19	3	Peripheral T-cell lymphomas, unspecified
Aggressive large granular	1	<1	Enteropathy-type T-cell lymphoma *OR* natural killer leukemia *OR* extranodal nasal type
Unclassified	21	3	Unclassifiable
Cutaneous lymphoma	72		
Cutaneous T cell, low grade	53	9	Mycosis fungoides / Sezary syndrome
Cutaneous T cell, high grade	19	3	Cutaneous T-cell lymphoma
Null-cell neoplasms	5	<1	Natural killer–cell leukemia

Figure 6: Updated Kiel Classification33 of 608 Canine Lymphomas With Possible Correlation With the World Health Organization Human Classification (Ponce et al., 2010)

In addition to the determination of the immunophenotype, the expression of specific markers as well as the quantification of the expression are found to be consistent predictors of poor outcome.

Rao et al. (2011) found an association between low class II MHC levels and poor survival, which is a parallel finding in people with diffuse large B-cell lymphoma, where Class II MHC expression is a strong predictor of outcome (Rimsza et al., 2007). Similar results have been reported for T cell CD4+ lymphoma in a recent study exploring

different immunotypes of T cell high grade lymphoma and their association with outcome (Deravi et al., 2017).

Lymphoid antibodies	
Anti-CD3	T lymphocytes
Anti-CD5	T lymphocytes
Anti-CD4	CD4 T lymphocytes, neutrophils
Anti-CD8	CD8 T lymphocytes
Anti-CD21	B lymphocytes
Anti-CD22	B lymphocytes
Anti-CD79a	B lymphocytes
Anti-Pax5	B lymphocytes
Myeloid antibodies	
Anti-CD14	Monocytes
Anti-CD18	Neutrophils, monocytes
Anti-CD4	Neutrophils, CD4 T lymphocytes
Anti-MPO	Neutrophils, monocytes
Anti-CD11b	Granulocytes, monocytes, some macrophages
Anti-CD11c	Granulocytes, monocytes, dendritic cells
Anti-CD11d	Macrophage subsets, T lymphocyte subsets
Other antibodies	
Anti-CD45	All leukocytes
Anti-CD11a	All leukocytes
Anti-CD34	Stem cells, early progenitor cells
Anti-class II MHC	Monocytes, B lymphocytes, T lymphocytes

Figure 7: Common antibodies used to phenotype canine hematopoietic neoplasms (Rout and Avery, 2017)

Again, a recent publication has explored the clinical relevance of CD25 marker in canine lymphoid tumors, finding that CD25-positivity in the lymph node was significantly higher in dogs with high-grade B-cell lymphoma and TZL, and the progression-free survival was significantly shorter in high-grade B-cell lymphoma with CD25-high expression (Mizutani et al., 2016). Also these results are partially in agreement with human's literature (Fujiwara et al., 2013).

Generally, studies taken together exhibit that in dogs high grade T-cell lymphomas are usually linked to a poor response to therapy (Wilkerson et al., 2005), with survival times being often shorter than the B-cell counterpart (Ponce et al., 2004; Morris and Dobson, 2001). However, some B-cell lymphoma like the Burkitt type result in short survival time, while some T-cell lymphomas, such as TZL, generally show a long-term survival (Seelig et al., 2014).

Studies focused on the prognostic value of aberrant phenotypes have also been proposed. (Williams et al., 2006; Gelain et al., 2006; Comazzi et al., 2008; Seelig et al., 2014). The most common aberrant patterns in canine lymphomas are reported to be the co-expression of T and B antigens (CD3 and CD21 or CD3 and CD79a), the expression of CD34, the co-expression of CD4 and CD8 on T cell lymphomas, and the under expression of CD45 and CD18 (Wilkerson et al., 2005; Guija et al., 2006; Riondato et al., 2006; Gelain et al., 2008; Comazzi et al., 2011).

Several works reported many T lymphocyte aberrancies detected by FC, thanks to the availability of different monoclonal antibodies for T-cell characterization (Wilkerson et al., 2005; Gelain et al., 2008) (Fig).

On the contrary, few antibodies are available for canine B-lymphocytes: consequently, a reduced number of B-cell antigens can be tested and many aberrancies would remain undetected. For this reason, validation of new anti-canine or cross-reacting antibodies is needed, and in the future may permit the identification of aberrancies in canine B-cell neoplasms too.

Neoplastic cells with aberrant antigen expression could be easily detected in different tissues, thus allowing for the evaluation, staging and a sensitively identification of minimal residual disease (MRD) by FC.

The WHO guidelines for canine lymphoma staging provide five stages for classify the spread of the tumor and include the involvement of lymph nodes (stages I-III), spleen/lever (stage IV), peripheral blood/bone marrow or any other non lymphoid tissue (stage V). Tumor at V stage is, according to some authors, correlated with a poorer outcome and a shorter survival time then stages I to IV (Vail and Young, 2013; Marconato et al., 2011). Information regarding the distribution of lymphoma makes it possible to assist in the formulation of therapeutic protocols, but also to accurately re-stage dogs at the end of a therapy session and to monitor the response to treatment, based on the evaluation of reduction in size of lymph-nodes and the assessment of a residual neoplastic population in LN, PB and BM (minimal residual disease, MRD) (Vail 2010; Marconato 2011). Despite the prognostic significance of BM infiltration in patients affected by large B cell lymphoma has been established by some studies (Marconato et al., 2011; Marconato 2013), to date the BM evaluation is not routinely performed (Davies et al., 2017). The issue of whether the examination of BM should be

performed or not is still under debate: one study declaim that BM involvement does not affect therapeutic decision and stage migration (Flory et al 2007); however, cases with BM involvement that do not correlate with PB abnormalities are reported (Martini et al., 2015) and, on the contrary, the finding of circulating blast cells does not necessarily imply marrow invasion as their presence may be attributable to 'overspill' (Chapel et al., 2009). Thus, BM evaluation should be included in routine staging in order not to miss infiltrated samples and to improve classification.

Several methods are used for the determination of neoplastic infiltration in tissues, mainly microscopic evaluation, FC and molecular biology techniques. The advantage of FC in tumor staging and MRD assessment is the high sensitivity in detecting small infiltration percentages: a recent publication reports the high performance of FC for the quantification of large B-cells, providing the cutoff of 0.56% and 2.45 % in peripheral blood and bone marrow, respectively, to safely discriminate between infiltrated and un-infiltrated (Riondato et al., 2016); however, specificity depends opon the type of lymphoma and the characteristics of the neoplastic cells. Large lymphomas or aberrant phenotypes are easily recognized (Martini et al., 2015; Riondato et al., 2016), while small cell lymphomas, especially those of B lineage or T CD4+, may be difficult to differentiate from normal lymphocytes. In these cases, molecular techniques such as polymerase chain reaction amplification of antigen receptor genes (PARR) are likely more sensitive and useful than FC for the determination of neoplastic infiltration (Lana et al., 2006), although PARR is yet unable to quantify the extend of infiltration. Thus, the use of FC and PARR is suggested in conjunction to improve the accuracy of clinical staging of lymphoma and detection of MRD in dogs (Aresu et al., 2014).

An example of a small cell lymphoma easily recognized by FC is small clear cell/T-Zone Lymphoma (TZL). TZL has a peculiar T cells immunophenotype CD45 negative, that can be used to readily identify this disease with 2 color flow cytometry, obviating the need for tissue biopsy (Martini et al., 2013; Seelig et al., 2014).

In human medicine the identification of persistent disease or relapse is currently based on imaging (positron emission tomography (PET) and computed tomography (CT) scanning are the principal tools) and clinical evaluations (Thompson et al., 2014; Huntington et al., 2015), but novel methods based on polimerase chain reaction (PCR)

and next generation sequencing (NGS) are now being studied with promising results: These innovative tools allow to detect and quantify circulating tumor DNA (cfDNA) released by neoplastic cells subject to destruction and necrosis. This method, also known as "liquid biopsy", offer MRD measurement with excellent sensitivity (Narayanet al, 2012; Newman et al, 2014; Roschewski et al, 2015, Rossi et al., 2017; Spina et al., 2018).

In veterinary medicine, we are still far away from these type of diagnostic investigation, although the potential value of cfDNA in the prognosis is reported in dogs affected by lymphoma and mammary tumor (Shaefer et al., 2007; Beffagna et al., 2017).

In conclusion, FC with a multi-parametric approach for the diagnosis of canine lymphoma, contributes to the evaluation of cell lineage as well as to antigen quantification and monitoring of the disease, and provides useful information for outcome prediction. The use of this technique will be further enhanced by wider panel of canine-specific marker antibodies and by a careful description of tumor entities.

Leukemia

A dog presenting with lymphocytosis may be a dilemma for clinicians. The diagnosis can be quite challenging, because leukemia is associated with nonspecific clinical signs and sometimes mild hematologic abnormalities may be difficult to interpret. To accurately diagnosed a lymphoid leukemia must be considered several factors, such as signalment, history, clinical findings, cell morphology and the immunophenotype.

Historically, the diagnosis of leukemia relied only on the morphological analysis. Lymphoid leukemias are divided into acute (ALL) or chronic leukemias (CLL), according the stage of maturation of neoplastic cells and the evolution of the diesease: CLL produces cells that are morphologically similar to normal small, mature lymphocytes. ALL, on the other hand, originates from more immature cells and shows cells morphologically resembling large blast cells.

Because the diagnosis and precise classification of leukemia by morphologic criteria alone is limited (Davis et al., 1997), immunophenotyping via FC is to date the preferred method for the characterization of leukemic cells in veterinary, and is the gold standard for classification of human leukaemias (Avery and Avery, 2007).

Leukemias in dogs are heterogeneous disease comprising approximately 10% of all haematopoietic tumor, with survival ranging from days to years, depending on the subtype. However, the real incidence of these tumors in the dog is imprecise, most likely because underdiagnosed due to the rapid clinical course (in ALL) and/or nonspecific clinical signs (Weiss and Wardrop 2011; Vail and Young, 2013).

Dogs with CLL are typically older than those with ALL: from several studies it emerges that the average of dogs with CLL is 10-12 years (range 3-19) (Comazzi et al., 2011; Bromberek et al., 2016; Moore and Vernau, 1999), compared with 7-8 for ALL cases (range 7 months-16 years) (Novacco et al., 2015; Adam et al., 2009; Bennett et al., 2016; Tasca et al., 2009). Interesting, this is in agreement with human medicine, where the prevalence of acute lymphoblastic leukemia is observed in children and chronic leukemia affects older patients (Chiaretti et al 2013; Rai et al., 1975). Some case reports and retrospective studies also suggest that large-breed dogs are more susceptible to acute leukemia (Tasca et al., 2009; Adam et al., 2009; Novacco et al., 2015), with a significant over-representation of Golden Retrievers and German Shepherd (Adam et al., 2009; Novacco et al. 2015; Bennett et al., 2016; Tasca et al., 2009), while one study shows that B-cell CLL is over-represented in small-breed dogs (Bromberek et al., 2016).

Regarding clinical signs, they may vary dramatically, also depending on clinical stage. The most frequent owner complaint at presentation is lethargy; other common abnormalities include reduced appetite, polyuria, polydipsia, and sporadic vomiting. Usually, dogs with CLL present with a long history of recurrent nonspecific signs. Physical examination may reveal splenomegaly, hepatomegaly, fever or generalized lymphadenopathy (Vail and Young, 2013). Lymphadenopathy associated with leukemia is usually milder than that of lymphoma, in which the lymph nodes are often massively enlarged (Couto 2003).

CBC at diagnosis could shows hematological abnormalities referred to leukocytosis, anemia and thrombocytopenia; lymphocytosis is usually much more extreme then that associated with other conditions, with lymphocyte counts ranging from 6,000 to well over 500,000/µl (Vail and Young, 2013; Novacco et al., 2015). Anemia is found in 80% of cases; in some studies it is reported to be significantly more severe in ALL cases then in CLL (Tasca et al., 2009) and to be related to a shorter survival time (Novacco et al.,

2014). Thrombocytopenia is another well-recorded haematological abnormality: it is reported that dogs with ALL had significantly higher thrombocytopenia grades than their counterparts with CLL (Tasca et al. 2009; Leifer and Matus, 1986).

Generally speaking, acute leukaemias present with more profound cytopenias than their chronic counterparts (Morris and Dobson, 2001).

From a clinical perspective, it is crucial to identify neoplasms of precursor from those of mature lymphoid cells: the prognosis of acute leukemia is very poor compared to CLL, that is generally a slowly progressive disease (Novacco et al., 2015; Adam et al. 2009; Comazzi et al., 2011; Bromberek et al., 2016).

For this purpose, immunophenotyping via FC is a valuable tool to distinguish between acute and chronic leukemia, as well as the differentiation between acute leukemias and a leukemic phase of lymphoma.

The knowledge of circulating lymphocyte of normal dogs is fundamental for determining if a particular subset has expanded. The major fraction of lymphocytes in peripheral blood are of T lineage (80%), with T helper cells (CD4+) outnumber the citotoxic T cell (CD8+), while the B cell are about 15%. The remaining fraction is usually composed by NK cells and double negative T lymphocytes (CD4-CD8-) (Byrne et al., 2000; Workman and Vernau 2003).

Based on the above statement, lymphoid leukemia should be considered when lymphocytes present an homogeneous immunophenotype, when atypical lymphoid cells are observed or when a phenotype typically less represented has increased (Vail and Young, 2013). However, in certain situation could be complicated distinguishing between reactive proliferation and neoplastic expansion. Some infectious or inflammatory diseases can also, occasionally, result in expansion of a phenotypically homogeneous population of lymphocytes. Differential diagnosis should be taken into consideration, including chronic ehrlichiosis, postvaccinal response, IL-2 administration, transient physiologic lymphocytosis. However, in cases in which the phenotype data are equivocal, clonality testing using PARR assay could be a useful second step (Burnett et al., 2016). On the other hand, an aberrant antigen expression can provide a definitive diagnosis of leukemia because reactive lymphocytes generally retain expression of the normal constellation of antigens.

The surface glycoprotein CD34, which is expressed on immature hematopoietic progenitors and not present on more mature and differentiated cells may help the distinction between CLL, ALL and lymphoma. Therefore, ALL (CD34+) can be distinguished from CLL (CD34-) and lymphoma (CD34-) (Vernau and Moore 2009; Wilkerson et al., 2005; Gelain et al., 2008). However, some lymphoma may occasionally show CD34 positivity. This expression is considered aberrant when blood and bone marrow are not infiltrated by neoplastic cells and the remaining antigen pattern suggests a neoplasm of mature lymphoid cells (Gelain et al., 2008). Moreover, a proportion of ALL are recognized to be CD34 –; the expression of the marker is reported to range from 37 to 76% of ALL (Adam et al., 2009; Bennett et al., 2016). In these cases, other laboratory and clinical features can help discrimination between lymphoma or leukemia, based on the fact that the published criteria for acute leukaemia diagnosis required >30% morphologically blast cells in either peripheral blood or bone marrow (Modiano and Helfand, 2000; Grindem and Neel, 2002; Adam et al., 2009). Therefore, the expression of CD34 marker is not conclusive of ALL.

To investigate new markers for leukemia diagnosis and classification, our group evaluated CD44 expression on peripheral blood of dogs affected by precursor cell and mature cell leukemia, finding a significantly higher MFI of the antigen in leukemic samples compared to controls and a higher expression was found in AL in respect with CLL, underling the utility of this marker for the identification of canine leukemias (Gelain et al., 2014)

Both types of lymphoid leukemia may arise from B, T, or NK cell clones. In humans, B-cell neoplasia predominates and represents 95% of CLL cases; in contrast with human counterpart, B-cell neoplasia is less common than T-cell neoplasia in canine CLL, representing less than 30% of cases (Moore and Vernau, 1999; Tasca et al., 2009). T-CLL with CD3+ CD8+ phenotype has been reported as the most frequent form, representing over 70% of CLL, mainly showing LGL morphology (80% of T-CLL). T-CLL shows a classic indolent behavior and long survival time, atypical CLL is characterized by very aggressive behavior and short survival, while B-CLL may be considered an intermediate behavior, with old dogs surviving quite long, and young dogs showing a more aggressive course. From a study emerged that the range of survival time for 17 dogs affected by B-cell CLL was from 20 to >800 days (Comazzi et al. 2011).

On the contrary, the true prevalence of B- vs T-cell ALLs in dogs should be considered uncertain. Bennett et al. (2016) found a predominance of T-cell ALLs (15 of 20). In contrast, in 2014 we published that of 71 dogs with acute leukemias, when myeloid leukaemias were excluded from analysis, B-cell origin constituted 43% and T-cell origin constituted 20% (37% were undifferentiated) (Novacco et al., 2015). In other two publications reporting on flow cytometric assessment of canine leukaemias, 15 of 25 and 47 of 51 ALL cases were B-cell-origin (Adam et al., 2009; Tasca et al., 2009).

Regarding the prognostic aspects, while it is well established that for the chronic counterpart the T-cell lineage have a better behaviour, it is instead unknown whether the same holds true for ALL, since few studies are currently present in literature and insufficient dogs had definitive immunophenotyping.

Despite the advances in classification, a separate clinical staging system has not been developed for chronic lymphocytic leukemia. Currently, all dogs with leukemia are classified as stage V based on the WHO staging system for lymphoma. Although a specific prognostication system for CLL has been used in humans, comprising clinical-pathological (Rai et al., 1975; Binet et al., 1981), molecular (IgVH mutation status) and flow cytometric (ZAP70 and CD38) parameters (Damle et al., 1999; Capello et al., 2004; Sulda et al., 2012), it has not been evaluated in the dog. The group of Colorado State University recently published a study aimed to describe the IgHV-D-J rearrangements of 55 canine patients with CLL, including non-Boxer and Boxer dogs. The majority of non-Boxers (75%) had mutated IGHV genes, whereas the majority of Boxers (79%) had unmutated IGHV genes. Despite the prognostic significance was not investigated, the results provide bases for further studies aimed to the risk stratification of patients (Rout et al., 2018).

However, both CLL and ALL are poorly characterized in dogs and few pathogenic and prognostic studies are available in the literature (Usher et al., 2009; Comazzi et al., 2011; Aricò et al., 2013; Giantin et al.,2013).

In conclusion, the application of FC in canine leukemia is useful for diagnosis and cell lineage. In CLL may bring also prognostic information. However, the strong heterogeneity of leukemias makes necessary to find possible molecular biomarkers associated with different survival times or disease evolution.

2. Aim

The utility of flow cytometry and its importance in the diagnostic pathway of hematopoietic tumors is even more relevant in both human and dog. Several studies have demonstrated that some flow cytometric findings may play also a role in the prediction of treatment free survival and survival time.

The last 15 years of literature in the issue of canine lymphoproliferative tumors demonstrates that strides have been made to improve classification and understanding of pathogenesis through immunophenotyping, yet classification, prognostic relevance and choosing appropriate therapy remains challenging.

Diffuse large B cell lymphoma is the most diffuse lymphoma subtype and, in the past years, several studies from our lab and international research groups have been performed to better characterize this tumor in terms of immunophenotyping (including flow cytometric aspects), pathological and molecular aspects. In the present thesis we decided to focus on some less investigated lymphoma/leukemia subtypes, starting from the experience collected during the last 10 years of FC diagnostic service, and trying to investigate some aspects that could be interesting from a biological point of view.

In particular, the aim of the present research project was to characterize specific canine lymphoma and leukemia subtypes within the application of flow cytometry, to describe the power of this technique for diagnostic purpose and to identify new biomarkers for risk stratification of patients, thus helping the oncologists to undertake the better manage of the patient.

To the aim, either retrospective e prospective approaches were adopted, depending on the nature of the studies. To draft a complete biological profile of tumors, an integration of immunophenotypic, clinical and pathological data was performed.

The main technique reported and utilized in the present thesis is flow cytometry, but other molecular tools have been used to better identify antigens of interest (when monoclonal antibodies were not available) and to explain the origin of phenotypic aberrancies (within the analysis of the gene and the transcript of the antigen).

Specific objectives were:

- to define preanalytical variables which could affect the power of flow cytometry for the evaluation of lymph node samples in lymphoma diagnosis;

- to define lymphoma entities from a pathological, phenotypic and clinical point of view and to improve the definition of the prognosis, with particular attention on T-zone Lymphoma and Marginal Zone Lymphoma. These two entities were chosen for the paucity of information available in literature, in spite of the relative frequency among canine lymphomas and the peculiar biological behavior;
- to evaluate the application of new markers (ZAP70, CD25, ki67 and CD38) for B-CLL diagnosis and prognosis.

To better exhibit the studies, the following chapter will be subdivided into four sections:

- EFFECTS OF PRE-ANALYTICAL VARIABLES ON FLOW CYTOMETRIC DIAGNOSIS OF CANINE LYMPHOMA: A RETROSPECTIVE STUDY
- CANINE T-ZONE LYMPHOMA: CLINICAL AND PATHOLOGICAL CHARACHTERIZATION
- NODAL MARGINAL ZONE LYMPHOMA: INSIGHT INTO THE BIOLOGICAL BEHAVIOUR
- EVALUATION OF PUTATIVE PROGNOSTIC MARKERS IN CANINE B-CLL: A PRELIMINARY STUDY

3. Studies

3.1 EFFECTS OF PRE-ANALYTICAL VARIABLES ON FLOW CYTOMETRIC DIAGNOSIS OF CANINE LYMPHOMA: A RETROSPECTIVE STUDY (2009–2015)

Background

In human medicine flow cytometry is widely used to immunophenotype leukemia and lymphoma cells. In veterinary medicine this technique is in rapid developmente and spread but, in contast with human medicine, a low number of facilities offer a flow cytometric services with an adequate skill in canine onco-haematology. As a consequence, it constrain to use reference labs to which samples coming from different institutions and private vets are shipped. We hypothesized that different sampling techniques, shipping and storage conditions might bias the results and influence the diagnostic performance of FC. To the authors' knowledge, only one study on the influence of pre-analytical variables on diagnostic performance of FC is available in cats (Martini et al., 2017), but a study evaluating the effects of such variables in a high number of dogs is still lacking. The aim of this retrospective study was to assess whether pre-analytical variables may influence the diagnostic utility of lymph node (LN) FNA samples from dogs with clinically suspected lympho-proliferative disease analysed by FC. The goal was to create recommendations for sampling techniques, sample storage and shipping, in order to decrease pre-analytical errors, and to increase the diagnostic utility of FC for the diagnosis of lymphoma and leukaemia in dogs.

