UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Immunophenotypic classification of canine malignant lymphoma in formalin-fixed, paraffin wax-embedded specimens using CD3 and CD79a cell markers.

by

Joyce Pearson

Submitted in partial fulfillment of the requirements for the MMedVet (Path) degree in the Faculty of Veterinary Science, University of Pretoria, Pretoria

1999

Promoter: Prof J W Nesbit

Department of Pathology

Faculty of Veterinary Science

University of Pretoria

© University of Pretoria

SUMMARY

Inconsistent use of nomenclature in different canine malignant lymphoma (CML) classification systems, which lead to incorrect diagnosis and prognosis, necessitated a retrospective study of 103 cases of CML. Histological classification was done according to the Working Formulation, on H&E sections, after standard processing. Immunophenotyping, using CD3 (T cell) and CD79a (B cell) markers, was carried out on the same sections.

Intermediate grade lymphomas were the largest category (49.5%), with 16.5% high grade lymphomas. More than half (53.3%) of the lymphomas were of B cell immunophenotype, and 36.5% were T cell lymphomas. Only 9.7% of the total number of lymphomas exhibited double negative staining. Only two categories, the immunoblastic and medium-sized macronucleolated (MMC) category (Fournel -Fleury *et al.*), exhibited constant (B cell) immunophenotype. All the other categories exhibited mixed immunophenotype.

The Working Formulation, with omission of the follicular tyoes (due to the rarity thereof in CML) and addition of the MMC category and immunophenotyping, is best for classifying CML.

TABLE OF CONTENTS

1.Introduction and literature review 1
2.Objectives 7
3.Material and methods 8
.1 Case selection 8
.2 Processing 9
.3 Classification 11
.4 Immunophenotyping 15
.5 Correlation of the Working Formulation and immunophenotyping with the REAL classification 16
.6 Statistical analysis 16
4.Results 17
.a Histological classification 17
.b Immunophenotypic classification 19
5.Discussion 25
.1 Introduction 25
.2 Sample frequency 25
.3 Architectural frequency 25
.4 Grading of canine malignant lymphoma 26
.5 Categories 26
.6 Immunophenotyping 30
6.Conclusion 35
7.Acknowledgements 36
8.References 37

1. INTRODUCTION AND LITERATURE REVIEW:

Malignant lymphoma in animals and man is a well recognised, often fatal, clinical and pathological entity. [2-28] These neoplasms may consist of different subsets of lymphocytes, namely T- and B- lymphocytes and natural killer (NK) cells. [2-28] Reliable distinction may be facilitated by immunophenotyping. [2-28] The Avidin - Biotin - Complex (ABC) immunoperoxidase technique forms the basis of most of the immunophenotypic methods to distinguish between T and B lymphocytes in formalin-fixed, paraffin wax-embedded tissues derived from cases of canine malignant lymphoma (CML).[5-9 11 19 20 23-26] Although a pan T cell marker (CD3) has been in use for several years [2 3 5-9 14 21-26], B cells could only by identified utilising a panel consisting predominantly of immunoglobulin-based markers. [14 20 21 24-26] Recently, a new B cell marker (CD79a), which would eliminate the use of multiple markers has been identified. [8 16 18-20] Correct identification of the immunophenotype is of critical importance in establishing an accurate diagnosis, which has both prognostic and therapeutic implications.[24 26] In human medicine, several classifications such as the Rappaport-[12 24 26], Kiel-[8 9 12 24-26], Luke Collins - [12 16 24 26], National Cancer Institute Working Formulation [8 24 26] - and Revised European-American Lymphoma (REAL) classifications of lymphoid neoplasms[13] have been in use at different times.[4 8 13 24 25 26 27] Although histopathological descriptions of the different entities were adequate in most of the lymphoma classification systems, the same biological entities often go under different names in the various classification systems.[4 8 13 24-26] The inconsistent use of nomenclature is both confusing and frustrating and may be the cause of incorrect prognosis and treatment.[13 24-26] Of the histopathological classification systems that have been in use for malignant

lymphoma in man, only the Working Formulation and the Kiel classification have been widely utilised in veterinary medicine.[8] [9] [24-26] There appear, however, to be several lymphoma subtypes that cannot be categorised into the existing classification system based on the Working Formulation.[8] Thus, a modification of the Working Formulation, based on the immunophenotype may be necessary, to increase the effectiveness of the classification of CML. Alternatively, a modification of the REAL classification which incorporates immunophenotyping in addition to the histopathological component may also be useful.[8]

The Working Formulation, which is now the most widely used classification system in human pathology in the United States, was initially designed as an inclusive system in which most of the classification systems then in use, could be incorporated. Except for the Kiel - and Luke Collins classification systems, most of the classification systems fell into disuse since the publication of the Working Formulation in 1982. The morphological classification of the Working Formulation was based on the histopathological features of haematoxylin and eosin (H&E) stained sections alone, since no immunological stains were available at that time. For this reason Harris *et al.* [13] considered the Kiel classification, which also incorporates immunohistochemistry, to be a more appropriate classification system. Although the Kiel classification used both detailed morphological criteria to define specific entities, it excludes primary, extra-nodal forms, and T-cell associated malignant lymphomas.[13] [26] A proposal from the International Lymphoma Study Group partially solved the above-mentioned problems by suggesting a combination and modification of these systems, namely the Revised European-American classification (REAL classification)[13], and has gained considerable support among

medical pathologists since its introduction in 1994 (personal communication: PM Close, Department of Anatomical Pathology, University of Cape Town School of Medicine).

The situation in veterinary science is unsatisfactory. In the past, either no subclassification was applied, or there was direct extrapolation from the human classification systems. Canine malignant lymphoma is considered to be the equivalent of non-Hodgkins lymphoma in man [8 24 26] and it is therefore logical that a comparable classification system should be adhered to. CML is the most frequently encountered lymphoid tumour, with a prevalence of between 13 to 24 cases per 100,000 dogs [9 24-26], although this is probably an underestimation.[24] As in man, an accurate histological diagnosis in cases of CML is an essential prerequisite not only for an accurate prognosis, but also for selection of a suitable treatment regime.[3 9 25 26] It is accepted by several researchers that clinical staging and histopathological classification are the two most reliable variables in CML in dogs for determining prognosis, response to treatment, remission periods and overall survival times.[3 9 24 25] Use of the Kiel- and Working Formulation classifications, have been implemented by several veterinary investigators[4 8 9 25 26], either individually or together, with success. However, opinions differ as far as the usefulness of the different classification systems are concerned. Greenlee *et al.* was of the opinion that the Working Formulation was of limited use, mostly due to the prominence accorded follicular lymphomas in this classification, and the scarcity thereof in dogs.[9] Teske, however, concluded in a multivariate analysis of lymphomas in 254 dogs, that both the Working Formulation and Kiel classification are readily applicable to CML but that the Rappaport classification is of limited value.[24] Furthermore, in a more recent study, Teske *et al.* concluded that the

Working Formulation with added immunophenotyping was best suited to the classification of CML.[26] Fournel-Fleury *et al.*, on the other hand, had a different opinion as to the usefulness of the different classification systems and concluded that whereas an updated Kiel classification was most suitable, the Working Formulation had limited value, and that a modified REAL may have some potential as a future classification system.[8]

Immunohistochemical markers have been used in recent years in the identification of the origin of the lymphocyte (B or T) in CML.[3 5-8 21 24 26] Identification of the cell of origin, B- or T cell, based on the histological appearance of the lymphoma (as in the Luke Collins classification used on humans)[15] is not reliable.[9 26] This viewpoint has been substantiated in a recent investigation where it was proved in a prospective, multivariate analysis, using cellular markers, that unexpected high frequencies of T cell lymphomas in dogs may exhibit B cell morphology histologically.[26]

