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CICLO XXXII

FLOW CYTOMETRY AND SUPPORTIVE TECHNIQUES FOR THE DIAGNOSIS AND CHARACTERIZATION OF FELINE LYMPHOPROLIFERATIVE DISEASES

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RIASSUNTO

La citometria a flusso è una tecnica di immunofenotipizzazione utilizzata comunemente in medicina umana per definire l'origine delle neoplasie ematopoietiche, anche se non si tratta del suo unico utilizzo in questa specie.
L'utilizzo in medicina veterinaria, nella pratica clinica, riguarda quasi esclusivamente lo studio delle neoplasie di origine ematopoietica. Tuttavia, la tecnica è molto più diffusa e studiata nel campo dell'oncologia canina, mentre nella specie felina sono presenti diverse limitazioni (Wilkerson et al, 2012; Guzera et al, 2016).
Una delle limitazioni più rilevanti per la specie felina è la scarsa disponibilità di anticorpi specie-specifici o cross reattivi per i cluster di differenziazione (CD) leucocitari del gatto. Questo non permette un'indagine sufficientemente approfondita delle cellule neoplastiche.
Altra importante limitazione nella specie riguarda la localizzazione delle lesioni neoplastiche: un'elevata percentuale di linfomi nel gatto sono di origine intestinale (Lowerens et al, 2005). Questo rende difficilmente campionabili le lesioni, soprattutto se si tratta di infiltrazioni uniformi del tessuto intestinale o con basso coinvolgimento delle stazioni linfonodali, e il campionamento non è agevole a causa della richiesta, spesso, di anestetici a causa dell'indole dei pazienti.

L'obiettivo di questo progetto di dottorato è quello di prendere in considerazione vari aspetti della diagnosi di linfoma e leucemia nella specie felina, utilizzando la citometria a flusso come tecnica principale e confrontandola anche con altre metodiche. Data la scarsità di dati in letteratura si è inteso procedere a partire dalla valutazione della fattibilità analitica e dalla determinazione dei valori di normalità per poi procedere ad esaminare alcuni particolari tipi di linfomi e soffermarci sull'eventuale significato clinico-patologico di alcuni specifici markers antigenici considerati buoni candidati per arricchire i pannelli antigenici citofluorimetrici nella specie felina.

A questo fine, verranno di seguito presentati quattro studi. Il primo è uno studio retrospettivo riguardante i fattori preanalitici che possono influenzare la qualità dei campioni per la citometria a flusso e quindi la loro possibilità di essere processati. Dal database del servizio di citometria dell'Università di Milano, sono stati selezionati, tra il 2009 e il 2016, casi

di sospetto linfoma con lesione primaria o versamenti disponibili per la citofluorimetria. Le variabili preanalitiche considerate riguardavano l'animale, il campionamento, la lesione e il clinico che ha effettuato il campionamento. All'arrivo dei campioni veniva valutato l'aspetto macroscopico e la cellularità. La cellularità è risultata la variabile che maggiormente influenzava la possibilità di un campione di essere processato per la citometria ed essa stessa è risultata influenzata dalle dimensioni dell'ago usato per il campionamento (maggiori performance con 21 G). Il 22% dei campioni processati non è risultato diagnostico; non sono state evidenziate differenze di cellularità tra i campioni toracici/addominali e periferici, né riportati effetti collaterali post campionamento, eccetto in un caso.

Il secondo studio è una raccolta prospettica di linfonodi di gatto non neoplastici, analizzati mediante citometria a flusso, citologia e istopatologia. L'obiettivo dello studio era quello di descrivere le sottopopolazioni linfocitarie in linfonodi di gatto non neoplastici. 16 linfonodi sono stati campionati da 11 pazienti, sono state ottenute delle sospensioni cellulari e dei preparati citologici per ciascun campione. Una metà di ogni linfonodo è stata conservata la diagnosi istologica. I risultati osservati per la citometria e la citologia erano molto simili a quelli descritti nel cane, con una maggiore proporzione di cellule di medie dimensioni. L'esame istologico ha rivelato la natura iperplastica di 5 campioni, che considerati separatamente hanno mostrato un'espressione significativamente maggiore di CD8 (P=0.008). Ulteriori studi sono necessari per comprendere potenziali differenze tra linfonodi neoplastici e non neoplastici nella specie felina.

Il terzo studio è una raccolta retrospettiva di masse mediastiniche di pazienti felini, analizzate mediante citometria a citologia. Lo scopo del lavoro era quello di comparare l'immunofenotipo di lesioni linfomatose e non, per valutare l'utilità della citometria a flusso nella distinzione tra i due tipi di lesione. Sono stati inclusi 19 casi, di cui 13 linfomi e 6 lesioni non linfomatose. Tra i linfomi, il fenotipo più comunemente riscontrato è stato CD4+CD8+ doppio positivo, mentre nella popolazione linfocitaria delle lesioni non linfomatose (policlonali mediante PARR) sono state riscontrate tutte le sottopopolazioni, tranne in un caso in cui i linfociti CD4+CD8+ doppi positivi erano il 78.8%. Secondo i nostri risultati quindi, la citometria non è sufficiente a discriminare in modo attendibile le due entità, perché nel gatto i linfomi CD4+CD8+ doppi positivi sembrano essere decisamente comuni. Perciò il cut-off proposto per il cane (altamente specifico per timoma), non è applicabile per il gatto.

Il quarto studio è un'analisi retrospettiva dei marker panleucocitari nelle popolazioni di leucociti in pazienti felini in salute, con patologie infiammatorie e neoplastiche. L'obiettivo dello studio era quello di delineare la modalità di espressione dei marker CD18 e CD44 nei leucociti nel sangue periferico di pazienti sani e fornire anche dei dati preliminari sulle possibili variazioni conseguenti al diverso stato funzionale (quiescente, reattivo, neoplastico). CD18 e CD44 sono stati valutati mediante citometria a flusso in 16 campioni di gatti sani e 21 con diverse condizioni patologiche. Nei gatti sani, entrambe le molecole erano espresse ad un livello maggiore nei monociti, medio nei polimorfonucleati e basso nei linfociti. L'intensità di fluorescenza mediana (MFI) del CD18 discriminava bene le tre popolazioni, mentre quella del CD44 era in gran parte sovrapponibile tra monociti e polimorfonucleati. Le cellule linfoidi reattive avevano un'espressione di CD18 maggiore rispetto ai linfociti quiescenti, mentre nessuna differenza è stata rilevata nella MFI del CD44 tra questi due gruppi. Entrambe le molecole erano espresse in maniera variabile sulle cellule neoplastiche di diversi soggetti, ma la MFI del CD44 tendeva ad essere maggiore rispetto a linfociti quiescenti e reattivi.

Nell'insieme, i risultati del mio progetto portano a considerare la citometria a flusso come una tecnica ancora molto immatura per l'oncologia nella specie felina, che necessiterebbe di ulteriori indagini, soprattutto riguardanti lo sviluppo di nuovi marcatori, nonché di una maggiore collaborazione da parte del mondo clinico per poter effettuare valutazioni clinico-patologiche e cliniche su numeri maggiori, essendo il linfoma una patologia estremamente diffusa nella specie felina.

ABSTRACT

Flow cytometry (FC) is an immunophenotyping technique routinely applied in human medicine to assess the origin of hematopoietic malignancies, even if this is not its only use in human species.

In veterinary medicine, its use in clinical practice mainly concerns with the evaluation of hematopoietic malignancies. However, this diagnostic tool is more widely and commonly applied in canine oncology, whereas in feline species there are many limitations for an extensive and routine use of this technique (Wilkerson et al., 2012; Guzera et al., 2016).

One of the most relevant limitations for feline species is the poor availability of feline species-specific and cross-reactive antibodies to the leukocyte clusters of differentiation (CDs). This doesn't allow a sufficiently detailed investigation of neoplastic cells.
Another important limitation for the species concerns with the localization of lymphomas: great part of lymphomas in cat are of intestinal origin hence intra-abdominal (Lowerens et al., 2005). This makes lesions harder to be sampled, especially when it is a homogeneous infiltrations of the bowel or without a significant involvement of regional lymph nodes. The sampling is even not encouraged by the frequent need of general anaesthesia because of the temperament of feline patients.

The aim of this PhD project is to consider different aspects of diagnosis of lymphoma and leukaemia in feline species, using flow cytometry as main tool, supported by more widely used and strong techniques.
Due to the paucity of data in literature on the topic we planned to start to evaluate analytic feseability and the evaluation of normality data then going on with the evaluation of some specific lymphoma subtypes and the potential clinic-pathological meaning of some candidate markers that could be inserted in FC panels for cats.

To this aim, four studies will be illustrated. The first one is a retrospective study about pre-analytical factors possibly affecting the quality of samples submitted for flow cytometry and thus their likelihood of being processed. Between 2009 and 2016, samples of suspected lymphoma with primary lesions/lymph nodes/effusions available for flow cytometry were selected from FC service's database of the University of Milan. Pre-analytical variables that were considered were related to the patient, the sampling procedure, the lesion and the clinician who performed the sampling. At their arrival in the lab, gross aspect and cellularity of the samples were assessed. Total nucleated cell count (TNCC) came up to be the variable that mostly affected the likelihood of a sample to be processed for FC and TNCC itself was influenced by caliber of the needles used for sampling procedure (21 G being the most performing). 22% of samples analysed were not conclusive; differences in cellularity between thoracic/abdominal and peripheral samples were not identified. No side effects following sampling were reported by vets, except in one case.

The second study is a prospective collection of feline non-neoplastic lymph nodes, analysed by FC, cytology and histopathology. The aim of the study was to describe the lymphocyte subsets in feline non-neoplastic lymph nodes to create the basis for comparison in neoplastic samples. Sixteen lymph nodes from 11 patients were collected, cellular suspensions were obtained and cytological smears were done. A half of each lymph node was preserved in order to perform histology. The results observed for the FC and cytological analysis were very similar to those described in the dog, with a higher proportion of medium size lymphocyte. Histological examination revealed the hyperplastic nature of 5 samples which considered separately from the others, showed a significantly higher expression of CD8 ($p=0.008$). Further studies are needed to understand the potential differences between non-neoplastic and neoplastic lymph nodes in feline species.

The third study is a retrospective collection of feline mediastinal masses, analysed by FC and cytology. The aim of the study was to compare the immunophenotype of lymphoma and non-lymphomatous lesions in order to assess if FC could reliably support the distinction between these two entities. 19 cases were finally collected: 13 lymphomas and 6 non-lymphomatous lesions. Among lymphomas, the most common immunophenotype detected was CD4+CD8+ double positive T-cell, while in non-lymphomatous lesions, lymphocyte population was composed by heterogenous T-lymphocyte subsets, except in one case in which CD4+CD8+ subset was dominant, reaching 78.8%. According to our results, FC is not enough to discriminate these two entities, since in cats, CD4+CD8+ double positive lymphomas are likely very common, thus the cut-off proposed for canine species (which was highly specific for thymomas) was not applicable to cats. Anyway, a larger caseload would be warranted, and histopathology should be available for every sample in order to reach a final diagnosis.

The fourth study is a retrospective analysis of pan-leukocyte markers on feline WBC populations in healthy, reactive and neoplastic samples. The aim of the study was to depict the pattern of expression of CD18 and CD44 on WBC subclasses on peripheral blood (PB) of healthy cats, and to provide preliminary data on possible variations with different functional states (resting, reactive and neoplastic) similarly to what already done in the dog. Samples from 16 healthy cats and 21 cats with different pathological conditions were tested by FC for CD18 and CD44 expression. In healthy cats, both molecules were expressed at higher level on monocytes, medium level on PMNs and lower levels on lymphocytes. CD18-Median Fluorescence Index (MFI) discriminated well the three population, whereas CD44-MFI mostly

overlapped between monocytes and PMNs. Reactive lymphoid cells had higher CD18 expression compared to resting lymphocytes, whereas no difference was detected in CD44-MFI between the two groups. Both molecules were variably expressed on the neoplastic cells from different individuals, but CD44-MFI tended to be higher than in resting and reactive lymphoid cells.

Overall, the results of this PhD project lead to account FC as an “immature” technique for the study of hematopoietic malignancies in feline species, that would need further investigations, mainly concerning the development and application of new markers, as well as a greater cooperation from clinicians in order to set better clinicopathological and clinical assessment on a greater number of patients, being lymphoma a quite common neoplastic disease in feline species.

1. INTRODUCTION

Feline lymphoma represents a real diagnostic challenge in veterinary oncology to date. Lymphadenopathy in feline species can be investigated with cytological examination (Ku et al, 2017), but flow cytometry isn't part of the routine diagnostic work-up. Moreover, peripheral lymphadenopathy in cats has seldom a neoplastic origin, unlike canine species. Frequently, feline lymphoma originates in extra-nodal sites: intestinal and mediastinal lymphoma are the most frequent among them (Louwerens et al, 2005; Barrs and Beatty, 2012; Moore, 2013; Fabrizio et al, 2014; Wolfesberger et al, 2016).

As mentioned above, among the techniques available to investigate lymphoma, FC isn't frequently used in the cat, like PCR for antigen receptor rearrangements (PARR), since both are recent tools about which a poor scientific literature is available to date (Moore et al, 2005; Werner et al, 2005; Guzera et al, 2016; Hammer et al, 2017) concerning feline species.

Thus, clinicians usually prefer to investigate suspect neoplastic lesions by histology and immunohistochemistry (IHC), being the gold standard for the diagnosis of lymphoma in feline species (Gabor et al, 1999; Valli et al, 2000; Wolfesberger et al, 2016). But, unlike FC, IHC (and Immunocytochemistry too) doesn't allow to assess the expression of more markers at the same time on the same cells, which is the multi-colour approach allowed by the flow cytometric technology (Comazzi et al, 2006).

In the last years, FC became a routinely used technology even for diagnostic and prognostic purpose in human oncology (Widen, 1992; Weir and Borowitz, 2001; Varma and Naseem, 2011; Woo et al, 2014; Lee et al, 2015; Grimwade et al, 2017). In veterinary medicine, its use is mainly limited to laboratories in academia, since costs are high and specifically trained staff is required; in this field, its main application concerns diagnosis, staging, and monitoring in hemato-oncology (Chabanne et al, 2000; Culmsee and Nolte, 2002; Wilkerson MJ et al, 2005; Aresu et al, 2014; Martini et al, 2015; Comazzi et al, 2017).

In the following sections, FC is described in all its technical aspects.

1.1 FLOW CYTOMETRY

The actual application of flow cytometry as a diagnostic tool in veterinary oncology is quite recent. Its usefulness concerns diagnosis of hematopoietic malignancies, immunophenotyping and evaluation of minimal residual disease of lymphoma and

leukaemia. The qualities that make FC a good diagnostic technique are the following: the mini invasiveness of sampling, the cost-effectiveness of the test and the possibility to obtain a quick result (usually in 24 h from sampling) (Comazzi and Gelain, 2011).

The general concept of the functioning of FC is that cells in suspension are labeled with monoclonal antibodies to bind specific antigens (Cluster of Differentiation, CD); fluorochromes used for the labeling phase are detected by a laser system and reported to an electronic device together with morphological features of the cells labeled.

1.2 TECHNICAL ASPECTS

The cytometer is an analyzer that interrogates cells/particles suspended in a solution through a hydraulic system, a light system (lasers) and an electronic system (detectors and computer). The functioning of a cytometer is resumed in *figure 1.*

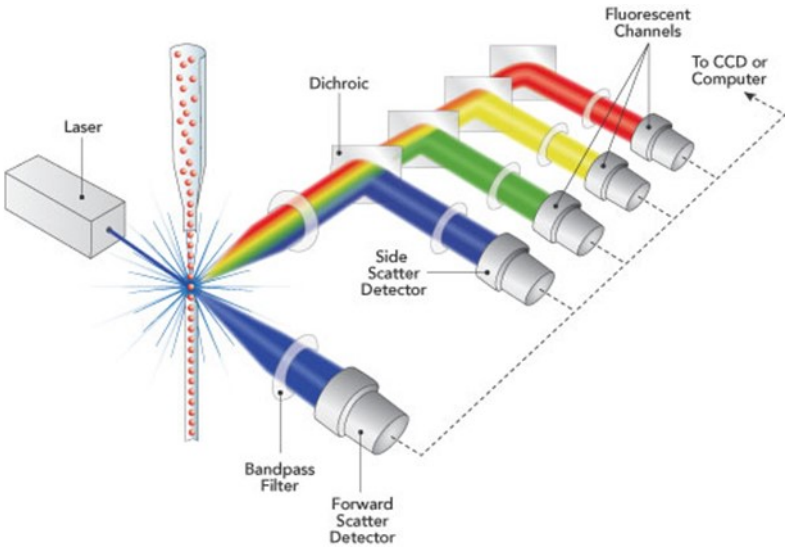

Figure 1: Flow Cytometer functioning.
(Source: *http://flowcytometry.med.ualberta.ca)*

Cells are conveyed by a liquid stream in a single line through a light source, which is able to excite fluorochromes that are attached to cells. Once that the fluorochrome has been excited, it returns to a quiescent state and emits a fluorescent signal which goes through an optic system to a detectors' system. The latter transforms the electronic signal into a

digital signal and transmits it to a computer: at this point the operator is able to analyze data which are usually reported in dot plots, even if other kinds of output are available. The forward-angle scatter (FSC) collects the light diffracted from the cell in proportion to its dimension, whereas the side-angle scatter (SSC) is proportional to cellular complexity (granularity of cytoplasm and lobulation of nucleus). These two parameters define the morphology of the investigated cells and this is represented by a morphological cytogram (FSC vs SSC). In *figure 2* a cytogram shows SSC along y-axis and FSC along the x-axis, representing larger and more complex cells upper right (granulocytes) and smaller and less complex cells lower left (lymphocytes).

Fluorescence is detected with the same angle as the side scatter: the fluorescence intensity is proportional to the quantity of antibody bound to the antigens of the cells and these light pulses are processed by a digital source and represented as dots on the software. The lasers can excite one or more fluorochromes with different wavelengths and these light pulses can be detected by different single detectors (photomultipliers) (Tarrant, 2005).