Material and Methods

Inclusion criteria

The FC database of the Department of Veterinary Medicine, University of Milan, Italy, was searched retrospectively and canine cases were identified over a period of six years (2009–2015). The inclusion criterion was a LN FNA submitted for flow cytometric immunophenotyping. If other sample types, such as peripheral blood (PB), bone marrow (BM) aspirates, body cavity effusions, FNA from mass lesions, spleen, liver or other tissues, were submitted, these cases were included in the study only if a LN FNA from the same animal was analysed, regardless of the diagnostic pathway used and

what tissue (e.g. PB or BM for leukaemias) was considered first in the diagnostic pathway. Exclusion criteria included cases composed of tissues other than LN aspirates and cases submitted for minimal residual disease analysis.

Sample collection

For flow cytometry analysis, LN FNA was resuspended in 1 ml RPMI 1640 with 5% fetal bovine serum (FBS) and 0.2% sodium azide, refrigerated at 4–6°C and shipped to the laboratory within 24 h of sampling with a cold pack in the envelope to maintain sample cooling. Peripheral blood (PB) and bone marrow (BM) were submitted in EDTA tubes. At admission, samples were examined visually and cellularity was evaluated using an automated analyser (XT-2000iV, Sysmex). Cellularity was considered to be suitable for analysis if the cell concentration in 1 mL was > 5 x 10^9 cells/L (corresponding to > 5 x 10^6 cells in total), whereas samples with < 1 10^9 cells/L (corresponding to < 1 x 10^6 cells in total) were excluded from processing, although slight variability may have occurred due to the preferences of the operator dealing with the sample. The erythrocyte lysis step was not considered to be necessary for LN FNA samples unless gross hemodilution was detected at visual inspection. For PB and BM samples, red blood cells were lysed by adding a lysis solution containing 8% ammonium chloride.

Flow cytometry

The panel of antibodies applied to samples was adapted over time as more conjugated antibodies against canine leucocytes became available. Samples processed before 2011 were analysed using mainly a two-colour approach. Starting from 2011, a multicolour approach was applied, with the addition of CD45 as tracking label in all tubes.

The diagnostic algorithm varied throughout the years, but a basic panel included antibodies against CD5, CD21, CD34, propidium iodide and CD45. On the basis of the staining results obtained with this panel, expression of other antigens was evaluated, including CD3, CD4, CD8, CD11b, CD14, CD18, CD20, CD25, CD44, CD79a, CD117 and major histocompatibility class (MHC) II.

All antibodies were previously titered to define correct working dilutions. For surface marker labelling, 50 µl cell suspensions, adjusted at 5 x 10^5 cells/tubes were incubated

for 20 min at 4-8°C with 50 µl primary antibody and 25 µl of RPMI 1640 medium plus 5% fetal bovine serum (FBS) plus 0.2% sodium azide. After incubation cells were washed and then resuspended in 500 µl PBS. If directly labelled primary antibodies were not available, 50 µl 1:400 FITC-conjugated rabbit anti-mouse IgG (Serotec, oxford, UK) or 3 µl of pure FITC-conjugated Goat anti-mouse Ig (BD Bioscience, USA) was added. After another 20 min incubation at 4-8°C, the cells were washed and resuspended in 500 µl of PBS.

For CD79a intracellular staining, cells were previously labeled with an anti-CD45-FITC, and then washed and permeabilized using BD FACS permeabilizing solution 2 (Becton Dickinson, San Jose`, CA). After washing, cells were incubated with anti-CD79a PE conjugated antibody, then washed and resuspended in 500 µl PBS. As a control, a separate aliquot was submitted for permeabilization and an appropriate isotype-matched negative control was added in order to distinguish between specific and nonspecific labeling.

The antibody panel used to stage lymphoma on PB and BM samples varied based on the phenotype of neoplastic cells identified in the LN sample. If the LN sample was not adequate for FC, the basic antibody panel was applied to PB and BM, with the addition of CD4 and CD8. Specificity, sources and clones of antibodies are listed b in Fig. 8:

Target molecule	Antibody clone	Source	Specificity
CD45	YKIX716.13	Serotec (Oxford, UK)	All leukocytes
CD3	CA17.2A12	Serotec	T cells
CD5	YKIX322.3	Serotec	T cells
CD4	YKIX302.9	Serotec	T-helper cells and neutrophils
CD8	YCATE55.9	Serotec	T-cytotoxic cells
CD21	CA2.1D6	Serotec	Mature B cells
CD79a	HM57	Serotec	B cells
CD11b	M1/70	eBioscience (San Diego, CA, USA)	Myeloid cells
CD14	TUK4	Serotec	Monocytes
MPO	2C7	Serotec	Myeloid cells
CD34	1H6	BD Pharmingen (San Diego, CA, USA)	Precursors

Figure 8: Antibodies used for the flow cytometric immunophenotyping of samples enrolled in the present study (Novacco et al., 2015)

Samples were acquired using a flow cytometer (FACScalibur, Becton Dickinson) and analysed using Cell Quest software (Becton Dickinson). A minimum of 5,000 events for LN and 10,000 events for PB and BM were gated to exclude dead cells. Neoplastic cells

were identified on the morphologic scatter cytogram or on CD45 versus FSC cytogram. For high-grade lymphomas or acute leukemias neoplastic cells were easily identified because of high FSC or CD45 expression. For low-grade lymphomas or chronic leukemias cellular scatter characteristics were not always enough to differentiate neoplastic from residual cells, thus neoplastic populations were identified according to a combination of scatter and immunophenotypic features (such as antigen under-expression or unusual phenotype). The percentage of neoplastic cells and mean FSC (evaluating mean cellular size) were evaluated on non-permeabilized samples. Residual non-neoplastic cells of the same lineage and residual non-neoplastic cells of another lineage (for instance B lymphocytes for T-cell markers) were used as positive and negative internal controls, respectively.

Criteria for diagnosis

The final diagnosis was based on a comprehensive evaluation of all the data provided by the referring veterinarian, including different combinations of history, presenting complaint, clinical signs, cytological smear, haematology data, LN/PB/BM FC analysis. Cases included in the present study were classified based only on the FC report, without any re-evaluation of the raw FC data.

Samples were considered to be 'non-diagnostic' if one or more of the following criteria were present: (1) insufficient cellularity (< 1 $x10^6$ cells in total); (2) sample composed primarily of dead cells; and (3) if flow cytometric results strongly differed from cytological evaluation, for example, when neoplastic cells were distrupted during processing and only a small residual non-neoplastic population was labelled. Dead cells were identified by gross evaluation of the sample (based on colour, odour or the presence of tissue debris) and/or with a viability stain (propidium iodide), which was included in 2013 in the antibody panel. Sample was considered adequate if >50% of cells was propidium negative.

Samples were considered to be 'negative for lymphoid tumour' if: (1) a mixed population of predominantly small cells was present with a cytology supporting a reactive/hyperplastic lymph node; or (2) other causes of lymphadenomegaly were identified by immunophenotyping and cytology, for example, LN metastasis from solid tumours, histiocytic tumours and plasma cell tumours. Samples were considered as 'likely lymphoid tumour' if: (1) flow cytometry from a lymph node showed a prevalent

population (> 65%) of lymphoid cells with a single phenotype, but only a reduced panel of antibodies was allowed and/or the lack of a good quality cytological smear precluded a definitive diagnosis; (2) flow cytometry from a lymph node was poorly cellular (< 1 x 10^6 cells in total) or provided equivocal results, but LN cytology was highly suggestive of lymphoma and immunophenotyping of PB and/or BM was suggestive of a lymphoid tumour. Cases were classified as 'lymphoid tumour' if a definitive diagnosis of lymphoma or leukaemia was made based on the results of immunophenotyping of LN, PB, or BM. In many cases, extended subtyping of lymphoid neoplasia was possible based on immunophenotyping of different tissues and cytological evaluation: (1) B cell lymphoma, irrespectively of the grade; (2) high grade T cell lymphoma, based on immunoreactivity to T cell markers and cytological aspects including high numbers of mitotic figures (≥5 mitotic figure/5 high power field (HPF) (×40) (Fournel-Fleury et al., 1997); (3) low grade T cell lymphoma, based on typical T zone pattern staining on FC and/or distinctive cytological features (4) acute leukaemia, starting with PB or BM immunophenotyping of precursor cells confirmed by LN infiltration and (5) chronic lymphocytic leukaemia, starting with PB or BM immunophenotyping confirmed by LN infiltration.

Data collection and statistical analysis

Statistical analysis was applied to identify pre-analytical variables possibly affecting the likelihood to reach a diagnosis; for this purpose, samples were grouped into 'diagnostic' (if any diagnosis had been made, regardless of the level of confidence) and 'non-diagnostic' (including samples not adequate for processing and samples processed but not diagnostic).

Pre-analytical variables investigated were related to: (1) animal variables, including breed (pure or mixed), age (years), sex (female, spayed female, male, neutered male) and size (toy/small, medium, large/giant); (2) sampling and shipping variables, including year (2009–2015), season (cool to cold from October to March, warm to hot from April to September), referring veterinarian (veterinarians who sent > 10 cases over the study period were considered individually, while veterinarians sending less than 10 cases in total were grouped in a single category), origin and shipping ('within institution', 'out of institution hand-delivered', 'out of institution delivered by express

courier'); and (3) sample variables, including type of sample (LN alone, LN plus PB, LN plus PB and BM, LN plus BM), LN cytological smear (present or not), cellular concentration of the LN sample and presence of gross artefacts (haemodilution, dead cells, none). Univariate and multivariate binomial logistic regressions were performed using SPSS v20.0 for Windows (IBM). Multivariate analysis was performed with a backward step selection, including only variables with $P < 0.3$ at univariate analysis. Significance was set at $P<0.05$.

Results

Out of 1273 samples, 264 cases were excluded due to lack of information regarding the sample (n = 88; 6.9%) or due to sampling of tissues other than LN (n = 176; 13.8%). Among the latter, 59 (33.5%) cases were represented by PB and BM, 49 (27.8%) cases by PB alone, 28 (15.9%) cases by extranodal masses (other than cutaneous), 18 (10.2%) cases by effusions, nine (5.1%) cases by spleen, seven (4.0%) cases by cutaneous masses, two (1.1%) cases by liver, and four (2.3%) cases by other tissues. The distribution of tissue type including samples excluded is shown in fig. 9.

Twenty-two cases (1.73%) were excluded because the final report was not available. Finally, 987 cases fulfilled the inclusion criteria and were included in the statistical analysis. Seventy-two (7.3%) samples were non-diagnostic. Among the 915 diagnostic FNAs, 839 (91.7%) were 'conclusive for lymphoid tumour', 61 (6.7%) were 'negative for lymphoid tumour', and 15 (1.6%) were 'likely lymphoid tumour'. A specific diagnosis was available for 812 cases; 596 (73.4%) cases were B cell lymphomas, 143 (17.6%) cases were high grade T cell lymphomas, 60 (7.4%) cases were low grade T cell lymphomas, eight (1.0%) cases were acute leukaemias, and five (0.6%) cases were chronic lymphocytic leukaemias. The results of univariate and multivariate analyses, and the odd ratios for diagnostic samples, are summarised in Table 1.

<u>Animal-related variables</u>

Breed was reported in 911 cases; there were 239 (26.2%) mixed breed dogs. Among the remaining 672 dogs, the most prevalent breeds included German shepherd (n = 59; 8.8%), Boxer (n = 56; 8.3%), Golden retriever (n = 46; 6.8%), Labrador retriever (n = 44; 6.5%), Rottweiler (n = 38; 5.7%) Doberman pinscher (n = 37; 5.5%), Beagle (n = 29;

4.3%), Bernese mountain dog (n = 23; 3.4%), English bulldog (n = 21; 3.1%) and Yorkshire terrier (n = 21; 3.1%).

Table 1: results of univariate and multivariate analysis for different pre-analytical variables examined for their effect on flow cytometric analysis of samples from dogs with clinically suspected lymphoma.

Preanalytical variable			Univariate analysis			Multivariate analysis
			Odds Ratio	95% confidence interval	p-value	p-value
Related to dogs	Breed		0.999	0.597-1.670	0.996	n.d.
	Age		1.035	0.959-1.117	0.378	n.d.
	Gender	Neutered male	ref	-	0.330	n.d.
		Male	2.672	0.803-8.888		
		Female	2.112	0.599-7.450		
		Spayed female	1.857	0.519-6.641		
	Size	Toy/small	ref	-	0.609	n.d.
		Medium	1.032	0.429-2.482		
		Large/giant	0.754	0.371-1.531		
Related to sampling and shipping	Year	2009	ref	-	0.031*	0.026*
		2010	0.681	0.174-2.664		
		2011	0.907	0.227-3.631		
		2012	1.550	0.336-7.146		
		2013	0.562	0.153-2.066		
		2014	0.775	0.212-2.839		
		2015	0.335	0.098-1.145		
	Season	cold	ref	-	0.154	130
		hot	0.703	0.433-1.142		
	Veterinarian				0.067	0.020*
	Provenience/shipping	Express courier	ref	-	0.247	0.590
		Hand-delivered	0.714	0.353-1.446		
		Within institution	0.453	0.160-1.286		
Related to the sample	Type of matrix	LN alone	ref	-	0.025*	0.049*
		LN and PB	2.877	1.346-6.150		
		LN, PB and BM	2.078	1.182-3.653		
		LN and BM	1.849	0.408-8.380		
	LN cytological smear	no	ref	-	0.009*	0.313
		yes	1.904	1.176-3.083		
	Necrosis	no	ref	-	0.000	0.000
		yes	0.133	0.054-0.326		
	Hemodilution	no	ref	-	0.210	1
		yes	0.234	0.024-2.274		
	LN sample cellularity		1.008	0.998-1.017	0.104	0.013*

Odds ratio for the probability to provide a diagnostic result are also given. LN, lymph node; PB, peripheral blood; BM, bone marrow; Ref, reference; ND, not detectable. * $P < 0.05$.

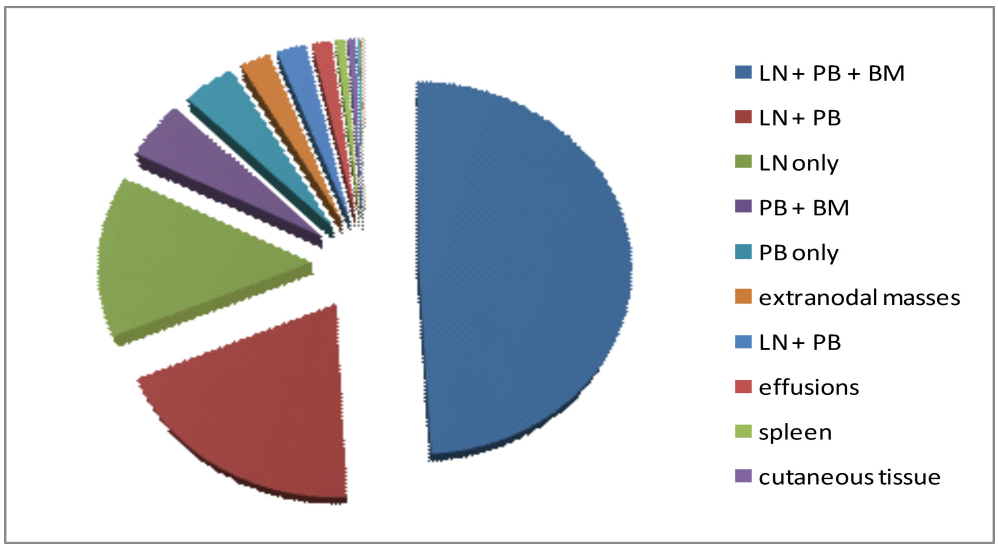

Figure 9: type of tissues received for immunophenotyping from 2009 to 2015.

Many other breeds were represented, with less than 20 cases each. In 76 cases, the breed was not recorded. Since the size of mixed breed dogs generally was not available, this variable was analysed only within the purebred dog group. Among the latter, 415 (61.8%) were of large/giant size, 115 (17.1%) were medium-sized and 142 (21.1%) were of toy/small size. Sex was reported in 901 cases; there were 213 (23.6%) intact females, 210 (23.3%) spayed females, 397 (44.1%) intact males and 81 (9.0%) castrated males. The male/female ratio was 1.15:1. Age was known for 899 dogs; the median age was 8 years (mean 8.4 ± 3.2 years, range 1-17 years). None of the patient-related variables significantly influenced the likelihood of having a diagnostic sample.

Variables related to sampling and shipping

The number of cases per year steadily increased during the study period, ranging from 63 cases fulfilling the inclusion criteria in 2009 to 200 cases in 2015 (Fig. 10). The highest percentage of diagnostic cases was reached in 2012 (124/128 cases, 96.9%) and the lowest in 2015 (174/ 200 cases, 87.0%). The likelihood of having a diagnostic sample varied significantly by years both with univariate (P = 0.031) and multivariate (P = 0.016) analyses. The percentage of samples collected in the cold and hot seasons was almost similar (51.2% and 48.8%, respectively) without any significant difference (P = 0.15). Thirteen veterinarians sent > 10 samples, ranging from 13 to 207 samples; veterinarians sending < 10 samples were grouped together (363 cases, 36.8%). Most of

the samples for which this information was available had been sent to the laboratory by express courier (559/816, 68.5%), 159 (19.5%) were sampled outside the institution and hand-delivered, and 98 (9.9%) were sampled within the institution. The likelihood of a having a diagnostic sample was not influenced by these variables on univariate analysis. However, the proportion of diagnostic samples varied significantly among veterinarians based on multivariate analysis (P = 0.004).

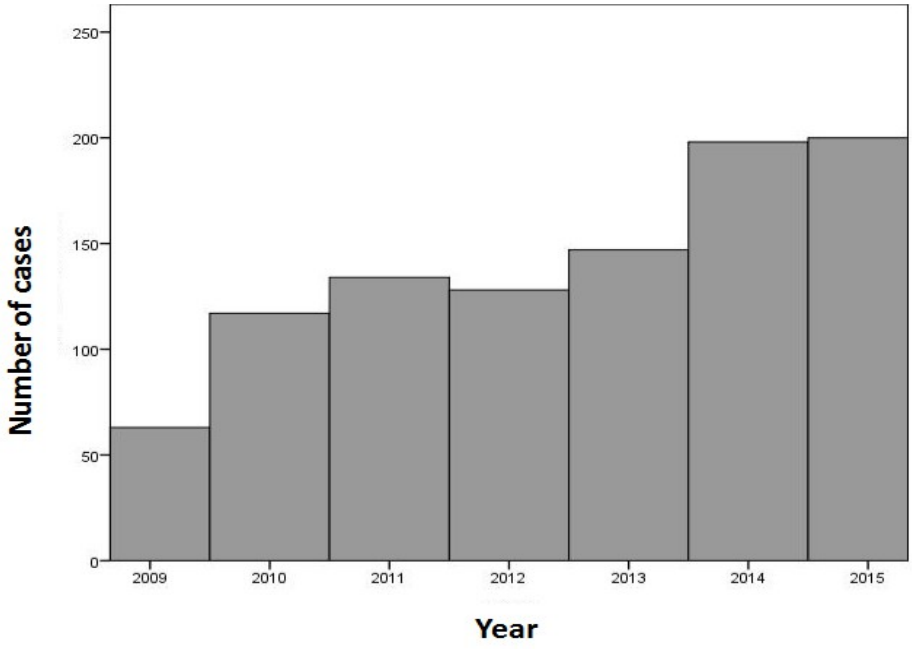

Figure 10: per-year distribution of the number of cases included in the present study.

Variables related to sample material

In the majority of cases (572; 58.0%), samples from all three sites (LN, PB and BM) of the same dog were available; LN and PB were sent in 225 (22.8%) cases, LN alone was submitted in 163 (16.5%) cases, and LN and BM were submitted in 27 (2.7%) cases. Sample material significantly influenced the likelihood of obtaining a diagnosis (P = 0.025). In particular, cases for which both LN and PB, or all three sites (LN, PB and BM) were obtained, had a higher likelihood of being diagnostic, compared to samples from LN alone (P = 0.011 and P = 0.006, respectively). The type of sample material also significantly influenced the likelihood of having diagnostic samples on multivariate analysis (P = 0.031). A LN cytological smear was provided in 634/987 (64.2%) cases, but the quality of these smears was not assessed in the present study. The presence of a

cytological smear significantly improved the likelihood of having a diagnostic sample (P = 0.009) only on univariate analysis. Lymph node sample cellularity was highly variable among cases, ranging from 0.02 to 436.19 x 10^9 cells/L (median 12.44 x 10^9 cells/L, mean 29.21-48.13 x 10^9 cells/L). Lymph node sample cellularity did not affect the likelihood of having diagnostic samples based on univariate analysis (P = 0.104), but was statistically significant on multivariate analysis (P = 0.013), with cellularity being higher in diagnostic than in non-diagnostic samples (median 13.1 x 10^9 cells/L and 2.09 x 10^9 cells/L, respectively). In the group of non-diagnostic cases, the cellularity was lower than the minimum adequate concentration (5.0 x 10^9/L) in 52.8% of cases, and lower than the lower cut-off value generally considered to be suitable for analysis (1.0 x 10^9/L) in 37.5% of cases. These percentages were much higher than those found in diagnostic samples (21.0% and 3.4%, respectively).

