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Molecular and immunohistochemical subtyping of canine lymphoma

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Abstract

Lymphoma is a broad term for a diverse group of neoplastic diseases of lymphocytes. In human oncology precise lymphoma subclassification, often based on immunohistochemistry (IHC) and molecular genetic abnormalities, allows accurate prognostication and treatment. Currently in canine oncology such precise subclassification is not possible. As a result, the prognosis and treatment of canine lymphoma is less tailored to a patient's specific disease. This study has two parts, each aimed at improving our ability to subclassify canine lymphomas. The first part involved investigating novel IHC stains in a group of canine lymphoma samples to identify their staining characteristics, with a focus on canine T-cell lymphomas (cTCL). The second investigated the prevalence and characteristics of *TRAF3* mutations in a group of canine B-cell lymphomas (cBCL) and assessed cTCL and non-lymphoma samples to see if *TRAF3* mutations are specific to cBCL.

For IHC investigation, 46 cTCL and 13 cBCL samples were assessed using c-Kit, CD30 and clusterin. Five, three and 11 cases were positive for c-Kit, CD30 and clusterin respectively. Three cases of cBCL were positive for clusterin and none were positive for either CD30 or c-Kit.

For *TRAF3* investigation, 49 dogs were included (n= 24 cBCL; n= 25 non-cBCL). Eleven dogs had matched non-tumour DNA assessed to determine if mutations were germline or somatic. All dogs had *TRAF3* assessed by Sanger sequencing. The prevalence of deleterious *TRAF3* mutations in cBCL was 36%. A deleterious *TRAF3* mutation was suspected to be germline in 1/5 cases with matched non-tumour DNA available for comparison. Deleterious mutations were not found in specimens from the non-cBCL group. Several synonymous variants were identified in cBCL and non-cBCL samples, which likely represent polymorphisms. These results indicate *TRAF3* mutations are common in cBC and may be important in the pathogenesis of cBCL.

These investigations have used novel IHC markers and *TRAF3* analysis to characterise a broad group of canine lymphomas. Further investigation is needed to investigate if these characterisations are useful in prognosticating or directing treatment in dogs with lymphoma.

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Declaration

I, Patrick Smith, declare that the work in this thesis is original, was carried out solely by myself or with due acknowledgements. It has not been submitted in any form for another degree or professional qualification.

Some of the work pertaining to TRAF3 mutations was presented as an abstract at the 2020 BSAVA Congress OnDemand and subsequently published in The Veterinary Journal (Smith et al., 2020).

Definitions/Abbreviations

ABC: activated B-cell

ABCA5: ATP-binding cassette A5

AKT: protein kinase B gene

ALCL: anaplastic large cell lymphoma

ALK: anaplastic lymphoma kinase

BCL: B-cell lymphoma

BCL-2: B-cell lymphoma 2 gene

BCL-6: B-cell lymphoma 6 gene

CARDII: caspase recruitment domain-containing protein 11

cBCL: canine B-cell lymphoma

CCDC3: coiled-coil domain containing protein 3

CCR7: chemokine receptor type 7

CD: cluster of differentiation

CLTC: clathrin

cIAP: cellular inhibitor of apoptosis

COX8A: cytochrome c oxidase subunit 8A

CSF: cerebrospinal fluid

cTCL: canine T-cell lymphoma

CXCL13: chemokine ligand 13

CXCR5: chemokine receptor 5

DLBCL: diffuse large B-cell lymphoma

DDX3X: DEAD-box helicase 3 X-linked

EATL-I: enteropathy-associated T-cell lymphoma type I

EATL-II: enteropathy-associated T-cell lymphoma type II

FBXW7: F-box and WD repeat domain containing 7

FDA: Food and Drug Administration

FFPE: formalin-fixed paraffin-embedded

GCB: germinal centre B-cell

GEP: gene expression profiling

HL: Hodgkin's lymphoma

ICC: immunocytochemistry

ICOS: inducible T-cell costimulator

IKKa: I kappa B kinase alpha

IFN-y: interferon gamma

IHC: immunohistochemistry

IL-2: interleukin 2

IRF4/MUM1: interferon regulatory factor 4/

JAK/STAT: janus kinase/signal transducer and activation of transcription

LBT: lymphoblastic T-cell lymphoma

MAP3K: mitogen activated kinase kinase kinase

MATH: meprin and TRAF homology

MHC: major histocompatibility complex

mTOR: mammalian mechanistic target of rapamycin

NFkB: nuclear factor kappa B

NGS: Next generation sequencing

NIK: nuclear factor kappa B inducing kinase

NLRP: Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing

NPM-ALK: nucleophosmin-anaplastic lymphoma kinase

NSCLC: non-small cell lung cancer

PARR: PCR for antigen receptor rearrangement

Pax5: paired box 5

PCR: polymerase chain reaction

PD1: Programmed death-1 receptor

PD-L1: programmed death-ligand-1

PI3K: phosphoinositide 3 kinase

PLC-y: phospholipase C gamma

POT-1: protection of telomeres 1

PSMA1: proteasome alpha subunit type 1

PTCL: peripheral T-cell lymphoma

PTCL-NOS: peripheral T-cell lymphoma, not otherwise specified

PTEN: phosphatase and tensin homolog

RAL/MAPK: ras-related protein/mitogen activated protein kinase

RelB: transcription factor RelB

RING: really interesting new gene

ROS1: proto-oncogene tyrosine protein kinase ROS

RTK: receptor tyrosine kinase

RT-PCR: reverse transcription polymerase chain reaction

SAP: serum amyloid protein

SATB1: special AT-rich sequence-binding protein-1

SMOC2: SPARC related modular calcium binding 2

TBC1D26: TBC1 domain family member 26

TBE: tris/borate/EDTA

TBL1XR1: transducin beta like 1 X-linked receptor 1

TCL: T-cell lymphoma

Tfh: follicular T-helper

TNF: tumour necrosis factor

TNFAIP3: TNF alpha induced protein 3

TRAF: tumour necrosis factor-associated factor

Treg: regulatory T-cell

TZL: T-zone lymphoma

WHO: World Health Organisation

1 Introduction

1.1 Lymphoma

Lymphoma is a broad term for a diverse group of neoplastic diseases of lymphocytes. Lymphoma is the most common haematopoietic malignancy in dogs and makes up 7-24% of all canine cancers (Vail et al., 2019).

Currently canine lymphomas are largely subclassified on an ad hoc basis, directed by owner factors (financial resources, willingness to treat etc) and availability of diagnostic tests and veterinary oncologic knowledge. Commonly canine lymphomas are subclassified primarily on anatomic location (eg multicentric nodal, alimentary, epitheliotropic etc), grade/cell size based on cytologic/histopathologic review of cell morphology and mitotic index, and basic immunophenotyping. No genetic profiling is used clinically in veterinary lymphoma diagnosis and management apart from PCR for antigen receptor rearrangement (PARR) to confirm clonality.

Immunophenotyping used in veterinary lymphoma management is almost exclusively limited to determining whether the neoplastic population is of B-cell or T-cell origin. This can be performed in several ways, including immunohistochemistry (IHC), immunocytochemistry, and flow cytometry. PARR results can also estimate immunophenotype, however this is considered less reliable due to reported lineage infidelity in PARR results (Burnett et al., 2003).

Precise lymphoma subclassification is essential to accurately prognosticate and direct therapy. There are several examples in veterinary lymphoma treatment where subclassification alters treatment and prognosis. Examples include: possibly improved outcomes in high-grade T-cell lymphoma (TCL) when treated with alkylator-rich multiagent chemotherapy, as opposed to standard doxorubicin-based multiagent protocols used for B-cell lymphoma (BCL) (Brown et al., 2018); and improved prognosis with T-zone lymphoma (TZL) as diagnosed by the absence of CD45 on flow cytometry (Seelig et al., 2016).

As outlined below, human lymphoma subclassification is far more advanced and over 80 lymphoma subtypes are recognised (Swerdlow et al., 2008). In canine oncology such precise subclassification is not yet possible, in part due to the absence of validated immunohistochemical and genetic markers, and due to the difficulty in performing large scale clinical trials to assess potential subclassification markers for prognostic or therapeutic significance. The driving aim behind this thesis is to try and identify new ways to subclassify canine lymphomas which can then be investigated for prognostic and therapeutic significance.

1.2 Classification of lymphoma

There have been several histological systems of classification for lymphoma in humans. The 2008 WHO classification of tumours of haematopoietic and lymphoid tissues (Swerdlow et al., 2008), revised in 2016 (Swerdlow et al., 2016), is the most common classification system currently used. This has been found to be a practical classification system for canine lymphomas (Valli et al., 2011) although an earlier study found that the previously used Kiel and Working Formulation lymphoma classification systems could also be applied to canine lymphoma (Fournel-Fleury et al., 1997). The most commonly diagnosed histological subtype of lymphoma in humans and dogs is diffuse large B-cell lymphoma (DLBCL) which is histologically similar to DLBCL in humans (Ponce et al., 2010, Seelig et al., 2016). Table 1-1 outlines the common canine lymphoma subtypes and their human equivalents.