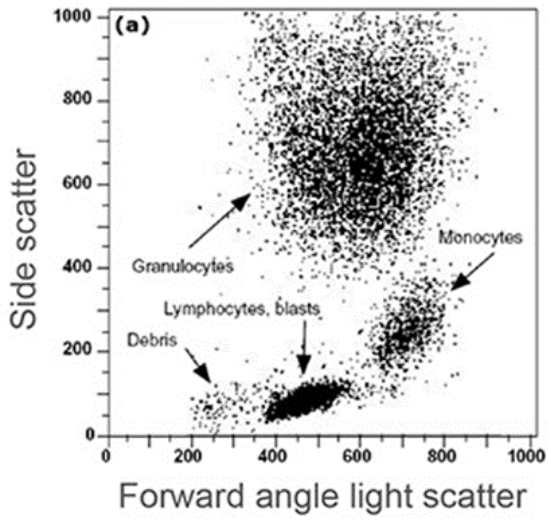

Figure 2: Dot plot: morphological cytogram.

1.3 ANTIBODIES

The available antibodies for FC application in veterinary medicine are limited (Weiss 2002, Aniolek 2014), especially in feline species (Brodersen et al, 1998; Meister et al, 2007). Nevertheless, in the last two decades the number of cell markers has increased and what is more important, pre-conjugated antibodies with different fluorochromes have become available for flow cytometry also for veterinary species. The relevance of this aspect is that two or more cell markers can be used simultaneously on the cells, in the same tube, because the fluorescence originated from labeled cells are different and can be read by different detectors concurrently. This also makes the analysis more efficient, because of a lower request of reagents and cells.

Fluorophore	Fluorescence color	Maximum excitation (nm)	Maximum emission (nm)
DyLight® 405		400	420
Alexa Fluor® 405		401	421
Pacific Blue™		410	455
DyLight® 488		493	518
Alexa Fluor® 488		495	519
FITC		490	525
DyLight® 550		562	576
PE		496, 546	578
Texas Red®		596	615
APC		650	661
Alexa Fluor® 647		650	665
Cy5		649	670
DyLight® 650		654	673
PerCP		490	675
DyLight® 680		692	712
Alexa Fluor® 700	Infrared	702	723
DyLight® 755	Infrared	752	778
DyLight® 800	Infrared	777	794

Table 1: List of commonly used fluorochromes for FC and their wavelength.

The most used fluorochromes in veterinary medicine are FITC (fluorescein isthiocyanate), PE (phycoerythrin), PerCP (peridinin chlorophyll protein), APC (allophycocyanine) and Alexa Fluo 647, which are listed in the following table (table 1).

As a general rule concerning the choice of antibodies, all the tubes should contain a common antibody to trace cells in the same way along the panel. The commonly used antibodies for this purpose are pan-leukocyte markers: CD45 for canine species and CD18 for feline species. When investigating a suspected neoplastic population, more markers of the same lineage might be needed, because maturation stages can be different and aberrancies could be present.

A good way to investigate neoplastic cells is to start with a basic panel, common to all the samples according to the tissue available and the tentative disease (for example leukemia vs lymphoma). After a first step, the panel will be enlarged addressing more antigens with a diagnostic, prognostic and sometimes research purpose. If a solid mass or a lymph node has been investigated with FC, blood and bone marrow can be investigated for the same cells, if they are available, in order to define the stage of lymphoma. Nevertheless, this is not so true for feline patients, where the antibodies' panel is reduced and it is usually applied fully on all samples. In veterinary medicine, there is no universally accepted diagnostic algorithm, but the panel to be used depends on the laboratory.

1.4 SAMPLES

Samples required for FC are cells in suspension. This makes peripheral blood, bone marrow, and body fluids the most suitable tissues for FC assay. Therefore, in order to analyze lymph nodes, solid masses, and other organs, they should be reduced to suspended cells.

Thus, the latter will be collected in tubes containing a preservation medium (usually RPMI 1640 + azide) (Liu et al, 2001).

The specimens intended for FC should be sampled, sent and delivered to the laboratory within 24 hours (Nguyen et al, 2007; Comazzi and Gelain, 2011), according to storage recommendations, which provide refrigeration temperatures (Jalla et al, 2004). The maximum storage that allows analysis is usually 48 hours, but neoplastic cells' integrity and antigens might be affected by storage times longer than 24 hours (Jalla et al, 2004).

Anyway, viability stains such as Propidium Iodide (PI) are useful to assess the quality of processed samples (Comazzi and Gelain, 2011), thus it is often included in the base panel or sometimes it is performed as a preliminary step to the complete panel.

A recent study concerning pre-analytical factors affecting canine samples (Comazzi et al, 2018) showed that clinicians, cellularity, tissue/s sampled and necrotic material were significantly related with the likelihood of samples to be diagnostic.

Another essential step for the analysis is red blood cells (RBCs) lysis, which is mandatory for peripheral blood and bone marrow and just if necessary, according to gross haemodilution, for lymph nodes, solid masses, and fluids. A water solution containing ammonium chloride provides osmotic damage to the red blood cells whereas preserves mostly leukocyte membranes from rupture. RBCs lysis can be performed before or after the labeling of white blood cells; in any case, a washing and centrifugation step afterwards is required to eliminate debris.

Prior to labeling step, it is mandatory to add a blocking agent to cells: this is normally fetal bovine serum (FBS) and it is necessary to block non-specific bindings. Afterwards, labeling phase can proceed. Specific antibodies (or sometimes cross-reactive antibodies,) are added to the samples and let incubate for 15-20 minutes at refrigeration temperature.

In some cases, antibodies might be unconjugated: this requires a two-steps procedure with a secondary antibody (normally polyclonal), pre-labeled with a fluorochrome.

At the end of each incubation phase, a washing step is required anyhow. Supernatant is thrown and cells are resuspended in phosphate buffer solution (PBS) 1X and are ready for acquisition.

1.5 DATA ANALYSIS

Consequently to acquisition, the data registered by the software have to be interpreted by a trained operator. The form in which data are represented depends on the operator, according exclusively to his/her preference. The most frequently used form is the two-parameters plot/cytogram (dot plots), in which results of physical parameters FSC and SSC are coupled (morphological scattergram), a single physical parameter is plotted with a fluorescent markers (for instance a common marker such as CD45) or two different fluorescences are plotted to evaluate co-expression a(Tarrant MJ, 2005). As a possible

alternative, a univariate frequency histogram can be used. *Figure 3* illustrates these two types of plots.

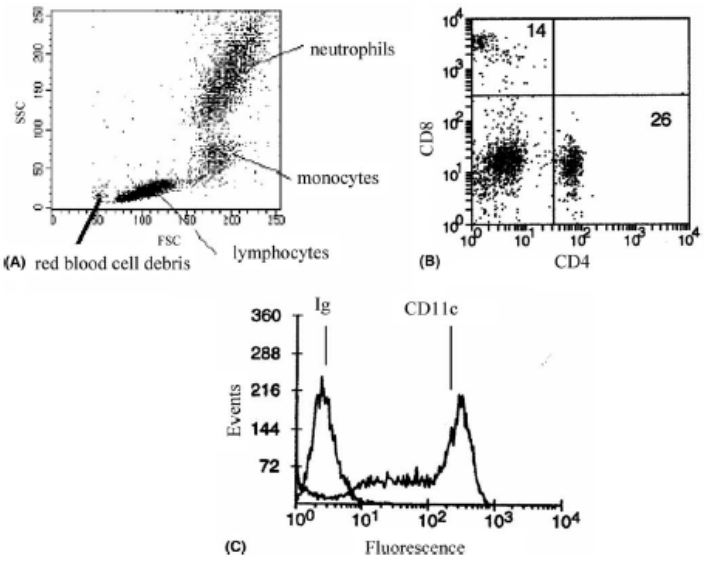

Figure 3 Dot plots (A, B) and frequency histogram (C) as a representation of FC data analysis. Tarrant MJ, 2005.

The dot plot representation is useful for the morphological evaluation of neoplastic population and, in blood samples, it is useful to separate lymphoid cells from granulocytes and monocytes (figure 3A). Drawing a dot plot scattergram is the starting step for creating a gate (usually oval to round in shape) to limit analysis just to the cells of interest. The *gate* might be done on the basis of morphological or fluorescence features, or both. This procedure is exemplified in figure 4 A-B.

This gating technique is also useful to exclude all but the population of interest from the analysis including debris, RBCs, other leukocytes, etc (Shapiro, 2004; Comazzi and Gelain, 2011). This procedure add a significant advantage to FC immunophenotyping since it allows to derive interesting clinical information from samples composed by a mixed population of cells without requiring a isolation/separation step.

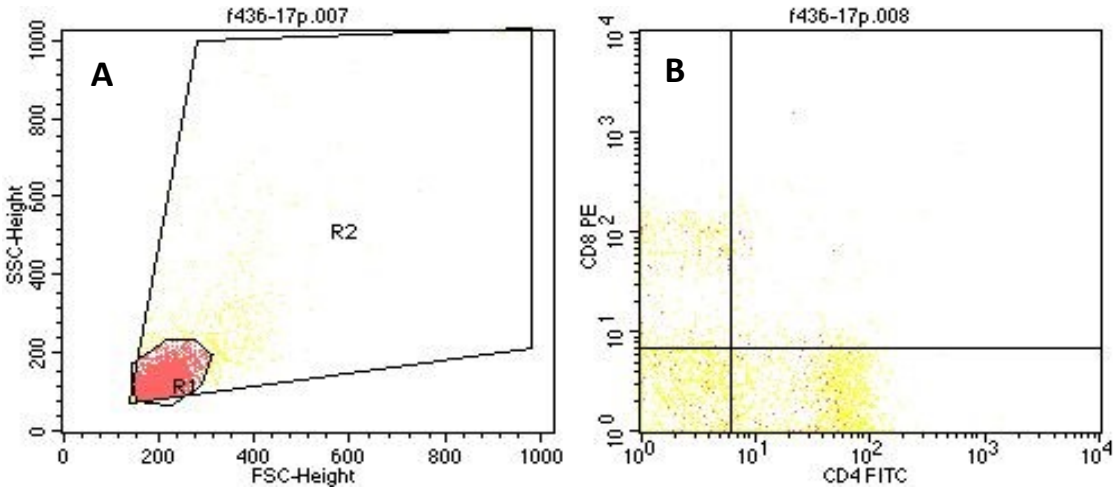

Figure 4 A, B. Scattergrams highliting the gating procedure. A is a morphological scattergram which allows to identify cells of interest on the basis of morphological features. B is the scattergram representing only the cells selected in R1, staining positive or negative for CD4, CD8 or both.

Usually, analysis is restricted to cells which stain positive for the panleukocyte marker (gating technique applied to CD18+ cells in feline species). The detected population is thus analysed with a multiparameter approach on further plots. The two-dimensional space of plots is divided into 4 quadrants: by moving the cutoff markers and information about the percentage of cells lying in each quadrant can be easily obtained. In addition for each cell population also fluorescence intensity of markers can be achieved.This data may have a diagnostic significance and in some cases also a possible prognostic significance: for example, in dogs, Major Histocompatibility Complex Class II (MHC II) is reported to be a positive prognostic factors when expressed as bright (high) (Rao et al, 2011).

2. FLOW CYTOMETRY APPLICATION TO FELINE LYMPHOPROLIFERATIVE DISORDERS

Lymphoproliferative disorder is a general term to denote a heterogeneous group of diseases of malignant origin, due to a clonal proliferation of lymphoid cells. Based on the prevalent solid or liquid presentation, lymphoid neoplasia are prevalently reported in two different groups *leukemia* and *lymphoma*. FC is widely applied nowadays in dogs for several purposes such as diagnosis, immunophenotyping, evaluation of prognostic markers and detection of minimal residual disease (MRD) (Williams et al, 2008; Comazzi and Gelain, 2011; Marconato et al, 2013; Riondato et al, 2016; Martini et al, 2013; Rao et al, 2011). Otherwise, its application is yet quite uncommon in cats, especially for diagnostic purpose (Gelain et al, 2006; Sharifi et al, 2007; Shirani et al, 2011; Guzera et al, 2016).

2.1 LYMPHOMA

Lymphoma is the most frequent hematopoietic cancer in dogs (representing 83% of hematopoietic tumors) and showing many common features with the human malignancy (Marconato, 2011). In both species, non-Hodgkin lymphoma is an heterogeneous group of malignancies with different subtypes and biological behaviours. Lymphoma in dogs appears to be a disease of middle-aged to older dogs, with some breeds being apparently more predisposed to develop the disease (Modiano et al, 2005). The 80% of dogs with lymphoma develops a multicentric form (Young and Vail, 2013). The most recent classification is based on the WHO scheme (figure 5).

Scientific literature concerning feline lymphoma is rather fragmentary compared to literature about lymphoma in dogs. Lymphoma was estimated to account for 30% of all tumours of feline species in the pre feline-leukaemia-virus (FeLV) era (Moulton and Harvey, 1990). According to the age and the retroviral status of the patient, lymphoma in cats may present in different anatomic forms (Louwerens et al, 2005). Mediastinal and multicentric forms are typical of younger cats and are often FeLV-related, whereas alimentary and cutaneous forms are typical of older patients and usually not related to a positive retroviral status. There are no specific breed studies about the predisposition to develop lymphoma, but Siamese cats were often over-represented in some case series

(Hardy, 1981; Gruffydd-Jones et al, 1979; Lorimer, 1999; Louwerens et al, 2005; Fabrizio et al, 2014).

Thus, involving different organs and sites, lymphoma in feline species can have different clinical presentations, unlike dogs where the most reported form is multicentric (nodal) lymphoma, presenting with generalized lymphadenopathy and often asymptomatic (Vail and Young, 2013).

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 5. Table summarizing 608 cases of canine lymphoma according to the updated Kiel classification compared to WHO classification according to Ponce et al, 2010.

Classification is grossly based on anatomical localization and further characterized in subtypes according to histopathological features (Valli et al, 2000; Vezzali et al, 2010; Wolfesberger et al, 2017). According to World Health Organization (WHO) cellular

morphology, histologic grade, immunophenotype, and localization allows to identify different subtypes. Histological evaluation is the gold standard for feline lymphoma since a cytological classification has not been applied on extensive case collections (Chino et al, 2013) in contrast with what has been done in dogs for Updated-Kiel classification (Ponce et al, 2010), in which different morphotypes has been reported also associated with prognosis (Sato et al, 2014).

Most of feline lymphoma were intermediate to high grade in an extensive study by Valli and colleagues of 2000, but most of cases were collected in the pre-vaccination era against feline leukaemia virus (FeLV). Since retrovirus-induced lymphoma may likely differ in sutbtypes and the retroviral status is not consistent among different studies, older case collections should be considered with cautions. Another more recent case series reported a greater number of ITCLs (intestinal T-cell lymphomas) than previously observed (Vezzali et al, 2010) and many studies are directly focused on alimentary lymphoma being apparently the most commonly observed form (Collette et al, 2016). In the study by Wolfesberger and colleagues of 2018, for example, 61 cases of alimentary lymphoma have been evaluated and 41% of these were classified as EALT type I (enterophathy associated T-cell lymphoma type I, namely a very aggressive type of malignancy) and 34% were classified as EATL type II (enterophaty associated T-cell lymphoma type II) according to the most recent definition (Valli et al, 2016).

A few studies considered the phenotype of lymphomas in feline patients in relation to the outcome (Patterson-Kane et al, 2004; Collette et al, 2016; Wolfesberger et al, 2017) showing no prognostic value for this feature. Otherwise, several studies have shown the aggressiveness and consequently poor prognosis of large-granular-lymphocyte lymphoma (LGLL) (Roccabianca et al, 2006; Krick et al, 2008; Finotello et al, 2017).

Unlike in canine species (Wilkerson et al, 2005; Gelain et al, 2008; Thalheim et al, 2013; Seelig et al, 2014; Martini et al, 2015), there are no studies in scientific literature assessing the prevalence of B or T immunophenotype in feline lymphomas by FC or eventual aberrant expression of leukocyte antigens. Flow cytometry is already a helpful diagnostic tool in veterinary oncology as mentioned above, but in feline species, it hasn't been applied frequently for a diagnostic purpose (Guzera et al, 2016). No data are published concerning the non-neoplastic counterpart of feline lymph nodes and that

should be the beginning for a deeper knowledge of the neoplastic samples,. As in dogs, a consensus recommendation should be warranted also in feline species.

In canine oncology, many studies are available relating flow cytometric features to diagnosis and prognosis of lymphoma (Gelain et al, 2006; Williams et al, 2008; Rao et al, 2011; Seelig et al, 2014; Martini et al, 2015; Martini et al, 2016; Mizutani et al, 2016; Cozzi et al, 2018). Such literature is not offered in feline oncology, thus much work for the application of this technique is yet to be done, enhancing its diagnostic potential with a wider panel of antibodies.

Unlike canine lymphoma, feline lymphomas are generally characterized by a restricted number of antibodies, due to the low number of antibodies available on the market, and in particular: B-cell lymphomas express CD21, whereas T-cell lymphomas express CD5 (Guzera et al, 2016) and in this latter case the neoplastic population can have a different commitment reacting positively to CD4 (T-helper lymphocytes) or CD8 (cytotoxic-T lymphocytes). Double positive CD4/CD8 phenotype in dogs has been shown to be highly specific for thymomas in mediastinal masses (Lana et al, 2006); otherwise, in cats such a study has never been proposed. CD18 is the commonly used pan-leukocyte marker which, in combination with forward side scatter properties, is useful to identify the neoplastic population. Anyway, small cellular size isn't forcedly sign of a benign origin; actually, there are no reports in feline species concerning a possible relationship between neoplastic behavior and cellular dimension.

Moreover, no studies are available using a multicolour approach, which is considered one of the most important advantages of using flow cytometry as a diagnostic tool (Comazzi and Gelain, 2011).

2.2 LEUKAEMIA

Leukemias are lymphohematopoietic disorders originating from bone marrow and characterized by prevalent bone marrow and/or peripheral blood involvement. Leukemias can be divided in lymphoproliferative and myeloproliferative, and further in chronic and acute disorders. This last categorization is clinically relevant since chronic leukemias are often associated with no clinical signs or mild non-specific clinical signs, thus diagnosed accidentally, running into a lymphocytosis possibly associated with other mild hematological features such as anaemia. Conversely, acute leukaemias are far more

aggressive in their clinical presentation, being primarily a disease of younger patients, often febrile, severely anaemic and/or thrombocytopenic with consequent asthenia/anorexia/lethargy (Adam et al, 2009; Novacco et al, 2016).

In normal conditions, canine or feline blood lymphocytes belong to different cell lineages having different functions. In particular, canine lymphocytes are 80% CD3/CD5+ T-cells (of which 45% CD4+ T-helper lymphocytes and 25% CD8+ cytotoxic-T-lymphocytes) and 15% CD21+ B-cells, the rest being double negative T-cells or natural killer (NK) cells. Feline lymphocytes have a similar distribution, but with more B-cells circulating (about 25%) compared to dogs (Byrne et al, 2000).

