

Lymphoproliferative disease. Diagnosis and more.

Definition: A neoplastic clonal expansion of lymphoid cells.

Disease primarily affecting bone marrow / blood is referred to as **lymphoid leukaemia**.

Disease primarily affecting other sites is referred to as **lymphoma**.

In dogs multicentric lymphoma accounts for approximately 80% of lymphoma cases, alimentary 7%, cutaneous 6% and mediastinal 3% with other miscellaneous cases in low numbers: CNS, bone, heart, nasal, ocular. It is commonest in middle aged to older animals. There is no known viral or infectious cause. Mutations in the tumour suppressor gene p53 have been identified in some cases.

Most cases present with generalised lymphadenomegaly but are otherwise well. 10-20% present with illness as a direct effect of neoplastic cell proliferation (interfering with organ function), cytopaenias secondary to myelophthisis or paraneoplastic disease. The most documented paraneoplastic disease in lymphoma is hypercalcaemia, most often associated with elaboration of parathyroid hormone related peptide (PTH-rP), especially by neoplastic T-cells. It has only rarely been documented in B-cell neoplasms and these may induce hypercalcaemia via osteolysis or activation of macrophages and increased activated vitamin D production.

Lymphoid leukaemias are less common than lymphoma and are classified as acute (proliferation of immature lymphoblasts) or chronic (proliferation of mature lymphocytes). They are commonest in middle to old aged dogs.

In cats data is harder to collate since the introduction of widespread FeLV vaccination. Prior to this multicentric and mediastinal lymphoma were commonest with a preponderance in young cats (median 4-6y). Now alimentary lymphoma is the most common form with a median age of 9-10 years and <5% FeLV positive. See Table 1.

As in dogs, lymphoid leukaemias are less common than lymphoma. Some cases of acute lymphoid leukaemia may be FeLV associated. Chronic lymphoid leukaemia is uncommon with no known association with FeLV.

Site	Frequency%	Age (y)	T-cell association	FeLV positivity
Alimentary	50-70	Aged	Low *	<5%
Multicentric	10-25	?FeLV	?FeLV	30%
Mediastinal	10-20	young	high	>80%
Nasal	10	aged	low	Low
Renal	5-10	mid	low	low to moderate

Table 1 Characteristics of feline lymphoma.

* This varies with exact site (eg LI and gastric lymphoma is usually B-cell, SI lymphoma is variable).

Diagnosis.

Diagnosis of leukaemia and lymphoma relies on ruling out other potential causes of the presenting signs and clinical data and on demonstrating clonality. For lymphoma a common presenting sign is lymphadenomegaly but signs may also relate to involvement

of certain organs and sites (eg alimentary, cutaneous, mediastinal). There is a wide range of non specific presenting signs for leukaemia but often the first indication of disease is finding lymphocytosis or circulating atypical/ectopic cells. Differential diagnoses for lymphoma and leukaemia are set out in Table 2.

Lymphoma	Differential diagnoses.
Lymphadenomegaly	Reactive lymph node enlargement secondary to infections or inflammation (including immune-mediated disease). Mineral associated lymphadenopathy. Other haematopoietic tumours (eg lymphoid / myeloid leukaemias, malignant / systemic histiocytosis). Metastatic tumours.
Alimentary	Inflammatory infiltrative disease (eg lymphocytic / plasmacytic histiocytic enteritis) Other intestinal neoplasms (eg adenocarcinoma, mast cell tumour in cats)
Cutaneous	Infectious / parasitic dermatitis. Immune mediated dermatitis. Other cutaneous neoplasms (eg squamous cell carcinoma)
Mediastinal	Thymoma. Heart base tumour (chemodectoma) Ectopic thyroid tumour. Inflammation, including granulomatous lymphadenitis/reactive lymphadenomegaly or mediastinal abscess / FB.
Leukaemia Lymphocytosis. In reality it is unusual to see lymphocyte counts >30/ul? (cat) and 10/ul?(dog (except Ehrlichia)) with non-neoplastic lymphoid disease.	Physiological (?adrenaline induced release from spleen) – not common in the dog. Age – mild lymphocytosis is not uncommon in immature animals, especially after vaccination. Lymphoma. Hypoadrenocorticism (mild lymphocytosis only) Thymoma. Chronic inflammation (more common in cats, mild in some dogs (eg CKCS). Infection. Uncommon in dogs but may occur with Ehrlichia, Brucella, Leishmania, rocky mountain spotted fever, Aspergillus and other fungal diseases, Spirocerca, Babesia, Dirofilaria immitis. More common in cats: FIV, Toxoplasma, FeLV, Mycoplasma felis, hyperthyroidism, immune mediated haemolytic anaemia..