Table 1-1. Common canine lymphoma subtypes and their proportion of all lymphoma cases in dogs and humans.

Lymphoma subtype		Proportion of canine cases (Vail et al., 2019, Ponce et al., 2010)	Proportion of human cases (Swerdlow et al., 2008, Thandra et al., 2021)	Comment
B-cell (59-79%)	DLBCL	22-52%	31%	Most common subtype in both species. Human DLBCL further subdivided into ABC and GCB based on GEP.
T-cell (25-52%)	PTCL-NOS	2-15%	4%	Heterogeneous group of diseases which cannot be further subclassified using existing techniques.
	Cutaneous TCL	2-12%	4%	Mycosis fungoides is the most common subtype of cutaneous TCL in both species.
	LBL	3-10%	2%	
	TZL	3-11%	Not recognised	Not recognised in humans

DLBCL: diffuse large B-cell lymphoma; PTCL-NOS: peripheral T-cell lymphoma, not otherwise specified; LBL: lymphoblastic (T-cell) lymphoma; TZL: T-zone lymphoma; ABC: activated B-cell; GCB: germinal centre B-cell; GEP: gene expression profiling.

Critical to the classification of lymphoma in humans is the use of IHC and molecular genetic tools to make a diagnosis of a specific lymphoma subtype.

IHC uses antibodies to demonstrate the presence of specific antigens within tissue sections (Ramos-Vara, 2005). Briefly, IHC involves the preparation of histology slides to expose specific tissue antigens ("antigen retrieval"). Antibodies are then applied to the slide for the purpose of binding specifically to the desired antigen. A second antibody is then applied which binds to the first antibody. This second antibody is conjugated to an enzyme (most commonly peroxidase) which then catalyses a colour-producing reaction which can then be visualised via light microscopy.

Often canine nodal and extranodal lymphoma is diagnosed using only samples that can be obtained using minimally invasive methods such as fine needle aspirates in order to reduce the financial cost to the owner and perceived invasiveness of general anaesthesia and biopsies on the dog. Fine needle aspirates produce cytological samples which can be assessed to determine cell size and morphology, however cannot provide information regarding the tissue architecture. Fine needle aspirate material can also be placed in a suspension and assessed using flow cytometry. Flow cytometry is able to sort cells in a suspension based on numerous criteria including size, granularity and fluorescent markers (Wilkerson, 2012). By attaching fluorescent markers to antibodies, flow cytometry can provide reliable immunophenotypic information (Wilkerson, 2012). Flow cytometry is able to differentiate canine lymphomas into distinct categories which often correlate with histopathologic and immunohistochemical features. In particular, flow cytometry can accurately predict a histologic/immunohistochemical diagnosis of T-zone lymphoma on the basis of T-cell marker positivity and CD45 negativity (Seelig et al., 2014, Comazzi and Riondato, 2021). Other examples, include flow cytometric immunophenotype CD45+CD3+CD8+CD4-MHC II- correlating with cutaneous T-cell lymphoma, and CD4+ lymphomas having a consistent histologic and gene expression profiles (Harris et al., 2019, Comazzi and Riondato, 2021). Flow cytometry is also prognostic in canine lymphoma (Avery et al., 2014). Flow cytometry is a non-invasive method that can correlate with histopathologic and immunohistochemical parameters, however histopathology and immunohistochemistry remain vital for definitive diagnosis, as there can be significant overlap between different canine WHO lymphoma subtypes when assessed by flow cytometry alone (Comazzi and Riondato, 2021, Riondato and Comazzi, 2021).

There are numerous molecular genetic tools available for use in investigating and subclassifying lymphoma. At their core these tools identify alterations in the genetic makeup of tissues, including chromosomal aberrations (eg copy number aberrations, fusions), mutations, and changes in gene expression. Polymerase chain reaction (PCR) based techniques allow the investigation of specific known DNA sequences to accurately interrogate the DNA sequence for abnormalities. Next generation sequencing (NGS), also known as massively parallel sequencing, is a more recent technology. NGS allows the entire genome, exome, or transcriptome to be sequenced and compared against a refence sequence to identify all mutations and abnormalities present. NGS such as RNA-Seq also allows for the quantification of gene expression and thereby allows the gene expression profile of different tumours to be measured. Identification of genes and metabolic pathways that are upregulated or downregulated can give insights into tumourigenesis and potential therapeutic targets for different tumours.

In humans accurate diagnosis of lymphoma into specific subclassifications requires the use of these tools. For example, the diagnosis of anaplastic large cell lymphoma (ALCL), a T-cell lymphoma, requires CD30 IHC positivity for diagnosis, and it is then further subclassified into anaplastic lymphoma kinase (ALK) positive ALCL or ALK negative ALCL on the basis of ALK IHC (Swerdlow et al., 2008), and human DLBCL is subclassified into germinal centre B-cell (GCB) subtype and activated B-cell (ABC) subtype on the basis of gene expression profiling (Swerdlow et al., 2008). Subsequent to the discovery of these DLBCL subtypes based on gene expression profiling, immunohistochemical panels (such as CD10, BCL6 and IRF4/MUM1) have been devised to more practically distinguish GCB from non-GCB DLBCL subtypes (Hans et al., 2004). The use of similar tools in canine lymphoma classification is less advanced; however it is progressing. In a seminal paper applying the WHO classification to canine lymphomas (Valli et al., 2011), the only diagnostic testing performed, beyond morphological assessment, was IHC immunophenotyping with the B-cell marker CD79a and T-cell marker CD3. Until further IHC markers and molecular profiling techniques are validated in veterinary medicine, precise lymphoma subclassification will remain beyond our grasp. Precise subclassification is critical for prognostication and for designing studies to determine optimal therapeutic strategies.

1.3 Immunohistochemical markers in lymphoma

IHC is integral in subclassifying lymphomas by differentiating morphologically identical populations of neoplastic cells into specific subgroups. No immunostain is 100% specific, and therefore panels of stains should always be used, and results correlated with the clinical picture.

Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) is a diagnosis assigned to a nodal or extra nodal mature T-cell lymphoma that cannot be assigned to any of the recognised specific T-cell lymphoma entities in the current WHO classification scheme (Swerdlow et al., 2008). In dogs PTCL-NOS is the second most common lymphoma subtype, making up about 15% of all lymphoma cases (Valli et al., 2013, Valli et al., 2011, Seelig et al., 2016). In humans, PTCL-NOS make up only 1% of lymphoma cases, and only 4% of non-Hodgkin's lymphomas (Jha et al., 2017, Seelig et al., 2016). This difference is no doubt in part due to the ability to differentiate human PTCL into numerous specific subtypes. These include angioimmunoblastic T-cell lymphoma, nodal PTCL with T follicular helper phenotype, adult T-cell leukaemia/lymphoma, ALK positive ALCL, and ALK negative ALCL among others (Swerdlow et al., 2008). These subclassifications are largely based on immunophenotyping via flow cytometry and IHC.

As previously mentioned, CD30 and ALK IHC are routinely evaluated in human PTCL, as CD30 positive cases can be classified as ALCL. ALK is prognostic in ALCL, with ALK positive cases having a significantly improved prognosis (Gascoyne et al., 1999, Swerdlow et al., 2008). Another routine distinction is whether the neoplastic cells are of follicular T-helper cell (Tfh) phenotype. Tfh phenotype is assigned if the cells are positive for at least two of the following Tfh markers: CD10, BCL6, PD1, CXCL13, CXCR5, ICOS, or SAP.

These markers have not been assessed in canine lymphoma. Particularly, subclassification of canine T-cell lymphomas remains difficult. A lack of methods to subclassify TCL results in most canine TCL being diagnosed as PTCL-NOS. From studies assessing canine TCL, inevitably a wide range of outcomes is seen (Moore, 2016, Goodman et al., 2016, Brown et al., 2018). This suggests that, like human TCL, canine TCL comprises a variety of specific subtypes, and if we were able to identify these subtypes we may be able to better prognosticate for, and treat, these patients.

With this goal in mind, we selected the following markers as being potentially useful for the subclassification of canine T-cell lymphomas: CXCR5, CD30, ALK, clusterin, c-Kit and PD-1. See Table 1-2 for a summary of an overview of these markers in human and canine diseases.

Table 1-2. Selected IHC markers used in human and canine lymphoma.

Marker	Role in human lymphoma	Use in canine disease
CXCR5	• Identify Tfh	• No role
CD30	• Required for diagnosis of ALCL • Reed-Sternberg cells positive	• Marker of ALCL • Marker of mast cells
ALK	• Prognostic in ALCL	• No role
Clusterin	• Not routinely used, however is a possible marker for ALCL	• No role
c-Kit	• No defined role	• No role in lymphoma • Diagnostic and prognostic in mast cell tumours
PD-1	• Identify Tfh	• No role

Tfh: follicular T-helper cells; ALCL: anaplastic large cell lymphoma.

1.3.1 CXCR5

CXCR5 is a chemokine, which are small peptides that direct the migration of leucocytes. It is a marker of Tfh, and is used in the subclassification of PTCLs (Swerdlow et al., 2008). It was first identified in Burkitt's lymphoma and is also termed Burkitt's lymphoma receptor 1.