When one of these subsets expands in a considerable fashion or atypical cells are observed, a neoplastic origin of the cells can be suspected. Immunophenotyping by flow cytometry is one of the available tools for diagnosis and characterization of leukaemia in small animals and humans. Concerning CLL, in dogs, T-cell subset is the most commonly involved (Ruslander et al, 1997; Vernau and Moore, 1999), whereas in humans B-cell CLL is far more common representing the 95% of all cases of CLL (Jennings and Foon, 1997), having a fair prognosis whilst T-cell CLL in humans seems to have an aggressive behavior and poorer prognosis, according to some reports (Hoyer et al, 1995). While chronic leukemia is typical of elder patients in both species, acute leukemias are frequently described in younger patients, especially kids (Adams et al, 2004; Novacco et al, 2016; Iacobucci and Mullighan, 2017). Historically, morphology was used to make distinction between these two entities, considering mature lymphocytes produced by a chronic disease (CLL) and immature or blastic lymphoid cells the sign of an acute process ongoing (ALL). Anyway, morphological studies aren't enough to classify leukemias, but today FC is considered the gold standard for classification of leukemias in human oncology (Jenning and Foon, 1997;). Moreover, in human oncology, genetic studies are fundamental for the definition of cancer therapy (Iacobucci and Mullighan, 2017). The prevalence of B- vs T-cell ALLs in dogs is unknown to date: scientific data are contrasting, showing sometimes a predominance of B-cell ALLs (Adam et al, 2009; Tasca et al, 2009; Novacco et al, 2016), as in humans (Iacobucci and Mullighan, 2017), and sometimes a predominance of T-cell ALLs (Bennett et al, 2016).

Chronic lymphocytic leukaemia (CLL) is likely the most common form of chronic leukaemia in feline patients, even though statistical studies to assess the prevalence of the different types of leukaemia are not available. Thanks to flow cytometry, CD4+ T-cell lymphocytic leukaemia has been shown to be the most commonly detected in feline species (Workman and Vernau, 2003; Avery and Avery, 2007; Campbell et al, 2013) and typically not FeLV associated. No studies concerning the prognosis of chronic leukemia are available, but from some case series, it generally emerges as a disease of older patients also living 1 or 2 years after diagnosis (Workman and Vernau, 2003; Campbell et al, 2013). The clinical presentation is similar to that in dogs and humans, with few or no symptoms detectable in many cases. Whilst reviews and case series concerning chronic feline leukaemia are available (Workman and Vernau, 2003; Weiss, 2005; Avery and Avery, 2007; Campbell et al, 2013), occasional case reports concerning acute leukaemia are described in literature (Bounous et al, 1994; Comazzi et al, 2000; Nagashima et al, 2005; Mylonakis et al, 2008; Shirani et al, 2011). Just recently, a small case series of six cats with acute lymphoblastic leukaemia (ALL) has been published (Tomiyasu et al, 2018), reporting a very poor prognosis for the patients affected.

As mentioned above, immunophenotyping by flow cytometry is a valuable tool in the distinction of CLL from ALL or from stage V lymphoma. This is possible for canine species, since an antibody anti-CD34 is available to define the maturity of the cells involved. Specifically, this glycoprotein is expressed by immature hematopoietic cells, thus it is typically shown by cells in acute leukaemia (AL) (Vernau and Moore, 1999; Workman and Vernau, 2003; Gelain et al, 2008; Williams et al, 2008). It can happen that some lymphomas express CD34 as well (Gelain et al, 2008), but in these cases, as cells should have a mature immunophenotypic pattern and appearance, CD34 is considered an aberrancy. Conversely, ALs could show negativity to CD34 (Adam et al, 2009; Bennett et al, 2017) in a variable percentage of cases. Thus, expression of CD34 is not forcedly diagnostic of AL. Anyway, this marker is unfortunately unavailable in feline species;

. Another marker, CD44, has been evaluated in dogs and humans, showing a higher fluorescence (MFI) comparing controls to leukaemia groups and also chronic and acute leukaemia groups (Gelain et al, 2014) and being available, it might reveal useful for feline species too.

As for lymphomas, flow cytometry is a good investigation tool also for leukaemia in veterinary medicine. Nevertheless, a restricted number of studies are available and a few of these aim at assessing the possible prognostic role of immunophenotyping, especially talking about cats.

3. AIM

As illustrated, the application of flow cytometry carries many advantages and many information about behaviour, prognosis, stage of disease, cell lineage involved. This is true for canine and human species, but not for feline species. In cats many limitations are present towards the application of FC as a routine diagnostic tool, first of all the limited panel of commercially available antibodies. To date, *alimentary lymphoma* seems to be the most common form of lymphoma in feline species and this often prevents clinicians from sampling such lesions.

Thus, the general aim of this project was to assess the applicability of FC to lymphoproliferative disorders of feline species. To this general aim, four studies were developed. Specific objectives were:

- to evaluate pre-analytical factors that could affect flow cytometric analysis on lymph node samples, masses, body fluids and urine;
- to describe lymphocyte subsets in feline non-neoplastic lymph nodes;
- to assess if FC is a powerful tool in the distinction of lymphoma vs. non-lymphomatous lesions in feline mediastinal masses
- to describe the expression of CD18 and CD44 on white blood cells of healthy individuals and provide some data about inflammatory and neoplastic status.

To address these aims, the following four studies are going to be illustrated:

1. Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples.
2. Cytological and flow cytometric analysis of lymphocyte subsets in non-neoplastic feline lymph nodes.
3. Feline mediastinal masses: flow cytometrical analysis of lymphomas and non-lymphomatous lesions.
4. The pattern of expression of CD44 and CD18 molecules on the cell surface of White Blood Cells in cats: preliminary results on healthy and diseased animals.

4. FLOW CYTOMETRY FOR FELINE LYMPHOMA: A RETROSPECTIVE STUDY REGARDING PRE-ANALYTICAL FACTORS POSSIBLY AFFECTING THE QUALITY OF SAMPLES

Background

Since lymphoma is rather a common neoplastic disease in small animals (Vail et al, 2013), canine oncology claims to date one of the most useful diagnostic tools in terms of mini-invasiveness, cost-effectiveness, rapidity, characterization, prognosis and staging (Comazzi and Gelain, 2011). Otherwise, to date this is largely missing in feline species, since one study have been published concerning the topic so far (Guzera et al, 2016).

The application of FC in feline species leads a few limitations, first of all the localization of most of the lesions which is intra-abdominal, and namely often considered a difficult sampling procedure. The other important limit is the absence of a wide panel of antibodies, that are conversely available and increasing in canine species, to better investigate the disease and enhance the possibility to find prognostic association between markers and outcome.

Anyway, there are no studies documenting or contradicting the difficulty of sampling abdominal lesions in feline patients, and specifically not applied to FC. Thus, the aim of the present study was to evaluate pre-analytical factors which could possibly affect the quality of samples destined to FC analysis, trying to provide some useful indications for the sampling of suspected lymphomatous lesions in feline species.

Materials and methods

The database of the FC service of the Department of Veterinary Medicine, University of Milan, from January 2009 to February 2016 was interrogated and feline cases were extracted. Among them, only cases sent to the laboratory for suspect lymphoma were selected. Finally, cases were included in the present study only if the primary lesion had been sampled and sent to the laboratory for FC immunophenotyping. Effusion samples were also included. Cases were excluded from the study if only peripheral blood and/or bone marrow samples had been sent to the laboratory.

For each case, data concerning subject, lesion, sampling technique, ancillary tests performed and data concerning the clinician who collected the sample were asked to the

referring veterinarian, if not provided at the time of sample submission (Table 2). If samples from multiple sites were available for a single animal, the data were recorded for each sample independently. Additional data were retrieved from the FC database (Table 2).

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

Feature group	**Specific variables**
Animal	*Breed*
	Sex (male, neutered male, female, spayed female)
	Age (years)
	FIV/FeLV status (positive or negative)
	Presenting complaint
	Clinical findings
Sampling procedure	*Sample catching* (free hand, ultrasound-guided, computed tomography-guided, surgical approach, any other)
	*Pharmacological restraint (*none, sedation, general anesthesia)
	Sampling technique (fine needle capillary biopsy, fine needle aspiration, any other)
	Needle size (G)
	Occurrence of side effects (yes or not)
Lesion	*Site* (peripheral lymph node, thoracic mass, intra-abdominal mass, effusion, any other)
	Size (≤2 cm, between 2 and 5 cm, ≥5 cm)
	Cytological diagnosis
	Histopathological diagnosis

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

Table 2: Pre-analytical data asked to the referring veterinarians or reported in the laboratory record for 97 samples of suspected feline lymphoma, sent to the laboratory for flow cytometric immunophenotyping. Italics: variable included in the statistical analyses

Flow cytometry

FC was performed on tissue aspirates collected in a liquid medium (either saline solution or RPMI 1640) or on effusions collected in EDTA-tubes.

Prior to labelling, all samples were counted via an automated hematology analyzer (Sysmex XT-2000iV, Sysmex, Kobe, Japan) to assess cellularity. For aspecific antibody binding blocking, $1x10^6$ cells were put in each FC tube, together with 25 µl of RPMI 1640 medium containing 5% fetal bovine serum. The samples were then incubated with the antibodies for 20 min at 4°C. The antibody panel varied among samples: indeed, for samples processed between January 2009 and December 2010, a single-color approach was used, whereas a multi-color approach was used for samples processed between January 2011 and February 2016 (CD5-FITC/CD21-PE/CD18-AlexaFluor647; CD4-FITC/CD8-PE/CD18-AlexaFluor647). Antibody clones and source are listed in Table 3. All antibodies had been titered before use, to determine the best working dilution. After incubation, samples were washed twice in 500µl PBS 1x, and finally resuspended in 500µl PBS 1x for final acquisition.

Samples labelled with primary unconjugated antibodies, were washed once after the incubation. Then, a 20 min incubation at 4°C was performed, with a FITC-conjugated secondary antibody (rabbit antimouse IgG-FITC, polyclonal, Serotec). At the end of this second incubation step, samples were washed twice and resuspended for acquisition.
All samples were acquired with a FACScalibur flow cytometer (Becton Dickinson, San Josè, CA, USA) and analyzed with a specific software (CellQuest, Becton Dickinson).

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

Table 3: Antibodies used for flow cytometric analysis of suspected feline lymphoma samples.

Statistical analysis

Statistical analyses were performed to assess whether the cellularity of FC samples and the likelihood of samples to be finally processed for FC were influenced by any pre-analytical variable (Table 2).

To this aim, continuous variables were arbitrarily categorized as follows: age (≤1 or >1 year); timespan between degree and sample collection (≤15 or >15 years). Effusions were grouped together irrespective of their primary location (abdominal or thoracic): thus,

lesions were classified into five groups based on their site: peripheral lymph nodes (pLN), abdominal lymph nodes or masses (aLN), thoracic lymph nodes or masses (tLN), effusions, any other site.

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

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

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

Finally, Cohen's Kappa was calculated to assess the level of agreement between cytological reports provided by the referring veterinarian, and the diagnosis made by the blinded reviewer (conclusive for lymphoma, negative for lymphoma, suggestive but not conclusive for lymphoma, not diagnostic). Results were evaluated according to Landis and Koch (Landis, J.R., Koch, G.G., 1977).

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

Results

Of 105 suspect lymphoma samples retrospectively selected from the FC service's database of DIVETLAB (Department of Veterinary Medicine, University of Milan), eight were then excluded from the study for different causes: three were delivered to the laboratory 48 hours after sampling, three were sampled after the administration of chemotherapy. Finally, in two cases the sampling was repeated twice because the first one had a low cellular concentration and had not been processed: in these two cases, only the first (poorly cellular) sample was included in the study. Thus, 97 were finally

included in the present study, from 86 different feline patients: 73 (75.3%) out of these samples were analysed for FC, while the remaining 24 (24.7%) were discarded by the operator for poor quality. Thirty-one (32%) samples were collected before 2011 and were analysed by a single-color approach and 66 (68%) samples were collected subsequently and analysed by a multi-colour approach.

Total nucleated cell count (TNCC) was reported for 91 samples, with a mean of 12.96 ± 21.19 x 10^3 cells/µL (median: 3.11 x 10^3 cells/µL; minimum-maximum: 0.01-89.88 x 10^3 cells/µL). In particular, it was significantly higher in samples finally processed for FC (mean: 14.78 ± 22.12 x 10^3cells/µL; median: 4.09 x 10^3 cells/µL; minimum-maximum: 0.16-89.88 x 10^3 cells/µL) than in discarded samples (mean 7.26 ± 17.20 x 10^3cells/µL; median: 0.54 x 10^3 cells/µL; minimum-maximum: 0.01-58.02 x 10^3 cells/µL; P=0.000).

Breed was known for 75 cats: 64 (85.3%) domestic shorthair (DSH), 4 (5.3%) Maine Coon, 2 (2.7%) British shorthair, 2 (2.7%) Chartreux, 2 (2.7%) Persian and 1 (1.3%) Norwegian Forest. Sex was known for 81 cats: 18 (22.2%) were intact females, 21 (25.9%) were neutered females, 9 (11.1%) were intact males and 33 (40.7%) were neutered males. Age was known for 85 cats, who aged from 5 months to 16 years, with a median age of 8 years. FIV/FeLV status was known only for 16 patients: 7 (43.8%) were negative for both, 7 (43.8%) were FeLV+ and 2 (12.5%) were FIV+. Signalling data did not affect the likelihood of the samples to be processed for FC, nor the TNCC.

The site of the lesion was known for all 97 samples: 24 (24.7%) pLN, 21 (21.6%) aLN, 21 (21.6%) tLN, 17 (17.5%) effusions and 14 (14.4%) other sites, including skin, kidney, spleen, liver and urine. Lesion's size was known for 49 samples: 13 (26.5%) were beneath 2 cm, 22 (44.9%) were between 2 and 5 cm and 14 (28.6%) measured more than 5 cm. The lesion's characteristics didn't affect the likelihood of the samples to be processed for FC, nor the TNCC.

The method of sample withdrawal was known for 66 samples: 16 (24.2%) were made by free-hand, 41 (62.1%) ultrasound-guided, 7 (10.6%) computed tomography-guided, 1 (1.5%) was obtained by surgical access and 1 (1.5%) by urethral catheterization. Patient waking condition was known for 53 samples: 24 (45.3%) were awake, 17 (32.1%) needed mild sedation, 12 (22.6%) needed general anaesthesia. None of these variables affected the likelihood of the samples to be processed for FC, nor the TNCC.

Of 52 samples for which this information was available, 6 (11.5%) were collected with a 18 G needle, 1 (1.9%) with a 20 G needle, 4 (7.7%) with a 21 G needle, 30 (57.7%) with a 22 G needle, 8 (15.3%) with a 23 G needle, 2 (3.8%) with a 25 G needle and 1 (1.9%) with a 27 G needle. Among them, the 21 G needle gave the most cellular samples, with a statistically significant difference from the other needles (P=0.045). Size of the needles with relative average cellular concentration are listed in Table 4. However, the size of needle didn't affect the likelihood of samples to be processed for FC.

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

Transport medium was known for 58 aspirates: 6 (10.3%) were collected in saline solution and 52 (89.7%) were collected in culture medium (RPMI or DMEM). All effusions were collected in EDTA tubes. Transport medium didn't affect the likelihood of samples to be processed for FC, nor their TNCC.

Concerning the experience of the clinician performing the sampling, the timespan between graduation and sample collection was < 15 years for 25 (46.3%) samples and > 15 years for 29 (53.7%) samples. Of 37 samples for which this information was available, 33 (89.2%) were collected by veterinarians with post-graduate qualifications. None of these two variables affected the likelihood of samples to be processed for FC, but clinicians who got postgraduate qualifications collected samples with significantly lower TNCC (P=0.027): qualified veterinarians collected samples with an average cellular concentration of 9.42 ± 19.86 $x10^3$ cells/µL (median 1.71 $x10^3$ cells/µL; minimum-maximum 0.01-87.54 $x10^3$ cells/µL), whereas unqualified veterinarians collected samples with an average cellular concentration of 33.16 ± 29.5 $x10^3$ cells/µL (median 34.01 $x10^3$ cells/µL; minimum-maximum 5.35-59.26 $x10^3$ cells/µL).

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

Table 4. cellular concentration of 52 samples of suspected feline lymphoma sent to the laboratory for flow cytometric immunophenotyping, according to the size of the needle used for sampling

Concerning cytology, 67 samples were sent with a cytological report: of these, 30 (44.8%) were conclusive for lymphoma, for 16 (23.9%) lymphoma was suspected with different confidence levels (diagnostic but not conclusive), for 9 (13.4%) lymphoma was excluded and 1 (1.5%) was diagnostic for thymoma (for a total of 10 lymphoma-negative samples). Moreover, 11 preparations (16.4%) were considered non-diagnostic because of poor cellular concentration, high haemodilution or poor quality of the preparation. 41 samples had cytological preparations available for review: 7 (17.1%) were conclusive for lymphoma, 10 (24.4%) were negative for lymphoma, for 17 (41.5%) diagnostic but not conclusive for lymphoma and 7 (17.1%) were considered non-diagnostic because of the reasons mentioned above. Thirty samples had both the cytological report and the slide available for review. The level of agreement between previous report and attached cytological preparations was poor (Kappa=0.118).

As a second step, the statistical analyses were performed including samples according to the five different lesion sites (pLN, aLN, tLN, effusions, and other sites). Results are

reported in Tables A – E at the end of the study. Significant differences were noted only within the tLN group: clinicians without postgraduate qualifications collected more cellular samples (P=0.036).

In the end, cellular concentration was evaluated according to the FC diagnosis. TNCC was not recorded for 4 samples. The remaining 69 processed samples were divided in three categories: positive for lymphoma, negative for lymphoma and non-diagnostic. TNCC significantly varied among the three groups (P=0.022; Table 5): in particular, non-diagnostic samples were less cellular than lymphoma and non-lymphoma samples (P=0.009 and P=0.040, respectively). The difference in TNCC according to FC diagnosis remained significant also within the pLNs and effusions groups (P=0.029 for both): the significant difference was between lymphoma and non-diagnostic samples for both groups (P=0.016 for pLNs and P=0.036 for effusions). TNCC values are shown in Table 6 and 7.