In man immunophenotyping utilising specific cellular markers, has become more common and widely used in recent years. Most immunophenotyping, has been performed using fluorescent immunocytochemistry on frozen sections and fine-needle aspirates. More recently an immunoperoxidase technique on formalin-fixed paraffin wax-embedded tissue sections has been employed for similar typing. [3 5-26] In both instances a panel of human leukocyte surface markers directed against various antigens including CD45A, Thy-1, CD49d, CD3, CD4, CD8, CD21 and anti-IgM, -IgG and -IgA have been utilised. The use of these cell markers in dogs is not practical for immunophenotypic diagnosis of CML, because many positive reactions are inconclusive [9], and application of such a wide range of cell markers is too expensive for general use in veterinary medicine.[20]

Some human cell markers are, however, suitable for use in dogs. Human polyclonal CD3 antiserum ([1]*DAKO A452 - rabbit anti-human) is effective on canine formalin-fixed paraffin wax-embedded tissues and is suitable as a pan T-cell marker in CML.[5 7 8 20 22 26] The advantages include high specificity and sensitivity and resistance of the antigen to formalin fixation.[6 13] CD3 is an intimate intracytoplasmic portion of the T-cell transmembrane receptor (TCR) that mediates signal transduction and is conserved across species lines.[14 22] The CD3 antibody reacts with the intracytoplasmic portion of the CD3 antigen. [2] The use of this cell marker to confirm the presence of a T cell neoplasm is important, because the diagnosis of a T cell lymphoma is indicative of a poor prognosis. [9 24 25]

Before the availability of the B cell marker, CD79a [16 19], no reliable single marker for B cells in dogs on formalin-fixed, paraffin wax-embedded canine tissues, apart from markers for the immunoglobulin chains, [3 5 14] was available. The lack of availability of a reliable B-cell marker that could be used on similar preparations precluded ready application of the modified Kiel classification system and the REAL classification systems. Recent identification of a polypeptide (encoded by the mb-I and ß29 genes) which is closely associated with the plasma membrane associated immunoglobulins, led to the development of an antibody against the mb-I gene product (designated CD79a at the Fifth International Workshop on Human Leucocyte Differentiation Antigens in 1993).[16 19] CD79a has a similar function in B cells as CD3 in T cells, namely, transmembrane signal transduction.[17 19] The monoclonal antibody JCB117 (DAKO CD79αcy M7051) which stains CD79a and cross-reacts widely across species lines, has been identified as a pan-B-cell marker in man.[14 17 19] This cell marker

---

[1]*DAKO: Southern Cross Biotechnologies, Cape Town, SA

appears in the pre-B cell stage of development. [19] The prevalence of B cell lymphomas may therefore have been underestimated because the markers previously used measured the production of immunoglobulin by B cells. [3] The more primitive, precursor cell clones that do not yet produce antibodies, would not have been identified. CD79a has recently been successfully applied to formalin-fixed paraffin wax-embedded tissue sections in the identification of NHL and Hodgkin's lymphoma in man.[16 17 19] In the more primitive forms, such as pre B cell lymphomas, the antibody showed intracytoplasmic positivity.[19] This cell marker has been utilised in the positive identification of canine B lymphocytes in CML in two recent trials. [8 20]

2. OBJECTIVES:

- Immunophenotyping of 103 canine malignant lymphomas using CD3 and CD79a cell markers to establish the involved lymphocyte subset.
- Histological classification of these lymphomas according to the Working Formulation.
- Application of a modification of the Working formulation that could be of clinical value, in terms of prognosis and survival times, considering the histological classification and immunophenotype.
- Determination of the suitability of the REAL classification in CML entities because of the all-encompassing nature of the REAL classification in terms of histological classification systems (includes Rappaport-, Luke Collins-, Working Formulation and Kiel classification systems) in conjunction with immunophenotyping.

3. MATERIALS AND METHODS:

1. *Case selection*:

Cases: One hundred and three canine lymphoma cases were selected from biopsy and necropsy material submitted to the Department of Pathology, and from biopsy material from Dr W S Botha, a veterinary pathologist in private practice in Pretoria, and Dr R D Last, a veterinary pathologist in private practice in Pietermaritzburg.

Biopsy and necropsy material comprised lymph nodes (peripheral and splanchnic), splenic, intestinal and skin specimens. Only necropsy material with an interim of two hours and less was used to exclude the potential for autolytic artefact.

Fixation in 10% buffered formalin did not exceed one week to optimise tissue preservation and reduce denaturation of the antigenic sites.

Controls: Positive and negative controls for immunophenotyping were selected from material emanating from a previous investigation. [20]

<u>Positive controls</u>: Cases that stained positively for either CD3 (positive controls for CD3), or CD79a (positive controls for CD79a) was included with each batch of specimens.

<u>Negative controls</u>: Samples of canine tonsil, which served both as a positive and negative control for both markers due to the normal distribution of B and T lymphocytes, was also included with each batch of specimens.

2. *Processing*:

a) Histopathology samples for classification:

The tissue was processed and embedded in paraffin-wax.
Sections were cut at 4µm from the blocks and mounted on poly-l-lysine ([2]*SIGMA P1274)-coated glass slides.
The slides were dried overnight in an oven at 60°C to enhance section adhesion.
Routine dewaxing and rehydration were carried out (15 minutes in xylol, and 3 minutes each in 100%, 96% and 70% alcohol)[1]

Histological classification was done on routine H&E stained sections from each case.

b) Immunophenotyping:

CD3: Blocking of endogenous peroxidases with 3% hydrogen peroxide (3ml hydrogen peroxide in 100ml methanol) for 15 minutes at room temperature, followed by 5 minutes rinsing in tap water. Unmasking of the antigenic sites was accomplished by using proteolytic enzymes, specifically, pronase E (Protease Type XIV SIGMA P-5147) for 30 minutes, after which the sections were rinsed in tap water for 5 minutes. After cooling in buffer (pH7.6 - made up from 2g BSA - SIGMA A2153- per 2 litre of PBS buffer after pH determination), treatment with normal serum (Goat SIGMA G-9023) at a dilution of 1:20 for 10 minutes at room temperature. The primary antibody (DAKO A0452) was then applied at a

[2]*SIGMA: PO Box 12202, Vorna Valley 1686, SA

dilution of 1: 100. After incubation at room temperature for 120 minutes, and jet washing in phosphate buffered saline (PBS) (2 X 5 minutes) at room temperature, the secondary biotinylated antiserum (diluted 1:500) (goat antirabbit - DAKO E432)) was applied for 45 minutes. Following another jet washing in PBS, peroxidase conjugated avidin ([3]*VECTOR Vectastain ABC Kit PK-4000) at a dilution of 1:500 was applied and incubated on the sections for 30 minutes. After further washing in PBS and exposure to strong DAB ([4]*MERCK BDH Prod 13033) for 16 minutes at room temperature, the sections were again rinsed (in deionised water) and counterstained with haematoxylin (monitored by light microscopy for adequate staining). After another washing in water for 5 minutes, followed by routine dehydration through increasing alcohol concentrations and xylol, the sections were cover-slipped.[10-12 20]

CD79a: The technique was similar for that of CD3, with the following exceptions: Unmasking of antigenic sites was done using citrate buffer, with microwave heating for 10 minutes after start of boiling point; the normal serum used was rabbit (SIGMA R-9133); the primary antibody (DAKO M7051) was used at a dilution of 1:25, and incubation was for 120 minutes at room temperature; the secondary antibody (rabbit anti-mouse - DAKO E 354), used at a dilution of 1:250 was incubated for 45 minutes.