Table 2 Differential diagnoses for lymphoma and leukaemia.

A feature of many of the differential diagnoses for leukaemia and lymphoma listed above is the non-clonal proliferation of lymphocytes. In contrast, and by definition, neoplastic lymphoproliferative disease is the result of clonal expansion of a (usually) single transformed lymphoid cell. Clonal lymphocyte proliferation secondary to antigenic stimulation is uncommon. Ehrlichiosis is a documented example in dogs in which no direct transforming effect on the genome has yet been identified. Helicobacter associated MALT lymphoma in humans is another example. These examples appear to induce clonal lymphoid proliferation by means other than genomic transformation. In contrast transformation secondary to viral disease is well documented in humans and some animals (eg FeLV / FIV cats, HTLV / EBV in humans, BLV in cattle).

The concept of clonality is central to the diagnosis of lymphoproliferative disease by both conventional and more recent molecular diagnostic techniques.

Cytology

In the cytological evaluation of lymph node aspirates identifying an excessively expanded population of lymphoid cells sharing similar morphology is one of the major criteria for the diagnosis of lymphoma. Here the “evidence” for clonality relies on detailed morphological assessment of lymphoid cells and the reliability of this judgment depends on factors including the nature of the lymphoproliferative disease, the quality of the smears / staining and the experience of the cytologist. In general cytology allows excellent morphological assessment of individual cells but limited appreciation of tissue architecture.

In most cases of lymphoma the cytological findings of a monomorphic proliferation and disruption / depletion of the expected normal lymphoid cell populations allow a confident diagnosis of lymphoma. In some types of lymphoma (eg follicular), where the disease has been detected early or where there is confounding disease (eg reactivity in submandibular nodes) these changes may not be so clear cut. This problem is especially heightened where a single smear representing a single node has been submitted but can be overcome by evaluation of several smears from several enlarged nodes. The chances of obtaining a non-representative sample of the nodal population are greater the fewer aspirates (from fewer nodes) are performed / submitted. When I am in doubt about the diagnosis of lymphoma I recommend further investigation with histopathology, preferably of an excised node.

Histopathology

The principles of lymphoma diagnosis in histopathology are broadly similar to those of cytology. Clonal proliferations of lymphocytes may be appreciated in histological sections from lymphoid tissues but it may be harder to accurately assess individual cellular morphology. However histopathology, especially of whole excised nodes, allows an interpretation of architecture. This may be useful in certain types of lymphoma and in early disease where the proportion of clonally proliferating lymphocytes in the whole node is not markedly increased but where their abnormal distribution allows a diagnosis to be made (eg mantle cell, marginal zone, follicular lymphomas).

In both cytology and histopathology, especially as the (early) diagnosis of lymphoma improves, differentiating ‘reactive variation’ from developing neoplasia may become more difficult, requiring either serial sampling or further more specific and specialised investigations.

Immunochemistry.

This technique may be applied to cytological and histological specimens. It uses monoclonal antibodies or polyclonal sera specific for certain key membrane, cytoplasmic or nuclear markers to distinguish lymphocyte subtypes (eg CD3 in T-cells, CD21 in B-cells). The antibody bound cells are detected by virtue of the labelling of antibodies with fluorochromes or, more traditionally, enzymes (eg horseradish peroxidase) to induce a colour change in the presence of a substrate. Histopathology lends itself to this technique due to the availability of tissue for staining (more sections are simply cut from the tissue

block). However, the number of antibody markers available for use in formalin fixed tissue is limited. The technique may be applied to cytological smears but preferably not after conventional staining (ie you have to submit plenty of good quality smears and specificity in advance that you require immuno staining). This detracts from immunocytochemistry, even though a wider range of antibodies is available.