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

Table 5: cellular concentration of 69 samples of suspected feline lymphoma sent to the laboratory for flow cytometric immunophenotyping, according to the flow cytometric diagnosis

Diagnosis [number of samples]	**Cellularity (x 10^3 cells / µl)**			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lymphoma [5]	45.96 ± 38.05	21.99	13.65	87.54

Non-Lymphoma [11]	13.32 ± 13.77	7.3	1.68	43.59
Non-Diagnostic [5]	4.78 ± 7.52	2.61	0.16	18.02

Table 6: cellular concentration of 21 feline peripheral lymph node aspirates sent to the laboratory for flow cytometric immunophenotyping for suspected lymphoma, according to the flow cytometric diagnosis

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

Table 7: cellular concentration of 11 feline effusions sent to the laboratory for flow cytometric immunophenotyping for suspected lymphoma, according to the flow cytometric diagnosis

Discussion

FC is widely used in human medicine and its use in veterinary medicine has been increasing in the last years, especially for canine lymphoproliferative diseases. In the canine species, this diagnostic tool turned out to be very helpful for a rapid and non-invasive lymphoma diagnosis (Comazzi and Gelain, 2011): these are two of the most important advantages that make this assay always more required by clinicians in their daily practice. Moreover, some studies have been published in the last years about the prognostic value of the flow cytometric immunophenotype (Comazzi et *al*, 2011; Rao et *al*, 2011; Marconato et *al*, 2013) in this species.

Nevertheless, in the feline species, FC is not commonly used; to the authors' knowledge, it was never described in the last decade until last year, when Guzera *et al* published the first scientific study about the application of FC in the diagnosis of feline lymphoma, highlighting the diagnostic accuracy of this technique (Guzera et al., 2016).

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

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

Based on our results, cellular concentration is a key-point in the discrimination between samples suitable or unsuitable for FC. Still, some poorly cellular samples were admitted to processing and, *vice versa*, some highly cellular samples were not. The choice whether to process or not the samples was left to the operator dealing with the sample, and was likely based also on other features, including the volume in which the sample was diluted and its macroscopic appearance (presence of clotted undefined material, bad sample storage during transport), together with the TNCC. Gross inspection of the specimen is recommended, before admission to processing for FC.

In our case series, similar numbers of pLN, aLN and tLN were present, in spite of the higher prevalence of alimentary lymphoma reported in cats (Richter K, 2003; Patterson-Kane et *al*, 2004). One possible explanation for this discrepancy is the presence of many non-lymphoma cases. Another possible reason is that clinicians prefer not to sample hardly achievable lesions such as gastro-intestinal lesions, spleen, liver or aLN, in face of a

supposed poor quality sampling. Still, our data support the application of FC even for intra-abdominal lesions, as these had the same likelihood of being finally processed for FC than the aspirates taken from peripheral lymph nodes (which are expected to be more comfortably achieved).

Although sedation or anaesthesia of the cat may be of aid to the clinician during specimen collection, these did not ensure to catch higher quality samples. Thus, they are not essential and the choice whether to use them or not should only be based on the cat's character. The possible occurrence of side effects might worry the operator, as well. However, for the cases included in the present study, no side effects have been reported following intra-thoracic/abdominal fine needle aspiration (FNA), but just one patient had a transient swelling after FNA of a pLN.

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

The results show that 21G needle, a medium size needle, is related with a higher cellular concentration of the sample. On one hand, smaller needles could damage the cells, that are more fragile for their neoplastic origin, and necrotic or clotted material or connective tissue could plug the needle. On the other hand, larger needles could be more traumatic on the tissue, producing bleeding and thus contamination of the sample with too much blood and other surrounding tissues (necrosis, connective, fat). Following these results, the advice is to use 21G needle to have good quality samples.

The fact that less cellular samples came from theoretically more qualified veterinarians is surprising: the most probable explanation for this result is a statistical artifact due to few samples coming from operators without post-graduate qualification (4). Most of these clinicians regularly send canine samples to our FC service, so they have a steady practical experience in sampling for this purpose. Otherwise, new inexperienced operators may have asked to the FC service's staff for instructions about sampling, transport, medium and sample storage prior to sampling. However, this result is of questionable value and should be better addressed by future studies.

One of the most important result from the present study is that the likelihood of processing the sample and the cellular concentration are not affected by size and site of the lesion, unlike what has been thought until now. This makes FC appropriate for

application in the feline species, even if lesions are intra-abdominal or thoracic. Anyway FC cannot be used alone, but should always be matched with cytological evaluation and, if possible, with histological evaluation. Cytology was available only in subset of the samples included in this study, but the diagnostic agreement between different operators was poor. This underlines how cytology alone is not sufficient for a lymphoma diagnosis in cats, though it is a mandatory first step in the diagnostic workup and, in some cases, it can give additional information about the kind of lymphoma (e.g. Reed-Sternberg cells in Hodgkin's like lymphoma).

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

The retrospective formulation of the present study is its main limitation: information collected were often incomplete and there was no standard sampling procedure. Confirmation will be necessary in the future, through a prospective sample collection and a complete submission of the case. Another main limitation of the present study is the lack of a confirmation test, as histology was available only for few cases (data not shown) and PARR for none. This prevented us from assessing the diagnostic accuracy of FC for feline lymphomas; anyway, Guzera et al. already evaluated it in their study, though on a narrow sample (Guzera *et al.,* 2016).

In conclusion, the results of the present study show how FC can be used for immunophenotyping in feline lymphomas, regardless of site and size of the lesion sampled. The use of 21G needle may enhance the probability to catch high quality samples. This is a pilot study aimed at making FC more widely known in the feline medicine world, and future studies are necessary to make this tool as useful as it is currently in dogs, from both a diagnostic and a prognostic point of view.

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	5	2
2-5 cm	5	1
Sampling		
Blinded	12	3
US-guided	1	0
Anesthesia		
Awake	8	2
Mild sedation	1	1
Needel size		
18 G	2	0
22 G	6	3
23 G	1	0
25 G	1	0
27 G	1	0
Technique		
FNA	8	3
FNB	7	0
Transport medium		
Saline solution	2	1
Culture medium	11	2
Years since graduation		
<15	9	1
>15	5	1
Post-degree qualifications		
No	0	0
Yes	10	2

Table A: 24 peripheral lymph nodes samples (pLNs), processed or not for FC according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	4	1
>5 cm	3	2
Sampling		
Blinded	0	0
US-guided	9	5
Anesthesia		
Awake	2	2
Mild sedation	5	1
General anesthesia	1	0
Needle size		
18 G	1	0
22 G	6	1
23 G	2	1
Technique		
FNA	8	3

FNB	4	0
Transport medium		
Saline solution	0	0
Culture medium	10	2
Years since graduation		
<15	3	2
>15	6	1
Post-degree qualifications		
No	1	0
Yes	5	3

Table B: 21 abdominal lymph nodes samples (aLNs), processed or not for FC according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	6	0
>5 cm	4	3
Sampling		
US-guided	11	3
TC-guided	2	2
Anesthesia		
Awake	4	1
Mild sedation	6	0
General anesthesia	2	3
Needle size		
18 G	1	0
22 G	6	2
23 G	3	0
25 G	1	0
Technique		
FNA	10	2
FNB	4	2
Transport medium		
Saline solution	1	0
Culture medium	11	3
Years since graduation		
<15	5	1
>15	4	1
Post-degree qualifications		
No	2	1
Yes	5	1

Table C: 21 thoracic masses samples, processed or not for FC according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Sampling		
Blinded	1	0

US-guided	3	3
TC-guided	3	0
Anesthesia		
Awake	1	1
Mild sedation	1	1
General anesthesia	4	0
Needle size		
20 G	1	0
21 G	3	1
22 G	2	1
Technique		
FNA	5	0
FNB	1	1
Years since graduation		
<15	2	0
>15	3	2
Post-degree qualifications		
No	0	0
Yes	4	0

Table D: 17 effusions samples, processed or not for FC according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	3	0
2-5 cm	1	3
Sampling		
US-guided	3	3
Surgical access	0	1
Catheterization	1	0
Anesthesia		
Animale sveglio	2	1
Lieve sedazione	1	0
Anestesia generale	2	0
Needle size		
18 G	0	2
22 G	2	1
23 G	1	0
Technique		
FNA	5	4
FNB	2	0
Transport medium		
Saline solution	1	0
Culture medium	3	3
Years since graduation		
<15	1	1
>15	4	2
Post-degree qualifications		
No	0	0
Yes	2	1

Table E: 14 samples from different organs (skin, kidney, spleen, liver, urine), processed or not for FC according to different pre-analytical factors

The results of the present study were used for a degree thesis (*"Fattori preanalitici e qualità dei campioni per citofluorimetria nel linfoma del gatto"*) and then published on a peer review international journal (Martini V, Bernardi S, Marelli P, Cozzi M, Comazzi S. **Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples**. *J Feline Med Surg* 2018; 20(6): 494-501.

5. CYTOLOGICAL AND FLOW CYTOMETRIC ANALYSIS OF LYMPHOCYTE SUBSETS IN NON-NEOPLASTIC FELINE LYMPH NODES

Background

In canine oncology, cytology of lymph nodes potentially affected by lymphoproliferative disorders is a key tool providing many information and often paired with FC analysis (Rout and Avery, 2017). These routine investigations are rather uncommon in feline species, thus yet far from becoming routine diagnostic tools, together. Accuracy of FC has been evaluated in a work by Guzera and colleagues (Guzera et al, 2016), but the good amount of data and scientific production available for dogs is yet lacking in feline species. Since a narrow panel of monoclonal antibodies is available and lymphomatous lesions in feline patients are often intra-abdominal (Louwerens et al, 2005), histopathology and immunohistochemistry remain more popular, being the gold standard for the diagnosis of lymphoma and immunophenotyping.

Starting from the very beginning, and after having evaluated the factors affecting the likelihood of feline samples to be processed for FC analysis as illustrated in the previous *study,* we considered significant to study the non-neoplastic counterpart of feline lymph node populations. Thus, the aim of the present study was to describe lymphocyte subsets in non-neoplastic feline lymph nodes (histologically diagnosed), as previously assessed in peripheral blood (Byrne et al, 2000).

Materials and methods

Sample collection

Popliteal and/or abdominal LNs were collected from feline patients dead or euthanized for reasons other than hematological malignancies or severe inflammatory diseases. All samples were taken from pet cats that were destined to necropsy and the main suppliers were “Tibaldi” Veterinary Clinic, Milan and the Veterinary Pathology Service, Veterinary Medicine, University of Milan, between December 2016 and October 2018. Owners were requested for an informed consent and agreed with the procedures. LNs were collected within 6 hours from death. The whole LN was placed into at least 5 ml of liquid transport medium (RPMI + azide 0.2%), or eventually in saline solution 0.9%, delivered to our FC

service and stored at +4°C until further processing, which occurred within 48 hours from death.

In order to prepare the samples for FC analysis, LNs were isolated from adipose and connective surrounding tissues. Fine needle aspirates of each LN were performed, using a 21 G needle. Collected material were placed in 1 ml RPMI with the addition of Na azide 0.2% to obtain a cellular suspension. Total nucleated cell count was assessed with an automated hematology analyzer (Sysmex XT 2000-iV, Sysmex, Kobe, Japan), and aspirates were repeated until a cellular concentration of $2x10^3$ cells/µL was reached. Subsequently, each lymph node was cut in two halves, perpendicularly to the loop, and these were used to obtain tissue impressions for cytological evaluation.

Cytological evaluation

Cytological impressions were stained with May-Grünwald Giemsa and analyzed by a single operator (SB). Quality of cells and their state of integrity was evaluated morphologically and the differential cell count was performed on 300 cells, dividing the population in the following groups: small lymphocytes (nucleus < 2 RBCs), medium size lymphocytes (nucleus = 2 RBCs), large size lymphocytes (nucleus > 2 RBCs), plasma cells, macrophages, neutrophils, eosinophils and mastocytes.

Flow cytometric analysis

Adequate volumes of cellular suspensions collected from the LNs were aliquoted in tubes for labelling in order to have $5x10^5$ cells/tube.

Immuno-labelling was performed using monoclonal antibodies (mAbs) species-specific or cross-reactive with feline antigens, pre- labeled with fluorophores (see *table 8*). The antibodies' panel consisted of CD5-FITC/CD21-PE/CD18-alexafluor647, CD4-FITC/CD8-PE/CD18-alexafluor647 and CD44-FITC/CD18-alexafluor647.

Cells were incubated for 20 minutes with the antibodies, at +4°C temperature, added with *fetal bovine serum* (FBS) 5% in order to block nonspecific bindings. Thereafter, each tube underwent a washing phase with phosphate buffer solution (PBS) 1x, running an 8 minutes centrifugation at 1100 rpm. Supernatant was discarded, and cells were resuspended in 500 µL PBS 1X.

Sample acquisition was performed with a FACScalibur (Becton Dickinson, San Josè, CA, USA) or a BriCyte E6 (Mindray, Schengen, China) flow cytometer and analyses were performed with a specific software (CellQuest, Becton Dickinson or MRFlow, Mindray).

A first gate (R1) was set in a morphological scattergram (FSC-H versus SSC-H) in order to exclude platelet and debris. A second gate (R2) was set in order to include only CD18+ cells having low complexity index (lymphoid cells) (figure 6). Analyses were restricted to the population of cells included in both R1 and R2. The percentage of CD-positive cells out of CD18+ lymphoid cells was then recorded for each antigen tested. All analyses were performed by a single operator who was blinded to the histopathological diagnosis.

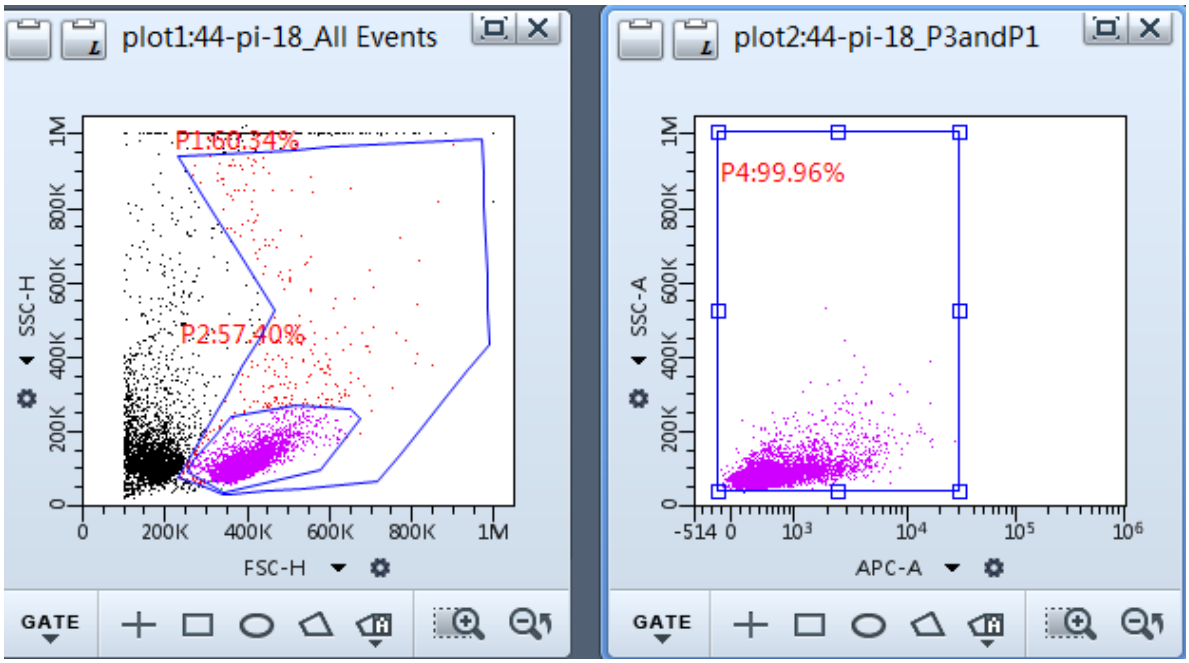

Figure 6: Scattergram representing the gating procedure to focus the analysis on CD18+ cell, with morphological properties suggestive of lymphoid cells.

Antibody	Specificity	Clone	Source
CD21-PE	B cells	CA2.1D6	Biorad, Oxford, UK
CD5-FITC	T cells	f43	SouthernBiotech, Birmingham, AL, USA
CD4-FITC	T helper lymphocytes	3-4F4	SouthernBiotech
CD8-PE	T- cytotoxic lymphocytes	fCD8	SouthernBiotech
CD18-AlexaFluor647	All leukocytes	CA1.4E9	Biorad
CD44 - FITC	All leukocytes	IM7	BD Pharmingen, San Diego, CA, USA

Table 8: Monoclonal antibodies for immuno-labelling of cellular suspensions of feline lymph nodes.

Histological evaluation

Tissue specimens, following cytological sampling, were fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin. Tissue blocks were utilized to obtain at least 2, 4–6-µm thick sections per case. Morphologic features were evaluated on tissue sections stained with routine Haematoxylin and Eosin stain.

PARR

When necessary, an aliquot of the LN cellular suspension was tested for clonality by PARR. Genomic DNA was purified by using Maxwell® RSC Tissue DNA Kit (Promega, Madison, WI) following manufacturer's instructions and the concentration of DNA in all obtained samples was evaluated by a fluorometric procedure using Quantus™ Fluorometer (Promega, Madison, WI).

T-cell receptor gamma (TCRG) and immunoglobulin heavy chain (IgH) locus were PCR-amplified using HotStarTaq Master Mix Kit (Qiagen, Valencia, CA), 100 ng of genomic DNA and primers previously designed and described (Moore et al, 2005; Werner et al, 2005) Concentration of all primers was 10 pmol/20µL of reaction mixture.

Amplification conditions used a 2-step modified touchdown protocol to increase specificity of the reactions. All PCR reactions were run in duplicate and heteroduplex analysis was performed in order to separate true clonal from false-positive results[7], PCR products (10 µl) were denatured at 95°C for 10 minutes, then allowed to reanneal at 4°C.

Native and heteroduplex samples were separated by polyacrylamide gel electrophoresis (PAGE).

A total of 10 µl of each native and heteroduplex samples were mixed with loading buffer and loaded directly into precast 10% nondenaturing polyacrylamide Tris-Borate EDTA (TBE) gels (BioRad, Hercules, CA). Polyacrylamide gels containing both native and denatured PCR products were run in TBE at 150 V for 2 hours. The gels were stained with ethidium bromide and visualized under UV light.

Statistical analysis

The percentages of each cell subpopulation were reported in an electronic sheet and descriptive statistics were calculated on the whole sample series and for each subgroup of LNs independently (popliteal, abdominal, histopathologically normal, reactive). Normally distributed data are reported as mean ± standard deviation, whereas not-normally distributed data are reported as median and range.

A Mann-Whitney U test was performed for each single variable to compare normal and reactive LNs, by means of a statistical software (SPSS v20.0 for Windows). Significance was set at $P \leq .05$.