---

[3]*VECTOR: BDS Diagnostics, PO Box 7312, Lynwood Ridge, Pretoria, SA

[4]*MERCK: Merck NT Laboratory Supplies, PO Box 1998, Halfway House, SA

The sections were stained with each specific marker in batches of 20.

One negative and one positive control slide, for each specific marker, was included with each batch of 20 cases.

c) Observations:
This was a single-blind study.

3. *Classification:*

The sections were examined under oil (IOOX) to evaluate the cellular morphology. The mitotic rate was determined under high dry (40X magnification). The following were recorded:

a) Type of tissue (eg. lymph node, spleen, skin).

b) Architecture of the neoplasm (follicular, diffuse).

c) Mitotic rate was established under 40 X magnification, the number of mitotic figures was counted in 10 fields and the mode determined. (The neoplasm was classified as low grade if between 0 and 3 mitoses were observed per field, as intermediate if between 3 and 5 mitotic figures were observed per field and as high grade if 6 and more mitotic figures were present[4], based on mode per 10 fields).

d) Cell size: The cells were classified as small if equal in size to that of an undistorted red blood cell, and as large if twice the diameter of a red blood cell. If it fell between these types, the size was recorded as intermediate.

e) Nucleus: The following features were recorded:
- Nuclear shape.
- Chromatin pattern.
- Nucleolar number and size relative to nuclear size.
- Mitotic rate.

f) Cytoplasmic volume and nuclear placement.

The lymphomas were classified into the following categories according to the histopathological characteristics as described by Carter and Valli (Working Formulation)[4], Valli[27] and Fournel - Fleury *et al.* [8], and the skin neoplasms as described by Walder *et al.*[28], as follows:

A. Low grade lymphoma

1. **Diffuse small lymphocytic** (DSL): diffuse pattern; small cells, round nucleus, clumped chromatin producing chromocentres, occasional nucleoli, scant cytoplasm (resemble normal, well-differentiated lymphocytes).
2. **Diffuse small lymphocytic (DSL) of intermediate type**: diffuse pattern, cells are larger than DSL, nucleus is round but may have an irregular outline, occasional nucleoli, scant cytoplasm.
3. **Diffuse small lymphocytic (DSL) plasmacytoid:** diffuse pattern, round nucleus, clumped chromatin (chromocentres), occasional nucleoli, abundant deeply basophilic/amphophilic eccentric cytoplasm, a mixture of DSL cells may be present. (Both DSL of intermediate type and DSL of plasmacytoid type are considered subtypes of DSL by some authors[4]).

4. **Follicular small cleaved** (FSC): follicular pattern, nuclei are angular/irregular/folded (cleaved), dense chromatin with inconspicuous nucleoli, scant cytoplasm.
5. **Follicular mixed** (FM): follicular pattern; nuclei are angular (irregular to cleaved-centrocytic), dense chromatin, inconspicuous nucleoli, scant cytoplasm.
6. **Macronucleolated medium-sized cells** (MMC's). These lymphomas were unclassifiable within the Working Formulation. Refer to the section on unclassifiable lymphomas for further clarification.

B. Intermediate grade lymphoma

1. **Diffuse small cell cleaved** (DSC): diffuse pattern, nuclei are angular/cleaved, hyperchromatic to coarsely stippled chromatin, inconspicuous nucleoli, scant cytoplasm.
2. **Diffuse mixed** (DM): diffuse pattern, two different cell populations: small and large cleaved or non- cleaved nuclei; *small cells*: hyperchromatic angular/cleaved nuclei, inconspicuous nucleoli; *large cells*: cleaved or non-cleaved nuclei, round to oblong in shape, hypochromatic, multiple prominent nucleoli (usually peripheral), abundant poorly demarcated cytoplasm.
3. **Diffuse large** (DL): diffuse pattern, round nuclei, inapparent chromatin (hypochromatic), prominent peripheral nucleoli, often fairly abundant cytoplasm.
4. **Diffuse large cleaved** (DLC): diffuse pattern, nuclei are angular/cleaved/folded several times (cerebriform),

finely dispersed chromatin, prominent nucleoli, scant basophilic cytoplasm.

C. High grade lymphoma

1. **Immunoblastic**: diffuse pattern; large, round nuclei, inapparent chromatin (hypochromatic), prominent central nucleolus, scant cytoplasm.
2. **Lymphoblastic:** diffuse pattern; small, round to angular nuclei, hyperchromatic to finely dispersed chromatin, inconspicuous nucleoli or multiple small nucleoli, scant cytoplasm.
3. **Small non-cleaved:** diffuse pattern; *non-Burkitt*: larger in size (1.5 red cells), coarse heterochromatin (chromocentres), multiple or single prominent nucleoli, cytoplasm abundant; *Burkitt*: homogenous population, round, hypochromatic nuclei, multiple small nucleoli, distinct cell borders. [4]

D. Miscellaneous lymphomas

1. **Mycosis fungoides** (epitheliotrophic lymphoma): primary epidermal neoplasm with secondary dermal involvement comprised of large cells, nuclei are folded several times (cerebriform), hypochromatic nuclei, inconspicuous nucleoli, scant cytoplasm.
2. **Nonepitheliotrophic lymphomas :** three types are recognised: *Large cell type*: Large vesicular round to folded nuclei, multiple nucleoli, scant amphophilic cytoplasm, moderate to high mitotic rate;

Immunoblastic : Very large cells, large round hypochromatic nuclei, single large central nucleolus, high mitotic rate; *Histiocytic*: Large ovoid to convuluted nuclei, large single nucleolus, abundant poorly defined cytoplasm.

3. **Unclassifiable lymphomas**: although unclassifiable according to the Working Formulation this category of miscellaneous CML were classified as macronucleolated medium-sized cells (MMC). This lymphoma subtype was identified and described by Fournel - Fleury *et al.* in 1996. Cells are small to intermediate in size (never more than 1.5 red blood cell in diameter) with hypochromatic nuclei and prominent (often multiple) nucleoli[8] (See Fig 3). Due to the low mitotic rate of these neoplasms, this group was allocated to the low grade lymphoma category.

Before placing a neoplasm in a particular category the histological morphology and mitotic rate were correlated.

4. *Immunophenotyping*

All the sections were examined in a single blind study, without taking the Working Formulation into consideration. Two sections from each case were stained with CD3 and CD79a, respectively. A positive and negative control were stained together with each batch of twenty cases (see above). A case was regarded as positive B or T cell in origin if at least 60% of the neoplastic cells exhibited positive staining for the specific cell marker. The staining pattern for CD3 was cytoplasmic membrane associated (See Fig 5) and for CD79a

was either perinuclear and intracytoplasmic or cytoplasmic membrane associated.

The results of this examination were recorded and correlated separately.

Light brown staining of lymphocytes, often intracytoplasmic with a smudged appearance, which was frequently seen in cases with abundant necrosis and/or haemorrhage, was regarded as non-specific. Cases in which CD3 stained the paracortical lymphocytes in a lymphnode[6], and CD79a the lymphocytes in the germinal centres[18], were regarded as hyperplasia, and removed and replaced by another case. The latter cases were not included in the 103 cases examined.

5. *Correlation of the Working Formulation and immunophenotyping with the REAL classification*

The neoplasms, together with their immunophenotype was correlated, as far as possible, with the categories as described by Harris *et al.* in the REAL classification.[13]

6. *Statistical analysis*

Statistical analysis was performed using Epilnfo (Vers6). The nature of the data only allows for frequency determination.