Flow cytometry.

A powerful variation of immunochemistry has recently been applied to canine lymphoproliferative disease. This is known as flow cytometry and may be applied directly to any fluids (including blood) and, after suitable preparation, to fresh aspirated or biopsied material. It allows rapid evaluation of cells using a large panel of antibodies specific for different cell markers. Cambridge University Veterinary School is the only UK laboratory to offer this technique. Fresh cells provide optimal results. Where samples are to be posted the addition of fixative to blood or resuspended aspirated / biopsied tissue is necessary. This can be supplied by the laboratory at Cambridge. Contact 01223 337625 for further information.

The flow system is designed to deliver particles (cells) in single file through a laser beam of blue light generated by an argon laser. As cells pass through the beam they scatter this blue light at different angles and can also lead to the generation of fluorescent light (green, orange and red) at longer wavelengths. A proportion of the blue light is refracted at a low angle, and is termed forward scatter. The amount of forward scatter correlates with cell size and is measured by a detector (photoelectric diode) in line with the laser beam (after blocking the direct laser beam). Some blue light is scattered at a high angle, termed side scatter, and the amount of side scatter is correlated with cell complexity (granularity and /or density). Side scatter and fluorescent light are measured at 90° to the direction of the laser beam using a system of dichroic mirrors. These are filters orientated at 45° to the direction of the light. The first mirror encountered by the beam reflects blue light onto a detector, but allows light of longer wavelength (green, orange and red) to pass through. The beam then encounters a mirror reflecting green light but transmitting orange and red light, and finally a 3rd mirror reflecting orange light but transmitting red light. Thus the amount of green, orange and red fluorescent light can be measured individually.

Unlabelled cells produce a low amount of green, orange and red fluorescence. However when a cell labelled with a fluorochrome (fluorescent marker) encounters the laser beam a large amount of fluorescent light is produced at a wavelength specific for that fluorochrome. Fluorescein isothiocyanate (FITC) produces green light, phycoerythrin (PE) produces orange light and peridinin-chlorophyll (PC) produces red light. For immunophenotyping, cells are stained with a monoclonal antibody (mab) conjugated with a fluorochrome, commonly FITC. If the antigen detected by the mab is present on the cell, the mab binds and the presence of FITC on the cell leads to a large amount of green fluorescence as detected on the FITC detector.

Cells may be dual stained with two, (or even three) antibodies, one conjugated with FITC and the second with PE (and the 3rd with PC). For dual staining if both of the antibodies bind, both orange and green fluorescence is detected. There is a degree of overlap in the wavelengths of light generated by FITC and PE, which means a cell labelled only with FITC will produce a small amount of light detected by the PE detector and vice versa, potentially creating false positive dual staining. This overlap must be corrected for, a process known as compensation. Cells labelled only with the FITC-conjugated antibody are analysed and the PE signal is altered electronically until it reads zero. The process is repeated with cells labelled only with PE-antibody adjusting the FITC signal to zero before running the dual-stained cells.

Aliquots of cells are stained with a panel of antibodies and suitable negative controls, the red cells being lysed by one of a number of reagents (eg. ammonium chloride). Single staining is routinely performed in veterinary medicine - one antibody in each tube. The first step in analysis is to locate the cells of interest by assessing their side scatter (SSC) and forward scatter (FSC) characteristics. A dot plot with FSC on the x-axis and SSC on the y-axis is produced (as is performed by flow cytometer haematology instruments). Knowledge of the normal position of neutrophils, monocytes and lymphocytes (or myeloid and erythroid precursors for bone marrow samples) on the plot is useful for identifying the position of abnormal blasts. Instrument settings must be kept constant if this is to be achieved. A “gate” is drawn around the population of interest and a histogram plot is produced, showing fluorescence on the x axis (log scale) and cell number on the y axis. This plot is formatted to show fluorescence of the gated area only and to show the fluorescent channel corresponding to the fluorochrome being used. The sample tubes are analysed in turn and the fluorescence of each antibody is compared to its negative control. The negative control samples show dim fluorescence (due to cell autofluorescence) with a peak in the first log decade. Positive staining (binding of the mab) is indicated by brighter fluorescence with a peak to the right of the negative control.