Results

To the aim of this study, 16 lymph nodes were collected, from 11 feline patients. Ten (63%) out of 16 were popliteal LNs, while the other 6 (37%) were abdominal.

Eight (73%) cats were domestic shorthair and the other 3 were a Persian, a Birman and one Siamese (9 % each). The median age was 12.5 years (range between 4 months to 18 years). One of the patients was adopted at unknown age but it was considered to be an adult cat. There were 6 spayed females (55%) and 5 males (45%), of which 3 (60%) were neutered. All cases are resumed in table 9, reporting the cause of death for each patient.

According to histopathology, 5 LNs were diagnosed as reactive based on the presence of moderate to severe follicular hyperplasia. The other samples were classified as atrophic or normal lymph nodes.

Sample	Breed	Sex	Age	Cause of death
Case 1 (popliteal)	DSH	NM	4 months	Trauma
Case 2 (popliteal)	Persian	SF	18 years	CKD
Case 3 (popliteal)	DSH	SF	18 years	CKD
Case 4 (abdominal+popliteal)	DSH	M	1 year	Trauma
Case 5 (abdominal+popliteal)	Birman	NM	15 years	CKD/AKI
Case 6 (popliteal)	DSH	NM	12 years	Thrombosis of aorta
Case 7 (abdominal+popliteal)	DSH	SF	17 years	Hypertyroidism
Case 8 (abdominal)	DSH	SF	13 years	CKD
Case 9 (popliteal)	DSH	SF	9 years	DKA
Case 10 (popliteal+abdominal)	Siamese	SF	1 year	Trauma
Case 11 (popliteal+abdominal)	DSH	M	Adult	Trauma

Table 9: Breed, sex, age and cause of death/euthanasia of the 11 cats from which the lymph node samples were collected. NM=neutered male, SF=spayed female, M=male. DSH=domestic shorthair; CKD=chronic kidney disease; AKI=acute kidney injury; DKA=diabetic ketoacidosis.

Cytological evaluation and differential cell count

One cytological specimen was excluded from analysis because of poor quality and high number of disrupted cells.

Differential cell count showed a prevalence of small lymphocytes, followed by medium size lymphocytes and large lymphocytes as shown in table 10. The other cell types were

seldom observed: eosinophils reached a maximum of 5%, plasma cells 4%, mast cells and macrophages 2% each, neutrophils 1%, being mast cells, macrophages and granulocytes observed in no more than 4 (27%) out of 15 cytological preparations.

Analysis of differential cell count was also performed on popliteal and abdominal LNs separately. In both groups, cell types distribution was very similar to those noticed considering all LNs together; cell types were mainly represented by small lymphocytes, followed by medium size lymphocytes. The other cell types were definitely less represented (table 11).

As shown in table 12, a higher number of medium size lymphocytes was observed in reactive LNs, but the difference wasn't statistically significant (P=.240).

	Small lymphocytes	Medium lymphocytes	Large lymphocytes	Plasma cells	Mast cells	Macrophages	Neutrophils	Eosinophils
Mean ± SD	71 ± 18%	25 ± 17%	2 ± 1%	1 ± 1%	0 ± 1%	0 ± 1%	0 ± 0%	0 ± 1%
median; min-max	75%; 21-95%	23%; 4-75%	1%; 0-4%	0%; 0-4%	0%; 0-2%	0%; 0-2%	0%; 0-1%	0%; 0-5%

Table 10. Distribution of cellular types in the cytological preparation from 15 feline non-neoplastic lymph nodes

	Small lymphocytes	Medium lymphocytes	Large lymphocytes	Plasma cells	Mast cells	Macrophages	Neutrophils	Eosinophils
Popliteal LNs (mean ± SD)	68 ± 21%	27 ± 20%	2 ± 1%	1 ± 1%	1 ± 1%	1 ± 1%	0 ± 0%	1 ± 1%
Popliteal LNs (median; min-max)	71%; 21-95%	25%; 6-75%	2%; 0-4%	1%; 0-4%	0%; 0-2%	0%; 0-2%	0%; 0-1%	0%; 0-5%
Abdominal LNs (mean ± SD)	77 ± 10%	21 ± 10%	1 ± 0%	1 ± 1%	0 ± 0%	0 ± 0%	0 ± 0%	0 ± 0%
Abdominal LNs (median; min-max)	79%; 61-88%	18%; 11-37%	1%; 0-2%	0%; 0-3%	0%; 0-0%	0%; 0-1%	0%; 0-0%	0%; 0-0%

Table 11. Distribution of cellular types in the cytological preparation from 15 feline non-neoplastic lymph nodes according to the anatomical site of the lymph node.

	Small lymphocytes	Medium lymphocytes	Large lymphocytes	Plasma cells	Mast cells	Macrophages	Neutrophils	Eosinophils
Normal LNs (mean ± SD)	75 ± 13%	21 ± 11%	2 ± 1%	1 ± 1%	0 ± 0%	1 ± 1%	0 ± 0%	0 ± 1%
Normal LNs (median; min-max)	77%; 55-95%	20%; 4-37%	1%; 0-3%	0%; 0-4%	0%; 0-1%	0%; 0-2%	0%; 0-1%	0%; 0-0%
Reactive LNs (mean ± SD)	59 ± 26%	37 ± 27%	2 ± 2%	1 ± 1%	1 ± 1%	0 ± 0%	0 ± 0%	1 ± 2%
Reactive LNs (median; min-max)	68%; 21-78%	31%; 12-75%	1%; 0-4%	0%; 0-2%	0%; 0-2%	0%; 0-0%	0%; 0-0%	0%; 0-5%

Table 12. Distribution of cellular types in the cytological preparation from 15 feline non-neoplastic lymph nodes according to histopathological diagnosis.

Flow cytometrical analysis

Results describing the whole population of LNs are summarized in table 13. Analysis of expression of different leukocyte markers were performed also in popliteal and abdominal LNs separately: mean ± SD, median, minimum and maximum values of expression for both groups are listed in table 14.

In two cases (case 7 and case 11), FC analyses revealed the presence of a discrete population with abnormal antigen expression. In case 7, 83.2% of cells were CD5+, but CD4 and CD8 double negative. In case 11, more than 40% of cells with lymphoid properties on morphological scattergrams stained negative for all lymphoid marker tested. PARR was performed in these two cases, to support the histopathological diagnoses and exclude a lymphoproliferative disease: a polyclonal result was obtained in both cases, definitively excluding lymphoid neoplasms.

In view of the histological findings, expression of CD antigens in normal and reactive LNs were evaluated separately. The results of the descriptive analysis are reported in table 15. No differences in the percentages of different lymphoid subtypes were evident except for CD8 expressing cells (T cytotoxic lymphocytes) which were significantly higher in reactive LNs compared to normal LNs (P=.008).

	CD44$^+$	**CD5$^+$**	**CD21$^+$**	**CD4$^+$**	**CD8$^+$**	**CD4$^+$CD8$^+$**
mean ± SD	98.9 ± 1.5	61.7 ± 14.1	26.1 ± 15.4	38.4 ± 13.2	15.9 ± 7.6	0.9 ± 0.5
Median; min-max	99.6; 94.9 – 99.8	60.7; 40.8 – 89.0	29.9; 2.61 – 53.2	37.6; 18.7 – 66.4	15.4; 3.7 – 29.1	0.8; 0.2 – 2.4

Table 13. Expression of leukocyte markers in non-neoplastic lymph nodes from 11 feline patients; values of mean ± SD, median, and range are expressed in % out of total CD18+ lymphoid cells.

	Value	**CD44$^+$**	**CD5$^+$**	**CD21$^+$**	**CD4$^+$**	**CD8$^+$**	**CD4$^+$ CD8$^+$**
Abdominal LNs	**Mean ± SD**	98.2 ± 2.3	64.8 ± 14.2	27.3 ± 12.3	43.6 ± 17.8	17.6 ± 8.8	0.7 ± 0.3
	Median; Min-max	99.6; 95.5-	60.8; 52.1-89.0	31.0; 6.5-41.8	36.0; 27.2-66.4	20.2; 3.7-	0.7; 0.2-1.2

		99.8				29.1	
Popliteal LNs	**Mean ± SD**	99.3 ± 0.7	59.8 ± 14.4	25.4 ± 17.6	35.2 ± 9.2	14.9 ± 7.1	1.0 ± 0.6
	Median; Min-max	99.5; 97.6-99.8	59.7; 40.8-83.2	28.9; 2.6-53.2	38.6; 18.7-48.6	13.0; 5.0-26.6	0.9; 0.4-2.4

Table 14. Expression of leukocyte markers in non-neoplastic lymph nodes from 11 feline patients according to the anatomical site of the lymph node; values of mean ± SD, median, and range are expressed in % out of total CD18+ lymphoid cells.

	Value	**CD44$^+$**	**CD5$^+$**	**CD21$^+$**	**CD4$^+$**	**CD8$^+$**	**CD4$^+$ CD8$^+$**
Normal LNs	**Mean ± SD**	99.1 ± 1.2	63.0 ± 15.0	27.1 ± 15.7	40.9 ± 14.9	12.6 ± 6.3	0.8 ± 0.3
	Median; Min-max	99.6; 95.5-99.8	62.8; 40.8-89.0	29.2; 6.5-53.2	39.2; 18.7-66.4	11.7; 3.7-25.3	0.8; 0.4-1.4
Reactive LNs	**Mean ± SD**	98.3 ± 2.1	58.8 ± 13.0	23.8 ± 16.4	32.9 ± 6.5	23.2 ± 4.4	1.0 ± 0.9
	Median; Min-max	99.6; 94.9-99.7	52.8; 46.7-79.7	30.5; 2.6-41.8	34.8; 25.1-40.2	20.4; 19.4-29.1	0.8; 0.2-2.4

Table 15. Expression of leukocyte markers in non-neoplastic lymph nodes from 11 feline patients according to the histopathological diagnosis; values of mean ± SD, median, and range are expressed in % out of total CD18+ lymphoid cells.

Discussion

Despite the usefulness of FC in a clinical diagnostic setup, FC for the diagnosis of hematopoietic malignancies (Guzera et al, 2016) has been seldom utilized in cats.

The aim of the present study was to describe non-neoplastic feline LNs through cytological and FC evaluation, similarly to what has been done and described for the dog by different research groups (Gibson et al, 2004; Wilkerson et al, 2005; Rütgen et al, 2015) in order to perceive a potential difference between neoplastic and non-neoplastic LNs in their FC appearance.

Of all the 16 non-neoplastic samples included, one abdominal LN did not yield an adequate specimen for differential cell count, compromising cytological evaluation, and thus removed from the caseload. Nevertheless, FC analysis of the same sample provided good quality results, thus suggesting a possible technical problem during smear preparation and excluding degenerative process.

Results from the cytological evaluation of 15 lymph node samples evidenced that small lymphocytes prevail in feline LNs, similarly to what has been observed in the dog (Rütgen et al, 2015). Medium-size and large lymphocytes were less frequent in the studied population (median values: 23% and 1% respectively). These cell types are expected to increase in inflammatory states but a concurrent increase of plasma cells over 5% is usually found (Raskin and Meyer, 2015); however, immature cells increase also in cases of neoplasia and for this reason cytology is often not sufficient to diagnose lymphoma in cats since lymphomas may be characterized by a variably mixed morphology compared to dogs. On the contrary, in dogs, cytology is fundamental in the diagnostic process of lymphoma and sometimes it could already provide a suggestion about the subtype (Rout and Avery, 2017). Comparing our results to what has been described in the dog (Rütgen et al, 2015), the composition of the leukocyte population in feline and canine non-neoplastic LNs largely overlaps, even if medium-sized lymphocytes were more represented in some of the cases included in the present study.

Providing that data regarding FC analysis of feline LN are missing, it is necessary to define the cell population that should be considered non-neoplastic against the histopathology goldstandard. The main lymphocyte subset identified by FC were T-cells, identified by CD5 expression. Next to T-cell population, a remarkable group of cells has been identified by anti-CD21 mAb, which labels the mature B-cell subset. The clone used in this case was canine-specific, but has been demonstrated cross reactive to feline CD21 (Brodersen et al, 1998). These same populations were also found in the dog, with a very similar distribution (Gibson et al, 2004).The distribution of T- and B-cell populations and of T-cell subsets (CD4+ and CD8+) seems to be very similar to what was formerly described in human patients, whose LNs are composed of 80% of T lymphocytes (almost the 70% being T-helper cells) and 15% of B lymphocytes (Tedla et al, 1999).

Within the T cell population, a very small subset of lymphocytes was double positive (CD4 and CD8): this is considered a physiologic resident population which has been identified

in other species and described in some studies as an extra-thymic population (Zuckermann, 1999; Bismarck et al, 2012), assuming a possible activation phase for this cell subset. In swines, dp-T-cells have been widely studied, because may represents the 60% of peripheral mononuclear cells and that may increase with age; otherwise, in humans, it represents only the 1-3% of peripheral T-cells and an increased number of dp-lymphocytes has been observed during viral infections and immune disorders (Rothe et al, 2017).Recently, it has been described also in canine lymph nodes (Rütgen et al, 2015) and in peripheral blood (Rothe et al, 2017); in this last report, the authors investigated the hypothesis of the presence of a heterogeneous subset including different subtypes and functions. This population has previously shown features of activated T cells (Bismarck et al, 2014) and the authors wanted to assess if, within this population, different specific functions of dp-T-lymphocytes could be identified, which is what they finally showed. To our best knowledge, no reports are available in literature concerning dp-T-lymphocytes of cats.

Expression of the pan-leukocyte marker CD44 was also evaluated. The reactivity of the anti-CD44 monoclonal antibody to feline antigens has been previously reported (Meister et al, 2007): it is a species-specific clone for the mouse, that in the present study showed a very similar expression to CD18, which is a pan-leukocyte marker commonly used in canine species and the clone is species-specific for the dog but cross-reactive with the feline antigen according to the manufacturer's datasheet.

In the present study CD18 was used in combination with morphology to define the lymphocyte population on which the analysis of other antibodies has been performed. CD44 is definitely less known in veterinary medicine and to the authors' knowledge, only one study is available examining expression of CD44 in dogs with chronic and acute leukemias (Gelain et al, 2014), showing how neoplastic cells express this marker in a significant higher fashion compared to health controls, and dog with acute forms showed higher expression compared to dogs with chronic forms. To our best knowledge, no information is available about expression of CD44 in feline hematopoietic cells. CD44 and CD18 seems to have a similar expression in lymphocytes from healthy patients, according to the present study, but their combined use could be helpful to understand if any variation occurs in neoplastic samples, like has been previously done for dogs with the expression of CD45/CD18 (Comazzi et al, 2006). This may be even easier using a

multicolour approach or considering the fluorescence intensity of stained cells, which in turn reveals the level of expression of the antigens tested. Multicolour approach is one of the most important advantages of FC, that no other technique can claim, neither IHC nor immunocytochemistry (ICC).

According to histological analysis, 5 non-neoplastic LNs were diagnosed as reactive based on moderate to severe follicular hyperplasia. As shown by our results, no statistically significant differences could be perceived between these two groups in the expression of CD antigens and cytological evaluation, except for the expression of CD8, being significantly higher in reactive samples. This may represent a cytotoxic immune response as a cause of reactive lymphoid hyperplasia in the cases included in the present study. Different causes of nodal reactivity may cause increase in different lymphoid lineages: FC evaluation of a larger number of reactive LNs would be beneficial to depict a more detailed scenario of immune responses in cats.

The unusual expression of CD antigens shown by cells of *case 7* and *11* could be the result of the poor preservation of samples which could have affected the antigenicity of cells or, particularly for *case 11* which was diagnosed as reactive by histopathology, negative cells might be hypothesized as a natural killer (NK) lymphocytes population for which no antibody is available either for feline or for canine species and no information about phenotype of these lymphocytes subset is known to date.

The data we provide about non-neoplastic population should be compared to an adequate number of lymphomas. In the largest case series of feline lymphomas investigated by FC up to date[3], 12 out of 13 lymphomas were characterized by a dominant population (>90%) of cells with the same pattern of antigen expression. Conversely, 1 case out of 13 showed a mixed population of B and T lymphocytes, similarly to the 6 control cases. Unfortunately, only this latter sample and 2 T-cell lymphomas were taken from LNs. None of the samples in the present study showed a single lymphoid population exceeding 90% of the cells. Clustering our results with those by Guzera and colleagues, it could be argued that FC might support the diagnosis of lymphoma also in feline species, although false negative results may be encountered.

The present study has some limitations, starting from the low number of cases included and the low number of antibodies being part of feline panel. These numbers should be expanded in future studies. Another pitfall of our work was the use of two different

cytometers, whilst ideally the same software should be use for data analysis and the presence of a single cytologist, whilst more would have strengthened the power of cytological data.

In conclusion, the present study evidences that the main lymphocyte subpopulations in non-neoplastic feline LNs is represented by T cells. A new pan-leukocyte marker, CD44, has been evaluated, showing good reactivity and binding on the surface of almost the whole gated $CD18^{+}$ lymphocyte population, showing thus good agreement between the two markers and making its application possible for feline leukocyte immunophenotyping. Further studies are needed to assess its behaviour in pathological conditions.

No relevant differences were noticed between reactive and normal LNs by FC and cytological analysis, except the higher percentage of CD8+ cells in reactive LNs. Further studies are needed to evaluate the neoplastic counterpart and a wider caseload is expected compared to previous studies (Guzera et al, 2016).

Preliminary results of the present study were used for a degree thesis (DATI PRELIMINARI SULLE SOTTOPOPOLAZIONI LINFOCITARIE IN LINFONODI DI GATTO: ANALISI CITOLOGICA E CITOFLUORIMETRICA) and presented to the 19^{th} international congress of ESVCP, London, UK. The present results have to be improved with a greater number of cases, thus we decided not to submit the present study to a journal, but to increase our caseload.

6. FELINE MEDIASTINAL MASSES: FLOW CYTOMETRICAL ANALYSIS OF LYMPHOMA AND NON-LYMPHOMATOUS LESIONS

Background

Mediastinal lymphoma is one of the forms that was historically associated with retroviral infections (especially FeLV) in feline species, generally of T-cell origin (Louwerens et al, 2005). It can be considered as a differential diagnosis when a mediastinal mass is detected, often occasionally or consequently to some clinical signs as dyspnoea, coughing or regurgitation (Fabrizio et al, 2014). Thymoma also is a possible and frequent diagnosis of mediastinal masses in feline species (Pintore et al, 2014) and other differentials are cystic lesions or other kind of neoplasms. These lesions can be investigated through diagnostic imaging, which is even helpful to guide sampling procedure of the mass. Cytology can be representative and thus diagnostic, especially for lymphomas; anyway, small cell lymphomas and thymomas can appear alike, since mature lymphocytes and thymocytes are morphologically the same and thymomas can have a prominent lymphoid component (Day, 1997; Raskin and Meyer, 2015). In these cases, histopathology is mandatory.