Artefacts were reported in nine non-diagnostic samples (12.5%), including one haemodiluted sample and eight necrotic samples, and in 18 diagnostic samples (2.0%), including three haemodiluted and 15 necrotic samples. Haemodilution did not affect the likelihood of having a diagnostic sample. On the contrary, necrotic samples were significantly less likely to be diagnostic on both univariate and multivariate analysis (P < 0.001 for both analyses).

Discussion

The results of the present study indicate that FC can be a suitable ancillary diagnostic aid in cases with a tentative diagnosis of canine lymphoma, since it provided diagnostic results in the majority of cases (about 90%). Among the pre-analytical variables investigated, no association was identified between signalment and the likelihood of obtaining a diagnostic result. In particular, dog size and breed did not influence the odds of obtaining a diagnosis. Fine needle aspiration of a lymph node is usually carried out without sedation, and, in theory, obtaining a good quality fine needle aspirate is more difficult in small-sized or aggressive dogs. However, the results of the present study indicate that FNA provides good quality s

The likelihood of obtaining a diagnosis varied among submitting veterinarians/institutions, possibly reflecting different technical skills of the operator

(veterinarian), supporting the necessity of an adequate technical training to increase the likelihood of obtaining a diagnostic result.

Regarding the method of delivery, no differences were found among samples submitted from the internal oncology service of the University of Milan, which were delivered within few minutes following sampling, those delivered in person within a few hours following sample collection and those shipped using an express courier. Moreover, no differences were found between samples shipped during the cold and hot seasons. The standard requirements of our laboratory include a maximum shipping time of 24 h and shipment on ice packs for FC samples. Therefore, our results are only valid under these conditions. In human medicine, refrigeration of FC samples generally is not recommended, since some antigens may be internalised and this may bias the final results (Ekong et al., 1993). Studies on this aspect in veterinary medicine are currently lacking, but it seems reasonable to refrigerate the samples during shipment to prevent cell disruption due to high temperatures, mainly in the hot season and in hot countries. It is currently unknown whether canine antigens vary their expression following refrigeration.

Several studies have been conducted on human samples using fixative solutions for stabilizing cells (Saxton and Pockley, 1998 ; Warrino et al., 2005; Davis et al., 2011); on the authors knowledge, one similar study has been performed on fixed samples of canine blood, showing that cells stored at room temperature were easily identifiable on both cell counter and cytometer up to 7 days, and marker expression was stable up to 3 days (Cian et al., 2014). However, the study was conducted on healthy dogs, thus currently there is no information in literature regarding the stability of neoplastic lymphoid cells.

Sample type and sample characteristics had the greatest influence on the diagnostic power of FC. The major factor leading to a non-diagnostic result was poor cellularity, with more than a half of non-diagnostic samples having a cell concentration lower than recommended, while haemodilution and dead cells have minimal effects on FC analysis. When a gross haemodiluition was evident, erithrocytes were removed within a rapid lysis step, while necrotic debris when present, may be easily identified by gross evaluation (based on colour, odour or the presence of tissue debris) or using a viability

stain, such as propidium iodide. Thus, artefacts, such as haemodilution and dead cells, made a minor contribution to non-diagnostic samples.

Having sample material from different sites (LN, PB, BM) and a concurrent LN cytological smear available improved the likelihood of establishing a diagnosis of lymphoid neoplasia. Therefore, complete cases, including FNA from LN, PB and BM, with cytology smears prepared before submission, are desirable for a more comprehensive evaluation by a clinical pathologist. Specifically, cytological evaluation of a smear may help to differentiate reactive/hyperplastic lymph nodes from lymphoid tumours in which the neoplastic population, which tends to be more fragile, has been disrupted. In the first case, samples would be classified as 'diagnostic, non-neoplastic'; in the second case, they would be classified as 'non-diagnostic'.

The presence of neoplastic cells detected via flow cytometry in PB and/or BM, together with a LN cytology compatible with lymphoma, may also permit a putative diagnosis of 'likely lymphoma', even if LN sample cellularity is too low for FC. This could be achieved when neoplastic cells can be differentiated from non-neoplastic cells, for example when the cells showed an aberrant phenotype (such as a T zone pattern) or when a high percentage of large CD21+ cells were detected.

Evaluation of PB and/or BM would also be important for differentiation of acute and chronic lymphoid leukaemias with secondary lymph node infiltration from nodal lymphoma with a haematogenous phase. The results of our study suggest that, when LN cellularity was too low to perform FC, flow cytometry of PB was most likely to be diagnostic. Our results indicate also that having a BM aspirate available for FC did not increase the likelihood of having a diagnostic sample if LN and PB were also submitted. This suggests that submitting peripheral blood together with a LN aspirate will facilitate interpretation of FC results and will assist with staging of lymphoma.

The limits of this study are inherent to its retrospective nature. Some possible sources of biases were not analysed, including the localization and size of the LN sampled, the technique used for sampling (free hand vs. ultra-sound guided, suction vs. capillary technique), the use of different transport media (RPMI vs. phosphate buffered saline vs. saline solution) and the quality of the cytological smears. Another limitation is the lack of a gold standard for the final diagnosis, which precluded us from defining the

diagnostic performances of both cytology and FC. This should be addressed in a future, prospective study.

In conclusion, flow cytometry performed on canine LN FNA specimens is a suitable diagnostic tool to confirm lymphoma in the majority of cases, regardless of animal characteristics, provided that the sample has sufficiently high cellularity, is submitted within 24 h following sampling and is shipped on ice packs. Packaging and shipping should be standardised to assure fast delivery within 24 h of sampling. Haemodilution has minimal effects on FC analysis, but veterinarians should make any possible effort to obtain a highly cellular sample. The addition of a PB sample and a LN cytological smear can be recommended to improve the likelihood of receiving a conclusive result.

The results of the present study were used for a degree thesis (FATTORI PREANALITICI CHE INFLUENZANO LA DIAGNOSI CITOFLUORIMETRICA DI LINFOMA NEL CANE) and then published on a peer review international journal (Comazzi S, Cozzi M, Bernardi S, Zanella DR, Aresu L, Stefanello D, Marconato L, Martini V. EFFECTS OF PRE-ANALYTICAL VARIABLES ON FLOW CYTOMETRIC DIAGNOSIS OF CANINE LYMPHOMA: A RETROSPECTIVE STUDY (2009-2015). Vet J 2018 Feb;232:65-69.)

3.2 CANINE SMALL CLEAR CELL/T-ZONE LYMPHOMA: CLINICAL AND PATHOLOGICAL CHARACHTERIZATION

Background

Small clear cell/T-zone lymphoma (TZL) represents a very peculiar canine lymphoma without a human counterpart and is identified as a single entity characterized by a small clear cell appearance by cytology (Fournel-Fleury et al., 2002), a nodular pattern of expansion from cells derived from the paracortex by histology (Valli et al., 2006) and a peculiar immunophenotype staining negative for CD45 by flow cytometry (FC), frequently associated with an aberrant CD21 expression (Martini et al., 2013; Seelig et al., 2014). In spite of the relative frequency of this tumor subtype, representing about 3-17 % of all canine lymphomas (Gelain et al., 2008; Ponce et al., 2010; Valli et al., 2011; Aresu et al., 2015) to date, the clinical presentation and characteristics of dogs with this lymphoma subtype have only been summarily described, reporting indolent nature with a long survival (Flood-Knapik et al., 2013; Ponce et al., 2004; Seelig et al., 2014).

CD45-negative hematopoietic neoplasms other than canine TZL have been reported both in humans (Ozdemirli et al., 1996; Ratei et al., 1998; Kumar et al., 2005; Wang et al., 2015) and dogs (Gelain et al., 2008; Williams et al., 2008), but the mechanisms underlying this phenotypic aberrancy have not been investigated. CD45 is a transmembrane protein tyrosine phosphatase expressed by the leukocytes surface in many different species, including dog. Two major CD45 isoforms (CD45RA and CD45R0) have been described in dog and expression patterns are different depending on leukocytes subclasses and activation status (Goto-Koshino et al., 2014).

The aim of the present study was to characterize TZL from a clinical and pathological point of view and to try to investigate some possible pathogenetic aspects. To the aim, the present work has been organized in two different tasks. The first purpose was to describe the clinical presentation and outcome in a retrospective series of canine CD45-negative small clear cell/TZL; the second purpose was to confirm the absence of CD45 at the protein level in a group of dogs with TZL, via the concomitant use of FC and immunohistochemistry, and to investigate the possible origin of CD45 protein loss, whether associated to genetic or transcriptional or post-translational modifications. Based on the absence of CD45 protein in canine TZL, subsequently the amount of CD45

transcript and the presence of CD45 gene will be investigated in the same cohort of dogs. For the purpose of the study a control group composed by normal sorted T cells, high grade T-cell lymphomas and normal lymph nodes has been selected.

Canine TZL: clinical presentation and outcome in a retrospective case series

Material and Methods

Case selection

The FC databases of the flow cytometric diagnostic services of Department of Veterinary Medicine (University of Milan, Milan, Italy) and Department of Veterinary Sciences (University of Turin, Grugliasco, Turin, Italy) were interrogated from January 2009 to December 2014, and the CD45-negative, small (FSC-height <400) T-cell lymphoma cases were selected. Additional inclusion criteria were a cytological diagnosis of small clear cell lymphoma (Fig. 11) and when available a confirmed histopathological diagnosis of TZL (Fournel-Fleury 2002; Valli et al., 2006) .

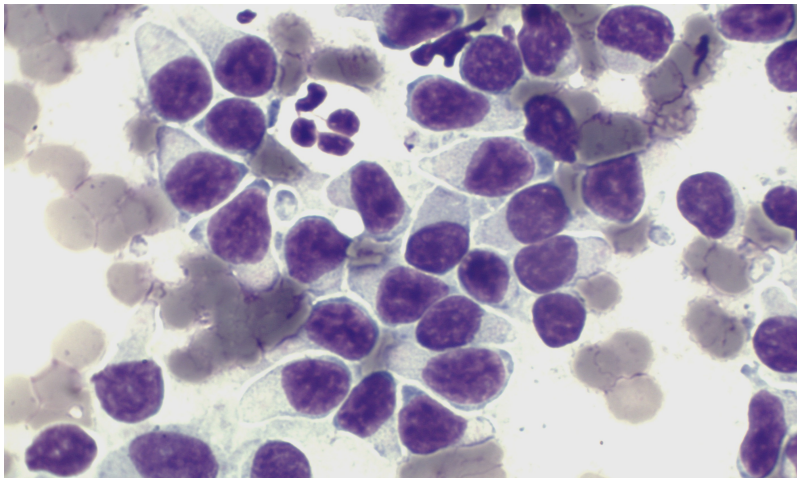

Figure 11: cytological smear of a TZL, carachterized by small lymphoid cells with clear cytoplasm and frequent 'hand-mirror' shape (small-clear cell appearance).

Flow cytometric immunophenotyping on LN was performed as previously described (first study of the thesis). If available, PB and BM were also examined via FC to quantify infiltration. Samples were acquired either with a FACScalibur or with a BD Accuri C6 (Becton Dickinson, San Josè, CA, USA) and analysed using the specific software CellQuest Pro or CFlow Plus (Becton Dickinson). PB and BM samples were considered infiltrated if a distinct CD45-negative population with similar phenotype shown in the

LN was detected. Infiltration was then quantified as the percentage of neoplastic cells out of the total events acquired, after exclusion of debris and platelets based on morphological scattergrams.

The neoplastic phenotype, PB and BM infiltration degree were recorded for each case. Clinical data were obtained from the medical records and by phone calls to the referring veterinarians. Background information collected for each dog included signalment, presence or absence of clinical symptoms, complete blood count (CBC) at diagnosis, treatment (if any), date and cause of death. Haematological abnormalities were defined as values out of the laboratory reference interval (RI).

Clinical stage was based on the World Health Organization (WHO) criteria for canine lymphoma; however, splenic, liver, PB and BM aspirates were not routinely performed and therefore it was not possible to definitively differentiate stage III from stage IV disease in some cases.

Median survival time was calculated via SPSS v17.0 for Windows. Survival time was defined as time between diagnosis and death. Dogs that died or were euthanized because of lymphoma were recorded as events; dogs lost to follow up, dead for unrelated causes or still alive at data analysis closure were censored. Because of the wide range of treatment protocols, prognostic factors were not investigated.

Results

Clinical presentation

51 dogs met the inclusion criteria. Cytology confirmed a small clear cell appearance in all cases, whereas histopathology confirmed TZL in the 8 cases also undergoing lymphadenectomy. Breed

was reported for 43 dogs, including 33 (76.7%) pure breed dogs and 10 (23.3%) mixed-breed dogs. The most represented breeds were Boxer (n=5), English bulldog (n=3), Labrador retriever (n=3) and Shih tzu (n=3). Sex was reported for 47 dogs. Among these, 23 (48.9%) were male (2 neutered) and 24 (51.1%) were female (12 spayed).

Age was reported for 44 dogs. Overall mean age was 9.9 ± 2.3 years (median 10 years; range, 5–14 years). In particular, 18 (40.9%) dogs were <10 years old and 26 (59.1%) were ≥10 years old. CBC data were available for 44 dogs. Four (9.1%) dogs had a mild anaemia, 3 (7.3%) had thrombocytopenia, 2 (4.5%) had leukopenia and 17 (38.6%) had

leukocytosis, 4 (9.1%) had neutropenia and 8 (18.2%) had mature neutrophilia, 2 (4.5%) had lymphopenia and 28 (63.6%) had lymphocytosis.

Overall mean white blood cell (WBC) count was 17.59 ± 10.52 × 10^3/µL (median 14.39 × 10^3/µL; range, 0.8–45.72 × 10^3/µL); overall mean neutrophil count was 8.24 ± 5.44 × 103/µL (median 6.73 × 10^3/µL; range, 0.59–27.2 × 10^3/µL); overall mean lymphocyte count was 8.31 ± 6.61 × 10^3/µL (median 6.95 × 10^3/µL; range, 0.12–30.76 × 10^3/µL). In 20 (39.2%) cases, neoplastic cells were CD8+, in 17 (33.3%) cases cells were CD4–CD8 double negative, in 8 (15.7%) cases cells were CD4+, in 4 (7.8%) cases cells were CD4+CD8+ double positive

and in 2 (3.9%) cases two distinct CD45-negative populations were identifiable, staining positive for CD4 and CD8, respectively. Forty-two (82.4%) cases stained positive for CD21.

Stage was reported for 43 dogs; however, staging procedures largely varied among veterinarians and were not standardized. One (2.3%) dog had stage I disease, 1 (2.3%) dog had stage III disease, 1 (2.3%) dog had stage IV disease and 40 (93%) dogs were classified as stage V because of PB and/or BM flow cytometric infiltration; in addition, skin and lungs were presumed to be involved in one case each.

The dog with skin involvement presented multifocal itchy alopecia of abdomen, neck and pinnae; histopathological examination of cutaneous biopsies from the neck and the abdomen revealed a diffuse subepidermal infiltration by small lymphocytes. Lung involvement was diagnosed based on thoracic radiographs, that revealed a generalized multifocal interstitial structured pulmonary pattern, and cytological examination of a percutaneous fine needle aspiration, that was suggestive of round cell tumour, whereas bronchoalveolar lavage was negative for lymphomatous cells.

Substage was reported for 22 dogs. Among these, 18 (81.8%) were asymptomatic (substage a), whereas 4 (18.2%) had clinical signs (substage b), mainly dyspnoea because of enlarged submandibular LNs. PB and BM samples were analysed via FC in 40 and 12 dogs, respectively, and all of them proved to be infiltrated. Overall mean PB infiltration was 34.6± 17.53% (median 35.07%; range,0.93–64.6%). Overall mean BM infiltration was 6.85± 7.88% (median 2.15%; range, 0.5–24.1%).

In the 12 cases that had BM analysed, mean PB infiltration was 24.64± 14.41% (median 27.1%; range, 1.8–41.65%); in particular, PB infiltration was higher than BM in all cases

but one. Flow cytometric scattergrams of LN, PB and BM from one representative case are shown in Fig. 12.

Clinical presentation is reported in Table 2.

Outcome

Follow-up data were available for 26 dogs. Four dogs received no therapy after diagnosis, 3 received corticosteroids alone and the remaining 19 dogs were treated with different protocols, including dose-intense (n=12) and metronomic (n=7) chemotherapy.

Overall median survival was 760 days (range, 15–1150 days). Eight of the 26 dogs died of lymphoma during the study period, with a median survival of 180 days (range, 15–760 days); among them, 3 dogs (37.5%) died within 3 months from the diagnosis, and 5 (62.5%) survived longer than 6 months. Among the remaining 18 dogs, 2 were lost to follow up after 120 and 386 days, respectively, 5 dogs died because of unrelated causes and 11 were still alive at data analysis closure, with a median follow-up time of 335 days (range, 50–1150).

Interestingly, three dogs (12%) developed a second malignancy during the study period (glioma, melanoma and oral carcinoma): two of them died because of the second malignancy, whereas the dog with oral carcinoma was still alive at the end of the study after 1150 days from the diagnosis.

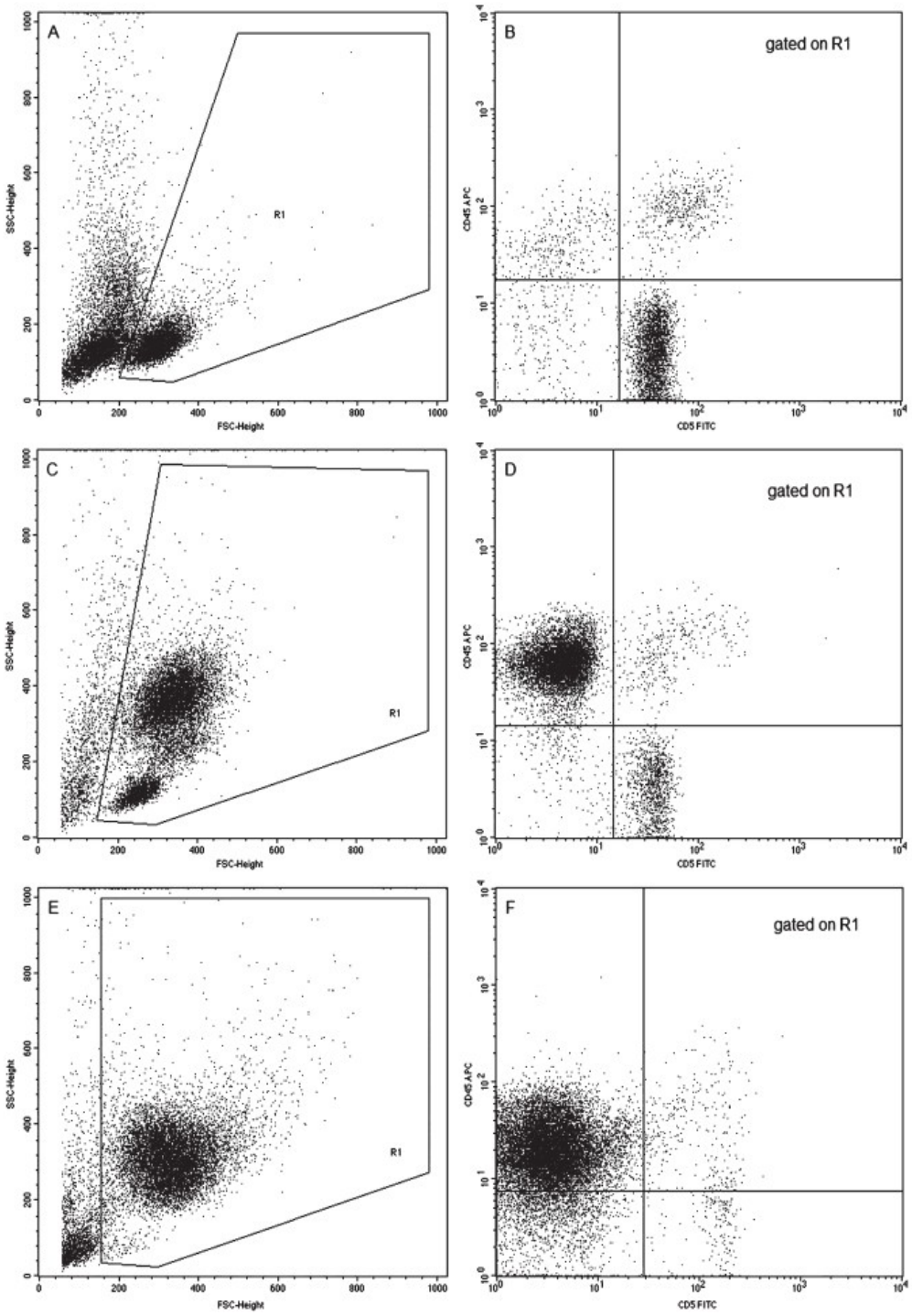

Figure 12: **f**low cytometric scattergrams representing lymph node aspirate (A and B), peripheral blood (C and D) and bone marrow (E and F) from a dog with small clear cell/T-zone lymphoma. Events were displayed at first based on morphological properties (A, C and E) and a gate (R1) was set to exclude platelets and debris. R1 cells were then displayed based on CD5-fitc and CD45-apc fluorescence. A distinct CD45-negative and CD5-positive population was identified in all samples (B, D, F, lower right quadrant), accounting for 98.6, 12.2 and 0.95% of all cells in the lymph node, peripheral blood and bone marrow, respectively.