CD3-12 (intracellular)	Early and mature T cells
CD3 (surface)	T cells (not primitive precursors)
CD4	Helper T cells, neutrophils and their precursors (canine)
CD5	T cells, (subset of B cells?)
CD8	Cytotoxic T cells
CD11a	Leukocytes, histiocytes
CD11b	Granulocytes and monocytes, histiocytes
CD11c	Granulocytes monocytes, and histiocytes
CD11d	Large granular lymphocytes & subset of monocytes
CD14	Monocytes
CD21	B cells (expressed @ later stage than CD79a)
CD79a	Early and mature B cells
Surface Ig	IgG, IgA and IgM on mature B cells and plasma cells
CD45	Leucocytes
CD45RA	B cells, early blasts of other lineages
CD90	T cells, monocytes -
MHC-II	B cells, monocytes, antigen presenting cells
MPO	Myeloperoxidase in neutrophils & precursors
MAC 387	Neutrophils and monocytes/macrophages
Neutrophil specific ab	Neutrophils and maturer precursors
CD34	Stem cells and early committed myeloid and lymphoid progenitors

Table 3 Showing some of the differentiation markers and corresponding cell expression employed at Cambridge University Veterinary School for characterization of leukaemias.

Immunochemistry methods do not necessarily detect clonality.

Even if 90% of lymphocytes in a node are found to be T-cell this is not synonymous with clonality. However clonality may be inferred where there is aberrant expression of cell markers by neoplastic lymphoid cells (eg concurrent expression of B and T cell markers). One interesting exception to this is the co-expression of CD4 and CD8 on T-lymphocytes. Such a cell population may well represent an atypical clonal proliferation but this phenotype also occurs in immature thymic T-cells prior to differentiation into helper T-cells (CD4+) or cytotoxic T-cells (CD8+).

How can we reliably detect clonality?

The PARR test (PCR Antigen Receptor Rearrangements) uses PCR to detect clonal rearrangements of immunoglobulin (B-cell) or CD3 (T-cell receptor). It is currently available only in the USA and has been shown to be a sensitive method for detecting neoplastic lymphoid cells in peripheral blood and tissues. This test may be applied to blood or to aspirated / biopsied tissue. The products of PCR amplification are separated electrophoretically and normal animals show a broad smear of bands corresponding to the myriad gene rearrangements in different lymphocytes. Most patients with lymphoma have single prominent bands because the majority of the cells present have arisen from a single transformed cell with a single gene rearrangement. It still suffers from certain infectious false positives (eg ehrlichia) and also apparent false negatives.

Why bother with immunochemistry?

The historical concept of lymphoma as a single disease entity belies the true nature and complexity of the disease. In human medicine serial attempts have been made to classify lymphoproliferative disease on the basis of distribution, morphology, molecular phenotypes and genetic alterations. A major purpose of classification is that it allows clinicians to take a sub-type of disease and predict optimal treatment regimens and prognosis. These attempts have culminated in the WHO (World Health Organisation) classification of human lymphoma in which there are currently approximately 35 sub-types with vastly different prognoses and therapies. This classification scheme is monitored and adjusted to incorporate new technologies, information, drugs and outcomes.

In veterinary medicine we are beginning to try to apply the same techniques to lymphoproliferative disease in dogs and, to a lesser extent, cats. Two classification schemes (modified Kiel and WHO) currently predominate but work is in progress to rationalise classification worldwide. As in the human field the classification is based on histopathological and cytological grades (which take into account architecture, mitotic rate and cell morphology) as well as immunophenotype. **It is hoped that this will culminate in a better understanding of lymphoproliferative disease allowing more accurate prediction of prognosis and, eventually, treatment.**

What do we already know about the impact of immunophenotype on prognosis in canine lymphoma?