In canine species, flow cytometry has been successfully applied to distinguish between lymphoma and non-lymphomatous lesions (Lana et al, 2006) and in particular thymomas were shown to have always >10% CD4+CD8+ double positive small T-lymphocytes (specificity = 100%).

Thus, the aim of the present study was to retrospectively compare the immunophenotype of lymphoma and non-lymphomatous mediastinal lesions in cats and to assess if FC could differentiate between the two groups.

Material and methods

The archive of the FC Service of the Veterinary Teaching Hospital of the University of Milan was retrospectively interrogated from January 2014 to June 2019, and samples from mediastinal masses in cats were extracted. Cases were retained in the study only if they fulfilled the following inclusion criteria: availability of FC data obtained on a mediastinal mass aspirate for blinded review, possibility to confirm or exclude lymphoma

based on different combinations of cytology, histopathology and PCR for antigen receptor rearrangement (PARR).

All cats were sampled for diagnostic purposes with an informed consent of the owner. Thus, according to the guidelines of the authors' Institution, a formal approval of the Ethical Committee was not required (EC decision 29 October 2012, renewed with the protocol n° 02-2016).

Flow cytometry

FC was performed on tissue aspirates obtained from mediastinal masses and collected in a liquid transport medium (RPMI 1640).

A gross inspection of the sample was performed to assess the volume of sample, the presence of clots, blood or necrotic material and the total nucleated cell count (TNCC) was performed either via a haematology analyser (Sysmex XT-2000iV, Sysmex, Kobe, Japan) or directly via cytometer (BriCyte E6, Mindray, Schengen, China) in order to assess the suitability of samples to be processed for FC analysis. Samples were processed as previously described (illustrated in "Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples"). In some cases, mass samples were submitted for FC analysis together with blood or other matrixes for staging purpose. Blood and effusion samples were collected into EDTA tubes, whereas fine needle aspirates were obtained from solid tissues and resuspended into tubes containing RPMI 1640. All matrices provided were processed for FC with the same operative procedure as mediastinal masses. If necessary, erythrocytes were lysed prior to acquisition at the cytometer by means of a solution containing ammonium chloride.

For the FC analysis, a multicolour approach was used; the following panel was applied to every sample: CD5-FITC/CD21-PE/CD18-alexafluor647 and CD4-FITC/CD8-PE/CD18-alexafluor18. Antibodies are listed in *table 16*. If low cellularity or volume were detected, the antibody panel was reduced to CD4-FITC/CD8-PE/CD18-alexafluor647.

Antibody	Specificity	Clone	Source
CD21-PE	B cells	CA2.1D6	Biorad, Oxford, UK
CD5-FITC	T cells	f43	SouthernBiotech, Birmingham, AL,

			USA
CD4-FITC	T helper lymphocytes	3-4F4	SouthernBiotech
CD8-PE	T- cytotoxic lymphocytes	fCD8	SouthernBiotech
CD18-AlexaFluor647	All leukocytes	CA1.4E9	Biorad

Table 26. Antibodies' panel for immuno-labelling of mediastinal masses of feline patients

Samples were acquired with FACScalibur flow cytometer (Becton Dickinson, San Josè, CA, USA) or with BriCyte E6 (Mindray, Shengen, China) and sample analyses were performed in a single session with a specific software (CellQuest, Becton Dickinson or MRFlow, Mindray, respectively)by a single operator who was blinded to all information about the cases.

PARR analysis

When necessary to solve differential and to support diagnosis, PARR was performed on stained slides or on archive material kept at -20°C.

Genomic DNA was purified by using Maxwell® RSC Tissue DNA Kit (Promega, Madison, WI) following manufacturer's instructions and the concentration of DNA in all obtained samples was evaluated by a fluorometric procedure using Quantus™ Fluorometer (Promega, Madison, WI).

T-cell receptor gamma (TCRG) and immunoglobulin heavy chain (IgH) locus were PCR-amplified using HotStarTaq Master Mix Kit (Qiagen, Valencia, CA), 100 ng of genomic DNA and primers previously designed and described (Moore et al, 2005; Werner et al, 2005). Concentration of all primers was 10 pmol/20µL of reaction mixture.

Amplification conditions used a 2-step modified touchdown protocol to increase specificity of the reactions. All PCR reactions were run in duplicate and heteroduplex analysis was performed in order to separate true clonal from false-positive results,[12] PCR products (10 µl) were denatured at 95°C for 10 minutes, then allowed to reanneal at 4°C.

Native and heteroduplex samples were separated by polyacrylamide gel electrophoresis (PAGE).

A total of 10 µl of each native and heteroduplex samples were mixed with loading buffer and loaded directly into precast 10% nondenaturing polyacrylamide Tris-Borate EDTA (TBE) gels (BioRad, Hercules, CA). Polyacrylamide gels containing both native and denatured PCR products were run in TBE at 150 V for 2 hours. The gels were stained with ethidium bromide and visualized under UV light.

Results

At first, 30 cases of mediastinal masses were retrieved in the FC database, from 2014 to 2019. Of these, 5 samples were excluded because of the low cellularity, and 6 because a final diagnosis could not be reached. Hence, 19 cases were included in the study (*table 17*), including 13 (68%) lymphomas (eg. Case 9 in *figure 7*) and 6 (32%) non-lymphomatous lesions.

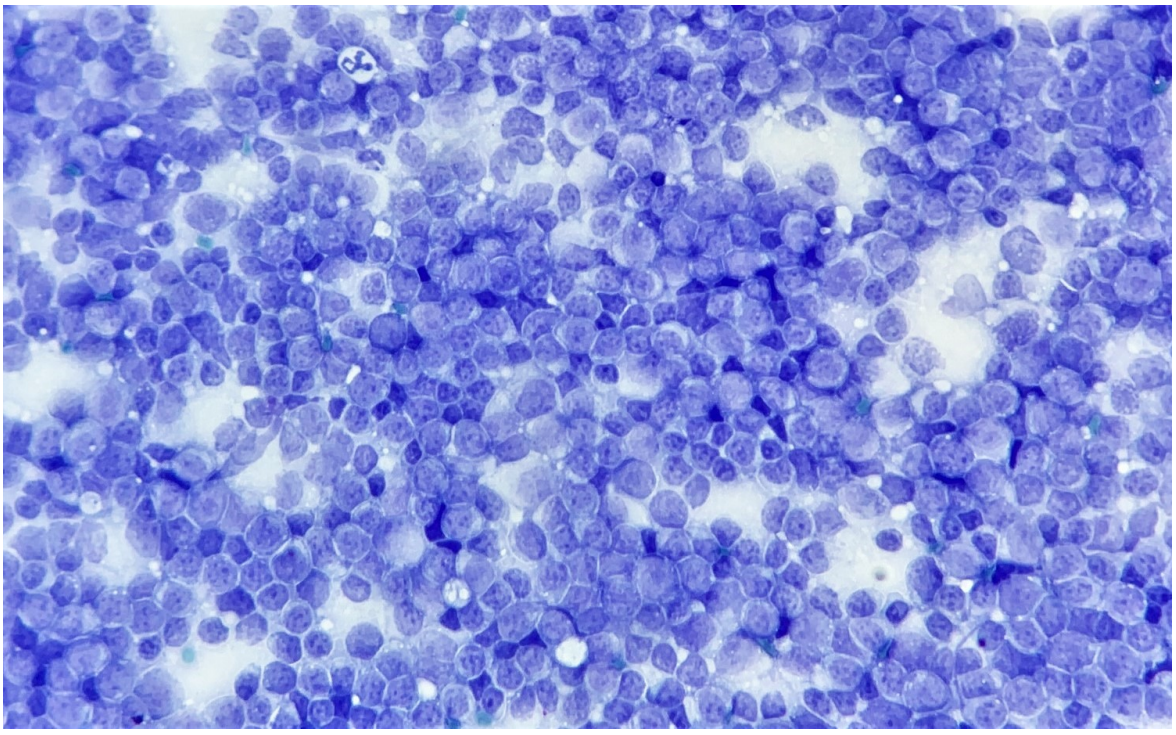

Figure 7. May-Grünwald Giemsa, 40x. Case 9, T-cell lymphoma.

CASE #	FINAL DIAGNOSIS	BREED	GENDER	AGE	FIV/FeLV	LYMPHOID CELL SIZE	CLINICO-PATHOLOGICAL FEATURES
1	Lymphoma dp	DSH	FN	3Y	Unknown	Medium	Histopathology
2	Lymphoma dp	DSH	FN	4Y	Unknown	Small	PARR (monoclonal)
3	Lymphoma dp	BSH	MC	9M	FIV/FeLV -	Medium	
4	Lymphoma dp	DSH	FN	4Y	FeLV +	Medium	
5	Lymphoma T4	DSH	FN	8Y	Unknown	Large	
6	Lymphoma dp	DSH	MC	8Y	FIV/FeLV -	Medium	
7	Lymphoma dn	SBI	MC	4Y	Unknown	Large	
8	Lymphoma dp	DSH	MC	2Y	Unknown	Medium	Neoplastic cells in PB
9	Lymphoma T4	DSH	FN	2Y	Unknown	Large	
10	Lymphoma T4	Unknown	FN	8Y	Unknown	Medium	
11	Lymphoma dp	DSH	M	2Y	Unknown	Medium	Neoplastic cells in PB and thoracic effusion
12	Lymphoma dp	DSH	MC	4Y	Unknown	Medium	Neoplastic cells in lymph nodes
13	Lymphoma dp	DSH	FN	10Y	Unknown	Medium	
14	Non-lymphoma	DSH	MC	5Y	Unknown	Small	PARR (polyclonal)
15	Non-lymphoma	DSH	FN	12Y	Unknown	Small	PARR (polyclonal)

16	Non-lymphoma	Unknow	Unknown	6Y	Unknown	Small	PARR (polyclonal)
17	Non-lymphoma	DSH	FN	10Y	Unknown	Small	PARR (polyclonal)
18	Non-lymphoma	DSH	FN	9Y	Unknown	Small	PARR (polyclonal)
19	Non-lymphoma	DSH	MC	8Y	Unknown	Small	PARR (polyclonal)

Table 17. Signalment and main clinico-pathological features in 9 cats with mediastinal masses. DSH: domestic shorthair; BSH: British shorthair; SBI: Sacred Birman; FN: female neutered; MC: male castrated; M: male; PB: peripheral blood.

Immunophenotype of non-lymphomatous lesions

FC results from cats with lymphoma and non-lymphoma are shown in table 18. All 6 non-lymphomatous samples had a polyclonal result by PARR and small lymphocytes (figure 8A) (nuclei diameter less than 2 RBC) were identified via cytology in all the cases. FC immunophenotyping revealed >79% of CD18+ cells in the samples to be lymphocytes expressing CD5 (pan-T-cell marker). No dominant lymphoid population could be identified in 5 cases (figure 8B), whereas the remaining sample was made up for 78.8% by CD4+CD8+ double positive cells. In particular, CD4+CD8+ double positive cells were >40% in 3 out of 6 cases and less than 10% in 1 case.

CASE #	FINAL DIAGNOSIS	CD5	CD4	CD8	DP	DN	CD21
1	Lymphoma dp	98.0	13.1	12.7	67.7	4.5	<1
2	Lymphoma dp	99.4	<1	<1	99.3	<1	<1
3	Lymphoma dp	99.7	<1	<1	99.8	<1	<1
4	Lymphoma dp	99.8	10.7	<1	89.1	<1	<1
5	Lymphoma T4	98.7	88.6	<1	11.0	<1	<1
6	Lympoma dp	100.0	25.8	<1	73.1	<1	<1
7	Lymphoma dn	94.6	11.8	8.6	1.3	72.9	<1
8	Lymphoma dp	ND	1.6	<1	96.7	<1	<1
9	Lymphoma T4	32.2	60.8	<1	<1	<1	<1
10	Lymphoma T4	9.7	87.0	1.9	1.2	<1	<1
11	Lymphoma dp	94.3	14.3	3.1	82.0	<1	<1
12	Lymphoma dp	98.2	1.4	20.6	77.3	<1	<1
13	Lymphoma dp	73.9	8.1	6.1	78.0	<1	<1
14	Non-lymphoma	84.3	24.1	30.7	2.7	26.8	<1
15	Non-lymphoma	92.2	22.8	43.8	17.9	7.7	1.9
16	Non-	86.8	18.2	28.1	45.5	<1	<1

	lymphoma						
17	Non-lymphoma	84.7	9.9	8.1	51.2	15.6	<1
18	Non-lymphoma	97.7	8.1	5.6	78.8	5.2	<1
19	Non-lymphoma	79.7	19.9	24.6	24.2	10.6	<1

Table 18. percentage of positive cells in lymphoid population from 19 cats with mediastinal masses. DP: double positive CD4+CD8+; DN: double negative CD4-CD8-

Immunophenotype of lymphomas

In lymphoma cases, neoplastic lymphocytes were large in size (figure 8C) (diameter of nucleus larger than 2 RBC) in 3 cases (23%) out of 13 while in the majority of cases lymphoid cells were small (8%) to medium (69%). In all cases, a prevalent lymphocyte subpopulation was found, representing more than 60% of CD18+ cells, whereas other residual lymphocyte subsets were poorly represented (figure 8D).

The most commonly established immunophenotype of lymphomas was CD4+CD8+ double positive (9 cases, 69%); while 3 cases (23%) showed a CD4+ T-cell phenotype and 1 case (8%) showed a CD4-CD8- double negative T-cell phenotype. In 2 of the CD4+ lymphomas (#9 and #10), an aberrant phenotype was found, with 60.8% and 87% of cells respectively, staining positive for CD4, but mostly negative for CD5.

In 1 case (#8) the sample was processed with a reduced panel because of the poor cellularity, which allowed labelling just with anti-CD4 and -CD8 antibodies, revealing 96,7% double positive cells, that were also detected in high proportions in thoracic fluid, thoracic lymph node and blood of the same animal.

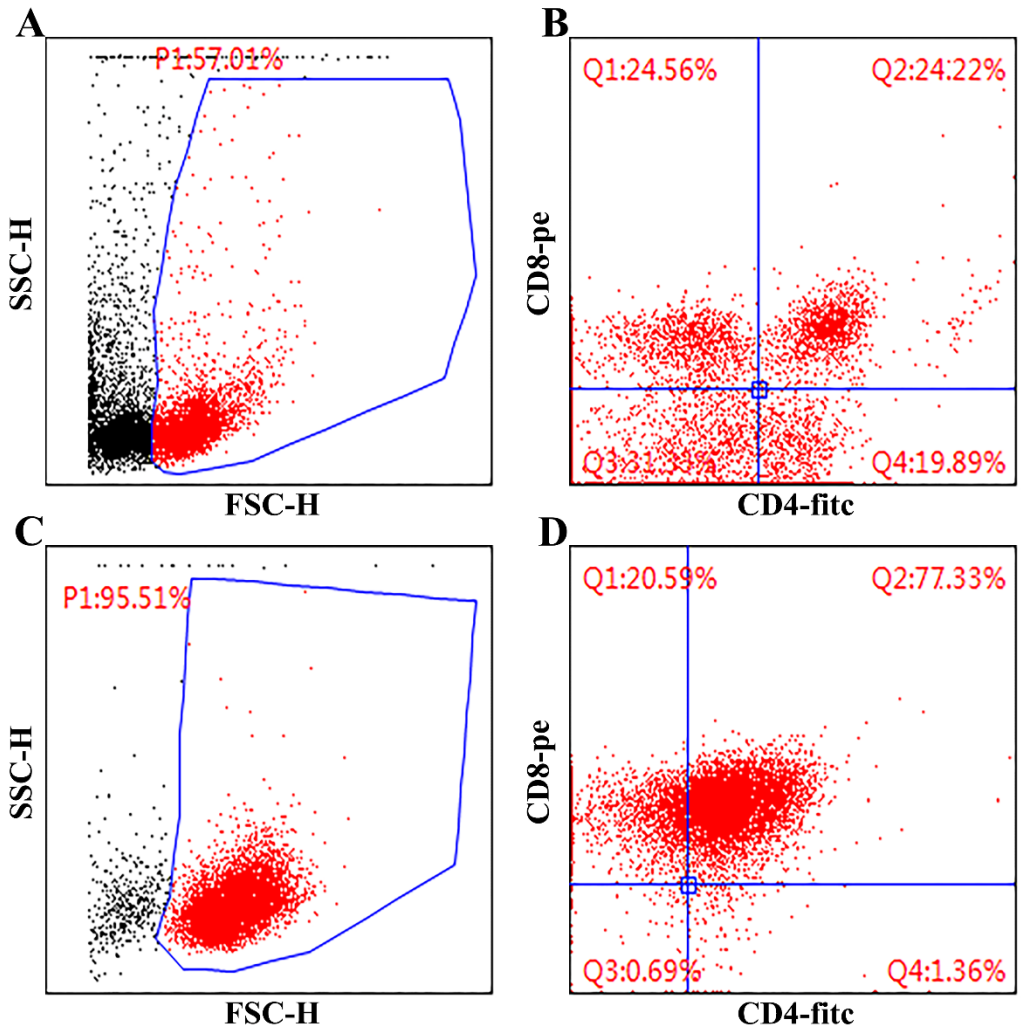

Figure 8. flow cytometric scattergrams of mediastinal masses in two cats. **A, C:** morphological scattergrams, a gate (P1) was set to exclude debris and disrupted cells. **B, D:** only P1 cells are shown, based on CD4 and CD8 staining. Fluorescence discriminators were set based on the level of fluorescence of unstained cells from the same sample. **A, B:** non-lymphomatous lesion; cells are small-sized and composed of mixed subpopulations of T-cells, including CD4+CD8+ double-positive cells (B, upper right quadrant). **C, D:** CD4+CD8+ double positive T-cell lymphoma; cells are medium sized and a dominant population of CD4+CD8+ double positive cells is present (D, upper right quadrant).

Discussion

Mediastinal lymphoma is quite common in young and FeLV positive cats (Gruffydd-Jones et al, 1979; Louwerens et al, 2005). Results of our caseload showed that the median age of cats with mediastinal lymphoma is lower than that of cats with non-lymphomatous mediastinal masses (4 years vs 8.5 years, respectively). Thymoma is by far the most frequent non-lymphoid tumor occurring in mediastinal space mainly in middle age to old cats (Patterson and Marolf, 2014).