4. RESULTS

a) Histological classification

Most of the specimens were lymph nodes (n = 92), with 8 skin specimens, 1 intestinal specimen and 2 splenic specimens. The nodal lymphomas were diffuse in the majority of cases (n = 83), with a pseudofollicular pattern in seven, and a true follicular pattern visible in two cases (See Fig 2). One of the splenic specimens exhibited a pseudofollicular pattern.

Using the Working Formulation, the 103 cases were classified in the categories as defined above and are tabulated according to frequency in Table 1. Seventeen cases (16.5%) were classified as high grade, 51 (49.5%) as intermediate grade and 27 (26.2%) as low grade CML. The skin neoplasms, all of which were nonepitheliotrophic, were classified in the miscellaneous category. The MMC lymphomas were included in the low grade category. No cases of FSC, FM, lymphoblastic and mycosis fungoides / epitheliotrophic lymphomas were represented in this study.

Diffuse large cell lymphomas made up the largest individual category and consisted of 31 cases (30.1%). The immunoblastic lymphomas were the second largest group (13 cases - 12.6%) with the mixed cell type (9 cases - 8.7%) being the third largest.

The skin neoplasms comprised 7.8% of the total number of cases. These neoplasms were subdivided into additional categories as described. Seven cases (87.5%) were classified as being of the large cell type, whereas one was categorised as being histiocytic.

Table 1. Classification of 103 cases of Canine malignant lymphoma according to the modified Working Formulation

CLASSIFICATION	FREQUENCY	PERCENTAGE
LOW GRADE	**27**	**26.2**
DSL	8	7.8
DSL(INTERMEDIATE)	8	7.8
DSL (PLASMACYTOID)	6	5.8
FSC	0	0
FM	0	0
MMC	5	4.9
INTERMEDIATE GRADE	**51**	**49.5**
DSC	5	4.9
DM	9	8.7
DL	31	30.1
DLC	6	5.8
HIGH GRADE	**17**	**16.5**
IMMUNOBLASTIC	13	12.6
LYMPHOBLASTIC	0	0
SMALL NON CLEAVED	4	3.9
MISCELLANEOUS	**8**	**7.8**
MYCOSIS FUNGOIDES	0	0
NONEPITHELIOTROPHIC LYMPHOMA	8	7.8
TOTAL	**103**	**100**

DSL = Diffuse small lymphocytic; FSC = Follicular small cleaved;
FM = Follicular mixed; MMC = Macronucleolated medium-sized cell;
DSC = Diffuse small cell cleaved; DM = Diffuse mixed;
DL = Diffuse large; DLC = Diffuse large cleaved.

b) **Immunophenotypic classification**

After immunophenotyping 38 cases (36.8%) were classified as being of T cell, and 55 cases (53.4%) as being of B cell immunophenotype. Ten cases exhibited no staining with either the CD3 or CD79a markers.

The frequency of positive staining in the different categories are tabulated in Table 2. High grade, immunoblastic lymphomas were consistently positive for the B cell immunophenotype, as was the MMC category. In the other categories the cell populations were variably positive with both the CD3 and CD79a markers. Double negative staining occurred in four categories, namely, the plasmacytoid variant of DSL, mixed small and large cell lymphomas, large cell lymphomas and nonepitheliotropic lymphomas. The low grade, plasmacytoid lymphomas had the highest prevalence of double negative staining (66.6%), and did not exhibit any positive staining with CD3.

Amongst the T cell lymphomas, low grade neoplasms made up 23.7% of the total percentage of T cell lymphomas, intermediate grade 52.6% and 5.3% were in the high grade category. In the case of B cell lymphomas the highest percentage (49%) occurred in the intermediate grade category; while the high grade cases made up 27.2% and the low grade 21.8%, respectively. Amongst the miscellaneous CML, nonepitheliotrophic lymphomas exhibited positive T cell staining in 62.5% of the cases while 12.5% were of B cell immunophenotype and 25 % were negative for both T and B cell immunophenotype. The histiocytic cell and large cell type in this

category stained negatively for both T and B cells. The total number of lymphoid neoplasms which could not be classified according to the Working Formulation was five. All of these neoplasms fitted into the MMC category[8], and all of these exhibited positive B cell immunophenotyping.

Table 2. Frequency of immunophenotype in the different categories of the modified Working Formulation in 103 cases of Canine malignant lymphoma

CLASSIFICATION	CD3 +	CD79a +	CD3 - and CD79a -	TOTAL
LOW GRADE	**11**	**12**	**4**	**27**
DSL	7	1	0	8
DSL (INTERMEDIATE)	4	4	0	8
DSL (PLASMACYTOID)	0	2	4	6
MMC	0	5	0	5
INTERMEDIATE	**20**	**27**	**4**	**51**
DSC	4	1	0	5
DM	4	3	2	9
DL	10	19	2	31
DLC	2	4	0	6
HIGH GRADE	**2**	**15**	**0**	**17**
IMMUNOBLASTIC	0	13	0	13
SMALL NON CLEAVED	2	2	0	4
MISCELLANEOUS	**5**	**1**	**2**	**8**
NONEPITHELIOTROPHIC	5	1	2	8
TOTAL	**38**	**55**	**10**	**103**

DSL = Diffuse small lymphocytic; MMC = Macronucleolated medium-sized cell;
DSC = Diffuse small cell cleaved; DM = Diffuse mixed;
DL = Diffuse large; DLC = Diffuse large cleaved.

b. 14444720
i. 14748241

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

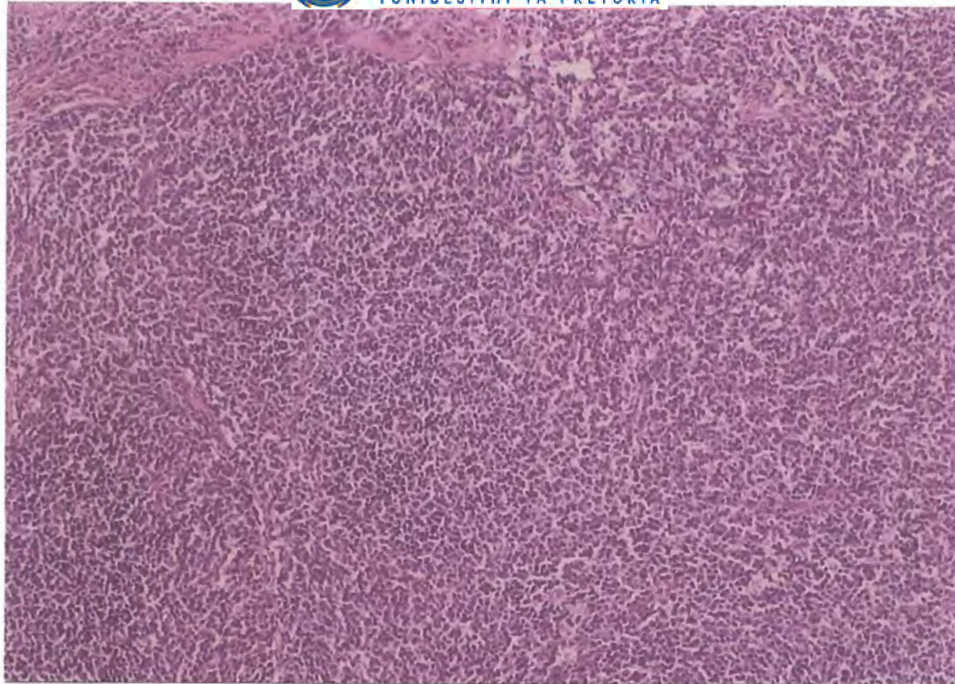

Figure 1: Lymph node showing a diffuse lymphoma. (Haematoxylin - eosin staining, original magnification : 100 X)

Figure 2: Lymph node showing a follicular lymphoma. (Haematoxylin - eosin staining, original magnification : 40 X)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