CXCR5 (as well as CCR7) acts as a homeostatic chemokine receptor and is a major regulator of the adaptive immune response (Murphy, 2019). CXCR5 plays a key role in lymphocyte migration through the lymph node microenvironment. It is expressed by mature recirculating B-cells (however not plasma cells) (Hargreaves et al., 2001), some dendritic cells, as well as a subset of CD8 positive and CD4 positive T-cells, most notably Tfh (Burkle et al., 2007). Tfh are able to migrate following activation from the T-zone to the follicles where they provide help for B-cell maturation and antibody production. The ligand for CXCR5, CXCL13, is a homeostatic chemokine that is constitutively secreted by stromal cells in B-cell areas of secondary lymphoid tissues (follicles) where B-cells encounter antigen and differentiate. CXCR5 activation results in the recruitment of naïve B-cells to follicles (Burkle et al., 2007).

Tfh malignancies, such as angioimmunoblastic T-cell lymphoma and ~20% of PTCL-NOS show high levels of CXCR5 expression (Ahearne et al., 2014). Some of these lymphomas morphologically appear similar to ALCL, however their derivation from Tfh suggest a separate pathogenesis.

CXCR5 is also widely expressed in B-cell neoplasms including B-cell chronic lymphocytic leukaemia, hairy cell lymphoma, mantle cell lymphoma, mucosal-associated lymphoid tissue lymphomas, follicular lymphoma, and DLBCL (Pals et al., 2007).

There have been no veterinary studies using CXCR5 IHC.

1.3.2 CD30

CD30 is a type one transmembrane receptor and member of the tumour necrosis factor receptor superfamily (Pierce and Mehta, 2017, Sotomayor et al., 2014), which is formed of intracellular, trans-membrane and extracellular domains (van der Weyden et al., 2017). During early gestation, CD30 expression is found within a variety of organ systems including the gastrointestinal tract, post pharyngeal foregut, urinary, musculoskeletal, reproductive, endocrine, nervous, haematolymphoid, and integumentary systems (Sotomayor et al., 2014). After three months of gestation CD30 expression becomes much more restricted, primarily to the haematolymphoid system (Sotomayor et al., 2014).

CD30 is involved in T-cell immune response and regulation. It plays a role in T-cell proliferation in response to T-cell receptor stimulation, acting as a costimulator in secondary T-cell response, as well as stimulating production of cytokines (IL-2, TNF, IFN-y) by T-cells (Horie and Watanabe, 1998, Pierce and Mehta, 2017). Studies in knock-out mice suggest CD30 has a role in immune-surveillance and cross-talk between B- and T-cells (van der Weyden et al., 2017).

CD30 transduces signals via the recruitment of TNF receptor-associated factor (TRAF) and TRAF-binding proteins. Binding of TRAF1, 2 and 5 has been documented, which stimulate the nuclear factor kappa B, mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. Activation of these pathways results in anti-apoptotic and pro-survival mechanisms within the cell (Watanabe et al., 2011, van der Weyden et al., 2017, Pierce and Mehta, 2017).

According to the WHO classification system for lymphoma (Sabattini et al., 2010), CD30 positivity is required for a diagnosis of anaplastic large cell lymphoma (ALCL). Nearly all cases of Hodgkin's lymphoma (HL) are also CD30 positive. Various other forms of lymphoma have shown variable CD30-positivity (Sabattini et al., 2010), including enteropathy-associated T-cell lymphoma I (EATL-I), although not EATL-II, as well as Epstein Barr virus positive DLBCL, PTCL-NOS, and mycosis fungoides (Pierce and Mehta, 2017).

ALCL can be classified according to the WHO guidelines as primary cutaneous or systemic (Swerdlow et al., 2016). The systemic form is most common however up to 60% have extranodal involvement. The systemic form can be further subdivided into ALK positive and negative. ALCL has a classic morphology showing atypical lymphoid cells with pleomorphic or horseshoe nuclei and abundant cytoplasm(2016).

CD30 expression has been assessed as a prognostic indicator in DLBCL with conflicting results (Hao et al., 2015, Hu et al., 2013), however it is correlated with other poor prognostic indicators such as bone marrow involvement, non-germinal centre B-cell like DLBCL, and BCL-2 and Ki-67 overexpression (Hao et al., 2015). It is thought that soluble serum CD30 levels may correlate with progression of disease and poorer prognosis in CD30 positive lymphomas (Sotomayor et al., 2014).

CD30 staining is obligatory in cases of human ALCL (Pierce and Mehta, 2017). In these cases the neoplastic cells display a membranous/golgi-associated staining pattern. In a case study using CD30 IHC in a dog, the neoplastic lymphocyte population also showed membranous staining (Pittaway et al., 2018) while in a case series of 13 dogs with intestinal ALCL, 50-100% of neoplastic cells showed strong membranous to cytoplasmic staining (Stranahan et al., 2019).

Several studies have reported using CD30 IHC in canine patients with neoplastic and non-neoplastic diseases (Park et al., 2007, Pittaway et al., 2018, Hohsteter et al., 2014, Bauer et al., 2017, Stranahan et al., 2019). CD30 IHC positivity was identified in a case report of a dog with pulmonary lymphomatoid granulomatosis (Park et al., 2007). Since in humans CD30 can be used to differentiate testicular embryonal carcinomas (Emerson and Ulbright, 2005) a 2009 study assessed CD30 positivity in canine seminomas and Sertoli cell tumours, however all 35 samples (20 seminomas, 15 Sertoli cell tumors) were negative (Yu et al., 2009). A later study assessed 105 testicular tumours and found 7% were CD30 positive (Hohsteter et al., 2014). Of those cases that were CD30 positive, only 5-10% of cells showed positivity (Hohsteter et al., 2014).

A study assessing atopic dermatitis in dogs successfully identified CD30 positive lymphocytes in circulation using flow cytometry (Olivry et al., 2011). Bauer *et al* (Bauer et al., 2017) assessed CD30 expression in canine mast cell tumours. As part of this study CD30 IHC was assessed in 31 canine mast cell tumour samples and in all cases the neoplastic cells showed diffuse positive staining.

Two studies exist using CD30 in canine lymphoma. The first was a case study which used CD30 to diagnose ALCL in a dog with CD3 and CD79a negative lymphoma (Pittaway et al., 2018). The second used CD30 to diagnose intestinal ALCL in a case series of 13 dogs (Stranahan et al., 2019). This study was the first to describe intestinal ALCL in dogs.

Valli *et al* described a cutaneous form of ALCL in cats associated with injection sites, and also a nodal form which typically also involves generalised skin disease and dependent oedema, however these reports did not include CD30 immunostaining. A recent study assessed CD30 IHC in a variety of feline lymphomas and found that 13% of T-cell lymphomas, 14% of B-cell lymphomas, and 71% of mixed-cell lymphomas were positive (Carminato et al., 2020).

CD30 is attractive as a therapeutic target due to the limited expression of CD30 by normal cells. Multiple methods have been trialled in human oncology to target CD30, including monoclonal antibodies and antibody-drug conjugates (Pierce and Mehta, 2017).

Brentuximab vedotin is an antibody-drug conjugate which targets the CD30 membrane receptor. It consists of a human monoclonal anti-CD30 antibody which is covalently linked to the microtubule disrupting agent monomethyl auristatin E (Scott, 2017). Binding of brentuximab vedotin to CD30 positive tumour cells initiates a cascade that ultimately results in apoptosis. Brentuximab vedotin currently has FDA approval for the treatment of relapsed HL, ALCL, primary cutaneous ALCL and CD30 positive mycosis fungoides, as well as first line treatment of advanced stage HL.

An *in vitro* study found that brentuximab vedotin induced growth inhibition and apoptosis in canine mast cell tumour cell lines, and that there was synergistic activity when brentuximab vedotin was combined with the tyrosine kinase inhibitor masitinib (Bauer et al., 2017).

In humans, it is unknown whether CD30 expression levels, quantified by staining intensity and distribution on IHC, relate to response to anti-CD30 therapies (van der Weyden et al., 2017).

No studies exist examining CD30 in a variety of canine lymphomas or as a therapeutic target in the dog.

1.3.3 ALK

Anaplastic lymphoma kinase (ALK), also known as CD246, is a receptor tyrosine kinase (RTK) of the insulin receptor superfamily. It plays roles in normal development and in oncogenesis. ALK has a similar structure to other RTKs, with an extracellular ligand-binding region, transmembrane region, and cytoplasmic tyrosine kinase domain. Within the insulin receptor superfamily, ALK is grouped with leucocyte tyrosine kinase due to homology between the receptors (Palmer et al., 2009). In humans the ligands known to bind to the ALK receptor are pleiotrophin and midkine (Webb et al., 2009). Heparin affinity regulatory peptide and heparin-binding neurotrophic factor are also activating ligands (Courty et al., 1991) although less is known about these ((Kovesdi et al., 1990).