Although the two diseases are different in terms of origin and pathogenesis they may resemble in terms of clinical symptoms, imaging and cytological appearance. In both cases small to medium lymphocytes are the prevalent population collected at fine needle biopsy since neoplastic epithelial cells (in thymoma) tend to poorly exfoliate because of their high cohesivity. In one retrospective study, only 7 of 17 mediastinal masses could be definitively diagnosed by cytology (Atwater et al, 1994). In contrast, the two diseases tend to exhibit a different prognosis and to benefit of different therapeutic approach being chemotherapy the preferred option for lymphoma while surgery and/or radiotherapy are often suggested for thymoma (Fabrizio et al, 2014; Roher Bley et al, 2018). Histopathologic biopsy may help to solve differential but tru-cut biopsy, which is the easier technique to sample mediastinal masses, may provide inconclusive results and surgical biopsy are often considered invasive procedures. PARR may also help to differentiate lymphoma and non-lymphoma, but false negative results may occur (Hammer et al, 2016) and it is time consuming. Moreover, PARR doesn't allow important morphological evaluation which should be complementary to other assay and although the lineage determination is possible (even if with some limitations, Hammer et al, 2016), it is not possible to determine the detailed immunophenotype of T-cell neoplasia. In 2006, Lana and collaborators tried to apply FC characterization of lymphoid population from mediastinal masses in dogs and found that a population of more than 10% of double positive (CD4+CD8+) thymocytes is highly suggestive of thymoma. We describe the application of FC to feline mediastinal masses, highlighting some differences with the canine counterpart.

CD4+CD8+ double positive T-cell phenotype is by far the most common in mediastinal lymphomas in our caseload (9 cases, 69% of lymphoma cases), whereas was it represented in one case only (out of 7) in the study by Lana and colleagues. Double positive lymphoma is relatively rare in dog and the majority of mediastinal lymphoma exhibit CD4+ T-cell phenotype (Lana et al, 2006). Because of this high percentage of double positive lymphoma samples in feline species, the cut-off value applied in dogs with a 100% specificity is not valid for cats. In the present case collection, the final diagnosis was challenging in few cases and histopathology was not available to confirm the final diagnosis of thymoma. PARR analysis was performed to assess clonality in all

non-lymphoma cases and to support lymphoma in case #2 which was characterized by a homogeneous expansion of small double positive T lymphocytes.

From our results it emerges that the FC diagnostic criteria suggested to discriminate lymphoma and thymoma in dogs cannot be applied in cats, due to the high prevalence of double positive mediastinal lymphoma. However, some bullet points may be derived, which should be taken into account when dealing with FC analysis of mediastinal mass aspirates in cats: 1) a dominant (>60%) population of lymphoid cells with a phenotype other than double positive CD4+CD8+ cells, is strongly suggestive of lymphoma; 2) lymphoma is highly probable if the dominant population is large-sized, irrespectively from the phenotype; 3) a dominant population of small to medium sized double positive lymphoid cells may be encountered in both lymphomas and non-lymphomatous lesions; 4) a mixed population of T lymphoid cells composed by CD4+, CD8+, double positive CD4+CD8+ and double negative CD4-CD8- lymphocytes, may be considered strongly suggestive of thymoma or other thymic lesions. If a dominant population of small-medium sized double positive cells is encountered at FC, other clinical-pathological features may help to solve the differential, such as the detection of double positive lymphoid cells circulating in peripheral blood (thus confirming lymphoma), the slightly larger size of cells in cytology in lymphoma compared with thymoma (Day, 1997), the positivity to FeLV infection (often related to mediastinal lymphoma, Louwerens et al, 2005) and the young age of cat. PARR could be also useful to confirm clonality and lymphoma, but histopathology remains mandatory for a definitive diagnosis.

The present study has some limitations, starting from the low number of cases (but in line with the only study available in the dog, Lana et al, 2006) and from its retrospective nature which may limit the power of the results and their potential application in a clinical setup. Second, histopathology was not available for all the cases. This is particularly important for confirming the final diagnosis of thymoma vs other non-lymphoid diseases and that is why, to the aims of this research we preferred to split our cases in lymphoma vs non-lymphoma cases rather than lymphoma vs thymoma.

Finally, FC data were obtained using two different instruments during the study period due to the acquisition of a new instrumentation in the lab. The use of different instruments, although adequately controlled and standardized, prevents the comparison between scatter properties and fluorescence intensity but minimally affects results in

terms of percentage of positive cells. This is the reason why we could not compare data regarding cellular size in flow cytometry and just referred to microscopic evaluation of lymphocyte size compared with erythrocytes (Fournel-Fleury et al, 1997) in cytologic smears. A prospective study on a larger caseload using a standardized approach and a single instrument may be useful to confirm if FC cell size (derived by forward scatter properties) could help to differentiate lymphoma vs non-lymphomatous lesions.

In conclusion, FC of mediastinal masses in cats may be of help to diagnose mediastinal lymphoma in case of large lymphoid cells or if an expansion of cells other than double positive CD4+CD8+ lymphocytes. Unfortunately, double positive mediastinal lymphoma is frequent in cats, thus limiting the diagnostic power of FC compared to dogs. A comprehensive evaluation of all anamnestic and clinico-pathological features may help in some cases, but PARR and histopathology are strongly suggested to solve differential in case of expansion of small-medium sized double positive lymphoid cells.

The results of the present study have been submitted to a peer review international journal.

7. THE PATTERN OF EXPRESSION OF CD44 AND CD18 MOLECULES ON THE CELL SURFACE OF WHITE BLOOD CELLS IN CATS: PRELIMINARY RESULTS ON HEALTHY AND DISEASED ANIMALS

Background

The panel of antibodies potentially used in diagnostic of feline lymphoproliferative diseases is very limited, in comparison with what happens in humans and dogs. The poor availability of monoclonal antibodies for feline leukocytes is partly due to the low cross-reactivity of humans/canine antibodies in cats and partly to the limited development of specific antidobies against feline antigens. This strongly under-power the potential use of FC in feline medicine and in diagnosis of feline lymphoma and leukemias. Pan leukocyte markers CD18, CD45 and CD44 are antigens that are generally expressed on all leukocyte subsets with different extent. In canine species, pan-leukocyte markers CD18 and CD45 have shown to give precious information about cells, especially if combined or assessed from a semi-quantitative point of view (Comazzi et al, 2006a, b). CD44 has also formerly shown to be differently expressed in dogs with acute or chronic leukaemia and in healthy controls (Gelain et al, 2014). Possibly, the semi-quantitative analysis of pan-leukocyte markers available in cats might allow to get more information about feline neoplastic samples as well.

Thus, the aim of the present study was to assess the expression of both CD18 and CD44 on white blood cells subpopulations in peripheral blood of healthy feline patients and to provide preliminary information about possible alterations observed in reactive and neoplastic status of lymphoid population.

Materials and methods

Samples for the present study were recruited at the Clinical Pathology Laboratory of the Veterinary Teaching Hospital (University of Milan), according to the following inclusion criteria: 1) feline samples analysed between July 2018 and July 2019; 2) no ongoing treatment with corticosteroids or chemotherapeutic drugs; 3) availability of a final diagnosis; 4) good quality samples with cellularity sufficient to test the whole Ab panel. All samples were delivered to the laboratory and processed within 24 hours from sampling. The tissue type sampled for each cat varied according to the clinician

preferences and diagnostic convenience. All cats were privately-owned and sampled for diagnostic purposes or routine health check. Thus, according to the regulations of the authors' institution, a specific approval of the Ethical Committee was not required for research use of the leftover specimens (EC decision 29 October 2012, renewed with protocol n°02-2016). Peripheral blood (PB) and effusion samples were collected in EDTA tubes. Solid tissues or lesions were sampled via fine-needle aspiration (FNA): the material obtained was partially used to prepare a cytological sample, and partially suspended in tubes containing 1 ml of culture medium (RPMI) for FC analysis. If not provided by the referring veterinarian, PB and effusion smears were prepared by the laboratory staff. All smears and cytological samples were stained with May-Grünwald Giemsa stain.

Flow Cytometry

Samples cellularity was assessed by means of an automated hematology analyser (Sysmex XT 2000-iV, Sysmex, Kobe, Japan) for PB and effusion samples, and by means of a flow cytometer (BriCyte E6, Mindray, Shenghen, China) for FNA samples, enabling the absolute count function. Thereafter, different volumes of each sample were put into FC tubes, according to the total nucleated cell count (TNCC), in order to reach a concentration of $5x10^5$ nucleated cells/tube. In order to reduce non-specific Ab binding, 25 µL of an RPMI solution containing 5% FBS and 0.2% sodium-azide were added to each tube. One tube served as negative control (unstained cells), whereas other three tubes were investigated by means of the following Ab cocktails, respectively: CD5/CD21/CD18, CD4/CD8/CD18, CD44/CD18. The antibodies were reported to be cross-reactive with feline antigens by the manufacturer or were specific for feline species. Ab clones are listed in *table 8*. All antibodies had been titered before use to detect the best working dilutions. After 20 minutes of incubation at room temperature 1ml of solution containing 8% ammonium chloride was added to each tube to lyse erythrocytes. Cells were then washed twice and finally resuspended in 500 µL of PBS 1X for final acquisition.

All samples were acquired immediately after staining by means of a flow cytometer (BriCyte E6). The cytometer status was controlled and, if needed, calibrated at the beginning of each laboratory session by means of specific controls (SPHERO Spura Rainbow Fluorescent Particles Mid-Range, Spherotech, Chicago, IL, USA). Laser voltages

and compensation matrices were kept constant during the whole experiment. For each tube a minimum of $10x10^3$ nucleated cells were acquired.

Analyses were performed using a specific software (MRFlow, Mindray) by a single operator in a single session. For each sample, the median fluorescence intensities (MFI) of unstained cells (FL-1 and FL-4 channels), CD18 and CD44 were recorded. CD18 and CD44 expression were recorded separately for PMNs, monocytes and lymphoid cells, via a back-gating strategy based on the morphological properties of the cells (FSC-H versus SSC-H). PMNs were considered as a whole, with sub-grouping into neutrophils, eosinophils and basophils, since no morphological or phenotypic property was able to distinguish the three subpopulations by FC. CD18-MFI and CD44-MFI were finally calculated for each population by dividing the MFI value of Ab-stained cells for MFI value of unstained cells, in the corresponding fluorescence channel.

All data were included in an electronic sheet. Descriptive statistics (mean, standard deviation, coefficient of variation -CV-, median and range) were calculated for each variable. Neoplastic samples were not included in the descriptive statistics because of the high variability in the cell lineage involved.

Results

Overall, 37 samples from 37 different cats fulfilled our inclusion criteria. These were grouped into healthy cats (16 cats), cats with inflammatory conditions and reactive lymphoid cells (6 cats) and cats with hematopoietic neoplasia (15 cats).

Healthy cats

PB samples from 16 healthy cats were analysed. Thirteen (81.3%) were Domestic Shorthair cats (DSH), 2 (12.5%) were Maine Coon and 1 (6.2%) was a Sphynx. Ten (62.5%) were females (of which 6 spayed) and 6 (37.5%) were males (of which 4 neutered). Mean age was 8.5 $\pm$ 4.5 years, median 8 years, min-max 5 months-18 years. One cat had a history of FeLV infection. At the time of testing, the cat was clinically healthy and tested for annual check, was not receiving any drug and had a normal CBC count and biochemical profile: thus, it fulfilled our inclusion criteria and was retained in the study. Also, CD18- and CD44-MFI of each PB population in this case overlapped with the mean values of the healthy cats' group.

Mean CD18-MFI on monocytes was 1406.4 ± 712.7 (median 1103.5; min-max 492.2-2887.0); mean CD18-MFI on PMN was 536.7 ± 357.3 (median 404.6; min-max 172.5-1361.0); mean CD18-MFI on lymphocytes was 50.6 ± 44.5 (median 37.0; min-max 5.7-169.7). The CV of CD18-MFI was 50.7% for monocytes, 66.6% for PMN and 87.9% for lymphocytes.

Mean CD44-MFI on monocytes was 355.9 ± 97.6 (median 366.5, min-max 212.4-507.7); mean CD44-MFI on PMN was 342.2 ± 82 (median 339.9, min-max 189.0-505.2); mean CD44-MFI on lymphocytes was 81.8 ± 27.6 (median 76.7, min-max 34.5-125.6). The CV of CD44-MFI was 27.4% for monocytes, 24.0% for PMN and 33.8% for lymphocytes.

Monocytes showed the high level of expression of both CD18 and CD44, whereas lymphocytes showed the lowest one for both antigens (*figure 9*). CD18-MFI was 3-fold higher in monocytes than in PMN (mean CD18-MFI ratio between monocytes and PMN = 3.4 ± 2.0), 17-fold higher in PMN than in lymphocytes (mean CD18-MFI ratio between PMN and lymphocytes = 17.2 ± 17.5) and 42-fold higher in monocytes than in lymphocytes (mean CD18-MFI ratio between PMN and lymphocytes = 42.4 ± 28.3). CD44-MFI did not differ between monocytes and PMN (mean CD44-MFI ratio = 1.0 ± 0.2) and was 4-fold higher in monocytes and PMN than in lymphocytes (mean CD44-MFI ratio = 4.4 ± 0.7 and 4.3 ± 0.7, respectively).

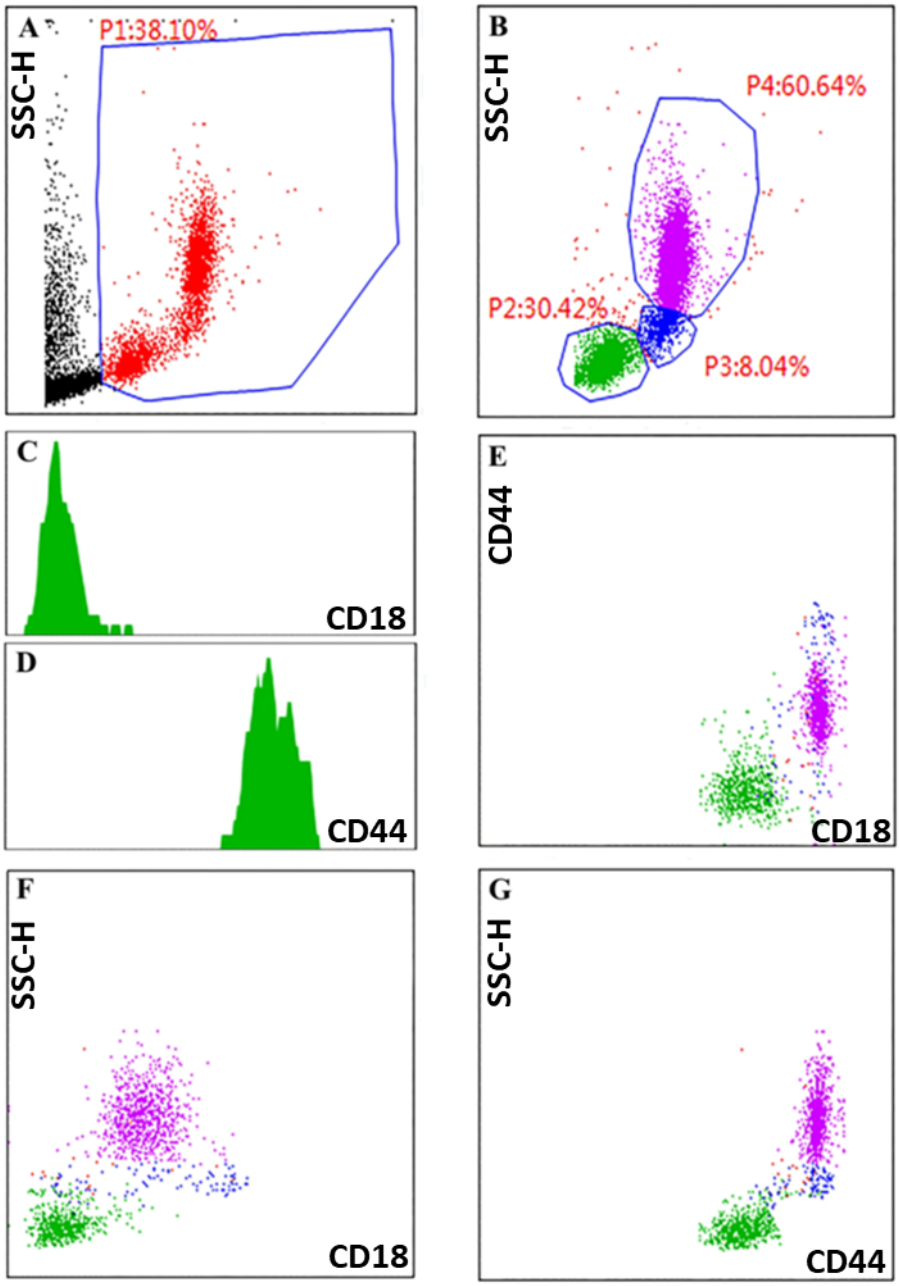

Figure 9: FC analysis of a PB sample from a healthy cat. **A:** cell size (FSC-H, x-axis) VS cellular complexity (SSC-H, y-axis); a gate (P1) was set to exclude platelets and debris. **B:** only P1 cells are shown; three gates were to include lymphocytes (P2, green dots), monocytes (P3, blue dots) and PMN (P4, purple dots). **C:** only lymphocytes are shown; x-axis shows the fluorescence level of CD18-stained cells. **D:** only lymphocytes are shown; x-axis shows the fluorescence level of CD44-stained cells. **E, F, G:** scattergrams showing CD18 and CD44 expression in the three WBC subclasses; colour code is the same of figure 1B. **E:** fluorescence level of CD44 (x-axis) VS CD18 (y-axis). **F:** fluorescence level of CD18 (x-axis) VS cellular complexity (SSC-H, y-axis). **G:** fluorescence level of CD44 (x-axis) VS cellular complexity (SSC-H, y-axis).

Diseased cats: reactive conditions

Six samples were composed of a mixed population of reactive lymphoid cells, including 2 (33.3%) reactive effusions secondary non-neoplastic diseases and 4 (66.7%) lymph node FNA diagnosed as reactive hyperplasia (supported by histopathological examination). Signalment data were lacking in one case. Among the remaining 5 cats, 3 (60%) were DSH, 1 (20%) was a Maine Coon and 1 (20%) was a Norwegian Forest Cat. Three (60%) were intact males and 2 (40%) were spayed females. Mean age was 6.9 $\pm$ 2.7 years, median 7, min-max 2-10 years.