Table 2. Clinical presentation of 51 dogs with small clear cell/T-zone lymphoma

	Breed	Sex	Age (years)	Stage	Substage	Anaemia	Thrombocytopenia	WBC count	Neutrophil count	Lymphocyte count	CD21	CD4/CD8	PB infiltration (%)	BM infiltration (%), (% sample purity)	Outcome (days)
1	Boxer	SF	9.5	V	a	No	Yes	WRI	Low	High	+	−/+	61.95		Dead (270)
2	Boxer	M	10	V	a	No	No	WRI	WRI	High	+	−/+	47.91		Alive (180)
3	Boxer	M	6	V	b	Yes	No	High	WRI	High	+	−/+	64.6		Alive (834)
4	Boxer	F	8	V		Yes	Yes	WRI	WRI	High	+	−/−	58.31		Dead (15)
5	Boxer	M	8	V		No	No	WRI	WRI	High	+	−/+	48		
6	English bulldog	F	10	V	a	No	No	High	WRI	High	+	+/−	34		Dead for unrelated causes (335)
7	English bulldog	M	10	V	b	No	No	High	High	WRI	+	−/−	12.2	0.95 (95.6)	Dead (17)
8	English bulldog	SF	12	V		Yes	No	High	WRI	High	+	+/−	57.4		
9	Labrador retriever	M	9	V	a	No	No	WRI	WRI	High	−	−/+	36	9 (83.4)	Dead (270)
10	Labrador retriever	SF	12	V	b	No	No	WRI	WRI	High	−	−/−	31		Lost to follow up (386)
11	Labrador retriever	F	6	V		No	No	WRI	WRI	WRI	−	−/+	2.5		
12	Shih tzu	SF	12	V	a	No	No	WRI	Low	High	+	−/−	41.65	2.3 (97.9)	Dead for unrelated causes (426)
13	Shih tzu	M	11	V	a	No	No	High	WRI	High	+	−/+	62		
14	Shih tzu	M	14		a	No	No	WRI	WRI	High	+	+/−			Lost to follow up (120)
15	Cane corso	F	5	V		No	No	High	High	High	+	−/−	26.4		
16	Cane corso	M	9			No	No	WRI	WRI	WRI	+	+(30%)/+(60%)			
17	Jack russell	F	9	V	a	No	No	WRI	WRI	WRI	−	−/+	17	1.21 (94.2)	Alive (1150)
18	Jack russell	M	7	V		No	No	WRI	WRI	WRI	+	−/−	21	2 (95.1)	
19	Cocker spaniel	SF		V	a	No	No	High	WRI	High	+	−/+	48.5		Alive (318)
20	Greyhound	SF	9	V	a	No	No	WRI	WRI	WRI	+	−/−	35.36		Alive (65)
21	Maltese	F	7	V	a	No	No	High	High	High	−	−/+	34.35	0.86 (96.2)	Alive (359)
22	Poodle	M	7	V	a	No	No	WRI	WRI	WRI	+	−/+	35.1		Alive (50)
23	Italian greyhound	SF	10	V	a	No	No	High	WRI	High	−	+/−	46.6		Dead for unrelated causes (210)
24	Miniature schnauzer	M	14	V	a	No	No	Low	Low	Low	+	−/+	1.8	0.5 (98.5)	Dead for unrelated causes (365)

Table 2. continued

	Breed	Sex	Age (years)	Stage	Substage	Anaemia	Thrombocytopenia	WBC count	Neutrophil count	Lymphocyte count	CD21	CD4/CD8	PB infiltration (%)	BM infiltration (%), (% sample purity)	Outcome (days)
25	Dachshund	M	12	I							+	−/+			
26	Bullmastiff	SF	9	V		No	No	High	WRI	High	+	−/−	45.35		Dead (760)
27	Chihuahua	M	12	V		No	No	High	High	High	−	+/+	60		
28	Australian shepherd	M	12	V		No	No	WRI	WRI	High	+	−/+	39.2	24.1 (88.3)	Dead (365)
29	German shepherd	NM	11	V		No	No	WRI	WRI	Low	−	+/+	0.93		
30	Pittbull	M		V		No	No	WRI	WRI	WRI	+	−/+	33		
31	Samoyed	M	11	V		No	No	WRI	WRI	High	+	−/−	22		
32	Springer spaniel	F	13		a						+	−/−			
33	Yorkshire terrier	M	10	III	b	No	No	High	WRI	High	+	−/−			Dead (84)
34	Mixed	F	13	IV	a	No	No	WRI	WRI	High	−	+/−			Alive (330)
35	Mixed	SF		V	a	No	No	WRI	WRI	WRI	+	+(30%)/+(60%)	4.5	0.7 (82.5)	Alive (343)
36	Mixed	M	9	V	a	No	No	High	High	High	+	+/−	35.9		Dead for unrelated causes (330)
37	Mixed	M	10	V		No	No	WRI	WRI	WRI	+	−/−	24.26		Dead (180)
38	Mixed	M	11	V		No	No	High	WRI	High	+	−/−	35.03		
39	Mixed	NM	9	V		No	Yes	WRI	WRI	High	+	−/−	33.2	11.3 (87.1)	
40	Mixed	F	13	V		No	No	WRI	WRI	WRI	+	+/+	25		
41	Mixed	SF	11	V		No	No	High	High	High	+	−/+	29		
42	Mixed	F	5								+	−/+			
43	Mixed	SF	9								+	−/−			Alive (365)
44	Unknown	F	10	V		Yes	No	Low	Low	WRI	+	−/+	14.8	17.3 (99)	
45	Unknown	F	12	V		No	No	High	High	High	+	−/−	40	12 (92)	
46	Unknown	M	12	V		No	No	WRI	WRI	High	+	+/−	60.4		Alive (360)
47	Unknown			V		No	No	WRI	WRI	WRI	+	−/−	18.7		
48	Unknown			V		No	No	High	High	WRI	+	+/−	39.17		
49	Unknown	SF	9								+	−/+			
50	Unknown										+	−/+			
51	Unknown										+	+/+			

F, female; M, male; NM, neutered male; SF, spayed female; WRI, within reference interval.

Loss of CD45 cell surface expression in canine T-zone lymphoma results from reduced gene expression

Materials and methods

Case selection

Samples from the present study were collected from the flow cytometric diagnostic services of Department of Veterinary Medicine (University of Milan, Milan, Italy) and Department of Veterinary Sciences (University of Turin, Grugliasco, Turin, Italy). Consecutive cases with a final diagnosis of TZL based on cytological appearance and FC immunophenotype (Martini et al., 2013) were included. In addition, cases of non-neoplastic lymph node and of high grade T-cell lymphoma showing CD45 expression were included as controls. When available, histopathological sections of the same cases were considered to confirm the diagnosis and perform immunohistochemical analyses.

Cell sorting

Neoplastic cells (CD45-negative and CD5-positive) from a subset of TZLs and normal T-cells (CD45-positive and CD5-positive) from non-neoplastic lymph nodes were sorted using a fluorescence-activated cell sorter (BD FACSVantage, BD Biosciences, San Josè, CA, USA). A minimum sorting yield of 1 $\times 10^5$ with more than 95% purity was considered suitable for quantitative real-time PCR analysis.

Histopathology and immunohistochemistry

For histological examination, three µm sections were stained with haematoxylin and eosin (HE).

For lymphoma phenotyping, a panel of primary antibodies was applied including: monoclonal mouse anti-human CD3 (Clone F7.2.38, Dako, Atlanta, Georgia, USA, T cells; diluted 1:100), monoclonal mouse antihuman CD5 (Clone CD5/54/F6, Dako, T cells; diluted 1:100), monoclonal mouse anti-human CD79acy (Clone HM57, Dako, all stages of B cells; diluted 1:100) and CD20 epitope-specific rabbit antibody (RB-9013-P, Thermo Fisher Scientific Inc., Cheshire, UK; mature B cells; diluted 1:800). Immunohistochemical analysis for CD45 was performed using an anti-canine CD45, clone CA12.10C12 (Leukocyte Antigen Biology Laboratory, UCDavis, Davis, CA, USA) (dilution 1:100).

Antibody was detected using an avidine-biotine-peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, California, USA). For negative controls, the primary antibody was excluded during the process. Membranous immunolabelling of the antibodies was evaluated in neoplastic cells in lymphomas and in the control lymph nodes. Evaluation consisted of qualitative assessment of cell types and location.

Quantitative real-time RT-PCR for CD45 transcript

The transcript analysis was performed on lymph node aspirates and on normal sorted T lymphocytes, diluted in RNAlater® (Applied Biosystems, Life Technologies, Carlsbad, CA) and stored at −20 °C until processing. The total RNA was isolated from cell pellets using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. To avoid genomic DNA contamination, on-column DNase digestion with the RNase-Free DNase (Qiagen) set was performed. First-strand cDNA was synthesised from 100 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The generated cDNA was used as template for quantitative real-time RT-PCR (qRT-PCR) in a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) using standard PCR conditions. The primers were designed using Primer Express 2.0 (Applied Biosystem, Life Technologies, Carlsbad, CA). Canine CD45 was amplified using the primer pair 5'- ATG GAG ATG CAG GGT CAA AT-3' (forward) and 5'-GCA ATG TAT TTC CTG GGT TCT T-3' (reverse). Primer pairs were designed on exon 19 and 20, shared by the two CD45 variants. In addition, these exons spare an intronic region of>3800 bp thus excluding the amplification of contaminant genomic DNA. Calibration curves using a 7-fold serial dilution (1:2) of a cDNA pool revealed PCR efficiencies of 99.5%. Canine transmembrane BAX inhibitor motif containing 4 (CGI-119) was chosen as reference gene for the absence of pathological state dependent differences in mRNA expression, as reported by Aricò et al. (2013). ΔΔCt method (Livak and Schmittgen, 2001) was used for the relative quantification of mRNA, ultimately expressed as Relative Expression (RE).

Quantitative real-time PCR for CD45 DNA

Lymph node aspirates and CD45-negative/CD5-positive cells sorted from TZLs were stored at −20°C until processing for DNA load analysis. Genomic DNA was extracted from cell pellets using DNeasy® Blood & Tissue kit (Qiagen) following the manufacturer's instructions. Specific primers for CD45 were designed using Primer3web (primer3.ut.ee) as follows: primer pair CD45 5'-AGCAAAGACACACGAAAGCC-3' (forward) and 5'-GCAATGTATTTCCTGGGTTCT −3' (reverse) for the amplification of a fragment of 257 base pairs. In order to detect reference genes for normalization of samples, primers targeting canine CGI-119 and canine GAPDH was designed as described: primer pair CGI-119 5'-GGATTTTGTGCTTGTCAGGAA-3' (forward) and 5'-CACTGGGAGCTTAGCAATTACA-3' (reverse) for the amplification of a fragment of 279 base pairs; primer pairs GAPDH 5'-GGAGAAAGCTGCCAAATATG-3' (forward) and 5'-ACCAGGAAATGAGCTTGACA-3' (reverse) for the amplification of a fragment of 194 base pairs (Mortarino et al., 2009).
In order to evaluate the PCR efficiency using a relative standard curve, dilution series were prepared by performing four fold serial dilution (1:10) of a control sample.
Efficiency was 100.47% for CD45, 102.16% for CGI119 and 98.70 for GAPDH. ΔΔCt method (Livak and Schmittgen, 2001) was used to describe the CD45 DNA load to the reference genes, expressed as Relative Quantification (RQ).

Amplicon sequencing

The specificity of the amplification for CD45 transcript and DNA was checked sequencing the DNA amplicon by Sanger chemistry (Eurofins Genomics S.r.l., Vimodrone, MI, Italy). The obtained sequences were aligned with the expected target sequence using BLAST (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

All analyses were performed with standard statistical software (SPSS v20.0 for Windows) and significance was set at $p \leq 0.05$ for all tests. Data distribution was assessed via Shapiro-Wilk test for each subset. Kruskal-Wallis test was used to compare CD45 transcript amount among TZL, cells CD5-positive sorted from reactive lymph nodes and CD45-positive high grade T-cell lymphomas. A Mann-Whitney test was used to compare CD45 transcript amount between TZL samples with< 95% and

>95% neoplastic cells. The Kruskal-Wallis test was also performed to compare CD45 DNA load among TZL, reactive lymph nodes and cells CD45-negative and CD5-positive sorted from TZL.

A non-parametric Spearman correlation analysis was used to determine the potential relationship between CD45 transcript amount and percentage of neoplastic cells in the TZL samples.

Results

Fine needle aspirates from an enlarged lymph node were obtained from 57 dogs and included in the study. Diagnoses were as follow: 40 (70.2%) TZLs, 7 (12.3%) high-grade T-cell lymphomas and 10 (17.5%) hyperplastic lymphadenopathy. Normal and neoplastic cells were obtained with cell sorting from 3 non-neoplastic lymph nodes and 3 TZLs, respectively. In addition, paraffin embedded lymph nodes from 2 cases diagnosed as reactive hyperplasia, 2 as Peripheral T-cell lymphomas (PTCL) and 2 as TZL were available for immunohistochemical analysis.

According to FC analysis, the percentage of CD45-negative neoplastic cells in the TZLs was 80.54 ± 14.9% (median 82.6%; min-max 30.77 -98.04%). The composition of the residual CD45-positive population was heterogeneous. One sample had a predominant (94.5%) population of granulocytes and the lymphoid population was composed of T-cytotoxic (4.8%), T-helper (1.4%) and B-cells (0.34%): this sample was highly hemo-contaminated at cytological examination. For the remaining samples, the residual CD45-positive population was composed of lymphoid cells alone, with a mean percentage of 36.57 ± 17.31% (median 33.22%; min-max 1.40–67.41%) of B-cells, 34.72 ± 19.45% (median 33.63%; min-max 1.89–92.09%) of T-helper cells, and 18.75 ± 14.64% (median 12.91%; min-max 0.72–60.15%) of T-cytotoxic cells. As expected, the CD45-negative and CD5-positive cells were not detected either in the reactive lymph nodes or in the high-grade T-cell lymphomas.

Immunohistochemical results showed a diffuse CD45 membranous expression in PTCLs. In the reactive lymph nodes, CD45 positive cells were located both in the paracortex and in the germinal

centres; cells in the marginal and mantle zones were also CD45 positive, even with a lower intensity (Fig. 13). TZLs were diffusely negative (Fig. 14).

Data concerning CD45 transcript amount were obtained from 41 cases, including 31 (75.6%) TZLs, 7 (17.1%) high grade T-cell lymphomas, and 3 (7.3%) sorted normal T cells. The mean neoplastic cells percentage in TZLs undergoing transcript analysis was 80.23 ± 15.61% (median 82.6%; min max 30.77–98.04%); in particular, 26 (83.9%) cases had <95% neoplastic cells, whereas the remaining 5 (16.1%) cases had >95% neoplastic cells. Results of transcript analysis are shown in Fig. 15.

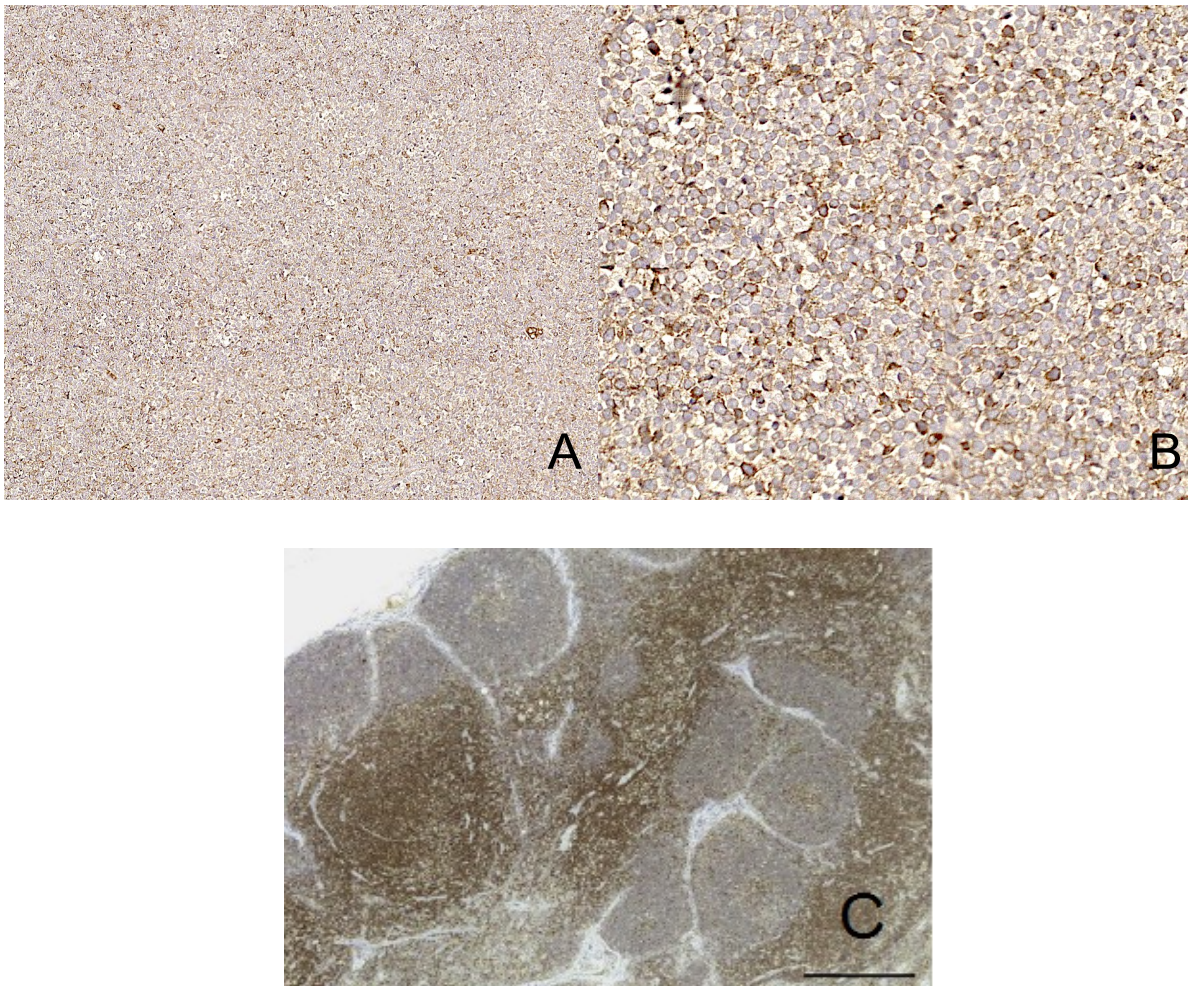

Figure 13: representative image of CD45 protein positive staining. A and B: Peripheral T-cell Lymphoma. C: reactive lymphoid hyperplasia. T-lymphocytes within the paracortex and B-lymphocytes in the follicles show a diffuse
CD45immunolabelling.

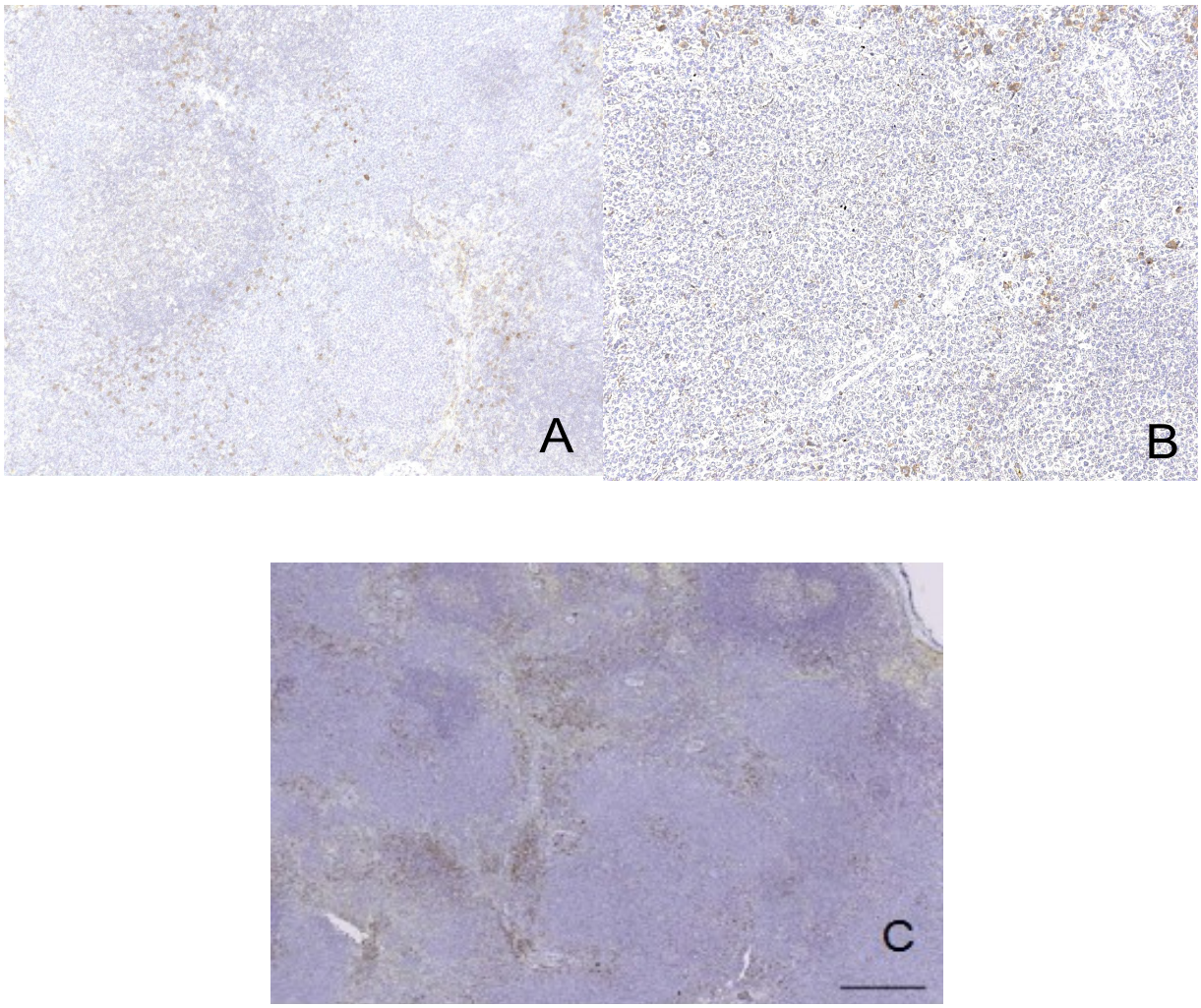

Fig.ure 14: representative image of CD45 protein staining in a T-zone lymphoma. Neoplasticlymphocytes are negative to CD45 immunolabelling.