In various non-human species, ALK expression is restricted to specific regions of the nervous system, eyes, tongue, testis and ovaries (Iwahara et al., 1997) (Hurley et al., 2006). ALK levels decrease at the end of gestation and only very small quantities are found after birth, with minimum levels reached at three weeks of age (Iwahara et al., 1997). Anti-ALK immunocytochemical studies in adult humans found only rare staining in neural cells, pericytes and endothelial cells of the brain. ALK is predominantly found in the nervous system of neonates, which suggests it has a role in the development of this system (Webb et al., 2009). However mice engineered without the *ALK* gene still live a normal lifespan with no apparent abnormalities (Webb et al., 2009).

ALK and its ligands have a role in the regulation of cell survival. Pleiotrophin and midkine regulate apoptosis, likely at least in part through ALK activity. Pleiotrophin, through ALK binding, leads to activation of anti-apoptotic proteins (serine/threonine kinases PI3-kinase and protein kinase B). Pleiotrophin plays a role in angiogenesis, possibly through ALK activation (Webb et al., 2009). Pleiotrophin can also activate ALK without direct pleiotrophin-ALK binding. Midkine-mediated ALK activation leads to increased proliferation (via insulin-like receptor substrate-1 and Shc interaction) by increasing nuclear factor-kappa B (NFkB) levels (Palmer et al., 2009).

Pleiotrophin and midkine have been linked to the proliferation, survival, metastasis and angiogenesis of tumours, however it is not clear how much of these effects are due to ALK-mediated signal transduction (Webb et al., 2009).

ALK overexpression and gain-of-function mutations have been implicated in oncogenic progression (Palmer et al., 2009). ALK overexpression has been documented in several human neoplasms, including lymphoma, non-small cell lung tumours, thyroid carcinomas, breast cancer, retinoblastoma, astrocytomas, Ewing sarcomas and rhabdomyosarcomas (Dirks et al., 2002, Kwak et al., 2010).

ALK is involved in oncogenesis as both a full-length ALK receptor and as ALK fusion proteins. Full-length ALK receptor involvement in oncogenesis is due to ALK over-expression and activating point mutations (Palmer et al., 2009), as well as paracrine/autocrine loops involving pleiotrophin and midkine (Webb et al., 2009), leading to increased ALK activation. However, ALK fusion proteins are the most common ALK abnormalities seen in cancers (Webb et al., 2009). ALK fusion proteins result from chromosomal translocations interrupting the normal ALK gene (at 2p23). The fusion protein displays self-association which mimics normal ligand binding and leads to constitutive activation (Webb et al., 2009). The most common fusion protein, nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), signals via the PLC-y, PI3K, RAK/MAPK and JAK/STAT pathways (Palmer et al., 2009).

ALK positivity is also seen in a rare subpopulation of human DLBCL patients and is correlated with an aggressive disease and poor response to treatment (Reichard et al., 2007). The fusion protein CLTC-ALK is more commonly encountered in ALK positive DLBCL cases, as opposed to the NPM-ALK fusion protein most commonly seen in ALCL (Choung et al., 2008).

ALCL is a T-cell non-Hodgkin's lymphoma characterised by CD30 expression (Palmer et al., 2009). ALK expression is associated with prognosis in human ALCL. ALK positive patients have an improved prognosis with significantly higher 5-year survival rates of 80%, compared to only 48% in ALK negative cases (Gascoyne et al., 1999, Swerdlow et al., 2016). Intestinal ALCL is most commonly ALK-negative (Savage et al., 2008).

It is not clear to what extent ALK IHC highlights ALK fusion proteins as well as full-length ALK receptors (Webb et al., 2009). In ALK positive ALCL, neoplastic cells show intense cytoplasmic and nuclear staining.

There have been no studies looking at ALK expression or mutations in canine lymphoma. A previous publication (Mariotti et al., 2014) assessed 12 canine pulmonary adenocarcinomas and found significantly increased ALK gene expression (measured with RT-PCR) in the neoplastic tissue compared to adjacent non-neoplastic lung tissue. This study also attempted to characterise ALK expression in the adenocarcinomas with IHC, however no samples were positively stained. It is unknown whether this was due to lack of ALK expression (below detectable level), suboptimal antigen retrieval, or lack of homology between the antibody used and canine ALK antigen.

Patients with human non-small cell lung cancer (NSCLC) with ALK fusion proteins have been shown to benefit from specific ALK inhibitors (Cabezon-Gutierrez et al., 2012) such as crizotinib (Kwak et al., 2010). Currently there are no commonly used ALK-targeted therapies for human patients with ALK-positive ALCL (Palmer et al., 2009), and no veterinary products targeting ALK.

1.3.4 Clusterin

Clusterin, also known as glycoprotein III, testosterone-repressed prostate message-2, apolipoprotein J and sulphated glycoprotein-2, is a 80kd glycoprotein composed of two alpha and beta subunits (Saffer et al., 2002). Clusterin is highly conserved across species and is present in varying amounts in numerous tissues. It is also frequently identified in body fluids. It is believed to play roles in numerous functions including reproduction, lipid transport, complement regulation, hormone secretion, and apoptosis (Jones and Jomary, 2002).

Clusterin is expressed in human lymphoma as well as various carcinomas, including mammary, renal, bladder, prostate, and ovary. Most cases of ALCL reveal clusterin IHC positivity, leading to some suggesting it as a diagnostic marker of ALCL in cases of poorly differentiated round cell tumours (Nascimento et al., 2004).

Clusterin has been assessed as a CSF biomarker for chronic spinal disorders in dogs (Shafie et al., 2014). This study found that CSF clusterin concentrations were increased in cases of degenerative myelitis and intervertebral disc disease compared to normal dogs and dogs with meningoencephalitis and idiopathic epilepsy. Clusterin IHC in this study revealed neuronal positivity was not significantly different between subgroups.

Clusterin has been evaluated in canine lymphoma in two studies. Both studies evaluated soluble clusterin in serum. The first study assessed the serum proteome of 21 dogs with lymphoma compared to healthy dogs. This study found measurable quantities of clusterin in 1/21 lymphoma dogs and in none of the healthy control dogs (Atherton et al., 2013). The second study specifically evaluated soluble serum clusterin levels as a potential biomarker for canine lymphoma (McNaught et al., 2020). This study found that serum clusterin levels were significantly lower in dogs with multicentric lymphoma compared to healthy controls, and no difference was found between dogs before treatment and after complete remission was achieved. The authors concluded that clusterin had limited potential as a biomarker due to the wide variation in clusterin value between individuals.

Clusterin IHC in canine lymphoma has not previously been assessed.

1.3.5 C-Kit

C-Kit is expressed in myeloid progenitor cells, dendritic cells, mast cells as well as pro-B- and T-cells. Normal B- and T-cells lose c-Kit expression during differentiation.

C-Kit mutations have been noted in numerous human haematopoietic neoplasms, however results in lymphoma have been contradictory (Giantin et al., 2013, Rassidakis et al., 2004, Pinto et al., 1994, Brauns et al., 2004, Rassidakis et al., 2003) with some studies finding frequent expression of c-Kit in specific CD30 positive lymphoma subtypes (HL, ALCL) (Pinto et al., 1994), others finding the opposite, with very infrequent expression in CD30 positive lymphomas (Rassidakis et al., 2004, Rassidakis et al., 2003), and some finding infrequent expression in other specific subtypes (PCTL, mycosis fungoides, Sezary syndrome) (Brauns et al., 2004). In a study assessing 56 cases of human DLBCL, 37% showed some c-Kit positivity (Vakiani et al., 2005). Another study assessing c-Kit IHC in 1166 cases of human lymphoma found only two cases positive for c-Kit (one TCL and one follicular lymphoma) (Zimpfer et al., 2004).

A study assessing c-Kit expression in canine lymphoma found that BCLs had low expression, as measured by mRNA PCR sequencing and flow cytometry, whereas TCLs had variable expression, with c-Kit expression increased in high grade more than low grade TCLs (Giantin et al., 2013). This study assessed various lymphomas with c-Kit immunocytochemistry (ICC) and found that 6/14 BCLs, and 7/11 TCLs were positive. IHC was not assessed (only ICC). A study investigating the use of masitinib in the treatment of epitheliotropic T-cell lymphoma found no expression of c-Kit when 8 tumours were assessed using IHC(Holtermann et al., 2016). A case report of a dog with cutaneous epitheliotropic lymphoma identified moderate cytoplasmic to membranous c-Kit positivity (Shiomitsu et al., 2012).

Overall the utility of c-Kit expression in human and canine lymphomas is unknown, however large scale studies of c-Kit expression in canine lymphomas are lacking. It remains to be determined if c-Kit IHC can be used for lymphoma subtyping and prognostication, or to predict response to therapy (e.g. with c-Kit inhibitors).

1.3.6 PD-1 and its ligand (PD-L1)

Programmed death-1 receptor (PD-1) and its ligand, programmed death-ligand-1 (PD-L1) are immune checkpoint molecules involved in maintaining T-cell tolerance to self-antigens. They act to limit autoimmunity by restricting T-cell activity in peripheral tissues (Hartley et al., 2018, Gravelle et al., 2017). PD-1 expression is induced by T-cell activation. Ligation of PD-1 by PD-L1 delivers an inhibitory signal which reduces T-cell response. It does this by promoting apoptosis in antigen-specific T-cells and reducing apoptosis in regulatory T-cells (Tregs) (Hartley et al., 2018). PD-L1 is expressed by immune cells, particularly antigen presenting cells. PD-L1 and its expression is frequently upregulated in tumour cells.