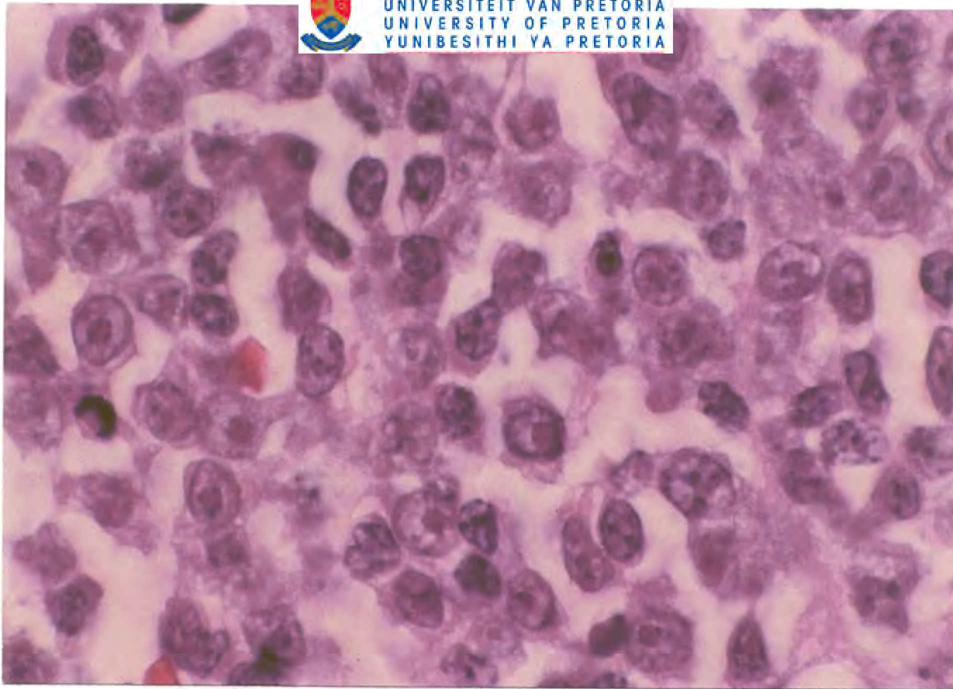

Figure 3: Lymph node showing a macronucleolated medium-sized cell (MMC) lymphoma. Note the cell size (compared to a red blood cell), hypochromatic nuclei and large nucleoli (Haematoxylin - eosin staining, original magnification : 1000 X)

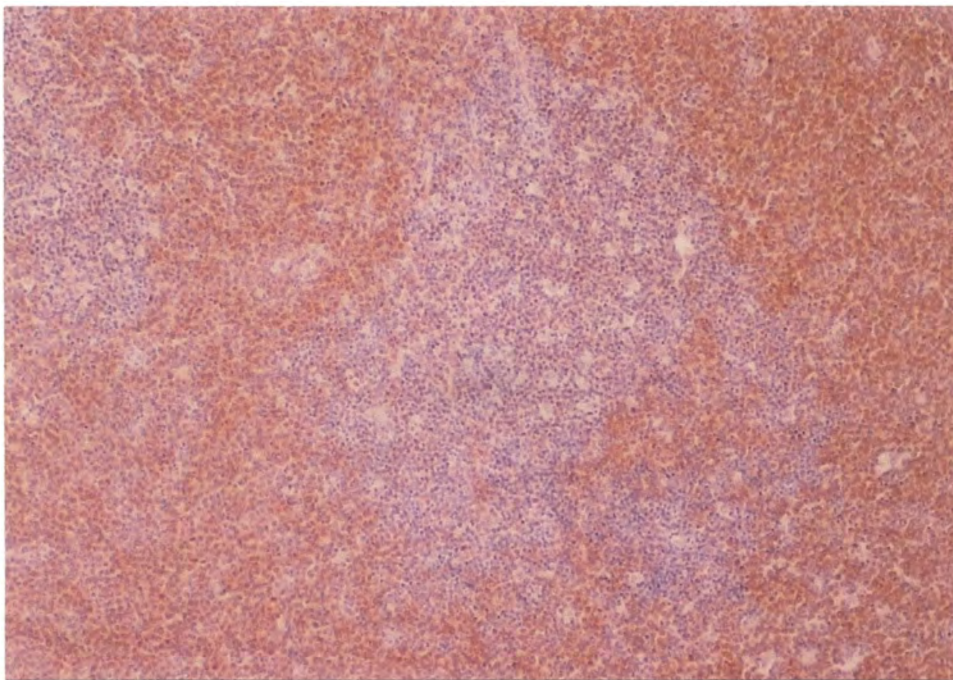

Figure 4: Immunohistochemical staining of a lymph node with CD79a showing positive staining of neoplastic lymphocytes, adjacent to unstained lymphocytes (DAB, counterstained with haematoxylin, original magnification : 200 X)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

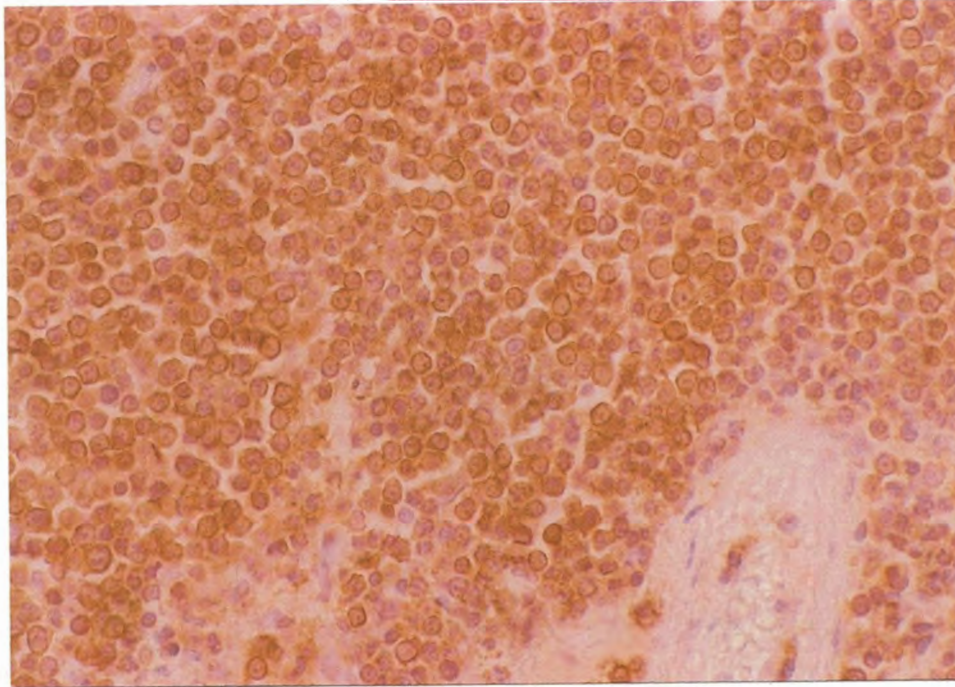

Figure 5: Immunohistochemical staining of a lymph node with CD3 exhibiting positive cytoplasmic membrane staining of neoplastic lymphocytes (DAB, counterstained with haematoxylin, original magnification : 400 X)

5. DISCUSSION

5.1 INTRODUCTION

Accurate histological classification of CML is fraught with difficulties. Although it has been applied in several investigations[4 5 8 9 26], only one detailed histopathological description of canine malignant lymphomas according to the Working Formulation is in existence.[4] Furthermore, the criteria for distinguishing between the different categories are often applied subjectively with respect to grading and cell size in determination of the category of CML. It is inevitable that a degree of subjectivity shall be introduced into an investigation of this nature due mostly to differences in investigative approach compounded by differences in CML prevalence in different geographic centres.