Low percentages of PMN and monocytes were detectable in 1 sample only. Thus, analyses were restricted to lymphoid cells in all reactive samples.

Mean CD18-MFI was 89.0 $\pm$ 71.9 (median 84.5, min-max 9.0-201.2). Mean CD44-MFI was 85.2 $\pm$ 33.4 (median 74.3, min-max 52.4-148.6). The CV for CD18-MFI and CD44-MFI was 80.8% and 39.2%, respectively.

Diseased cats: hematopietic tumors

Fifteen samples from cats with different hematopoietic neoplasms were analysed for CD18 and CD44 expression. Tissue sample included: 5 (33.3%) lymph node FNA, 4 (26.7%) PB samples, 2 (13.3%) effusions, 2 (13.3%) mediastinal mass FNA, 1 (6.7%) splenic FNA and 1 (6.7%) FNA of an intestinal mass. In all cases, a round cell neoplasia was diagnosed based on clinical presentation and cytological examination and phenotyped via FC. In a subset of cases, confirmatory histopathology was also available. All cats were DSH; 10 (66.7%) were males (of which 7 neutered) and 5 (33.3%) were females (of which 2 spayed). Age wasn't reported in 2 cases; for the remaining 13 cats, mean age was 6.5 $\pm$ 4.6 (median 4 years, min-max 2-14 years). Three cats had a history of FeLV infection.

For 9 samples, the final diagnosis was T-cell lymphoma. These included 4 (44.4%) lymph nodes FNA, 2 (22.2%) effusions and 1 (11.1%) each of mediastinal mass, intestinal mass and infiltrated PB. Mean CD18-MFI on neoplastic cells was 119.4 $\pm$ 118.3 (median 48.7, min-max 35.7-354.0). Mean CD44-MFI was 111.0 $\pm$ 45.9 (median 115.3, min-max 32.1-171.4). The CV for CD18-MFI was 99.1%, for CD44-MFI was 41.4%.

Two PB samples with T-cell leukaemia were analysed: one was chronic lymphocytic leukaemia (T-CLL) and one an acute lymphoblastic leukaemia (T-ALL). CD18-MFI and

CD44-MFI on neoplastic cells were 209.0 and 200.1 for T-CLL and 175.3 and 55.6 for T-ALL, respectively.

B-cell lymphomas were diagnosed on a lymph node FNA in two cases, and CD18-MFI and CD44-MFI of neoplastic cells were 53.6 and 46.4, respectively.

Finally, three cats had a diagnosis of round cell tumour, staining negative for the four lymphoid markers tested. Tissue types includean intestinal mass, a splenic FNA and a PB sample. CD18-MFI and CD44-MFI of neoplastic cells were 9.3 and 383.6, 2.3 and 47.6, 1044.9 and 461.0, in the three samples respectively.

Results obtained on circulating lymphocytes in healthy cats, reactive lymphoid cells and neoplastic cells are shown in *figure 10*.

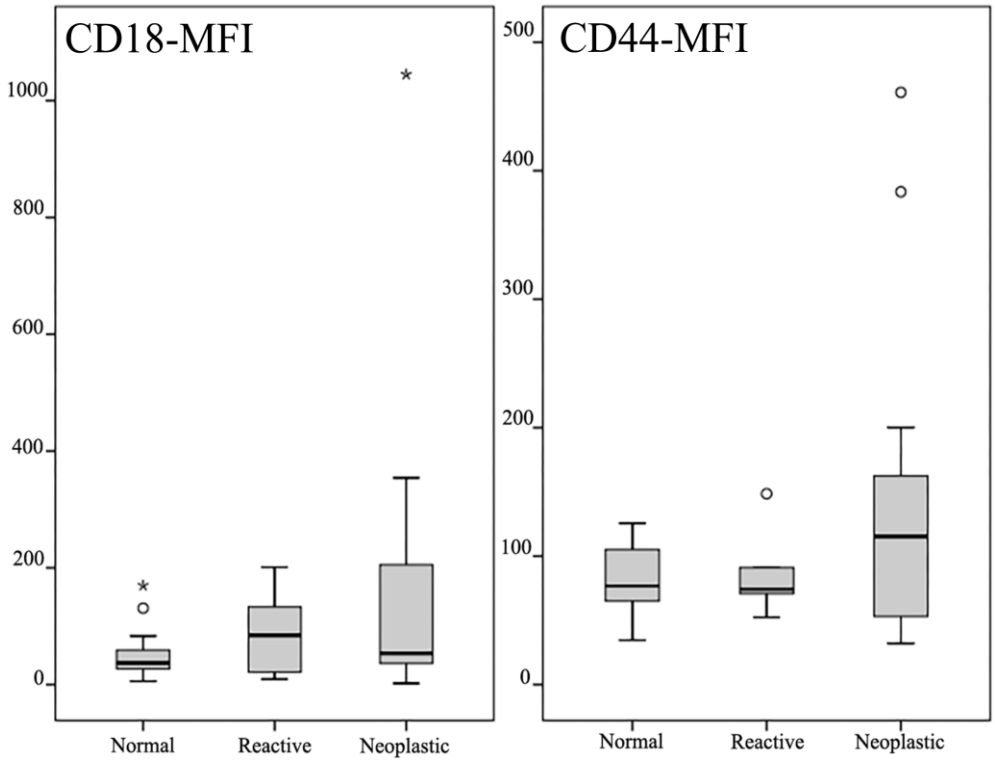

Figure 10: Median fluorescence intensity (MFI) of CD18- and CD44-stained cells from 16 PB samples from helathy cats (analyses restricted to the lymphoid population), samples with reactive lymphoid cells and 15 samples with different hematopoietic neoplasms.

Discussion

The present study describes the pattern of expression of two pan-leukocyte markers (CD18 and CD44) on the cell surface of feline WBC, as detected via FC in the PB of a set of

healthy animals. Preliminary data on reactive lymphoid cells and hematopoietic cells are also provided.

Although both markers stain positive on all leukocytes in each sample, different comments may derive when analysing the level of expression in the different cell populations.

Regarding PB samples from healthy cats, both proteins are expressed at higher levels on monocytes than on PMNs and lymphocytes. However, CD18-MFI allows a better discrimination than CD44-MFI among the three subclasses, as documented by the higher rations obtained when coupling CD18-MFI on monocytes with either CD18-MFI on PMNs or lymphocytes. On the contrary, despite the higher mean and median CD44-MFI shown by monocytes compared with PMNs, the ratio between the two values was close to 1, thus complicating the discrimination between the two classes based on fluorescence level. As a result, lymphocytes are easily identified in a dot plot coupling the intensity of fluorescence of CD18 and CD44, being a discrete population with low intensity of fluorescence of both antigens. Conversely, monocytes are located at the edge of a smear, with homogenous CD44-MFI, without a clear separation from the PMNs cloud (*figure 9*). Monocytes and PMNs are more easily discriminated by coupling CD18-MFI with a complexity index (SSC-H): this type of scattergrams seems to be the most appropriate to distinguish among WBC subclasses in cats. Unfortunately, CD18-MFI suffers from a great variability within each WBC subclasses, as documented by our results on healthy cats: monocytes had the lowest CV for CD18-MFI, still being >50%, and a peak of about 89% was reached for lymphocytes, whereas the CV for CD44-MFI was consistently <35%.

Besides serving as pan-leukocyte markers and being of aid in FC analysis, both molecules have biological roles that may explain the different degree of expression detected in the present study in reactive and neoplastic samples.

CD18 is a component of β2 integrins, which are adhesion molecules involved in leukocyte extravasation. It is expressed on the cell surface of all WBC subclasses, with variable levels of expression according to cellular activation and differentiation status and is primarily involved in leukocytes rolling on the endothelium and subsequent diapedesis (Tan, 2012). Indeed, genetic deficiency of CD18 cause impaired leukocyte extravasation and immunodeficiency in many different species, including cats (Bauer et al, 2017). The slightly higher mean CD18-MFI we encountered in reactive cells compared to resting

lymphocytes in the PB of healthy cats may be linked to the activated status of the cells or their location out of blood vessels or both. Conversely. Neoplastic cells had a more variable CD18-MFI with a mean value close to the one of resting lymphocytes. The different lineages and maturation status of the neoplastic cells may account for this variability and the degree of expression when compared to resting lymphocytes.

Interestingly, one neoplastic sample had an extremely high level of CD18 expression. This was a PB sample from a cat with severe cytopenias and circulating cells with cytological immature appearance. No Ab reacting with feline myeloid antigens was available in the laboratory at the time of testing this sample, and all lymphoid markers stained negative. Thus, the cat was diagnosed with acute leukaemia of unknown lineage. Neoplastic cells also showed an unusually high expression of CD44. Both CD18-MFI and CD44-MFI overlapped the values obtained on normal myeloid cells. This may suggest a myeloid origin of the neoplastic cells. However, the potential usefulness of pan-leukocyte marker as a tool for acute leukaemia classification in cats should be further investigated.

CD44 is a hyaluronan receptor ubiquitously expressed on the cell surface and involved in many processes requiring interaction with the extracellular matrix. This molecule is considered a cancer stem cell marker and has been studied extensively because of its role in tumorigenesis and development of metastasis (Morath et al, 2016). Based on our results, the degree of expression of this molecule is minimally variable within each WBC subpopulation. Data obtained on resting circulating lymphocytes and reactive lymphoid cells outside blood vessels completely overlap, thus excluding any variation with activation status and extravasation. Conversely, CD44-MFI was higher and more variable among neoplastic samples. On one hand, its higher expression is highly linked to the neoplastic nature of cells supporting the role of CD44 in hematopoietic cancer development in the feline species. On the other hand, the high variability of expression among neoplastic samples is likely dependent on cell lineage and maturation stage of the cells involved. When considering the homogeneous group of T-cell lymphomas, the variability of CD44-MFI was only slightly higher than the one obtained on resting lymphocytes in healthy cats (CV=41.1% and 33.8% respectively). The only B-cell lymphoma we included had relatively low expression of both CD18 and CD44, although both values were included in the range of expression of T-cell lymphomas. Unfortunately, we were not able to assess any possible difference in pan-leukocyte markers expression

among different lymphoid subclasses even in healthy conditions, because of conflicting combinations of antibodies and fluorochromes. Of notice, however, only one peak was noted on the histograms when assessing CD expression on resting lymphocytes (figure 9), suggesting a homogeneous expression within the whole population, likely not dependent from the composition of the population itself. Whether different degrees of expression of CD44 may play a diagnostic role in discriminating between neoplastic and non-neoplastic samples, and among different neoplasms, is still has to be elucidated.

The present study has some limitations. The first one is the small number of samples analysed; our results are only preliminary and should be validated on a larger caseload. Second, the combination of antibodies and fluorochromes prevented us from assessing CD18 and CD44 expression within single lymphoid subclasses. Third, few cases in the study had a history of FeLV infection. The only FeLV-infected cat included in the healthy cat group had CD18- and CD44-MFI values overlapping with the mean of the group: serological positivity alone in an otherwise healthy cat seems not to affect the two molecules. Still, we cannot exclude that active viral replication causing leukopenia and clinical signs may influence antigen expression.

In conclusion, this is the first study describing the pattern of CD18 and CD44 expression on feline WBC in healthy and pathological conditions. Our results highlight similarities and differences in the expression of the two antigens. We support the combined assessment of both molecules in FC practice, in order to amplify the information gained with a single analysis. Future studies should assess whether the inclusion of CD45 in the antibody panel may further improve the amount of information gained with the analysis of pan-leukocyte markers expression on feline WBC.

The results of the present study have been submitted to a peer review international journal.

8. CONCLUSIONS

The present PhD project had the aim of providing support to the application of FC in the diagnosis of feline hematopoietic malignancies similarly to what already done in the dog. The four studies illustrated above, showed, each, fair results.

In particular, the first study highlighted the sample cellularity to be the major factor affecting likelihood of samples to be processed for FC analysis and no differences in sample quality was detected between peripheral and intra-abdominal lesions, supporting once again the possibility to apply this diagnostic tool in feline species.

An important step was achieved with the description of non-neoplastic counterpart (reactive or not) of feline lymph nodes lymphoid subsets. Subsets identified were really similar to those observed in the dog. The only significant difference revealed by our results was the higher percentage of CD8+ cells in reactive samples. The major limitation of this study is the low number of samples and a comparison with neoplastic counterpart in the future should be considered. The low number of samples is the reason why we decided not to submit this study as manuscript yet, but to proceed with the sample collection to improve the significance of our observations and data.

The third study showed that the criteria suggested to discriminate lymphoma and thymoma in the dog aren't suitable to feline counterpart because of the high number of double positive mediastinal lymphomas, making histopathology mandatory to solve differential in these cases. Otherwise, large lymphocyte masses or clonal expansions other than double positive lymphocytes are strongly suggestive of lymphoma.

The last study revealed important preliminary data about the expression and MFI of CD18 and CD44, which resulted to be of aid in discriminating WBC subclasses. Individually, CD18-MFI had a high variability between monocytes, PMNs, lymphocytes and was higher in reactive lymphocytes, whereas CD44-MFI had a lower variability between the populations but was higher in neoplastic cells.

The present PhD project offers preliminary data that should be developed. Thus, further studies are warranted to better define the possible use and application of FC as a routine diagnostic test in feline species.

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10. OTHER STUDIES

During my PhD I was involved in some studies which didn't concern my PhD project and that will be described in this small section.

- Comazzi S, Cozzi M, **Bernardi S**, Zanella DR, Aresu L, Stefanello D, Marconato L, Martini V. *"EFFECTS OF PREANALYTICAL VARIABLES ON FLOW CYTOMETRIC DIAGNOSIS OF CANINE LYMPHOMA: A RETROSPECTIVE STUDY (2009-2015)".* Vet J 2018 Feb; 232: 65-69.

Flow cytometry (FC) is increasingly being used for immunophenotyping and staging of canine lymphoma. The aim of this retrospective study was to assess pre-analytical variables that might influence the diagnostic utility of FC of lymph node (LN) fine needle aspirate (FNA) specimens from dogs with lymphoproliferative diseases. The study included 987 cases with LN FNA specimens sent for immunophenotyping that were submitted to a diagnostic laboratory in Italy from 2009 to 2015. Cases were grouped into 'diagnostic' and 'non-diagnostic'. Pre-analytical factors analysed by univariate and multivariate analyses were animal-related factors (breed, age, sex, size), operator-related factors (year, season, shipping method, submitting veterinarian) and sample-related factors (type of sample material, cellular concentration, cytological smears, artefacts). The submitting veterinarian, sample material, sample cellularity and artefacts affected the likelihood of having a diagnostic sample. The availability of specimens from different sites and of cytological smears increased the odds of obtaining a diagnostic result. Major artefacts affecting diagnostic utility included poor cellularity and the presence of dead cells. Flow cytometry on LN FNA samples yielded conclusive results in more than 90% of cases with adequate sample quality and sampling conditions.

- Martini V, Melega M, Riondato F, Marconato L, Cozzi M, **Bernardi S**, Comazzi S, Aresu L. *"A RETROSPECTIVE STUDY OF FLOW CYTOMETRIC CHARACTERIZATION OF SUSPECTED EXTRANODAL LYMPHOMAS IN DOGS".* J Vet Diagn Invest 2018 Nov; 30(6): 830-836.

Flow cytometry (FC) is widely applied to characterize and stage nodal lymphomas in dogs because it has a short turnaround time, requires minimally invasive sampling, and allows contemporary evaluation of neoplastic cells in the primary lesion and of blood and marrow involvement. We investigated advantages and limitations of FC in suspected extranodal lymphomas in dogs. The likelihood of obtaining a suitable FC sample was significantly lower for aspirates of extranodal lesions than for lymph node aspirates. However, we noted no differences among different extranodal lesion sites. We also describe FC results for 39 samples compatible with extranodal lymphoma. A dominant population of large cells was easily identified on morphologic FC scattergrams in many cases. Phenotypic aberrancies were frequently present, mainly in T-cell lymphomas. Lymphoma cells were distinguishable from normal residual lymphocytes in >85% of cases, facilitating the quantification of putative blood and marrow involvement by FC. Despite the high percentage of non-diagnostic samples (32 of 73, >40%), we support the inclusion of FC in the diagnostic workup of suspected extranodal lymphomas in dogs, in conjunction with histopathology. Histopathology is the gold standard for diagnosing lymphoma, provides relevant information, including tissue invasion and epitheliotropism, but has a longer turnaround time.

- Martini V, **Bernardi S**, Russo V, Guccione J, Cmazzi S, Roperto S. *"BLOOD LYMPHOCYTE SUBPOPULATIONS IN HEALTHY WATER BUFFALOES (BUBALUS BUBALIS, MEDITERRANEAN LINEAGE): REFERENCE INTERVALS AND INFLUENCE OF AGE AND REPRODUCTIVE HISTORY".* Vet Immunol Immunopathol. 2019 May; 211: 58-63.

There is an increasing interest toward infectious diseases and mechanisms of immune response of water buffaloes, mainly because of the growing economic impact of this species and of its high-quality milk. However, little is known about the immune system of these animals in physiological conditions. Recently, a wide number of antibodies cross reacting with buffalo antigens has been validated for use in flow cytometry (FC), allowing detailed characterization of the lymphocytic population in this species. The aim of the present study was to describe the lymphocyte subpopulations in a large number of healthy water buffaloes, providing reference intervals (RIs), and to assess whether the composition of blood lymphocyte population significantly varied with age and reproductive history. Our final aim was to lay the ground for future studies evaluating the role of host immune response in water buffaloes. One-hundred-twelve healthy buffaloes from four different herds in the South of Italy were included in the study. All animals had been vaccinated for Infectious Bovine Rhinotracheitis (IBR), Salmonellosis, Colibacillosis and Clostridiosis, and all herds were certified Brucellosis- and Tuberculosis-free. Venous blood collected into EDTA tubes was processed for FC, and the percentage of cells staining positive for the following antibodies was recorded: CD3, CD4, CD8, CD21, TCR-δ-N24, WC1-N2, WC1-N3 and WC1-N4. Absolute concentration of each lymphoid subclass was then calculated, based on automated White Blood Cell (WBC) Count. Reference Intervals were calculated according to official guidelines and are listed in the manuscript. The composition of the lymphocyte population varied with age and reproductive history, with animals <2-years-old and heifers having higher concentration of most of the subclasses. The present study provides RIs for the main lymphocytic subclasses in healthy water buffaloes, highlighting gross differences between young and old animals. Establishment of age-specific RIs is recommended in water buffaloes. The data we present may be useful as a basis for further studies concerning mechanisms of immune response toward infectious agents in water buffaloes.