Results vary a little between studies. In general B-cell lymphoma (50-70%) is more common than T-cell (approx 30%). Some do not fit into either category either having dual staining or no staining (null cells). The single commonest sub-type of lymphoma is diffuse large B-cell, accounting for approximately 40% of canine multicentric lymphomas.

There have been few case series to date but initial reports are revealing. In general prognosis is poorer for T-cell lymphomas than for B-cell. In one study involving identical CHOP therapy in 55 dogs the median survival for T-cell lymphomas was 159 days versus 389 days for B-cell lymphomas **although this is an over-simplification**.

In another recent study involving 57 dogs, again given identical chemotherapy, a T-cell lymphoma (designated Small Clear Cell or T-zone lymphoma) in fact had the most favourable prognosis in terms of duration of first remission (median 12 months) and overall survival (median 21 months) time and a B-cell lymphoma (sub-type Burkitt) effectively had no remission time and a median survival time of < 1 month. **Relying on simplistic immunophenotyping alone may be misleading (ie. CD3 versus CD79a, T versus B cell differentiation)**. Overall, in this study, first remission duration and overall survival time were significantly longer for B-cell lymphoma largely due to the prevalence of the commonest sub-type (diffuse large B-cell). This sub-type accounted for 20 out of 57 lymphoma cases and (despite classification as high grade) had a median duration of first remission of 12 months and an overall survival time of 17 months.

Lymphomas which are currently considered indolent in dogs include follicular lymphomas (FL), mantle cell lymphoma (MC) and marginal zone lymphoma (MZL) which are all B-cell. Together with TZL these are uncommon, probably accounting for less than 10% of all canine lymphomas, although the exact number is unknown and these are possibly under-diagnosed. With the exception of the B-cell Burkitt subtype, lymphomas with especially poor prognosis are generally T-cell (eg T-plasmacytoid, T-lymphoblastic).

Another fact worthy of note is the relationship between grade, prognosis and treatment. Many of the indolent lymphomas are of low grade with long survival times but often have poor responses to chemotherapy (eg reduction in size of lymph nodes). Conversely, lymphoma classified as high grade (eg the commonest lymphoma sub-type: diffuse large B-cell) may result in short survival time without treatment but may respond very well to chemotherapy.

Summary of diagnosis.

- Signalment.
- History (ie ill?) – this affects stage.
- Clinical examination (part of staging).
- Imaging (eg mediastinum, liver, spleen, kidneys – a further part of staging).
- Appropriate lymph node aspirates / biopsies.
- Full biochemistry and blood count with blood film examination to allow staging (I-V and a or b).
- Retroviral status in cats.
- Further investigations: immunohistochemistry, PARR, flow cytometry, genetic analysis.

Prognosis

Once a diagnosis of lymphoma has been made we should attempt to give an idea of prognosis. What factors affect prognosis in canine and feline lymphoma?

The two factors in canine lymphoma which have the strongest association with prognosis are **WHO substage** ((a) – not clinically ill or (b) – clinically ill) and **immunophenotype**. The study involving 55 dogs outlined above (Keller) assessed the impact of WHO substage. Substage (a) patients had median survival times of 345 days, those of substage (b) 44 days. Factors with moderate association include sex (males have poorer prognosis?), hypercalcaemia. Factors with weak association include clinical stage (I-V) and histologic grade (eg high grade tumours may be more responsive to chemotherapy). Many of these factors are not independent, eg hypercalcaemia is dependent largely on immunophenotype.

It may be a shock that clinical stage of lymphoma in dogs is so weakly associated with prognosis but it is the case with the exception of advanced Stage V lymphoma where heavy infiltration of the bone marrow results in cytopenias. Why bother staging? It allows for a more accurate assessment of relapse and, to a lesser extent, remission. It also allows for the detection of intercurrent disease. Human lymphoma staging has been modified to more accurately reflect clinical presentations and appears to have a greater prognostic input.

In cats the impact of immunophenotype is uncertain and the distribution of lymphoma quite different from dogs. Factors associated with a favourable prognosis include FeLV negativity, early clinical stage, addition of doxorubicin to the treatment regime, stage(a) and a good initial response to chemotherapy (unfortunately this currently cannot be pre-judged). Many cats with FeLV negative lymphoma which initially respond well to chemotherapy may survive well in excess of 1.5 years.