5.2 SAMPLE FREQUENCY

The preponderance of lymph nodes (89.3%) is a reflection of the type of biopsy specimens which are usually received in this facility, and are consistent with the type of biopsy specimens received in Europe and America.[26] Tissue samples from necropsy material (which would include splenic and intestinal samples) were mostly unrewarding, since most had to be discarded due to a prolonged interim.

5.3 ARCHITECTURAL FREQUENCY

Follicular lymphomas, by definition, should have evidence of more or less circular expansion of neoplastic centres throughout the node, with demarcation by a cuff of lymphocytes, and should not contain high endothelial venules[4] (See Fig 2).The apparent scarcity of follicular cell type lymphomas in this study (1.9%) was in accordance

with findings by Carter *et al.* (0.8%) [4], Greenlee *et al.*(4.5%) [9], and Fournel - Fleury *et al.* (2.2%) [8], and undoubtedly reflects the scarcity of follicular lymphomas in dogs. "Pseudofollicular" lymphomas, however are slightly more common.[4 8] These are expansions of neoplastic cells (usually paracortical in location) which contain high endothelial venules, may be surrounded by connective tissue bands, and cause compression of the surrounding tissue.[4] Seven nodal and one splenic specimen exhibited these features in this study (7.8%); a finding comparable to that of Fournel - Fleury *et al.* (8.7%).[8] The high prevalance of follicular lymphomas (14.9%) recorded by Teske *et al.* in 1994 could be the result of inclusion of both follicular and pseudofollicular lymphomas in a single category.[26]

5.4 GRADING OF CANINE MALIGNANT LYMPHOMA

Grading was based on the mitotic rate and the mode was utilised for determination of the mitotic rate, because of its greater reliability and higher accuracy compared to averaging which could be adversely affected by very low and very high numbers. Furthermore, it is believed that this method is an improvement upon the figure derived from the average number of mitoses in 5 fields at 1000X magnification as proposed by Valli, 1993[27] and the estimated number of mitoses per high power (40X) field in 5 fields as followed by Fournel- Fleury *et al.* in 1997.[8]

5.5 CATEGORIES

Intermediate grade lymphomas were the largest category in this study. The high prevalence of intermediate grade lymphomas (49.5%) is similar to findings previously reported by Greenlee *et al.*

(1990)[9], Teske *et al.* (1994)[26] and Fournel-Fleury *et al.* (1997)[8], but differs from the findings of Carter *et al.*[4], in which high grade lymphomas had the highest prevalence (66.8%).[4] The latter study recorded a prevalence of 28.4% for intermediate grade lymphomas. The reason for the discrepancy between the findings of Carter *et al.* [4] and the findings reported by other investigators is unclear, but could be related to subjectivity of categorisation in the different grades. Carter *et al.*[4] and Greenlee *et al.*[9] reported a low prevalence of low grade tumours (5.3% and 11.4% respectively) as did Teske (16.4%)[26] whereas the finding of 21% reported by Fournel - Fleury *et al.*[8], approximates the finding in this study.

The most commonly encountered individual categories in this study were diffuse large cell lymphomas (30.1%), immunoblastic lymphomas (12.6%) and mixed small and large cell (intermediate grade) lymphomas (8.7%). With the exception of the latter category, this is commensurate with the investigations reported by Carter *et al.* [4], Greenlee *et al.* [9], Teske *et al.* [26] and Fournel - Fleury *et al.* [8] No lymphoblastic lymphomas were encountered in this study, which corresponds with a similar absence in one of the investigations reported by Teske *et al.* [26] and the low prevalence in investigations reported by Greenlee *et al.* [9] and Fournel - Fleury *et al.*[8], and is in contrast to the findings of Carter and Valli [4] in which a high prevalence of 17.2% was noted.[4] Although speculative, this discrepancy may be attributable to the fact that the findings reported by the latter authors is a reflection of the large numbers of mediastinal tumours included in their investigation.[26] The absence of mycosis fungoides (epitheliotrophic lymphoma) amongst the skin

neoplasms was unusual. In part, this may have been influenced by the small sample size, but may also have been influenced by geographic differences.

5.5.1. LOW GRADE LYMPHOMAS

DSL and intermediate DSL lymphomas were the most prevalent of the low grade lymphomas (7.8% of each type). This corresponded to the findings of Carter *et al.* (4.9%)[4], Greenlee *et al.* (10.2%)[9] and Fournel - Fleury *et al.* (12%) [8] in the DSL category. The intermediate DSL category was not identified in their reported investigations. The plasmacytoid variants made up 22% of the low grade lymphomas in this investigation, in contrast to the findings of Fournel - Fleury *et al.* in which 100% of the DSL low grade lymphomas were comprised of the plasmacytoid variant.[8] According to Teske *et al.* (1994), follicular lymphomas, consisting predominantly of small cleaved cells, were the most common type of lymphoma (12.1%) in the low grade category. This finding may be related to the high prevalence of follicular lymphomas in his study.[26] Neither DSL, nor the plasmacytoid variant, were reported in this investigation by Teske *et al.*[26]. The plasmacytoid variant was categorised in the DSL category in the studies reported by Carter *et al.* [4] and Greenlee *et al.* [9], without any further subclassification.

The MMC lymphomas were categorised in the group of

low grade lymphomas based on their mitotic rate; after initial grouping with the unclassifiable lymphomas in the miscellaneous category because they could not be classified according to the Working Formulation. They made up 4.9% of the total number of lymphomas. Fournel - Fleury *et al.*[8], who introduced the term for this lymphoma subtype in 1997, reported a comparable prevalence of 9.7% for this subtype in their investigation.

5.5.2. INTERMEDIATE GRADE LYMPHOMAS

The most common group of lymphomas in the intermediate grade in this study was the DSL lymphomas, which comprised 30.1% of the lymphomas. This is similar to the findings of 20% by Carter *et al.* [4], 48.3% by Greenlee *et al.* [9], 40.2% by Fournel - Fleury *et al.* [8] and 30.2% by Teske *et al.* [26]. Teske *et al.* [26] also found that this group of lymphomas and follicular lymphomas were almost equally represented, 30.2% and 31% respectively.[26] The prevalence of the mixed small and large cell lymphomas in this study was, however, higher than in other, comparable investigations.[4 8 9 26] This may, in part, be due to the subjective criteria adopted to distinguished between DL lymphomas and mixed cell lymphomas in the investigations of Carter *et al.* [4] , Greenlee *et al.* [9], Teske *et al.* [26] and Fournel - Fleury *et al.* [8] Diffuse small cleaved cells (4.9%) and diffuse large cleaved cells

(5.8%) made up a small proportion of the total number of lymphomas, which is again comparable to previously reported findings by Carter *et al.* [4], Teske *et al.* [26] and Fournel - Fleury *et al.* [8]

5.5.3. HIGH GRADE LYMPHOMAS

The most prevalent group in the high grade lymphoma category, was the immunoblastic type, which comprised 12.6% of the total number of lymphomas. This corresponds with findings reported by Carter *et al.* [4], Greenlee *et al.* [9], Teske *et al.* [26] and Fournel - Fleury *et al.* [8] These large cells, with their large, prominent nucleoli within large round nuclei are distinctive and do not allow for misinterpretation. No lymphoblastic lymphomas were encountered in this study, a finding which correlates with that of Teske *et al.* [26]

5.5.4. MISCELLANEOUS GROUP

The high prevalence of the large cell type of the nonepitheliotrophic lymphomas was dissimilar to previous findings by Day (1995), in which the immunoblastic and histiocytic cell types predominated.[5] The significance of these differences are difficult to determine, since the number of cases were small; 8 and 10, respectively.