A significant difference in the CD45 transcript amount was detected among TZLs, normal T cells and high grade T-cell lymphomas ($p < 0.001$). mRNA levels were significantly lower in TZLs than in normal T cells and high grade T-cell lymphomas ($p < 0.001$), respectively. No significant difference was found between normal T cells and high grade T-cell lymphomas ($P = 0.138$).

When exploring the correlation between the amount of CD45 transcript and the percentage of the neoplastic cells in TZL, a significant inverse correlation was detected between CD45 mRNA data and the percentage of neoplastic cells ($p = 0.010$). In addition, the amount of CD45 transcript was higher in the 26 TZL samples with < 95% neoplastic cells (mean = 0.37 ± 0.29) than in the 5 TZL samples with > 95% neoplastic cells (mean = 0.23 ± 0.16), although the difference was not statistically significant.

Data concerning CD45 DNA load were obtained for 33 samples, including 24 (72.7%) TZLs, 3 (9.1%) CD45-negative cells and CD5- positive cells sorted from TZLs and 6 (18.2%) non-neoplastic lymph nodes. The mean neoplastic cells percentage in TZLs undergoing DNA analysis was 81.95 ± 9.88% (median 83.26%; min-max 30.77–96.5%).

Amplicon DNA sequencing confirmed (99–100%) the specificity of the amplification for both CD45 targets.

Results are shown in Fig. 15. The differences among subsets were not significant irrespective of the reference gene considered (p =0.165 for CGI-119 and p = 0.895 for GAPDH).

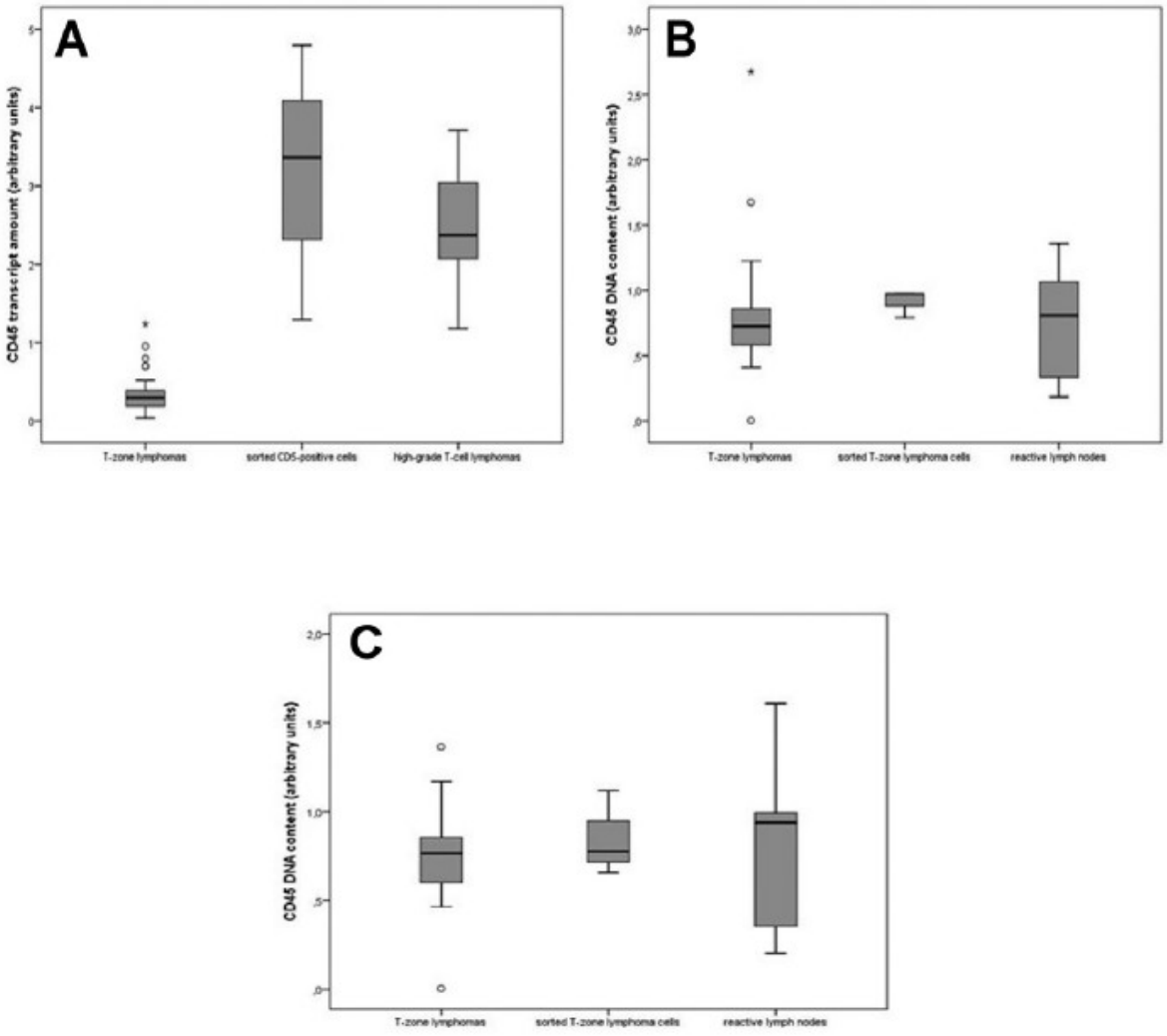

Figure 15. CD45 transcript and DNA amount in canine T-zone lymphomas and in controls.Panel A: CD45 transcript amount in canine T-zone lymphomas, sorted CD5-positive cells from reactive lymph nodes and high-grade T-cell lymphomas; CGI-119 was used as a housekeeping gene; CD45 transcript amount was significantly lower in T-zone
lymphomas than in the other two groups, respectively ($p < 0.001$). Panel B: CD45 DNA load in canine T-zone lymphomas, sorted neoplastic cells from T-zone lymphomas and reactive lymph nodes; CGI-119

was used as a reference gene; no significant difference in CD45 DNA load was found among groups. Panel C: CD45 DNA load in canine T-zone lymphomas, sorted neoplastic cells from T-zone lymphomas and reactive lymph nodes; GAPDH was used as a reference gene; no significant difference in CD45 DNA load was found among groups.

Discussion

Until now, only few studies have described the clinical presentation of dogs with TZL. In agreement with previous data, adult dogs are usually affected, and there seems to be no sex predilection (Flood-Knapik et al., 2013; Seelig et al., 2014).

In previous reports, Golden retrievers were the dominant breed, suggesting a possible genetic risk factor (Seelig et al., 2014). Interestingly, no Golden retrievers were present in our case series, possibly being attributable to a different genetic background among countries or, less likely, to a lower prevalence of this breed in Italy. Further studies should be performed to highlight possible similarities and differences in the genetics within the Golden retriever population but in a recent study that we conducted with the European Canine Lymphoma network emerged (Comazzi et al., submitted) that no breed prevalence for TZL were found in European Golden retriever population.

Although staging was not undertaken in every dog, the majority of dogs in the present case series had an advanced clinical stage at presentation, in agreement with a previous study (Aresu et al., 2013). Despite this and in contrast to high grade T-cell lymphomas, prognosis was not inexorably poor, thereby questioning the utility of the WHO system to stage indolent lymphomas. It is possible that other variables may show a more useful prognostic significance for this lymphoma subtype.

When available, PB and BM were always infiltrated. Neoplastic cell percentages were mostly higher in PB samples compared with BM samples. This may be because of an overspill phenomenon, when nodal neoplastic cells are likely released into the blood circulation, rather than to BM invasion and homing.

A role might be played by the lack of CD45, since the inhibition of this phosphatase impaired motility and homing of both normal and leukemic humans cells in a recent study (Shivtiel et al., 2011). The small degree of BM infiltration might also explain the low prevalence of peripheral cytopenias reported in the present study and in the study by Seelig et al. (2014).

The median survival in this study is comparable with those already reported in the literature (Seelig et al., 2014; Ponce et al., 2004)

Interestingly, three dogs died within a short period from the diagnosis. Two of them showed clinical symptoms at diagnosis, whereas substage was not reported for the third case. Substage b is a well-known negative prognostic factor in canine high grade lymphomas. The same prognostic significance may be held true in indolent lymphomas, thereby explaining the short survival of these dogs.

About 10% of the cases included here developed a second malignancy. This event has already been reported in dogs (Marconato et al., 2011) and people (Xu et al., 2013; Tajika et al., 2014) with lymphoma.

The causes underlying the development of second malignancies are still unclear, but treatment, above all alkylating agents, has been associated with subsequent malignant neoplasms in human medicine (Daniels et al., 2013; Ahmadzadeh et al., 2014; LeMieux et al., 2015). Two of three dogs developing second malignancy in this study had received alkylating chemotherapy.

Another possible explanation for second malignancy development is a genetic predisposition to cancer. Indeed, neoplastic transformation is based on genetic abnormalities of many different genes (oncogenes, tumour-suppressor genes and stability genes), which can occur in the germ line, resulting in hereditary predisposition to different types of cancer or in somatic cells, resulting in sporadic tumours (Vogelstein et al., 2004).

The major limits of the first phase of the study are inherent to its retrospective nature. Indeed, for almost half of the included population, clinical data were missing because not reported or retrieved by the referring veterinarians. Also, staging workup varied among veterinarians, possibly leading to under-staging of cases. Finally, we were not able to perform survival analysis because of the huge variety of treatment regimens adopted. Unfortunately, treatment in dogs with lymphoma are not standardized yet and also the choice whether to treat or not is left to referring vets.

Interestingly, all the studies assessing the expression of CD45 protein in TZL by FC use the same antibody clone (YKIX716.13), although conjugated with different fluorochromes (Martini et al., 2013; Seelig et al., 2014). In the present study, we analysed two cases of TZL via immunohistochemistry using a different antibody clone

and results confirmed FC analysis. Both techniques and clones could not detect CD45 protein, thus confirming the lack of CD45 protein expression in canine TZL.

Although we can not completely exclude partial modification of the protein during FC and IHC processing, these preliminary results supported the hypothesis of a complete loss of CD45 in TZLs.

To investigate this scenario, we inquired whether CD45 transcript was also reduced in canine TZL and consequently if CD45 gene was still represented in the genome of the neoplastic cells. To answer this hypothesis, we designed two quantitative real-time PCR experiments in order to investigate the transcript amount and DNA load, respectively. In the study setting, a major limiting factor was related to the selection of control lymphocytes for both transcript and DNA analyses. Indeed, CD45 is differently expressed in the various canine leukocyte subclasses, also depending on the activation status (Goto-Koshino et al., 2014), and CD45 transcript amount may vary accordingly. In particular, CD45 is expressed at lower levels in B-cells than in T-cells (Comazzi et al., 2006): thus, inclusion of B-cells in the control group for transcript amount analysis would have affected results concealing possible differences between TZLs and controls. Respect to this, we selected as control high grade T-cell lymphomas and a pure T-cell population obtained by cell sorting from non-neoplastic lymph nodes.

Based on our results, CD45 transcript amount resulted significantly lower in TZLs compared to high-grade T-cell lymphomas and normal T-cells. Albeit a small CD45 transcript amount was detected in TZLs, this might be associated to the contamination of relevant residual normal lymphocytes in the samples obtained. This was well demonstrated when comparing the CD45 gene expression to the number of neoplastic cells in these tumors. The lowest CD45 transcript amounts were associated to TZLs with the highest percentage of neoplastic cells and consequently the lowest percentage of residual lymphocytes. Taken together, these results suggest that CD45 transcript is virtually absent in TZL neoplastic cells.

For DNA load analysis, high grade T-cell lymphomas were excluded as controls due to the possible presence of genetic abnormalities that occur in cancer cells, leading to unpredictable biases. In contrast, the selection of non-neoplastic lymph nodes as controls, potentially should not interfere with this result, as variations in genes copy number are not expected in reactive lymph nodes, at least in the CD45 gene. However,

the in silico identification of the reference gene for DNA load analysis was complicated by two biological variables. First, copy number variations may eventually occur at gene level biasing the results and second, no relevant data are published about genetic modifications of canine TZL. Thus, primers for two different genes were designed for our analysis and results showed a similar load in TZLs and controls, irrespective of the reference gene used, demonstrating that CD45 gene is not deleted in canine TZL.

Primers design was also challenging, as different isoforms of CD45 exist obtained by alternative RNA splicing (Dupéré-Minier et al., 2010) and the gene comprises many introns (www.ensembl.org). Furthermore, we designed primers that were adapted to amplify a transcript fragment common to all CD45 isoforms, and a gene segment specifically encoding for the transcript fragment was amplified. By doing this, we reached a double goal: first, we included all CD45 isoforms in our analysis; second, we described the different fate of a single sequence at two different levels (transcript and DNA). Many different mechanisms may cause CD45 gene transcription switch off, including genetic, epigenetic, and genomic abnormalities.

Still, gross chromosome or gene deletions are unlikely. Indeed, we were able to detect the presence of CD45 gene in TZL cells. In addition, Seelig et al. (2014) reported that there is no evident loss of the telomeric end of chromosome 7, where CD45 is located, although no scientific data are described in their publication. All other mechanisms are still possible and should be evaluated via further studies.

Different studies in human medicine investigated the importance of CD45 in normal T- and B-cells, highlighting its role in cell maturation, signal transduction and apoptosis mediator (Pingel and Thomas, 1989; Kishihara et al., 1993; Byth et al., 1996; Lesage et al., 1997; Fortin et al., 2002; Pang et al., 2009). Its involvement in neoplastic transformation may be suspected, but this has never been demonstrated. Interestingly, different oncogenes are located near CD45 gene in canine chromosome 7, including ABL2, AKT3, YES1 and some members of the Ras family (www.ensembl.org). These oncogenes may play a role in cancer development in human leukemias and lymphomas (Mao et al., 2003; Huang et al., 2010; Kim et al., 2015; Roberts and Mullighan, 2015). Perturbation of methylation on the promoter regions of these genes or genomic abnormalities on a large segment of canine chromosome 7 may cause impaired transcription of any of these oncogenes

participating to the tumorigenesis. Thus, it cannot be excluded that the absence of CD45 protein is only a phenotypic epiphenomenon, with minimal involvement in the tumor biology.

Further studies are needed, assessing possible alterations in oncogenes activation. The whole CD45 gene sequencing would probably solve the limitations of the second phase of the study, where only a fragment of CD45 gene specifically encoding for the transcript fragment selected was amplified. Thus, mutations, short deletions or base pair variations may occur upstream of the amplified fragment, remaining unnoticed to our analysis, but still preventing gene transcription.

In conclusion, the first phase of the present study reports the clinical presentation and the outcome of a series of dogs diagnosed with CD45- negative small clear cell/TZL. The majority of dogs had stage V disease, were not symptomatic (substage a) and peripheral cytopenias were uncommon. The reported predisposition of Golden retrievers to develop TZL was not confirmed by our results.

Canine TZL is known to bear a good prognosis with long survival times, even if a subset of dogs in our study died within few weeks. Further prospective studies on larger case series, with standardized staging workup and treatment regimens, and longer follow-up times are needed to confirm our results.

The second phase confirms the lack of CD45 protein in canine TZL, irrespective of the different techniques and antibody clones used. Based on our results, this phenotypic aberrancy is likely due to the absence of gene transcription, as CD45 DNA was present, whereas CD45 transcript was virtually absent in the neoplastic cells.

The results of the present study were preliminary presented in poster form at the 14-ICML (Martini V, Cozzi M, Aricò A, Poggi A, Riondato F, Marconato L, Aresu L, Comazzi S. EVALUATION OF CD45 PROTEIN EXPRESSION AND TRANSCRIPT IN CANINE SMALL CLEAR CELL / T-ZONE LYMPHOMA) and then published on two separate papers on peer review international journals (“V. Martini, L. Marconato, A. Poggi, F. Riondato, L. Aresu, M. Cozzi and S. Comazzi. CANINE SMALL CLEAR CELL/T-ZONE LYMPHOMA: CLINICAL PRESENTATION AND OUTCOME IN A RETROSPECTIVE CASE SERIES. Vet Comp Oncol. 2016 Aug;14 Suppl 1:117-26”. “V. Martini, M. Cozzi, A. Aricò, G. Dalla Rovere, A. Poggi, F. Albonico, M. Mortarino, E. Ciusani, L. Aresu, S. Comazzi. LOSS OF CD45 CELL

SURFACE EXPRESSION IN CANINE T-ZONE LYMPHOMA RESULTS FROM REDUCED GENE EXPRESSION. Vet Immunol Immunopathol. 2017 May;187:14-19".)

3.3 CANINE NODAL MARGINAL ZONE LYMPHOMA: INSIGHT INTO THE BIOLOGICAL BEHAVIOUR

Background

In human and veterinary medicine, the Marginal Zone Lymphoma (MZL) group is divided into 3 subtypes according to the World Health Organization (WHO) classification (Swerdlow et al., 2016): Mucosal Associated Lymphoid Tissue Lymphoma (MALT), splenic Marginal Zone Lymphoma (sMZL) and nodal Marginal Zone Lymphoma (nMZL). These 3 entities are described as separate diseases in terms of biology, clinical presentation and behaviour. The major diagnostic criterion is the site of presentation (Ferreri and Zucca, 2007). MALT is relatively common, encompassing 5% to 8% of all NHLs and it most frequently involves the gastrointestinal tract (66% of all MALT cases), occurring

in patients with a history of autoimmune disorders and chronic inflammation (Olszewski and Castillo, 2013; Zucca and Bertoni, 2016). SMZL and nMZL are quite rare, each comprising less than 1% of NHL. sMZL is a symptomatic disease which at onset usually involves spleen, bone marrow (BM) and peripheral blood (PB). Many patients with nMZL show regional (head and neck) lymphadenopathy, but more than 70% present with stage III/IV disease (according to the Ann Arbor staging scheme) (Angelopoulou et al., 2014; Tadmor and Polliack, 2017). The prognosis is reported to be less favourable for nMZL than for MALT and sMZL (Nathwani et al., 1999).

The same classification is adopted for the dog; MALT lymphoma has not been well described in canine patients. It is rare, and predominantly involves the respiratory and intestinal tracts, but other locations have been occasionally reported, such as the salivary gland and eyelid (Valli 2007; Hong et al., 2011). Canine sMZL has been described in terms of presentation and outcome. Unlike human sMZL, the majority of canine sMZL represents an incidental finding during physical examination and abdominal ultrasound. Canine sMZL has an indolent clinical course and splenectomy, with or without systemic chemotherapy, is usually curative (Stefanello et al., 2011; O'Brien et al., 2013; Van Stee et al., 2015).

Canine nMZL is generally classified as an indolent lymphoma, having a low mitotic index and a slow clinical progression (Valli 2007). However, in spite of the putative indolent nature, some dogs with nMZL may experience an aggressive disease course

(Valli et al., 2006; Flood-Knapik et al., 2012; Valli et al., 2013; Aresu et al., 2015; Marconato et al., 2015). Specific studies focused on clinical presentation and behaviour of canine nMZL are lacking. The aim of this study is to describe the clinico-pathological features and outcome in a cohort of dogs with histologically confirmed MZL with a primary nodal presentation, thus better characterize this tumour as a single disease entity.

Material and Methods

Inclusion criteria

Medical records of dogs with lymphoma referred to the Centro Oncologico Veterinario between 2012 and 2016 were retrospectively reviewed for cases with a histopathological diagnosis of nMZL (Valli et al., 1981; Valli et al., 2011) To be eligible for enrolment, dogs were required to undergo a complete staging work-up, including complete blood count (CBC), serum biochemistry (including Lactate Dehydrogenase-LDH-activity and Ionized Calcium concentration), cytology and flow cytometric immunophenotyping on a lymph node (LN) aspirate, thoracic radiographs, abdominal ultrasound, fine-needle aspiration of liver and spleen regardless of their sonographic appearance, and PB and BM infiltration degree assessed by flow cytometry (FC). The abovementioned work-up is standard of care in the Centro Oncologico Veterinario. Previous lymphoma-directed therapy (including steroids) was an exclusion criteria.