The PD-L1/PD-1 axis is frequently dysregulated in cancer, leading to an upregulation of PD-L1 expression on tumour associated macrophages and tumour cells. This leads to an increase in PD-1 signalling in T-cells which leads to apoptosis, anergy and functional exhaustion of T-cells, aiding in immune escape of the tumour (Gravelle et al., 2017).

In human non-Hodgkin's lymphoma overexpression of PD-L1 by tumour cells and PD-1 by tumour infiltrating lymphocytes has been shown (Andorsky et al., 2011, Laurent et al., 2015).

A flow cytometric study of canine lymphoma patients found a significant number of malignant cells in B-cell lymphoma (20%) showed upregulation of PD-L1, compared to only 2% of non-neoplastic B-cells. Neoplastic and non-neoplastic T-cells showed very low PD-L1 expression. In the same study no difference was found between malignant and normal B-cells in terms of PD-1 expression, however no malignant T-cells showed PD-1 expression, compared to 50% of normal T-cells (Hartley et al., 2018). Shosu *et al* used PD-L1 IHC to assess various canine tumours and found that PD-L1 was expressed in many tumour types, including 15/15 of the lymphoma samples assessed (Shosu et al., 2016).

A study assessing PD-L1 expression in canine tumour cells macrophages via flow cytometry, Western blotting, immunofluorescence imaging and RT-PCR found that all tumour cells expressed PD-L1 to various degrees, and concluded that PD-L1-mediated T-cell suppression may be an important mechanism of immune evasion in dogs (Hartley et al., 2017).

Anti-PD-1 antibody therapy has shown favourable responses in several human cancers (Postow et al., 2015). PD-1 and PD-L1 antibodies had functional effects on canine T-cells in a preliminary study (Coy et al., 2017), and a second study which assessed the use of a PD-L1 monoclonal antibody as a therapeutic in canine oral malignant melanoma and undifferentiated sarcoma found that 1/7 melanomas and 1/2 sarcomas showed objective responses (Maekawa et al., 2017). PD-L1 IHC was not used in this study, however it opens the door for the use of anti-PD-L1 antibodies as a therapeutic in canine oncology in future.

A recent study (Choi et al., 2020) developed canine anti-PD-1 and anti-PD-L1 antibodies which were shown to be specific for the intended epitopes. The anti-PD-1 antibody was successfully used for IHC, and the anti-PD-L1 antibody demonstrated functional activity against PD-L1, suggesting it could potentially be developed into a therapeutic anti-PD-L1 treatment in future.

No studies have specifically assessed using PD-1 or PD-L1 IHC in a variety of canine lymphomas.

1.4 Molecular genetic classification of lymphoma

Many genes contributing to lymphomagenesis have been identified in human oncology, however relatively little is known about the molecular genetic abnormalities associated with the development, prognosis and treatment of canine lymphoma (Bushell et al., 2015). Recent advances in gene sequencing have led to an increase in identification of mutations. The high rate of lymphoma in specific breeds, and the propensity for specific breeds to get certain types of lymphoma (e.g. T-cell lymphoma in Boxers) indicates an as yet poorly understood genetic basis of lymphoma (Elvers et al., 2015).

Genomic instability is a key feature of all carcinogenesis (Hanahan and Weinberg, 2011) and there are numerous types of genetic abnormalities that can occur. Broadly, these abnormalities contribute to the formation of cancer by the activation of oncogenes or inactivation of tumour suppressor genes. The most commonly recognised abnormalities involve genetic mutations which alter the genetic sequence. Common types of mutations include point mutations, duplications, frameshift mutations, and chromosomal translocations. Sanger sequencing is an accurate method of sequencing DNA, although it has several limitations. These include the need to know the sequence of the target DNA (in order to make primers), only sections of DNA ~500-600 base pairs in length can be sequenced, and mutations can only be detected if the prevalence in the cells being assessed is over ~10% (poor sensitivity) (de Koning et al., 2015). Common mutations identified with Sanger sequencing include point mutations and frameshift mutations. Point mutations are the alteration of a single nucleotide. These mutations can have several effects including: being silent (no change in amino acid), missense (different amino acid), or nonsense (change to a stop codon which prematurely terminates the sequence). Frameshift mutations insert or delete a number of nucleotides that is not divisible by three, thereby altering the reading frame and completely altering the downstream sequence. Next generation sequencing (also termed massively parallel sequencing) is a powerful tool in assessing the genetic abnormalities in carcinogenesis as it is able to overcome many of the limitations of Sanger sequencing. RNA-seq (a form of next generation sequencing) sequences the transcribed RNA and so permits the identification of any post-transcriptional abnormalities and can quantify levels of gene expression and so give gene expression profiles.

1.4.1 Molecular profiling in lymphoma

Human diffuse large B-cell lymphoma (DLBCL) is a clinically heterogeneous group of lymphomas which cannot be further subclassified based on morphology alone. However when gene expression profiling is used, DLBCL can be subclassified into at least two different groups, including activated B-cell (ABC) lymphoma and germinal centre B-cell (GCB) lymphoma. This separation is prognostically significant, with GCB DLBCL having a significantly better prognosis. GCB DLBCL have a 76% 5-years survival, compared to just 16% with ABC DLBCL (Richards et al., 2013).

Constitutive activation of the NFkB pathway has been found in most human lymphoid malignancies (Mudaliar et al., 2013). Differential gene expression of the NFkB pathway can be prognostic as in the case of human DLBCL, with the gene profile of the more aggressive ABC DLBCL showing constitutive NFkB activity (Davis et al., 2001).

1.4.2 Molecular profiling in canine lymphoma

Frantz *et al* (Frantz et al., 2013) assessed gene expression profiles in six common canine lymphoma subtypes (LBT, TZL, PTCL-NOS, Burkitt-like B-cell lymphoma, DLBCL, marginal zone lymphoma) and found these subtypes could be separated into three distinct groups based on gene expression; high grade T-cell lymphoma (LBT, PTCL-NOS), low grade T-cell lymphoma (TZL), and B-cell lymphoma (marginal zone lymphoma, DLBCL, Burkitt-like lymphoma). This separation was prognostically significant. The study also identified four genes (*CD28, ABCA5, CCDC3, SMOC2*) whose expression could be measured to accurately categorise samples into one of the three groups. This study was not able to separate the DLBCL group into distinct ABC and GCB groups, however this may have been due to the small sample size (n=10).

Richards *et al* (Richards et al., 2013) attempted to separate canine B-cell lymphoma cases using immunohistochemical algorithms, gene expression profiling, and immunoglobulin heavy chain variable region mutational assessment. This study found that immunohistochemical algorithms used in human oncology to differentiate ABC DLBCL and GCB DLBCL were not useful in canine lymphoma. However, gene expression differences were able to separate the canine samples into two groups, similar to human GCB and ABC DLBCL. This distinction was then used to select a canine-specific set of differentially expressed genes. This canine-specific gene expression profile was able to identify two groups with significantly distinct progression free survival. Furthermore, these canine-specific "ABC/GCB" discriminating genes, while different from the human ABC/GCB gene list, are involved in the same pathways and processes (e.g. NFkB signalling and B-cell receptor signalling). Immunoglobulin heavy chain variable region mutational assessment was also able to separate out cases into two distinct groups with significantly different survival times.

A study specifically assessing gene expression in canine CD4 positive TCL found 5011 genes were significantly different in neoplastic CD4 positive T-cells compared to control CD4 positive T-cells from healthy dogs (Harris et al., 2019). This study assessed six dogs with CD4 positive TCL and compared them to six healthy dogs. It found that gene expression was consistent with flow cytometry results, with decreased expression of CD5, CD25 and MHC class II correlating with negative flow cytometry results for these markers. Parathyroid hormone-related peptide expression was increased in all cases, regardless of whether the patient was clinically hypercalcaemic. Three of the dogs with lymphoma were Boxers and it was found that their gene expression was overall not significantly different from the dogs of other breeds. However 82 genes were significantly different in the Boxers compared to other breeds, with ROS1 being one of the most overexpressed genes in the Boxer. *SATB1* and *PTEN* were identified as frequently mutated in a previous study (Elvers et al., 2015) however this was not seen in this study. Pathway analysis found significant upregulation of PI3K and mTOR (both associated with increased cell proliferation) and significant down regulation of PTEN (an antagonist of AKT/mTOR axis).

Elvers *et al* compared the somatic mutations of BCL and TCL in three dog breeds (Boxer, Golden Retriever, Cocker Spaniel) with different lymphoma immunophenotype predispositions (Elvers et al., 2015). This study used matched tumour and normal tissue samples to identify somatic mutations and compared mutations between BCL predisposed breeds (Cocker Spaniel and Golden Retrievers) and TCL predisposed breeds (Golden Retrievers and Boxers). On average 18 non-silent somatic mutations were identified in lymphoma cases, with TCLs having more mutations than BCLs. Most significantly mutated genes were only mutated in either BCL or TCL (not both), with only seven percent of mutations being in both groups. The most significantly mutated genes in BCL were *POT1, FBXW7* and *TRAF3*. The most significantly mutated genes in TCL were *SATB1, TBC1D26, PSMA1, COX8A,* and *PTEN*. Mutations in BCLs tended to be similar between breeds, however TCLs tended to have very little overlap of mutations between breeds. Of the 15 most commonly mutated genes in both breeds, only *SATB1* was shared. Forty-four percent of boxers had a mutation of at least one of the following genes: *PI3KCD, PIK3RI, MTOR,* and *PTEN*, but only six percent of Golden Retrievers had these mutations. Both breeds had different mutations affecting the NLRP family.