5.6 IMMUNOPHENOTYPING

The prevalence of T cell lymphomas (36.8%) in this study was higher, but comparable to that reported by Greenlee *et al.* (16.6%) in 1990[9] and Fournel-Fleury *et al.* (26%) in 1997[8] and almost identical to the 37.9% reported by Teske *et al.* in 1994.[26] More than half of the lymphomas (53.4%) in this investigation expressed the B cell immunophenotype, which is lower than that reported by Greenlee *et al.* in 1990[9] and Fournel - Fleury *et al.* (74%) in 1997[8], and almost identical to the 58.9% reported by Teske *et al.* in 1994.[26]

The number of cases which failed to react with either of the two antibodies, was higher than reported by Teske *et al.* in 1994[26] and Fournel - Fleury *et al.* in 1997.[8] However, it should be noted that these two studies were not quite comparable in that a panel of numerous B cell markers, including CD79a in the latter[8] but excluding CD79a in the former study[26], were used to determine the immunophenotype. Most lymphomas of the plasmacytoid subtype stained negatively with both CD3 and CD79a antibodies.

The number of T and B cell lymphomas within each of the major classification groups (high grade, intermediate grade and low grade) in this study could not be compared with existing data, because the immunophenotypic prevalence was established by application of the updated version of the Kiel classification system in those instances. 8 9

The exclusive B cell phenotype of the immunoblastic lymphomas is in accord with the findings reported by researchers such as Greenlee

et al. in 1990 [9] , Harris *et al.* in 1994[13] and Fournel - Fleury *et al.* in 1997.[8] Lymphomas in the MMC category were consistently positive when stained with CD79a; a finding similar to that reported by Fournel - Fleury *et al.* [8]

The high prevalence of double negative staining in the plasmacytoid variant of DSL; a consistent feature in both veterinary[8] and human medicine[13 19], may be related either to advanced or to disordered plasmacytic differentiation of these cells; in spite of the fact that normal human and dog plasma cells react with the CD79a cell marker. [8 18 19] Lymphoplasmacytoid lymphomas / immunocytomas in man exhibit similar histological characteristics to the plasmacytoid variant of CML.[13] Double negative staining is not a consistent feature in either the plasmacytoid variant in CML or the lymphoplasmacytoid lymphoma / immunocytoma lymphoma in man; some lymphomas in both these categories do stain positively for the B cell immunophenotype.[13 18 19]

Some DL cell lymphomas in this study also exhibited similar negative staining with both CD3 and CD79a; a phenomenon which is in accordance with the situation in man, first reported in the initial CD79a trials by Mason *et al.* in 1995[19], and validated by Harris *et al.* in 1994.[13]

No lymphoma in this study exhibited positive immunophenotyping for both CD3 and CD79a markers. This is in accordance with the findings of Teske *et al.* [26] and Fournel - Fleury *et al.* [8] , but at variance with the findings reported by Greenlee *et al.* in 1990[9], in

which some lymphomas stained positively with both B and T cell markers. This discrepancy is probably a reflection of the high specificity of the markers used in this study; as opposed to the degree of lower specificity of the markers utilised in the earlier investigation.[9]

The high percentage of the T cell immunophenotype amongst the nonepitheliotrophic lymphomas of the skin corresponds with the findings of Day (1995).[5] An interesting finding in this study by Day was that, in contrast to the exclusive B cell immunophenotype of the immunoblastic lymphomas in lymphoid tissue, those in the skin exhibited the T cell immunophenotype.[5] No immunoblastic nonepitheliotrophic lymphomas were present in this study. The fact that the histiocytic nonepitheliotrophic lymphoma in this investigation, exhibited double negative staining, is deserving of special note; especially since the morphology and immunophenotype of this lymphoma bears a strong resemblance to that of the anaplastic large cell lymphoma (T and Null cell types) in the REAL classification.[13] Further investigation of this finding is needed to determine the significance thereof.

All the other categories in this study exhibited mixed immunophenotypic characteristics. Mixed immunophenotypicity in many of the categories was reported before in an earlier investigation by Ferrer *et al.* in 1994.[7] Correlation of these findings with those of other investigations was hampered by the fact that the immunophenotype was determined in the latter instances in relation to the Kiel classification system. Meaningful interpretation is

therefore difficult, if not impossible. Furthermore, the number of cases which stained positively with each marker within each category is small. Statistical analysis of the frequency in the different categories, with the exception of the DL lymphomas, failed to show any statistically significant differences ($p <$ than 0.05). Furthermore, the subjectivity involved in distinguishing the DL lymphomas from mixed cell lymphomas rendered meaningful interpretation impossible.

Thus, it can be concluded that the Working Formulation in it's present format, where the histopathological features are the only criteria used to categorise CML, is of limited value to the veterinary pathologist and clinician. It is clear that the immunophenotype in the majority of categories, cannot be predicted with any accuracy from the histopathological features.

Since the immunophenotype is so important in terms of prognosis, as shown by Teske *et al.* [25 26], and since it cannot be accurately predicted from the histological characteristics [26], CD3 and CD79a markers should be utilised, prior to any attempts at classification in cases of CML. All T cell lymphomas carry a poor prognosis.[9 25 26] Grading of T cell lymphomas can, therefore, be considered superfluous [25 26] and regardless of the mitotic rate, should carry a poor prognosis.[25] However, the grading (low, intermediate and high) does have prognostic implications with respect to B cell lymphomas, since high grade tumours have a poor prognosis in terms of survival time.[25] All B cell neoplasms, should therefore be graded according to the Working Formulation. Simplification of this grading system,

would entail the omission of the separate categories for follicular lymphomas, due to the rarity thereof in CML.

The following abbreviated Working Formulation is, therefore, proposed for B cell lymphomas:

Low grade:
- Diffuse small cell (possibly with special note of the plasmacytoid variant)
- Macronucleolated medium-sized cell

Intermediate grade:
- Diffuse small cleaved
- Diffuse mixed
- Diffuse large
- Diffuse large cleaved

High grade:
- Immunoblastic
- Lymphoblastic
- Small non-cleaved

Miscellaneous:
- Mycosis fungoides
- Nonepitheliotrophic lymphoma

The nature of the REAL classification is such that it incorporates several different Working Formulation categories, into one REAL category; for instance: diffuse large cleaved, noncleaved , immunoblastic and diffuse mixed small and large cell categories are classified as diffuse large cell lymphomas. In addition, many

categories in the Working Formulation are also represented in more than one REAL category. Therefore, until such time as more data regarding prognosis and survival times become available for each of the categories within the Working Formulation, as exist for man, use of the REAL classification is not practical in the veterinary field.

6. CONCLUSION

There is good correlation between the findings of CML in South Africa and that of data collected in the US, Holland and France, in terms of prevalence of the different categories in the Working Formulation as well as in the prevalence of the different immunophenotypes. Application of the Working Formulation is relatively simple. However, some difficulty may be experienced when subjective criteria are employed to distinguish between some of the categories. Immunophenotyping, however, is essential and plays a key role in prognosis. Grading of B lymphocyte CML, by using the suggested modified Working Formulation, would enhance it usefulness and practicality to clinicians and pathologists, alike. The REAL classification is currently of limited value in the classification of canine malignant lymphoma.