Flow cytometry

FC was performed on fresh samples of LN aspirates as described before (first study of the thesis). PB and BM involvement were defined as the presence of cells of B-lineage (CD21+) of medium to large size. Although specific cut-off values for defining tumour infiltration in PB and BM have not been defined for canine MZL, these were set at 0.56% for PB and 2.45% for BM, respectively, out of the total CD45+ cells. These values were derived from a recent study on the analytical and diagnostic performances of FC to detect PB and BM neoplastic infiltration of large B-cell lymphoma cells in dogs (Riondato et al., 2016). Cytological smears of LN, PB and BM aspirates were evaluated in parallel with FC in order to confirm cell morphology, evaluate mitotic figures and detect neoplastic infiltration (Comazzi and Gelain, 2011).

Immunohistochemistry

A peripheral enlarged LN was surgically removed, formalin fixed and paraffin embedded, stained with haematoxylin and eosin, and examined by a veterinary pathologist. For immunohistochemistry, antibodies against CD3 (clone F7.2.38; Dako, Atlanta, Georgia, USA), CD5 (clone CD5/54/F6; Dako), CD79a (clone HM57; Dako) and CD20 (clone RB-9013-P, Thermo Fisher Scientific) were used on paraffin-embedded sections. The diagnosis of nMZL was confirmed according to the WHO classification (Valli et al., 2011).

Treatment and outcome

Dogs were treated with a 20-week combination induction chemotherapy, consisting of L -Asparaginase (week 1), vincristine (weeks 2, 3, 4, 13), cyclophosphamide (weeks 2, 13), doxorubicin (week 7, 16), lomustine (weeks 10, 19) and prednisone (weeks 1-20), as previously described (Marconato et al., 2015). Dogs whose owners wished to pursue immunotherapy, also received an intradermal injection of 0.5 mL autologous vaccine on weeks 4, 5, 6, 7, 12, 16, 20 and 24. The vaccines consisted of tumour-derived heat shock protein-peptide complex coupled with hydroxyapatite ceramic powder (Marconato et al., 2014; Marconato et al., 2015). Response to treatment was classified as complete remission (CR), partial remission (PR), stable disease (SD), or progressive disease (PD) (Vail et al., 2010). Response was evaluated at each therapeutic session and was required to last for at least 28 days.

Statistical analysis

Time to progression (TTP) was calculated as the interval between initiation of treatment and PD or relapse, whereas lymphoma-specific survival (LSS) was measured as the interval between initiation of treatment and lymphoma-related death. Dogs lost to follow-up or dead for lymphoma-unrelated causes before PD, as well as those still in CR at the end of the study, were censored for TTP analysis. Dogs alive at the end of the study, lost to follow-up or dead due to causes other than lymphoma were censored for LSS analysis. Response rate (RR) was defined as the sum of all dogs achieving CR and PR. Survival was analysed according to the method of Kaplan-Meier. Differences between survival curves were evaluated with the log-rank test. Multivariate analyses

were performed using a Cox stepwise proportional hazard model to identify variables that might be of independent significance influencing TTP and LSS. Variables considered were breed (mixed or pure), sex, age (cut-off arbitrarily set at 7 years), weight (cut-off arbitrarily set at 10 kg), packed cell volume (PCV) (normal, decreased, increased), platelet count (normal, decreased, increased), serum lactate dehydrogenase (LDH) activity (normal, decreased, increased), serum ionized calcium concentration (normal, decreased, increased), substage (a or b), spleen involvement (yes or no), PB infiltration (yes or no), total lymphocyte count in peripheral blood (as a continuous variable), BM infiltration (yes or no), and extranodal site involvement (yes or no). Binomial logistic regression was performed to investigate the independence of LDH activity and response to treatment with respect to the abovementioned variables. Statistical analysis was performed via SPSS v20.0 for Windows (IBM, New York, USA). Significance was set at $P \leq .05$ for all tests.

Results

Thirty-five dogs met the inclusion criteria. Among them, 29 have been included in a previous paper (Marconato et al., 2015). Nine dogs (25.9%) were mixed breeds, while 26 (74.3%) were pure breeds (Table 3).

The median age was 7.0 years (mean: 7.6 ± 3.1 years, range: 3.0-15.0 years). In particular, 15 dogs (42.9%) were younger than 7 years, while 20 dogs (57.1%) were 7 or more years. Median weight

was 24.6 kg (mean: 23.0 ± 12.5 kg, range 3.0-44.4 kg), with 7 dogs (20%) less than 10 kg and 28 (80%) 10 kg or more. There were 21 (60%) males (3 neutered) and 14 (40%) females (5 spayed).

All dogs were presented with generalized lymphadenopathy and this was the reason for initial presentation. Lymphadenopathy had been present for a median of 20 days (range: 2-120 days). At the time of diagnosis, 23 (65.7%) cases were asymptomatic, while 12 (34.3%) showed non-specific clinical symptoms. All dogs had stage V disease. Splenomegaly was detected during physical examination in 20 (57.1%) dogs. However, the percentage of cases with splenic involvement rose up to 97.1% (34 dogs) based on abdominal ultrasound and cytological evaluation. In these dogs, abdominal ultrasound revealed splenomegaly; the spleen showed abnormal echogenicity and echo-structure,

with a diffusely heterogeneous parenchyma. Splenomegaly was considered as moderate in 30% and severe in 70% of the cases. Focal lesions were often observed (70% of the dogs), represented by 1 to 2 cm large hypoechoic nodules or multiple small hypoechoic nodules, with a consequent spotted appearance of the parenchyma ("honey-comb appearance"). The sonographic findings suggested parenchymal infiltration, confirmed by cytological evaluation showing a homogeneous or highly prevalent population of medium sized blast cells, often with macronucleoli.

The liver was infiltrated in 27 (77.1%) dogs, as documented by sonographic changes and confirmative cytology. In addition, 10 (28.6%) dogs had extranodal involvement, with the lung present (9 cases), while only 1 dog had more than 1 extranodal site documented (eye and bladder). Lymphoma at extranodal sites was diagnosed by imaging and confirmative cytology in all but one cases; the dog with ocular involvement had a resolution of bilateral uveitis after the first chemotherapy administration, consistent with a presumptive neoplastic nature of the lesion.

Cytologically, the neoplastic cells were medium sized and characterized by nuclei of intermediate size (1.5-2 times the size of a red blood cell) with fine chromatin, prominent single central nucleoli and a moderate amount of weakly basophilic cytoplasm. Few residual mature lymphoid cells were also present. Sometimes a scant population of larger lymphoid cells, defined as centroblasts with anisocytosis and anisokaryosis, was observed. Mitotic index was low with less than 1 mitotic figure/5 high power field (HPF) (×40) (Fig. 16).

FC confirmed the B-cell lineage of the neoplastic cells. CD21 and/or CD79a+ cells represented the predominant cells in LN samples (median: 82.7%, range: 42.0-95.7, mean: 78.1 ± 15.6) (Wilkerson et al., 2005). They showed median forward scatter (FSC) of 432.8 (mean: 440.5 ± 46.2, range: 357.1-521.6) (Fig.17). An admixed population of small residual lymphocytes was also present, yet scarce in percentage (median: 6.4%, mean 12.4 ± 18.2%, range: 3.0%-22.8%). Regarding PB and BM infiltration, 34 (97.1%) dogs had PB involvement, with a median percentage of neoplastic cells of 6.4% (mean: 12.4 ± 18.2%, range: 0.7%-53.5%), while BM was infiltrated in 20 (57.1%) cases, with a median percentage of neoplastic cells of 8.1% (mean: 12.5 ±11.4%, range: 3.0%-51.6%).

Histology and immunohistochemistry were performed in all cases.

Table 3: Clinical presentation and outcome of 35 dogs with nodal marginal zone lymphoma

Breed	Sex	Age (y)	Thrombocytopaenia	Stage	Substage	Immunotherapy	Response	Cause of death (d)
Akita inu	NM	9	No	V	b	Yes	CR	Alive (613)
Australian shepherd	F	12	No	V	a	No	CR	Lymphoma (337)
Australian shepherd	M	11	Yes	V	b	No	PD	Lymphoma (5)
Bassethound	M	7	No	V	b	Yes	PR	Other (238)
Beagle	M	5	No	V	a	Yes	CR	Alive (1605)
Bernese mountain dog	M	3	No	V	a	No	PR	Lymphoma (35)
Border collie	M	4	No	V	b	No	PR	Lymphoma (340)
Boxer	M	6	No	V	a	Yes	CR	Lymphoma (544)
Dachshund	M	6	No	V	a	No	CR	Lymphoma (1042)
French bulldog	F	5	No	V	b	Yes	CR	Lymphoma (215)
German shepherd	F	8	No	V	a	Yes	CR	Other (93)
German shepherd	F	3	Yes	V	a	No	CR	Lymphoma (127)
Golden retriever	M	4	No	V	a	Yes	CR	Lymphoma (385)
Jack russel	M	5	No	V	b	Yes	CR	Lymphoma (125)
Jack russel	M	7	No	V	a	No	CR	Lymphoma (730)
Labrador retriever	M	3	No	V	b	Yes	CR	Lymphoma (259)
Labrador retriever	F	7	No	V	a	No	PR	Alive (1016)
Mixed	SF	15	No	V	a	No	PR	Lymphoma (133)
Mixed	M	9	No	V	a	Yes	PR	Lymphoma (156)
Mixed	SF	6	No	V	a	Yes	CR	Lymphoma (680)
Mixed	M	9	No	V	a	No	CR	Lymphoma (111)
Mixed	F	5	No	V	b	Yes	CR	Lymphoma (188)
Mixed	M	9	No	V	b	Yes	CR	Lymphoma (248)
Mixed	NM	9	No	V	a	No	CR	Lymphoma (632)
Mixed	F	9	No	V	b	No	PD	Lymphoma (20)
Mixed	SF	9	Yes	V	a	Yes	CR	Alive (601)
Petit bleu	SF	11	No	V	a	Yes	CR	Other (181)
Pinscher	SF	11	Yes	V	b	No	PD	Lymphoma (7)
Pomeranian	M	13	No	V	a	No	CR	Lymphoma (211)
Poodle	M	7	No	V	a	Yes	PR	Lymphoma (399)
Poodle	F	14	No	V	b	No	PD	Lymphoma (45)
Rottweiler	M	5	No	V	a	Yes	CR	Lymphoma (349)
Rottweiler	F	6	No	V	a	No	CR	Lymphoma (160)
Shih-tzu	M	5	No	V	a	Yes	CR	Lymphoma (152)
Yorkshire terrier	NM	8	No	V	a	No	CR	Lymphoma(1403)

Abbreviations: CR, complete remission; F, female; lymphoma, dead of lymphoma-related causes; M, male; NM, neutered male; other, dead of other causes then lymphoma; PD, progressive disease; PR, partial remission; SF, spayed female.

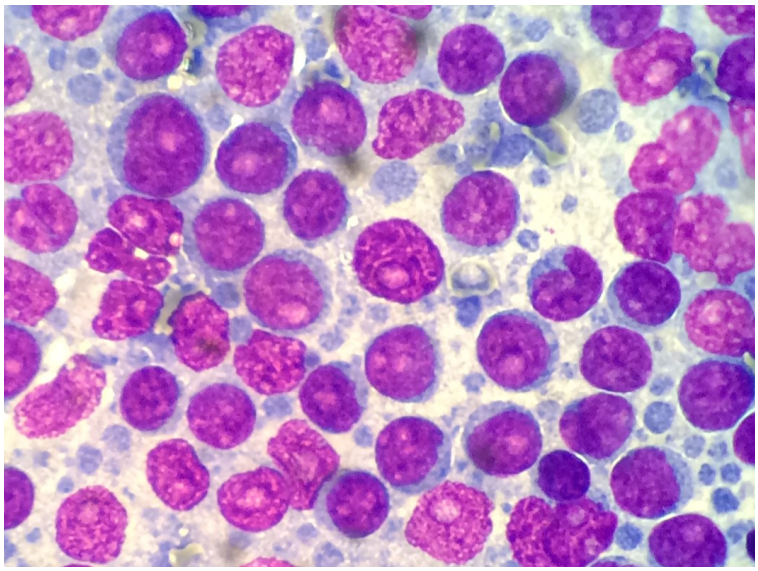

Figure 16: cytological smear of a nMZL, characterized by nuclei of intermediate size with prominent single central nucleoli and abundant lightly stained cytoplasm and with mitoses absent except in advanced cases. A mixture of some immunoblasts and centroblasts sometimes is present.

Histological grade was available for 31 cases; among them, 30 were at a late stage of development, characterized by a diffuse growth pattern and loss of follicle-related architecture. The capsule was documented to be thinned and taut. The greatest proportion (80%-90%) of cells was medium sized (1.5-2 times the red blood cell), with scant eosinophilic cytoplasm, round nucleus and single prominent central nucleolus. The remaining 10% to 20% of the LN population was represented by small mature lymphocytes. Sometimes, large cells defined as centroblasts and immunoblasts were observed; mitotic activity of these cells was variably, low to moderate and the mean mitotic index ranged from 0 to 5 in 10 HPF (×40) (Aresu et al., 2015). Tingible body macrophages were present. CD79a and CD20 immunohistochemical positivity confirmed B-cell origin (Valli 2007; Valli et al., 2011).

Regarding CBC at diagnosis, 3 (8.6%) dogs were anaemic (PCV < 37%), 31 (88.5%) had a PCV within the reference interval, and 1 (2.9%) dog had an increased PCV (57%); 4 (11.4%) dogs had thrombocytopaenia (platelet count $<200\times10^3/\mu L$ confirmed by smear evaluation), while 31 (88.5%) had a normal platelet count. Thirty-four dogs were normocalcemic, while 1 dog had a decreased

ionized calcium concentration. LDH activity (< or ≥300 IU/L) was increased in 14 (40%) cases, while it was normal in the remaining 21 (60%) dogs.

Binomial logistic regression revealed no significant correlation between LDH activity and all abovementioned variables. No correlation was found between PB lymphocyte count and TTP or LSS.

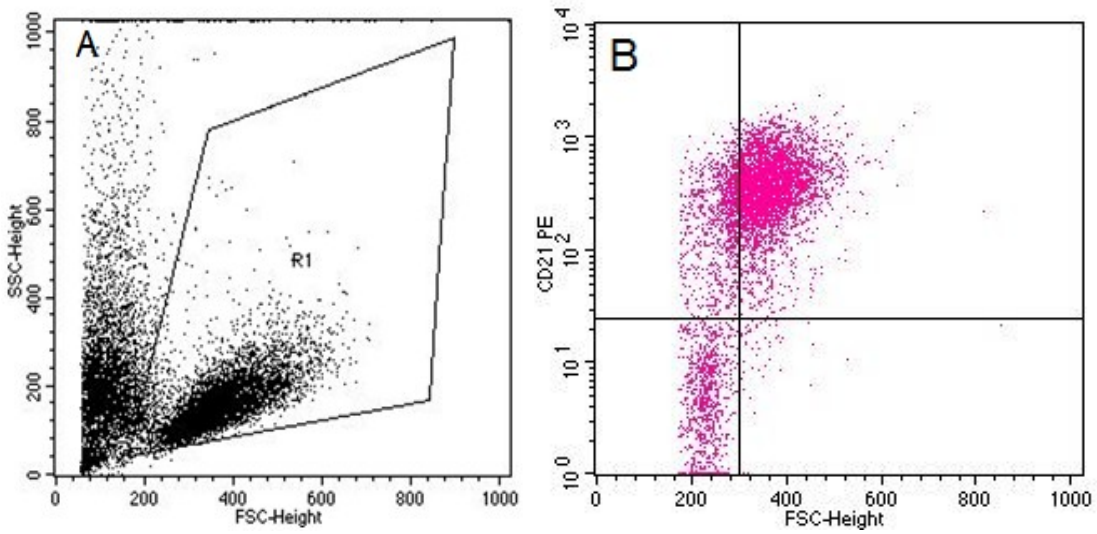

Figure 17: flow cytometric scattergrams representing lymph node aspirate of a nMZL. Events were displayed at first
based on morphological properties (IA) and a gate (R1) was set to exclude platelets and debris. R1 cells were then displayed based on CD21-alexa 647 fluorescence and FSC . Neoplastic cells were medium to large-sized positive for CD21 antibody (B, up and right quadrant). Small lymphocytes reffered to residual normal population were present (B, low and left quadrant.

Treatment and outcome

Thirty-four dogs were treated with chemotherapy, and 18 (52.9%) received concurrent immunotherapy. One dog received no treatment at all and was excluded from the survival analysis. TTP and LSS for this dog were 49 and 340 days, respectively. Of the 34 dogs that were treated, 25 completed the planned treatment schedule and 9 died during treatment due to PD. Among all others, 20 (80%) achieved CR (of those, 13 received concurrent immunotherapy) and 5 (20%) achieved PR (of those, 3 received concurrent immunotherapy).

Sixteen dogs having completed the planned protocol received rescue chemotherapy after documentation of PD: 12 received a CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone)-based protocol, whereas 3 were treated with DMAC (dexamethasone, D-actinomycin, melphalan, cytosine arabinoside). A second CR was obtained in 14 of them.

Binomial logistic regression revealed that platelet count was significantly associated with treatment response (P = 0.033). Thrombocytopenic dogs had a lower probability of responding to treatment (odds ratio: 0.071; 95% confidence interval: 0.006-0.810). RR was 50% for dogs with thrombocytopaenia and 80% for dogs with a normal platelet count. Overall median TTP was 149 days (range: 1-994 days). None of the investigated variables significantly influenced TTP. Overall, median LSS was 259 days (range: 5-1605 days). Four dogs were alive at data analysis closure after 601, 613, 1016 and 1605 days. Three dogs died of tumour-unrelated causes after 93, 181 and 238 days. Cause of death was due to lymphoma in the remaining 28 dogs. LDH activity (P = 0.025) and substage (P = 0.008) significantly influenced LSS. In particular, median LSS was 385 days (range: 111-1605 days, n = 20) for dogs with a normal LDH serum level, and 211 days (range: 5-601 days, n = 14) for dogs with increased LDH; asymptomatic dogs (substage a) had a median LSS of 399 days (range: 93-1605, n = 22), compared with 125 days (range: 5-613, n = 12) for symptomatic dogs (substage b). Multivariate Cox's proportional hazard regression analysis showed the influence of platelet count (P = 0.01) on LSS.

Discussion

This study describes the clinical presentation and outcome of 35 dogs with histologically confirmed nMZL. Despite the retrospective nature of the design, data concerning initial staging, treatment and follow-up were available for all dogs, thereby providing robust information. Canine nMZL is considered an indolent disease (Valli et al., 2011; Aresu et al., 2015), however the published studies describing the clinical and morphological features of indolent lymphomas suggest that a subset of nMZL cases may display a more aggressive clinical course (Valli et al., 2006; Flood-Knapik et al., 2012; Valli et al., 2013; Aresu et al., 2015; Marconato et al., 2015).

Indeed, based on our results, the indolent designation may not always be appropriate, as all dogs had generalized lymphadenopathy and one third of them were symptomatic at initial presentation. In contrast with previous reports, suggesting that nMZL typically does not cause any systemic involvement (Valli 2007), all dogs in the present cohort but one had PB involvement and 57.1% had BM involvement, while one-third of them had extranodal involvement. The cause for the discrepancy between

PB and BM infiltration is unclear, but an overspill of neoplastic cells from affected nodes in the absence of true BM invasion could be a possible explanation, similar to what has been described for T-zone lymphoma (second study of the thesis). Alternatively, it may be due to the different cut-offs used to define positive PB and BM samples and to the use of FC for staging, which is a very sensitive tool to detect BM and PB infiltration compared with standard light microscopy (Comazzi and Gelain, 2011). Although splenic involvement was detected in 97% of cases, a primary splenic MZL was considered unlikely, based on the integration of clinical and pathological data. Indeed, in canine primary sMZL, the spleen is usually the only site involved, and the diagnosis is frequently incidental (Stefanello et al., 2011; O'Brien et al., 2013; Valli et al., 2006). In this study, ultrasonographic findings including splenomegaly, diffuse heterogeneity and hypoechoic nodular lesions suggested diffuse secondary infiltration of the parenchyma. Conversely, primary splenic MZL is characterized by a solitary focal hypoechoic mass without any changes of the surrounding tissue (Stefanello et al., 2011; O'Brien et al., 2013; Valli et al., 2011).

In humans, nMZL is defined by the WHO classification as “a primary nodal B-cell neoplasm that morphologically resembles LNs involved by MZL of extranodal or splenic types, but without evidence of extranodal or splenic disease.” This implies that also in humans the diagnosis of nMZL is mainly based on the pattern of dissemination of the disease, essentially based on the fact that sMZL involves the spleen without concomitant peripheral lymphadenopathy, while nMZL does not have a clinical evidence of extranodal or splenic disease (Swerdlow et al., 2016; Van den Brand et al., 2013). In spite of these considerations, we cannot definitely rule out a primary splenic origin of the tumour with a secondary late dissemination to peripheral nodes.

For the cases included in this study, we were able to evaluate the neoplastic population by means of 3 different techniques, namely cytology, histopathology and FC. The different techniques gave concordant and overlapping information: samples mainly comprised medium-sized cells, but sometimes were accompanied by a population of centroblasts/immunoblasts and scant resident small lymphocytes.

These features also correspond to those previously described in the literature for humans, where the presence of sheets of centroblasts appears to be related to disease progression and tumour transformation into large B-cell lymphoma (Kaur 2011).