Bushell *et al (Bushell et al., 2015)* assessed *TRAF3* mutations in many cases of cBCL. Initial RNA sequencing of 14 dogs identified mutations predicted to alter TRAF3 function in 28.6% of dogs. *TRAF3* coding exons were sequenced in 63 confirmed cBCL cases and 30.2% were mutated (a further 4/21 lymphomas of unknown phenotype had *TRAF3* mutations). This study identified 27 mutations, of which 13 were single nucleotide variants (predicted to truncate the protein) and 13 were insertion-deletion mutations. Lymphoma samples were matched with normal skin to assess for germ-line mutations and interestingly 21 samples had germ-line mutation identified which was predicted to affect TRAF3 function. In total, 44.4% of B-cell lymphoma samples with a *TRAF3* mutation had more than one mutation (somatic or germ line) predicted to affect TRAF3 function. This study also looked at *TRAF3* mutations in 148 cases of human DLBCL using Affymetrix SNP 6.0 software and identified 13 (8.9%) cases with deletions affecting *TRAF3*. They then used FISH to confirm the deletion in five cases. RNA-sequencing data from 91 cases showed a significant reduction in TRAF3 expression in mutated individuals.

Some veterinary studies have suggested that deregulated NFkB activity is also a feature of canine B-cell lymphoma (Rowell et al., 2011, Richards et al., 2013, Mudaliar et al., 2013). Mutations to the *TRAF3* gene, which is involved in NFkB activity, have been shown in a number of studies assessing canine B-cell lymphoma (Bushell et al., 2015, Elvers et al., 2015). Richards *et al (Richards et al., 2013)* found that differences in NFkB pathway were among the variables that could be used to separate dogs into prognostically distinct groups, similar to human ABC DLBCL and GCB DLBCL. Genes involved in regulating NFkB activity have been found to be commonly mutated ABC BCL cases, which is consistent with the altered NFkB activity found on gene expression profiling. Mutations affecting NFkB activity include loss of function of the negative regulator *TNFAIP3*, activation on positive regulators including *CARD11* and others (Bushell et al., 2015). Based on these findings, NFkB inhibition is a promising therapeutic target for tumours with increased NFkB activity.

Previous research at our institution used next generation sequencing (RNA-Seq) to assess the mutational status in canine lymphoma in 18 BCL and five TCL (Waugh, 2015). This work identified several gene variants that were proportionately more frequent in BCL. These included mutations in *TRAF3, POT1, DDX3X* and *TBL1XR1*, in which mutations were only identified in BCL samples. *TRAF3* variants were unique in all cases and all mutations were within exons 9, 10 and 11. The majority of mutations identified in a previous study of *TRAF3* mutations in canine BCL were also within these exons (Bushell et al., 2015).

Based on these findings, *TRAF3* was selected for further investigation in this study.

1.4.3 TRAF3

TRAF3 is a TNF-receptor associated factor and is part of the CD40 signalling cascade which regulates proliferation, immunoglobulin class switching and apoptosis (Elvers et al., 2015). It is part of the tumour necrosis factor receptor-associated factor (TRAF) family of cytoplasmic adaptor proteins (Moore et al., 2015). TRAF3 signalling is used by a number of receptors, including TNF-receptors, pattern recognition receptors, and several viral proteins (Moore et al., 2015).

TRAF3 is considered a tumour suppressor gene and has been implicated in several tumours including several types of lymphoma, multiple myelomas, as well as other malignancies (Bushell et al., 2015). In the absence of stimulation, TRAF3 forms a complex with TRAF2, the E3 ubiquitin ligase cIAP1/2, and NFkB-inducing-kinase (NIK). In this complex, cIAP1/2 targets NIK for degradation, which leads to inhibition of NFkappa-B activation. B-cell activating factor (BAFF) stimulation results in trimerized BAFF-receptors or CD40 recruiting cytoplasmic TRAF2 and TRAF3. This releases NIK from the TRAF2-TRAF3-cIAP1/2 complex, leading to NIK accumulation in the cytoplasm. This leads to phosphorylation of I-kappa-B kinase alpha (IKKa), which subsequently causes the inactive NFkB2/p100 to convert to the active NFkB2/p52. This complex then forms dimers with RelB which translocate to the nucleus and induce the transcription of anti-apoptotic Bcl2 family proteins, ultimately leading to B-cell survival.

TRAF3 also acts with other proteins to target MAP3K14 for degradation. It negatively regulates NFkB by targeting NIK for constant ubiquitisation and degradation (Bushell et al., 2015, Sun, 2011). NIK is then recruited by the TRAF2-cIAP1/2 ubiquitin ligase complex and degraded (Vallabhapurapu et al., 2008). Therefore reduced TRAF3 activity leads to NIK stabilisation and an upregulation of NFkB activity.

TRAF3 in dogs is found on chromosome 8 and is composed of 568 amino acids in 11 exons. The *TRAF3* protein has several domains. The N-terminal part of *TRAF3* has a really interesting new gene (RING)-type binding zinc domain and several zinc fingers (Qian Yin, 2009). The RING domain mediates the interaction between E2 ubiquitin conjugating enzymes and their substrates. Zinc finger domains mediate interactions between proteins and DNA, RNA, lipids and other proteins. The C-terminal part of *TRAF3* can be divided into TRAF-N and TRAF-C domain. The TRAF-N domain includes two coiled coil regions which mediate the homo- and hetero-oligomerisation among TRAF family members. TRAF3 forms heterotrimers with TRAF2 (He et al., 2004) as part of its normal function. This TRAF trimerization enhances the otherwise weak interactions between the TRAF proteins and their substrates (Qian Yin, 2009). The TRAF-C domain contains a meprin and TRAF homology (MATH) domain which is necessary for receptor interaction and interactions with adaptor proteins (Qian Yin, 2009).

TRAF3 mutations can have a dominant negative effect, meaning heterozygous mutations can cause phenotypic changes. Mutations to RING-type domain or zinc finger domain can competitively displace normal TRAF3 if the C-terminal *TRAF3* domains on the mutated protein are normal. Mutations to the C-terminal *TRAF3* domains can prevent normal TRAF3 recruitment (Force et al., 1997).

Elvers *et al (Elvers et al., 2015)* found that *TRAF3* alone is mutated in 30% of Cocker Spaniel and Golden Retriever B-cell lymphoma, and 30% and 50% of either or both *TRAF3* and *MAP3K14* are mutated in Cocker Spaniels and Golden Retrievers respectively.

Aim of this study

This study had two main aims. These were:

1. To investigate the immunohistochemical characteristics of canine lymphoma using a variety of immunohistochemical markers, some of which have proven significance in human lymphoma subclassification, or other canine cancers, to see if this would assist in the subclassification of canine T-cell lymphoma in particular, and
2. To investigate the prevalence and characteristics of *TRAF3* mutations in canine B-cell lymphoma, and to assess dogs with other non-BCL diseases (including TCL) to see if *TRAF3* mutations are specific to BCL.

2 Materials & Methods

2.1 Immunohistochemistry

2.1.1 Samples

Tissue from 59 canine patients diagnosed with lymphoma at centres throughout the UK were included. All samples were submitted to Veterinary Diagnostic Services (VDS, Glasgow, UK) for either full diagnostic assessment (histology and immunohistochemistry by VDS pathologists), or for immunophenotyping after initial histology was performed by a separate laboratory (Nationwide Laboratory Services, Leeds, UK). All samples had initial immunophenotyping (B-cell/T-cell) performed at our institute (VDS) and all had histopathology and/or immunophenotyping reports available for review. All samples were diagnosed as lymphoma and were immunophenotyped by immunohistochemistry by a board-certified pathologist (or resident under Specialist supervision). B-cell/T-cell immunophenotyping involved immunohistochemistry with a panel of at least CD3 for T-cells and CD79a and/or Pax5 for B-cells, using standard IHC protocols. Samples were included if enough tissue remained for additional IHC to be performed. Unfortunately due to unexpected difficulties in optimising some of the IHC stains, some tissue samples did run out before the completion of the project. Samples were excluded if their lymphoma diagnosis and/or B-cell/T-cell immunophenotype was ambiguous, or if there was insufficient sample for additional IHC to be run.

2.1.2 Staining protocols used for IHC

See table 2-1 for details of the antibodies used. Staining was performed by the Veterinary Diagnostic Services laboratory (University of Glasgow, UK). Staining protocols had previously been validated by the laboratory for c-Kit (Webster et al., 2007) and clusterin (Shafie et al., 2014). CD30 staining required numerous trials based on previous publications (Park et al., 2007, Pittaway et al., 2018, Stranahan et al., 2019, Hohsteter et al., 2014) before a successful technique was found by following the process used in a publication by Bauer *et al (Bauer et al., 2017)*. CXCR5, ALK and PD-1 IHC staining was attempted as outlined below.