Classification of lymphoma incurs considerable expense and when funds are limited it could be argued that money is better spent on treatment. Where funds are not limited classification of lymphoma sub-type is possible. Currently this may enable more accurate prognostication and, in future, tailored therapy. In human medicine patients diagnosed with the counterparts of canine indolent lymphoma may not even be treated, avoiding the potential side effects of chemotherapy. Some veterinarians are already advocating this approach, or at least a less intensive chemotherapeutic regimen, for the small T-cell sub-type (TZL / SCC).

A more pragmatic approach, given the predominance of diffuse large B-cell lymphoma and its distinctive histological and cytological appearance, is to be guided by the pathologist as to which cases may benefit from a more definitive classification. T-cell lymphomas are quite readily distinguished cytologically from many B-cell lymphomas and the different sub-types may also be distinguished, depending on experience of the pathologist and the quality of the smears. The canine patients most likely to benefit from accurate lymphoma classification are those with indolent sub-types (eg TZL, FL, MZL) and those with aggressive sub-types (eg T-lymphoblastic, T-plasmacytoid) – both T-cell. The former could be spared unnecessary intensive chemotherapy, the latter could also be spared the potential side effects of (potentially ineffective) chemotherapy, given palliative care or promptly euthanased to avoid further suffering.

This principle is based on limited clinical experience and, partly, extrapolation from human lymphoma. Only after accurate staging, detailed immunophenotyping and standardised therapy may some 'short-cuts' be validated for veterinary use.

In reality we are therefore a long way from recommending euthanasia or withholding chemotherapy purely on the basis of phenotype in canine patients and information about immunophenotype has to be taken in conjunction with the equally important substage when making clinical decisions. Currently I recommend further investigation (either immunohistochemistry or flow cytometry) for many cases other than diffuse large B-cell lymphoma in dogs. This may allow some prognostic information to be given to the owner and will build up a greater body of knowledge about the behaviour of the large range of diseases in dogs called "lymphoma".

References

[Lana SE, Jackson TL, Burnett RC, Morley PS, Avery AC.](#)

Utility of polymerase chain reaction for analysis of antigen receptor rearrangement in staging and predicting prognosis in dogs with lymphoma.
J Vet Intern Med. 2006 Mar-Apr;20(2):329-34.

[Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K, Fournel-Fleury C.](#)

Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy.
Vet J. 2004 Mar;167(2):158-66.

[Fournel-Fleury C, Ponce F, Felman P, Blavier A, Bonnefont C, Chabanne L, Marchal T, Cadore JL, Goy-Thollot I, Ledieu D, Ghernati I, Magnol JP.](#)

Canine T-cell lymphomas: a morphological, immunological, and clinical study of 46 new cases.
Vet Pathol. 2002 Jan;39(1):92-109.

[Valli VE, Vernau W, de Lorimier LP, Graham PS, Moore PF.](#)

Canine indolent nodular lymphoma.
Vet Pathol. 2006 May;43(3):241-56.

[Flory AB, Rassnick KM, Stokol T, Scrivani PV, Erb HN.](#)

Stage migration in dogs with lymphoma.
J Vet Intern Med. 2007 Sep-Oct;21(5):1041-7

[J Comp Pathol.](#) 2007 Feb-Apr;136(2-3):186-92.

Retrospective study of 82 cases of canine lymphoma in Austria based on the Working Formulation and immunophenotyping.

[Burnett RC, Vernau W, Modiano JE, Olver CS, Moore PF, Avery AC.](#)

Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes.
Vet Pathol. 2003 Jan;40(1):32-41.

[Keller ET, MacEwen EG, Rosenthal RC, Helfand SC, Fox LE.](#)

Evaluation of prognostic factors and sequential combination chemotherapy with doxorubicin for canine lymphoma.
J Vet Intern Med. 1993 Sep-Oct;7(5):289-95.

Fournel-Fleury C et al: Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. J Comp Pathol 117:35, 1997