Indeed, histology revealed that all cases but one were at a late stage of development, characterized by a diffuse growth pattern and loss of the follicle-related architecture. The only dog with the classical histologic marginal presentation and a slight PB and BM infiltration experienced long LSS (680 days). More cases are needed to define if the histological architecture pattern may be independently associated with a differing clinical behavior.

Based on the above, it may be hypothesized that late-stage nMZL behaves clinically like high-grade lymphomas, with a tendency to spread systemically. Accordingly, Richards et al. (2013) found molecular similarities between nMZL and diffuse large B-cell lymphoma (DLBCL), suggesting that these conditions might represent a continuous spectrum of the same disease.

Approximately one-third of dogs died due to lymphomas within 6 months despite treatment, thereby exhibiting a poor outcome that contrasts with the “indolent” tumour designation. This discrepancy is likely due to the different inclusion criteria among studies. Equally, the case selection of the current study might be biased as dogs with generalized lymphadenopathy are more likely to be referred to a referral centre and undergo a full staging work-up. Indeed, this is the first case series focused exclusively on nMZL, with strict staging criteria. Most of the studies published in the veterinary literature include many different lymphoma subtypes or are limited to small case series with incomplete staging and follow-up data (Valli et al., 2006; Flood-Knapik et al., 2012; Valli et al., 2013). Overall, TTP and LSS were disappointingly low, suggesting that the CHOP-based protocol used in the current series of dogs may not be the best option.

Regarding prognostic factors, thrombocytopenic dogs had a significantly lower RR and shorter LSS. Thrombocytopenia is reported in 10%-13% of cancer-bearing dogs (Grindem et al., 1991; Botsch et al., 2009), and is generally considered to be a poor prognostic factor (Valli 2007; Zemann et al., 1998; Moore et al., 2001). Substage b was an additional independent risk factor, in agreement with previous studies, showing a correlation with a poor outcome (Teske et al., 1994; Jagielski et al., 2002). Increased LDH serum level was also significantly associated with a shorter LSS. An increased LDH level at diagnosis has been associated with a shorter survival in people with indolent

lymphoma (Bastion et al., 1991; Lopez et al., 1994). It may be possible that the same holds true in dogs.

Surprisingly, PB and BM involvement were not significantly associated with outcome. This is in contrast with what has been described for DLBCL (Marconato et al., 2013). The cut-off values may have influenced the definition of stage. It must be acknowledged that the cut-off values used in the current study and extrapolated from previously published data may be inappropriate for MZL, due to the smaller size of neoplastic cells that impede discrimination between neoplastic and reactive B-lymphocytes. A specific validation study is needed to define the correct FC approach for staging MZL (Riondato et al., 2016).

The main limitation of this study is the absence of cases with nodular presentation and earlier disease stages (I-IV). This prevents extrapolation about the clinical course of nMZL and the clinical significance of PB and BM involvement. As all dogs enrolled in the present study had generalized lymphadenopathy, it is possible that dogs were initially asymptomatic for a long time and during that time regional lymphadenopathy may have gone unnoticed.

In conclusion, dogs with nMZL may present at an advanced stage of disease, with an overall poor prognosis despite the indolent designation. Due to the significant clinical interest, the issue of dose intensity should be further explored in dogs with nMZL.

The results of the present study were preliminary presented as an oral presentation at the 18th

ESVCP, ESVONC congress in Nantes (FRA) (Cozzi M, Marconato L, Martini V, Aresu L, Riondato F, Rossi F, Stefanello D, Comazzi S. “CANINE NODAL MARGINAL ZONE LYMPHOMA: DESCRIPTIVE INSIGHT INTO THE BIOLOGICAL BEHAVIOUR”) and then published on a paper on peer review international journals (Cozzi M, Marconato L, Martini V, Aresu L, Riondato F, Rossi F, Stefanello D, Comazzi S. “CANINE NODAL MARGINAL ZONE LYMPHOMA: DESCRIPTIVE INSIGHT INTO THE BIOLOGICAL BEHAVIOUR”. Vet Comp Oncol. 2018 Jun;16(2):246-252)

3.4 EVALUATION OF PUTATIVE PROGNOSTIC MARKERS IN CANINE B-CELL CHRONIC LEUKEMIA: A PRELIMINARY STUDY

Background

B cell chronic lymphocytic leukemia (B-CLL) is a common hematological disorder in dogs, consisting in a clonal expansion of malignant mature B lymphocytes. Although B-CLL is not the most diffuse subtype of chronic lymphocytic leukemia in dogs this entity show a particular comparative interest for the strong similarity from clinical, clinico-pathological and biological behavior and outcome with human chronic lymphocytic leukemia (Comazzi et al., 2015; Rout et al., 2018). Up to 50% of affected dogs are asymptomatic at presentation and incidental lymphocytosis is often noted during routine blood work or senior health profiling. Similarly to humans, B-CLL affects patients of middle-elderly age, most often exhibiting indolent behavior, but some patients experience a more progressive form of the disease (Vail et al., 2001; Moore and Vernau, 2003; Comazzi et al., 2011; Workman HC and Vernau W., 2003; Williams et al., 2008; Comazzi et al, 2015; Bromberek et al, 2016).

A substantial heterogeneity exists in canine chronic lymphocytic leukemia with marked variation in classification and outcomes within and among subtypes. Our research group identified immunophenotype as a predictor of survival but the outcome, mainly of B-CLL remains highly variable (Comazzi et al., 2011) and, to date, no specific prognostic markers have been identified. In human medicine, several investigations have made to identify subgroups of B-CLL patients with different outcome using various panels of surface and cytoplasmic markers, including lymphocyte antigens, cell-adhesion molecules, integrins, or complement regulatory proteins (Huang et al., 2011). High expressions of Zeta-associated protein 70 (ZAP-70) and cyclic ADP ribose hydrolase (CD38) have been recognized as strong adverse risk factors in human's B-CLL (Moreno and Montserrat, 2008, Rossi et al 2010) being correlated with the mutational status of IgVH. Some studies have proven that these molecules are expressed also by canine cells, but the possible significance in canine B-CLL is still unknown (L.M. Smyth et al., 2006; Mortarino et al., 2009).

Other flow cytometric markers also have been published for their prognostic potential in human's CLL. Several studies have investigated the prognostic significance of Interleukin-2 receptor alpha chain (CD25) expression, with contradictory results. Two

earlier reports (Hjalmar et al., 2002, Tefferi et al., 1997) showed that patients with CLL whose cells expressed CD25 had a worse outcome than those with CD25-negative cells. In contrast, other studies reported the opposite situation, suggesting high expression of CD25 as linked to a good prognosis B-CLL (Zucchetto et al., 2005; Capello et al., 2006; Shvidel et al., 2012).

The expression of CD25 has been investigated also in cells from canine lymphoma and leukemia patients (Dickerson et al., 2002; Mizutani et al., 2016). In particular, one recent publication showed that the progression-free survival was significantly shorter in CD25-high group than that in CD25-low group. The study was conducted on acute leukemias and several lymphoma subtypes, but the clinical significance of CD25-positivity has not been explored in CLL (Mizutani et al., 2016).

The nuclear protein Ki67 has been widely used to assess clinical behavior and determining the outcome in various human malignancies and in some solid tumors of the dog (Brown and Gatter, 2000; Sholzen and Gerdes, 2000; Endl and Gerdes, 2000; Broyde et al, 2009; Fonseca-Alves et al 2015; Fonseca-Alves et al 2017; Brunetti et al 2017). A recent study on canine lymphoma suggested the determination of ki67 by flow cytometry for the early detection of lymphomas transforming from low grade to high grade (Poggi et al. 2013), while other studies found Ki67 as an independent predictor for survival in treated high-grade B-cell lymphomas (Poggi et al, 2016; Sierra et al, 2017). All these findings indicate that ki67 immunolocalization is related to proliferative activity in canine malignant tissue, but to date no data concerning canine B-CLL are available.

The identification of novel markers for B-CLL diagnosis in canine patients could contribute to the better characterization of the malignant clone and help to explain the heterogeneity in the clinical behavior of the disease.

Starting from the experience of human medicine the aim of the present study was to prospectively investigate the expression of ZAP70, CD25, and KI67 by flow cytometry and CD38 mRNA expression by quantitative real-time PCR on canine B-CLL cells. Then, we correlated the expression of the markers with some preliminary outcome data. The main purpose of the present work was to determine whether the markers are expressed or not by leukemic cells and if they could be putative prognostic markers with clinical utility.

Material and Methods

Eligibility

The present study included newly identified patients affected by B-CLL from 2015 to 2017 for ki67, ZAP70 and CD25 analysis, and whole blood samples at time of diagnosis from patients with B-CLL in the range of time 2010-2016 and stored into RNA later at -20 for CD38 transcript analysis in canine cells.

Clinical data were obtained from the medical records and by phone calls to the referring veterinarians. Background information collected for each dog included signalment, blood count (CBC) at diagnosis, date and cause of death. All dogs were privately owned and blood was collected for diagnostic purposes with the informed consent of the owners. Thus, a formal approval of the Institution Committee for Animal Care of the authors' Institutions was not required.

The diagnosis of B-CLL required a persistent lymphocytosis of more than 6×10^9/l and was based on standard morphologic and immunophenotyping criteria (Workman and Vernau, 2003; Adam et al., 2009; Comazzi et al., 2011). Routine laboratory studies consisted of complete blood count performed with an automated analyzer including leukocyte differential and platelet count. Automated differentials were validated by microscopic evaluation of blood smears stained with May Grünwald-Giemsa. Anemia was defined by PCV<37%. Thrombocytopenia was confirmed if both platelet count was <100,000/mL and blood smear showed low platelet estimation and no evidence of platelet clumping.

Exclusion criteria included:

(1) morphology suggestive of immature or blast cells in at least 30% of cells (medium to large cell size; round or indented, medium to large nucleus with poorly condensed chromatin; the presence of nucleoli or some combination of these),

(2) positivity to CD34,

(3) moderate or severe lymphadenomegaly, splenomegaly, or both with nodal, splenic, or both having cytological features compatible with lymphoma. Mild lymphadenopathy or splenomegaly

was not considered an exclusion criterion, except for those cases showing cytological features suggestive of specific lymphoma subtypes, and

(4) positive serologic titer for Ehrlichia or Leishmania or any other identifiable cause of lymphocytosis (eg, hypoadrenocorticism, postvaccinal lymphocytosis, stress lymphocytosis, others).

ZAP70 antibody validation

Since the use of this antibody in canine species has never been described before, the clone used was validated as follow:

1. reactivity on canine ZAP-70 protein was evaluated by computational analysis comparing the sequence of antigen polypeptide provided by the producer aligned with the homologous canine sequence. We performed a search of the non-redundant protein sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the reference protein sequence;
2. reactivity with canine cells was confirmed by Western Blotting using canine peripheral blood mononuclear cells as a target;
3. specific labeling on T circulating lymphocytes from canine blood was confirmed using a triple color staining for ZAP-70 and CD5 (T cells) and CD21 (B cells) on samples from healthy dogs.

Flow Cytometry

- Standard panel for CLL diagnosis

Flow cytometric immunophenotype was performed as previously reported (first study pf the thesis). The B-CLL cases were identified by a homogeneous expansion (>60%) of “small” CD21+ lymphocytes (Workman and Vernau, 2003). These cases were considered “cytology-confirmed” if the report described the majority of cells as mature, small, having condensed chromatin.

- Experimental panel

The experimental panel was performed on newly diagnosed cases using antibodies against ki67, CD25, and ZAP70.

For ki67 staining, peripheral blood samples were labeled with a specific FITC-conjugated antibody directed against Ki67 (Mouse anti-Human, clone MIB-1, DAKO, Glostrup, DK) using a fixation/permeabilization method with methanol as previously described (Poggi et al., 2013). Proliferative activity was expressed as percentage of Ki67 positive cells.

Cell surface expression of CD25 was evaluated by anti-CD25 monoclonal antibody PE-conjugated (Mouse anti Canine, clone P4A10, eBioscience) and processed as previously described for surface antibody stainings (Comazzi et al., 2006). The analysis was performed on gated lymphocytes and expressed as percentage of CD25 positive cells out of lymphocytes population.

For the intracellular protein ZAP-70, fixation and permeabilization procedures were performed using the Leucoperm reagents A and B (Serotec, Oxford, UK) according to manufacturer's instructions. ZAP70 antibody R-PE-conjugated (Mouse anti Human, clone SBZAP, Zebra Bioscience, Enschede, Netherlands) was used for the stain. Tubes were prepared as follow: negative control (only cells, without antibodies), one tube containing CD5-FITC/isotype control-PE/CD21-Alexa 647 (Fluorescence Minus One – FMO- control), one tube containing CD5-FITC/ZAP70-PE/CD21-Alexa 647. Afterward, the samples were washed in phosphate buffered saline (PBS), resuspended and immediately acquired at the flow cytometer. A minimum of 10000 events was acquired and analysis was performed on gated CD21 positive lymphocytes staining positively for ZAP70, using unstained cells. Threshold was defined on CD21 positive cells using the FMO tube.

Quantitative real-time RT-PCR for CD38 transcript

Since cross-reactive anti-CD38 antibodies are currently not available for dogs, we preliminary investigated this putative marker at RNA level. The transcript analysis was performed on whole blood sample diluted in RNAlater® (Applied Biosystems, Life Technologies, Carlsbad, CA) and stored at −20 °C until processing. The total RNA was isolated from cell pellets using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. To avoid genomic DNA contamination, on-column DNase digestion with the RNase-Free DNase (Qiagen) set was performed. Total RNA

concentration and quality were measured with Qubit Fluorimeter (Thermo Fisher Scientific, Waltham, MA USA) using a Qubit RNA HS Assay kit. First-strand cDNA was synthesized from 200 ng of total RNA using QuantiTec Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. The generated cDNA was used as template for quantitative real-time RT-PCR (qRT-PCR) in a BioRad CFX Real-Time PCR detenction System (BioRad, California, USA) instrument using standard PCR conditions. The qRT-PCR reactions consisted of 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Life Technologies, Carlsbad, CA), 0.3 µl of forward and reverse primers (10 µM) and 1 µl of diluted (1–40) cDNA. Primers were designed using Primer3web (primer3.ut.ee) based on the corresponding canine genome project gene sequences available through Ensembl web site (www.ensembl.org). Canine CD38 was amplified using the primer pair 5'- CCT GAT TGT GGT GGG CAT-3' (forward) and 5'-TCG CAC GAC TTG AGT GTA-3' (reverse) for the amplification of a fragment of 188 base pairs. Canine transmembrane BAX inhibitor motif containing 4 (CGI-119) and GAPDH were chosen as reference gene for the absence of pathological state dependent differences in mRNA expression, as reported by Aricò et al. (2013) and Mortarino et al (2009).

Calibration curves using serial dilution of a control sample revealed PCR efficiency of 100.1%. ΔΔCt method (Livak and Schmittgen, 2001) was used for the relative quantification of mRNA, ultimately expressed as Relative Expression (RE).

Statistical Analysis

Statistical analysis was performed via SPSS v20.0 for Windows. Statistical significance was set at $p \leq 0.05$. To assess whether the expression of the antibodies and the transcript could influence survival, we divided cases into low and high expression: an arbitrary cutoff was set at the median value calculated of the percentage of positive cells for each antibody and the median of relative expression of CD38 mRNA. For ki67 antibody, the cutoff value of 12.2% used for the discrimination between high grade and low-grade lymphomas was also evaluated, based on a previous published paper (Poggi et al. 2013). Then, curves were generated by the Kaplan-Meier method and compared with the log-rank test. Survival time (ST) was calculated from the date of diagnosis of CLL to death. Cases were censored if lost to follow-up, if they died or were euthanized because of unrelated causes, or if they were alive at the end of the study.

The following factors were investigated using multivariate Cox's proportional hazard regression analysis to assess association with long survival: breed (pure or mixed), sex (male or female), age (continuous variable), WBC (continuous variable), presence of anemia (yes or not), presence of thrombocytopenia (yes or not), total lymphocyte count (continuous variable), % of B cells (continuous variable), % of ZAP70 positive cells (continuous variable), % of CD25 positive cells (continuous variable), % of ki67 positive cells (continuous variable).

Results

Case description

The present analysis included peripheral blood samples from 37 newly identified patients affected by B-CLL.

Clinical data were obtained for 27 dogs. Among these, 20 dogs (74%) were of pure breed and 7 (26%) were of mixed-breed, with Boxer (n=4) as the most represented breed, followed by Maltese dog (n=2), Labrador Retriever (n=2) and American Staffordshire Terrier (n=2). 15 (55.5%) were male (1 neutered) and 12 (45.5%) were female (5 spayed).

Overall median age was 10.5 years (range 3–15 years). In particular, 10 (37%) dogs were <10.5 years old and 17 (63%) were ≥10 .5 years old.

Hematological data were obtained for 26 dogs. Eleven (42.%) dogs were anemic, 7 (26.9%) had thrombocytopenia. All dogs had lymphocytosis; the overall median lymphocyte count was 57,32 $\times 10^3/\mu L$ (range 6.87-885.15 $\times 10^3/\mu L$), with an overall median white blood cell (WBC) count of 69,9 $\times 10^3/\mu L$ (range 11.3-970.8 $\times 10^3/\mu L$). FC for CLL diagnosis was carried out for all patients. The percentage of B cells ranged from 47 to 98 % (median 82%).

Follow up data

Follow up data were available for 17 dogs. Overall median survival was 192 days (dd) (range 3-771 dd). Nine of the 17 dogs died of causes related to their leukemia, with a median survival of 59 dd (range 3-332). Among the remaining 8 cases, 3 dogs were lost to follow up at 187, 563 and 644 dd, 1 dog died of other causes at 206 dd, while 4 dogs were alive at data analysis closure at 75, 635, 691 and 771 dd.

Validation of ZAP70 antibody for flow cytometry

The cross-reactivity of ZAP70 human antibody against canine cells was investigated within sequence analysis of the target epitope. The peptide corresponding to the amino acids 280-309 of human ZAP70 was queried against canine sequences with NCB1 protein BLAST tool.

The antigen recognized by clone SB-ZAP used for the present research showed 86% of identity with ZAP70 protein of Canis Lupus Familiaris (Fig. 18). The substitutions of aminoacids don't change the structure of the epitope.

Western blotting analysis on canine PBMC showed a positive band at the expected molecular weight (70 kD). Flow cytometry on peripheral blood lymphocytes from healthy dogs showed a positivity to ZAP-70 just on CD5 positive cells (T cells) while CD21 positive cells (mature B cells) stained negative (Fig. 19).

ZAP70, ki67and CD25 analysis

24 cases were enrolled for the FC experimental panel.

ki67 was performed for 23 cases, CD25 was performed for 21 cases, while ZAP70 was performed for 16 cases. The percentage of positive cells ranged from 0.65 to 24.98 % (median 4.2 %) for ki67 antibody, from 3.27 to 97,8 % (median 68,4 %) for CD25 and from 7.6 to 96.4 % (median 41.6 %) for ZAP70 (Fig. 20-21) (Table 4).

The prognostic influence of ki67, CD25, and ZAP70 was assessed in our cohort. Follow up data were available for just 14 dogs included in the experimental panel. ST of patients with high expression was compared with low expression. An assessment of the Kaplan-Meier curves revealed a highly significant difference between expression levels and outcome (p=0.027) for ZAP70: ST was significantly shorter in the group of high expression of the marker. On the contrary, for ki67 and CD25 markers no significance was found, with p=0,287 and p=0,803, respectively, and p=0.07 for ki67 with the 12.2% cutoff (Fig.21). Median ST were as follow: median of ZAP70 low expression (n=6): nor reached, ZAP70 high (n=6): 59 dd; CD25 low expression (n=4): 329 dd, CD25 high expression (n=8): 86 dd; ki67 low expression (n=6): not reached, ki67 high expression (n=6): 86 dd; ki67 ≤12.2% (n=19): not reached, ki67 >12.2% (n=4): 86 dd.

Cox's multivariate analysis revealed that none of the variables considered was significantly associated with survival.

```
TR|F1PJS1|F1PJS1_CANLF  LKADGLIYCLKDACPNTNAS--SEAAAPTLPAHPSTFTQAHRRIDTLNSDGYTPEPARLA 138
SP|P43403|ZAP70_HUMAN   LKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYTPEPARIT 300
SP|P43403-2|ZAP70_HUMAN ------------------------------------------------------------
SP|P43403-3|ZAP70_HUMAN LKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYTPEPARIT 174

TR|F1PJS1|F1PJS1_CANLF  SSEKAQSMPMDTSVYESPYSDPEELKDKKLFLKRENLLMADIELGCGNFGSVRQGVYRMR 198
SP|P43403|ZAP70_HUMAN   SPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMR 360
SP|P43403-2|ZAP70_HUMAN -------MPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMR 53
SP|P43403-3|ZAP70_HUMAN SPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMR 234
                         ****************************:***:********************
```

Figure 18: Allignment of canine ZAP70 peptide to human ZAP70 corresponding the aminoacids 280-309. Red: un-conservative change. Green: conservative change.