Staining was performed using a Dako Autostainer (Dako, Santa Clara, USA).

2.1.2.1 Clusterin, c-Kit and CD30 protocols

Clusterin and c-Kit staining was performed according to the following protocol: 5μm sections were cut, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Antigen retrieval was performed using 10 mM sodium citrate buffer pH 6.0 in an automated pressure cooker. The samples were loaded onto the autostainer (Dako). The buffer was rinsed and endogenous peroxidase activity was quenched with 5 minute blocking solution (Dako Real Peroxidase, Dako). The samples were incubated with the primary antibody at the appropriate dilution for 30 minutes at room temperature. Sections were washed and incubated with the conjugated antibody. The immunocomplexes were detected with 3,3-diaminobenzidine (Dako K5007 DAB) substrate chromogen. Samples were counterstained with haematoxylin. Sections were dehydrated using a series of degraded alcohol baths and mounted in synthetic resin.

CD30 staining was performed by an identical procedure aside from the samples being incubated with the primary antibody for 16 hours at four degrees Celsius.

2.1.2.2 CXCR5, PD-1 and ALK protocols

The protocol outlined above was followed however the following variations were attempted:

- Antigen retrieval was performed on different samples using the following techniques:
  1. Heat induced epitope retrieval using Menarini Access Retrieval Unit. Buffers used: Sodium Citrate pH6, or EDTA pH8 or pH9, treated for 1 min 40 sec at 125^0C full pressure.
  2. Enzymatic antigen retrieval using Proteinase K RTU (Dako).
- The following antibody dilutions were trialled:
  - 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200.

2.1.3 Evaluation and scoring system used for clusterin IHC

Nascimento *et al (Nascimento et al., 2004)* previously described a grading system which examined the staining pattern of clusterin (golgi, membranous, cytoplasmic) and then divided cases into 3 categories (focal: <25%, intermediate: 25-75%, diffuse: >75%) based on the proportion of neoplastic cells showing golgi staining over the entire field. This system was adapted for this study. A further category was added (rare: <1% cells) due to the low numbers of positive cells in some samples. The term "focal" was changed to "mild" as "focal" suggests a geographic concentration of positive cells within the samples, whereas in all cases the positive cells were interspersed throughout the sample.

Samples were assessed by finding a representative area of neoplastic cells, if possible distant from any areas of stromal/background staining. Between 5-10 40x high power fields (hpf) were assessed in each case. All samples showed varying degrees (ranging from very mild to mild) of granular nuclear and cytoplasmic staining. This was not considered to be positive staining.

Grading scheme used:

- Negative: No lymphoma cells positive.
- Rare: <1% lymphoma cells positive
- Mild: <25% lymphoma cells positive
- Intermediate: 25-75% lymphoma cells positive
- Diffuse: >75% lymphoma cells positive.

Samples also had their pattern of staining (golgi, membranous, cytoplasmic) and intensity (weak, moderate, strong) recorded.

2.1.4 Evaluation of c-Kit IHC

Samples were categorised as positive or negative. Samples were considered positive if any of the neoplastic cells showed specific staining. Positive samples had the staining pattern (golgi, membranous, cytoplasmic), intensity (weak, moderate, strong), and geographic distribution (interspersed individual cells, regionally diffuse, diffuse) recorded. Samples were categorised as "regionally diffuse" if all lymphoma cells within a focal geographic region were positive while the remainder of the neoplastic cells outside this area were negative.

2.1.5 Evaluation of CD30 IHC

Samples were categorised as positive or negative. Samples were considered positive if any of the neoplastic cells showed specific staining. Positive samples had the staining pattern (golgi, membranous, cytoplasmic), intensity (weak, moderate, strong), and geographic distribution (interspersed individual cells, regionally diffuse, diffuse) recorded. Samples were categorised as "regionally diffuse" if all lymphoma cells within a focal geographic region were positive while the remainder of the neoplastic cells outside this area were negative.

2.1.6 Evaluation of ALK, CXCR5 and PD-1 IHC

Human Hodgkin's lymphoma, human tonsil and canine tonsil were used for antibody optimisation with standard methods and then modifications as outlined in 2.1.2.2. The samples were assessed for positive staining.

Table 2-1. Antibodies used for immunohistochemistry.

Antibody	Source	Antigen derived from	Dilution for IHC	Manufacturer	Positive control	Comments
CD30 (Ber-H2) (sc-19658)	Mouse	Human	1:20	Santa Cruz Biotechnology	Canine MCT	Incubated 16 hours at 4 degrees C
Clusterin (ab104652)	Goat	Human	1:4000	Abcam	Canine spinal cord	
C-Kit (CD117) (A4502)	Rabbit	Human	1:100	Dako	Canine MCT	
CXCR5 (ab46218)	Rabbit	Human	n/a	Abcam	n/a	
PD1 (ab52587)	Mouse	Human	n/a	Abcam	n/a	
ALK (D5F3)	Rabbit	Human	n/a	Cell Signalling Technology	n/a	

MCT: mast cell tumour.

2.2 TRAF3 mutation analysis

2.2.1 Samples

Samples were collected from canine patients with suspected/confirmed lymphoma presenting to the oncology service at the Small Animal Hospital, University of Glasgow, UK between 2010 and 2014. Samples included tissue biopsies for histopathology and tissue fine-needle aspirates. Aspirates had DNA extracted within 24 hours of collection and samples were stored frozen (-80 degrees Celsius) or, if submitted for histopathology, were formalin-fixed paraffin-embedded (FFPE). Matched non-tumour DNA samples were obtained from blood samples from the same patient. All matched blood sample were assessed cytologically by a clinical pathologist and had no cytological evidence of circulating neoplastic cells. Samples were submitted to Veterinary Diagnostic Services, University of Glasgow, UK for pathological assessment as part of routine diagnostic evaluation or collected post-mortem. Lymphoma diagnosis was based on cytology or histopathology results in combination with polymerase chain reaction for antigen receptor gene rearrangement (PARR) results, flow cytometry, and/or immunohistochemistry. PARR was performed at the University of Glasgow as previously described (Waugh et al., 2016). Immunophenotyping by flow cytometry was performed at the University of Glasgow using a panel of antibodies comprising: CD5, CD21, CD45, CD3, CD4, CD8, CD34, CD79a, MHC II, MAC387, and CD14. Immunohistochemistry antibody panels were decided by the attending pathologist, with all including at minimum Pax5 and/or CD79a, and CD3. DNA samples from non-lymphoma cases (from peripheral blood, lymph node aspirates, or lymph node FFPE tissue) were collected from samples submitted to the VDS from canine patients with a variety of diseases, in which there was no clinical suspicion of lymphoid neoplasia.

Sample collection and subsequent research activity were approved by the Faculty of Veterinary Medicine Ethics and Welfare Committee (License number: 1a/09; 32a/15) and written consent was obtained from owners at the time of initial presentation. The *TRAF3* polymerase chain reactions (PCR) performed in this study used DNA which had been previously extracted from blood and tissue samples.

2.2.2 DNA extraction

DNA was purified from blood and unfixed samples using DNeasy Blood and Tissue Kits (Qiagen, Manchester, UK), and from FFPE samples using QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. Briefly, FFPE samples had excess paraffin trimmed and one to eight 5-10um thick sections were cut. The sections were placed in a tube with xylene, vigorously vortexed, centrifuged, and supernatant removed. The pellet was dehydrated and resuspended in buffer ATL (Qiagen). Proteinase K (Qiagen) was added and the sample vortexed. The sample was incubated for one hour at 56 degrees Celsius, then a further hour at 90 degrees Celsius. Buffer AL (Qiagen) and ethanol were added and the sample immediately vortexed. The homogenate was transferred to a QIAamp MinElute column and centrifuged to bind the DNA to the column membrane, and prepared by passing Buffers AW1 (Qiagen) and AW2 (Qiagen) through the membrane with serial centrifugations. Buffer ATE (Qiagen) was then applied to the membrane for 5 minutes before the final centrifugation which eluted the DNA into the resulting flow through. For blood and unfixed samples, 20μL proteinase K was added to the blood or homogenated tissue sample. 200μL buffer AL (Qiagen) was added and incubated at 56 degrees Celsius for 10 minutes, then 200μL ethanol was added. The resulting mixture was pipetted onto a DNeasy Mini spin column and centrifuged to bind the DNA to the spin column. The DNA was prepared by serial centrifugations with Buffer AW1 followed by Buffer AW2. The DNA was then eluted by centrifugation after 200uL of water were applied to the membrane.

2.2.3 Polymerase chain reaction (PCR)

2.2.3.1 Primer design

Primers were designed using Integrated DNA Technologies® (IDT, Leuven, Belgium) online PrimerQuest® tool. Primers were designed to amplify exons 9, 10 and 11 of the canine *TRAF3* gene (Table 2-2). Exons were selected based on the location of the majority of variants identified in previous studies (Elvers et al., 2015, Bushell et al., 2015, Waugh, 2015). Primers were synthesised by IDT as a 10μM concentrate and were diluted to 1μM for use.