7. ACKNOWLEDGEMENTS

The author would like to acknowledge Dr Rick Last and Dr Willem Botha for making available case material from their various practices in Pietermaritzburg and Pretoria. Ms Marie Smit and the staff of the Histopathology Laboratory, Department of Pathology, Faculty of Veterinary Science, University of Pretoria are thanked for preparation of the sections, with particular ackowledgement of ms Marianne Rossouw for the immunohistochemical staining. The promoter prof Jon Nesbit is acknowledged for invaluable input in this project and time and effort spend on the preparation of the manuscript. Prof Jaco van der Lugt, Department of Pathology, Faculty of Veterinary Science, University of Pretoria, is acknowledged for taking the photographs, and mrs Heleen Smit of the Faculty of Veterinary Science, University of Pretoria, for development of the prints.

8. REFERENCES

1. Bancroft J. D. & Stevens A. (Eds) 1982. Immunohistochemistry. In: *Theory and Practice of Histological Techniques.* 2nd edition. Churchill Livingstone, Edinburgh.

2. Campana D., Thompson J. S., Amlot P., Brown S. & Janossy G. 1987. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. *Journal of Immunology*, 138 : 648 - 655.

3. Carter R. F. & Valli V. E. O. 1988. Advances in the cytological diagnosis of canine lymphoma. *Seminars in Veterinary Medicine and Surgery (Small Animals)*, 3 : 167 -175.

4. Carter R. F., Valli V. E. O. & Lumsden J. H. 1986. The cytology, histology and prevalence of cell types in canine lymphoma classified according to the National Cancer Institute Working Formulation. *Canadian Journal of Veterinary Research* 50 : 154 -164.

5. Day J. 1995. Immunophenotypic characteristics of cutaneous lymphoid neoplasia in the dog and cat. *Journal of Comparative Pathology*, 112 : 79 - 96.

6. Ferrer L., Fondevila D., Rabanal R. & Ramis A. 1992. Detection of T lymphocytes in canine tissue embedded in paraffin wax by means of antibody to CD3 antigen. *Journal of Comparative Pathology*, 106 : 311 - 314.

7. Ferrer L., Fondevila D., Rabanal R., Tarres J. & Ramis A. 1993. Immunohistochemical detection of CD3 antigen (pan T marker) in canine lymphomas. *Journal of Veterinary Diagnostic Investigation*, 5 : 616 - 620.

8. Fournel - Fleury C., Magnol J. P., Bricaire P., Marchal T., Chabanne L., Delvedier A., Bryon P .A . & Felman P. 1997. Cytohistological and immunological classification of canine malignant lymphomas : comparison with human non-Hodgkins lymphomas. *Journal of Comparative Pathology*, 117 : 35 - 59.

9. Greenlee P. G., Filippa D. A., Quimby F. W., Patnaik A. K., Calvano S. E., Matus R. E., Kimmel M., Horvitz A. I. & Lieberman P. H. 1990. Lymphoma in dogs: a morphologic, immunologic and clinical study. *Cancer*, 66 : 480 - 490.

10. Haines D. M .& Chelak B. J . 1991. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin fixed paraffin embedded tissue for diagnostic pathology. *Journal of Veterinary Diagnostic Investigation*, 3 : 101 - 112.

11. Haines D. M. & Clark E.G. 1991. Enzyme immunohistochemical staining of formalin fixed paraffin embedded tissue for diagnosis in veterinary pathology. *Canadian Veterinary Journal*, 32 : 295 - 302.

12. Harris N. L., Jaffe E. S., Stein H., Banks P. M., Chan J. K. C., Cleary M. L., Delsol G., De Wolf Peeters C., Falini B., Gatter K. C., Grogan T. M., Isaacson P. G., Knowles D. M., Mason D. Y., Muller - Hermelink H., Pileri T. M., Piris M. A., Ralfkaier E. & Warnke R. A.

1994. Revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group . *Blood*, 84 : 1361 - 1392.

13. Hsu S. M. 1990. Immunohistochemistry In: Wilchek M. & Bayer E.A. (Eds) *Methods in Enzymology : Avidin-biotin Technology*, 184:357 - 363 Academic Press Inc. San Diego & London.

14. Jones M., Cordel J. L., Beyers A. D., Tse A. G. D. & Mason D. Y. 1993. Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies. *The Journal of Immunology*. 150 : 5429 - 5435.

15. Korkolopoulou P., Cordell J., Kaklamanis L., Tsenga A., Gatter K. C. & Mason D. Y. 1994. The expression of the B-cell marker *mbl* (CD79a) in Hodgkin's disease. *Histopathology*, 24 : 511 - 515.

16. Luke R. J. & Collins R. D. 1992. Tumours of the hematopoietic system. *AFIP Fasicles* Vol 28 2nd Edition.

17. Mason D.Y.. Cordell J. L., Tse A. G. D., van Dongen J. J. M., van Noesel D. J. M., Micklem K., Pulford K. A. F., Valensi F., Comans-Bitter W. M., Borst J. & Gatter K. C. 1991. The IgM - associated protein mb-1 as a marker of normal and neoplastic B cells. *Journal of Immunology*, 147 : 2474 - 1482.

18. Mason D. Y., van Noesel D. J. M., Cordell J. L., Comans - Bitter W. M., Micklem K. & Tse A. G. D. 1992. The B29 and mb-1 polypeptides are differentially expressed during human B cell

differentiation. *European Journal of Immunology*, 22 : 2753 - 2756.

19. Mason D. Y., Cordell J. L., Brown M. H., Borst J., Jones M., Pulford K., Jaffe E., Ralfkaier E., Dallenbach F.& Stein H. 1995. CD79a: a novel marker for B cell neoplasms in routinely processed tissue samples. *Blood*, 86 : 1453 - 1459.

20. Milner R. J., Pearson J., Nesbit J. W. & Close P. 1996. Immunophenotypic classification of canine malignant lymphoma on formalin-fixed paraffin wax-embedded tissue by means of CD3 and CD79a cell markers. *Onderstepoort Journal of Veterinary Research*, 63 : 309 - 313.

21. Moore P. F., Olivry T. I. & Nayden D. 1994. Canine cutaneous epitheliotropic lymphoma (mycosis fungoides) is a proliferative disorder of CD8+ T cells. *Journal of Pathology*, 144 : 421 - 429.

22. Nash R. A., Scherf U. & Storb R. 1991 Molecular cloning of the CD3 subunit of the T cell receptor / CD3 complex in the dog. *Immunogenetics*, 33 : 396 - 398.

23. Rabanal R. M, Ferrer L. & Else R. W. 1995. Immunohistochemical detection of canine leucocyte antigens by specific monoclonal antibodies in canine normal tissue. *Veterinary Immunology and Immunopathology*, 47 :13 - 23.

24. Teske E. 1994. Canine malignant lymphoma: a review and comparison with human non-Hodgkins lymphoma. *Veterinary Quarterly,* 4 : 209 - 219.

25. Teske E., van Heerde P., Rutteman G. R, Kurzman I. L., Moore P. F. & MacEwen E. G 1994. Prognostic factors for treatment of lymphoma in dogs. *Journal of the American Veterinary and Medical Association*, 24, 205 : 1722 - 1728.

26. Teske E., Wisman P., Moore P. F . & van Heerde P. 1994. Histologic classification and immunophenotyping of canine non-Hodgkin's lymphomas: unexpected high frequency of T cell lymphomas with B cell morphology. *Experimental Hematology*, 22 : 1179 - 1187.

27. Valli V. E. O. 1993. Pathology of the lympho-hemopoeitic system. In: Jubb K. V. F., Kennedy P. C., Palmer N. (Eds) *Pathology of Domestic Animals*. 4th Edition Academic Press Inc., San Diego.

28. Walder E. J. & Gross T. L. 1992 Lymphocytic tumours. In: Gross T. L, Ihrke P. J. & Walder E. J. *Veterinary Dermatopathology: A macroscopic and microscopic evaluation of canine and feline skin disease*. Mosby Year Book, St.Louis