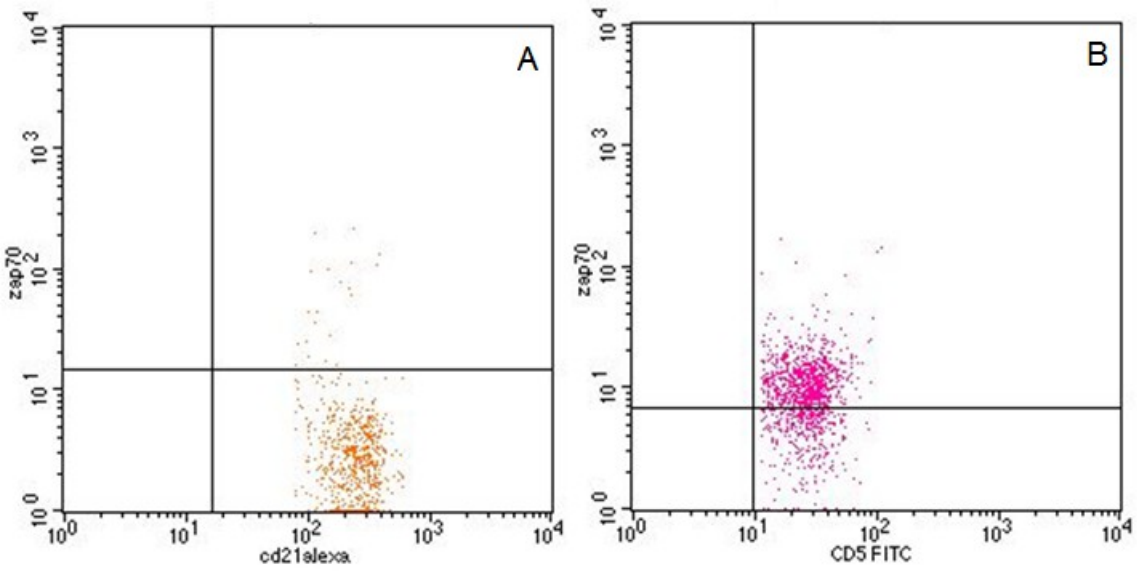

Figure 19: flow cytometric analysis on peripheral blood of a healthy dog. A: CD21-alexa 647 VS ZAP70-pe; circulating B lymphocytes are positive for CD21 and negative for ZAP70 (lower-right quadrant). B: CD5-fitc and ZAP70-pe; circulating T lymphocytes are positive for both CD5 and ZAP70 (upper-right quadrant).

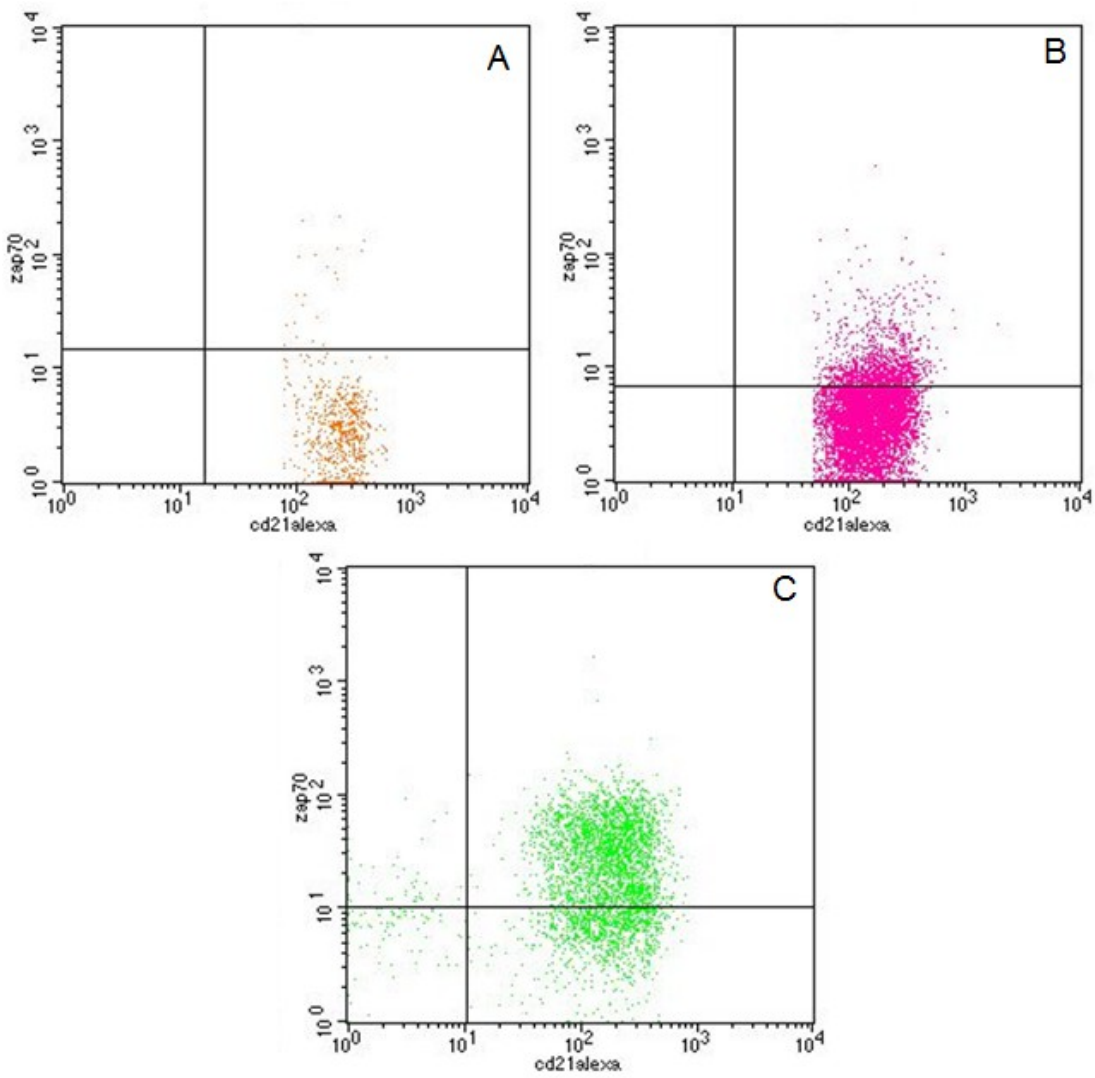

Figure 20: flow cytometric scattergrams representing CD21-alexa 647 VS ZAP70-pe of B-lymphocytes. Cells positive for both CD21 and ZAP70 fall in the upper-right quadrant of the scattergrams. A: B-lymphocytes of a healthy dog staining positive for CD21 and negative for ZAP70 . B: a sample of CLL-B characterized by low ZAP70 expression. C: a sample of CLL-B characterized by high ZAP70 expression.

Table 4: percentage of positivity for ZAP70, CD25 and ki67, and outcome of dogs enrolled for the FC experimental panel

CASE	ZAP70 %	CD25%	ki67 %	SURVIVAL	OUTCOME
1	34,94	88,14	6,14	644	Lost to follow-up
2	24,7	3,27	1,41	206	Dead (other)
3	35,97	78,76	2,61	771	Alive
4	69,9	46,5	21,4	329	Dead (leukemia)
5	92,03	90,2	4,16	59	Dead (leukemia)
6	96,4	97,8	7,29	49	Dead (leukemia)
7	63,8	88,8	2,84	691	Alive
8	41,6	93,31	12,08	635	Alive
9	41	73,4	12,5	86	Dead (leukemia)
10	41,52	25,6	3,52	na	Unavailable
11	66,6	84,2	24,98	4	Dead (leukemia)
12	7,6	12,07	0,65	na	Unavailable
13	na	na	1,58	187	Lost to follow-up
14	na	na	0,6	192	Dead (leukemia)
15	48,2	na	4,96	na	Unavailable
16	80,03	46,2	3,2	3	Dead (leukemia)
17	na	3,37	na	na	Unavailable
18	na	11,2	3	na	Unavailable
19	na	64,3	4,2	na	Unavailable
20	na	90,2	12,1	na	Unavailable
21	na	86,7	8,9	na	Unavailable

22	na	44,3	8,8	na	Unavailable
23	19,1	68,4	13,9	na	Unavailable
24	22,54	64,89	2,47	75	Alive

CD38 mRNA analysis

24 whole blood samples collected from patients diagnosed with B-CLL at time of diagnosis and stored into RNA-later at -20 were tested for CD38 mRNA expression in canine cells. The real-time analysis revealed that the marker was expressed by cells. The distribution of CD38 relative expression in the whole patient cohort was highly variable, ranging between 0.06 and 8.6 (median 2,34) (table 5). The percentage of leukemic cells ranged from 47 to 96 %. Survival time was available for 11 dogs included in the real-time PCR analysis. The association between patients with high and low expression was also evaluated but no association between the relative expression of the protein and survival was found. Median ST for CD38 low expression (n=5) group was 332 dd, while for CD38 high expression (n=6) was 86 dd, p=0,806 (Fig.21). Multivariate analysis revealed that none of the clinical and hematological variables was significantly associated with survival.

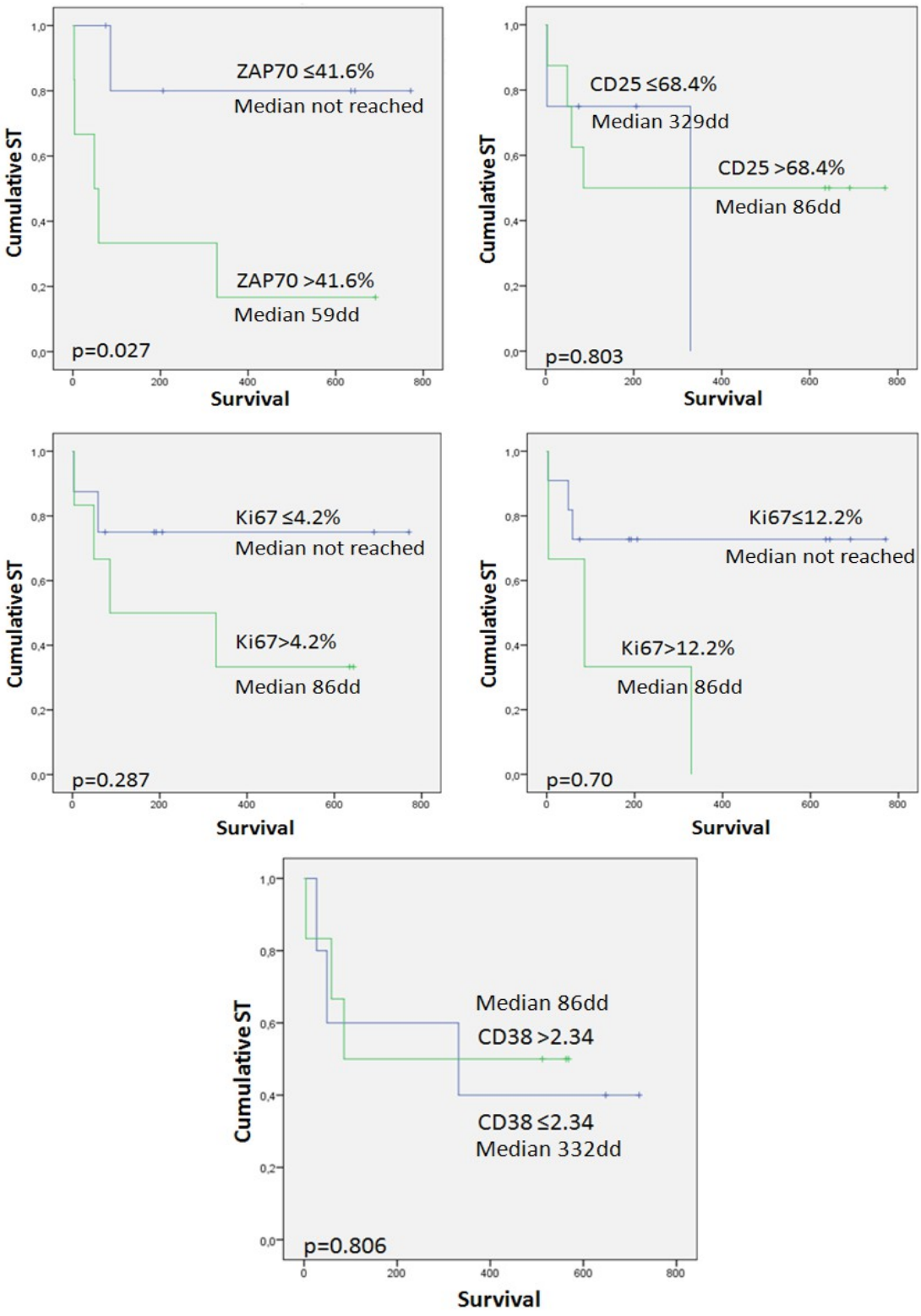

Fig.ure 21: Kaplan-Meier curves of the markers Zap70, CD25, ki67 and CD38.

Table 5: relative expression , expression group and outcome of dogs enrolled for CD38 analysis.

Case	CD38 RE	Expression group	Survival	Outcome
1	7,009823	high		Unavailable
2	4,358335	high		Unavailable
3	0,829491	low		Unavailable
4	4,919763	high	563	Lost
5	0,395959	low	332	Leukemia
6	2,25328	low		Unavailable
7	1,71247	low		Unavailable
8	0,275799	low		Unavailable
9	0,392655	low	27	Leukemia
10	2,341277	low		Unavailable
11	4,704521	high		Unavailable
12	0,351336	low		Unavailable
13	6,783545	high		Unavailable
14	0,062443	low	641	Lost
15	3,429273	high		Unavailable
16	1,568132	low	771	Alive
17	8,640013	high	59	Leukemia
18	6,975293	high		Unavailable
19	0,116931	low	49	Leukemia
20	5,820898	high	691	Alive
21	4,186288	high	635	Alive
22	5,967231	high	86	Leukemia
23	1,234057	low		Unavailable
24	4,646609	high	4	Leukemia

Discussion

In contrast with the human counterpart, B-Chronic lymphocytic leukemia are uncommon diseases in dogs in which CD8+ T-CLL is the most diffuse leukemia subtype. Recent studies (Brombereck et al., 2016) has described the clinical characteristics and breed predisposition of B-CLL in a cohort of 491 dogs but no prognostic information was recorded. In the present preliminary study, we prospectively investigated four putative prognostic markers of B-CLL (ZAP-70, Ki67, CD25, and CD38) for their possible use via flow cytometry on a prospective clinical study. The choice of these markers was based on studies in human CLL, since canine B-CLL shares many features with human CLL. In humans, ZAP-70 protein has been considered a predictor of IgVH mutational status in 93% of patients. ZAP-70 expression and IgVH mutation status were comparable in their ability to predict time to treatment requirement following diagnosis. CLL that is positive for the marker ZAP-70 has an average survival of 8 years while CLL that is negative for ZAP-70 has an average survival of more than 25 years (Wiestner et al., 2003; Rossi et al., 2010). The evaluation of ZAP-70 in flow cytometry assumed a diagnostic role for human CLL in order to predict prognosis, but its use has been currently replaced given the availability of feasible and cheap molecular tests to directly define mutational status. The molecular testing, in general, can be performed using a variety of methods. The methodology used for the test may vary from one laboratory to another, however RT-PCR of RNA and sequencing of cDNA of the IGVH detection of mutation from germline sequence is the preferable method (Capello 2004). IgVH mutation analysis combined with FISH, ZAP-70, and beta-2 microglobulin measurement provide comprehensive prognostic assessment and may be used to determine the approach to therapy for all CLL patients.

To our knowledge ZAP-70 expression on lymphoid cells has not been yet described in dogs but ZAP-70 has been evaluated by our research group at mRNA level showing a high expression in T lymphocytes and T-CLL neoplastic cells (Mortarino et al., 2009). In canine B-CLL a variable expression of ZAP-70 mRNA was found but its prognostic role has not been elucidated yet. In the present research, we evaluated ZAP-70 protein expression on B neoplastic cells from canine B-CLL using a monoclonal antibody (clone SB ZAP) designed for the human protein. The cross-reactivity of the antibody was first evaluated comparing “in silico” canine and human sequences via Blast tool then by

Western Blotting on peripheral blood mononuclear cells. In addition, reactivity was evaluated via FC on normal lymphocytes from healthy dogs, confirming that ZAP-70 is expressed in T-cell subtypes but not in normal circulating B-cells. In contrast, neoplastic B-CLL cells expressed variable positivities to ZAP-70 (Table 4). Preliminary assessment of the possible prognostic value of ZAP-70, using an arbitrary cut-off set at the median percentage value of all B-CLL cases (41,6%), showed that cases with high expression of ZAP-70 are statistically correlated to a shorter survival, similarly to what reported in humans. Results open interesting perspectives on the possible use of this marker in a clinical setup to predict prognosis, although our data need to be validated on a larger cohort of dogs with an adequate follow-up and a consistent therapy in order to define the best cutoff value to discriminate between long and short survivor dogs. In addition, more studies will be necessary to define if, similarly to humans, the positivity to ZAP-70 is related to IgVH mutational status that has been recently evaluated in a recent paper in dogs showing a breed predilection pattern (Rout et al., 2018).

Ki67 is a non-histone nuclear protein expressed in all active phases of the cell cycle and virtually absent in resting cells. Its use as a diagnostic and prognostic marker is well documented in various human malignancies and recently our research group developed a flow cytometric test to evaluate Ki67 in lymphoma cells showing a good correlation with tumor grading by using a cutoff value of 12,2% (Poggi et al, 2015). In the present paper we evaluated the percentage of Ki67 positive cells using both an arbitrary cutoff set at the median percentage of B-CLL cells (4,2 %) and the cutoff value suggested for lymphoma by Poggi et al. In both the cases no statistical association with survival time was found but the lower percentage value rendered a lower p value suggesting that this marker should be evaluated on a wider cohort of dogs to definitely rule out its possible use as a prognostic index or, as an alternative, to define the best cutoff value to be used in a clinical setup.

CD25 is an activation marker in lymphocytes that has been described as variably expressed by canine B-CLL and other lymphoid malignancies, with a predilection on some specific breeds. The rationale to test this antigen on canine B-CLL is that, since CD25 has been reported to be expressed in B memory cells (Amu et al, 2007), it could be related to a more mature development pattern that could be linked to a different

outcome. However, results from this preliminary study did not show any association between CD25 expression and ST, neither in terms of statistical analysis nor as a trend, thus suggesting that it is not a promising marker for predicting prognosis.

CD38 is a cell surface glycoprotein acting as an enzyme and a receptor for CD31.

In humans with CLL, the expression of CD38 is correlated to the one of CD49d, marking a CLL subset with very poor prognosis (Zucchetto et al, 2012). Similarly to ZAP-70, CD38 immunoreactivity has been also considered a surrogate of high-risk subtype of CLL and of un-mutated IgGVH. Since to date, neither specific or cross-reactive antibodies are available for canine CD38 we decided to use a molecular approach to preliminary investigate at RNA level if this molecule could be associated to a different outcome in dogs with B-CLL. Unfortunately, our preliminary results seems to discourage the use of such a target for prognostic purposes in dogs since no correlation was found among survival and CD38 mRNA. The limit of such an approach is that we evaluated mRNA for CD38 on all leukocytes population and not on sorted neoplastic cells and this could have biased results since gene expression could have been influenced by other subtypes than neoplastic cells. However, no correlation was found among CD38 mRNA relative expression and purity of neoplastic cells determined via FC, thus we consider CD38 as a less promising prognostic marker for canine B-CLL, also in light of the lack of a specific antibody for FC.

One of the major pitfalls of the present study is the low number of cases with a complete follow- up. However, to date, very few papers investigating the outcome of B-CLL are available (Comazzi et al., 2011) and the long survival of such diseases make difficult to reach the end point of new prospective studies. An increase of caseload with an adequate follow-up is necessary to confirm our preliminary results.

The second pitfall to be considered is related to therapy which is not consistent among cases enrolled. Therapies were highly variable among cases and were left to the clinician's and the owner's choice, varying from no therapy, to corticosteroids, to chlorambucil and this treatment may be variable also in the same patients during the time. In addition, a previous work (Comazzi et al., 2011) demonstrated that therapies have minimal or no effect on overall survival of dogs with CLL and that the choice for different protocols is generally based on the clinical status of the patient and aimed to improve the quality of life and to reduce complications.

Again an increase of caseload will be advisable to confirm these results in dogs treated in a consistent way.

The results of the present study suggest that ZAP-70 and Ki67 are likely the most promising target molecules to be investigated via FC to predict prognosis of B-CLL. Further prospective studies on a larger cohort of dogs, treated with the same protocol and with a long follow-up (in light of the indolent behavior of CLL) are needed not only to confirm our results but also to define the best cutoff values useful in a clinical setup to split population with different outcomes.

The results of the present study will be object of a specific paper to be submitted to peer review international journals.

4. Conclusions

Results achieved within the present project provide useful information for the definition of some specific tumor entities, and support the use of FC for the diagnosis of lymphoproliferative neoplasms.

In particular, from the first study emerge that flow cytometry performed on canine LN FNA specimens is a suitable diagnostic tool to confirm lymphoma, regardless of animal characteristics. Veterinarians should make any possible effort to obtain a highly cellular sample.

Regarding TZL, we can confirm that it is an indolent lymphoma characterized by a long survival, despite the V stage of the disease at presentation. We can also state that the absence of CD45 protein is likely due to the absence of gene transcription, providing a new tile for the comprehension of the pathogenesis of this tumor.

The issue of nMZL, disclosed that dogs may present at an advanced stage of disease with an overall poor prognosis, providing useful information for better understanding the behavior of this entity, usually classify as indolent, and opening a discussion of clinical interest about therapeutic approach.

Finally, the study on B-CLL encourages further investigations for the use of ZAP70 as novel marker for B-CLL stratification.

All the studies taken together underline, another time, how the use of FC could be an important resource in veterinary oncohematology, for both diagnosis and practical approach to the disease.