Table 2-2. TRAF3 primers

Exon	Direction	Primer Sequence	Amplicon size (bp)
9	Forward	5'- ACC AGA ACT TCC ATT TCC TGT AT -3'	387
	Reverse	5'- CAG CTC ACT GAG AAT ACC CAA A -3'	
10	Forward	5'- CAG GTA GAG ACC AGG ACA CA -3'	330
	Reverse	5'- TAA CTA GCC GGT GTC TCT TCT -3'	
11	Forward	5'- CCA GCC TTC CTG ACA CTT AC -3'	666
	Reverse	5'- AGA TGC CTT CTG AAT CC -3'	

Bp: base pairs

2.2.3.2 Reaction conditions

Amplification was performed in a 25µL reaction volume consisting of: 0.1 units of Invitrogen AccuPrime *TAQ* DNA Polymerase, High Fidelity (ThermoFisher Scientific, Paisley, UK); Invitrogen™ Accuprime™ PCR Buffer II (ThermoFisher Scientific); 0.5 µM forward primer; 0.5 µM reverse primer; and 20 ng DNA template. Template controls contained DNA-ase free water instead of template DNA and were included after every seven samples. Samples previously shown to contain amplifiable DNA served as positive controls.

Thermal cycling was conducted using a BIO-RAD C1000 Touch™ Thermal Cycler (BIO-RAD, Kidlington, UK) under the following reaction conditions: initial activation at 94°C for two minutes; 40 cycles of 94°C for 15 s, 58°C for 30 s, 68°C for 30 s; and 68°C for seven minutes.

2.2.3.3 Gel electrophoresis

Products were visualised using one percent agarose gel electrophoresis. The agarose was prepared by completely dissolving one gram of agarose powder in tris/borate/EDTA (TBE) buffer solution. Two microlitres of ethidium bromide (10mg/ml) were added to the solution and it was placed in a mould with 10-30 wells. Once set, the gel was placed in a gel box and submerged in TBE solution. Each sample was prepared by combining 9uL of sample and 1uL of loading gel. A DNA ladder was placed in the lateral most well and each test well was filled with 10uL of solution. The gel was run at 80V for ~one hour until the visible loading gel had run ~75% down the gel. The gel was then photographed under UV light and size of products estimated by comparing to the ladder solution in lateral most well.

2.2.3.4 DNA Purification

PCR products were purified to remove residual primers and nucleotides using the MinElute PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. Briefly, the PCR Reaction mixed was combined with Buffer PB (Qiagen). This mixture was placed in a MinElute column and bound to the membrane by centrifugation. The sample was washed by centrifugation with Buffer PE (Qiagen). Samples were then eluted in 20-40 µL of distilled water.

2.2.3.5 Sequence Analysis

Direct Sanger Sequencing of purified amplicons was performed by Source Bioscience (Bellshill, UK). Sequence files were imported into CLC Genomics Workbench 6.5.1 (Qiagen) software. Chromatograms were visually inspected, and samples with poor quality traces were re-sequenced. Contiguous sequences were made by aligning forward and reverse reads. These were then compared against the canine *TRAF3* reference nucleotide sequence CanFam 3.1(Hoeppner et al., 2014) and sequence variants identified. Mutated alleles were translated *in silico* to determine the effect of variants. Mutations were classed as deleterious if they were predicted to alter the amino acid sequence of the protein.

3 Results

3.1 Use of immunohistochemistry in lymphoma to identify specific subpopulations

In this part of the study, a range of antibodies were used on a broad range of canine lymphoma samples to see if any subgroups could be identified. The initial panel of antibodies were selected based on their role in subtyping human TCLs (CXCR5, PD-1, ALK, CD30) as there is currently a particular lack of canine TCL subtyping. The tissue samples chosen for the project were mainly TCL with a smaller number of BCL included for comparison. When it became clear that some of the antibodies could not be optimised for canine tissue within the timeline of the project, other antibodies were selected for assessment. These were: clusterin, which may have a role in subtyping TCL in poorly differentiated cases, and c-Kit, which currently does not have a role in human lymphoma, although it has not been assessed in a broad selection of canine lymphoma samples.

Successful staining was achieved with clusterin, c-Kit and CD30 and interpretation of the staining is detailed below and in tables 3-1 and 3-2.

Fifty-nine canine lymphoma samples were included in the study. These included 46 TCL and 13 BCL. Of the TCL samples, 9/46 were considered low grade (seven TZL, two low grade gastrointestinal TCL), and 37/46 were considered high grade (16 nodal PTCL-NOS, six cutaneous non-epitheliotropic TCL, five high grade gastrointestinal TCL, two cutaneous epitheliotropic TCL, two mediastinal TCL, two subcutaneous TCL, and one each of ocular, mesenteric, testicular and muscular TCL). Of the BCL samples 12/13 were considered high grade (10 nodal DLBCL, one high grade gastrointestinal, one high grade cutaneous non-epitheliotropic lymphoma) and 1/13 was considered low grade (splenic mantle cell lymphoma).

Overall, 21 samples were positive for at least one of the antibodies assessed. 18/21 were TCL and 3/21 were BCL; 20/21 were only positive for a single antibody. One case (case 28 – high grade subcutaneous TCL) was positive for both CD30 and clusterin. There was no obvious correlation between positivity for the novel antibodies and morphological subtype. Of positive samples, 15/18 TCL cases were high grade and 3/18 were low grade, and 2/3 BCL cases were high grade and 1/3 were low grade.

3.1.1 ALK, CXCR5 and PD-1 IHC could not be optimised

Despite testing a range of IHC protocols (including changing antibody dilution and antigen retrieval methods), antibody marker optimisation on canine tissue was not possible for ALK, CXCR5 and PD-1 within the timeframe of the project, so these antibodies could not be assessed. With all markers and using all the IHC techniques outlined in 2.1.2.2 the canine control tissue was universally negative, so these markers were not further investigated. If more time had been available for the project, more attempts would have been made to optimise these markers for canine tissue. Several additional methods could have been pursued, including using tissue which had been more recently formalin fixed (as prolonged formalin fixation can degrade tissue antigens), or trialling additional canine control tissues (for example for ALK, trying to obtain samples of the previously reported CD30 positive ALCL cases, as these may have been positive if canine ALK expression is similar to human ALK expression). Also, we could have assessed the expression of the target protein in the samples using RT-PCR or RNA-seq. This would have allowed us to confirm the presence of the target protein and then we could have focussed our attempts on those samples with the highest levels of expression, as these would be most likely to be positive. We could also have trialled additional antigen retrieval methods and staining methods. For example, our CD30 protocol involved incubating the samples at 4 degrees Celsius for 16 hours. This method was pursued due to a previous publication reporting this method to be effective. Unfortunately we were not able to trial prolonged incubations or different temperatures for all our samples due to the time limitations and financial constraints of the project.

3.1.2 Clusterin IHC

Fifty-six samples were stained with clusterin and a summary of clusterin positive patient and staining characteristics is shown in Table 3-3. See Fig. 3-1 for positive control sample.

14/56 (25%) samples assessed were considered positive. These included 11/43 (26%) TCL and 3/13 (23%) BCL. Results were not available for three TCL cases as insufficient tissue samples were available for staining.

Of the positive TCL cases, 3/11 were low grade, all of which were TZL, making 3/7 TZL cases assessed positive for clusterin. The remaining 8/11 positive cases were high grade, and included four PTCL-NOS, two cutaneous non-epitheliotropic TCL, and one each of high grade gastrointestinal and subcutaneous TCL. Of positive BCL cases, 2/3 were DLBCL and 1/3 was low grade splenic mantle cell lymphoma. No obvious patterns of staining could be linked to the histopathological grade or morphological diagnoses.

All positive samples had a golgi cytoplasmic staining pattern. No other staining pattern was seen in neoplastic cells. All samples had interspersed positive cells. In no samples was there diffusely positive staining. Nor were there any samples with geographic areas of diffuse staining. 9/14 samples were graded as rare staining (<1% neoplastic cells) and 5/14 were graded as mild staining (<25% neoplastic cells). Staining intensity was moderate and strong in 12/14 and 2/14 samples respectively. An example of a positive case is shown in Fig. 3-2.

In one case of testicular lymphoma, the normal testicular structures adjacent to the tumour showed positivity. Testicular clusterin staining has previously been described in humans (Liu et al., 2013).

A common feature noted in almost all samples was a fine granular, predominantly nuclear (with a lesser degree of cytoplasmic), staining (see Fig. 3-3). Given the ubiquity of this staining pattern, and the fact that it appeared independent of other staining patterns, it was not considered a positive result. Subjectively, the BCL seemed to have a greater intensity of this diffuse fine granular staining than the TCL samples.

3.1.3 C-Kit IHC

Fifty-eight samples were stained with c-Kit antibody and a summary of c-Kit positive patient and staining characteristics are shown in Table 3.4. See Fig. 3-4 for positive control sample.

5/58 samples were considered positive. All five were TCL (5/46; 11%). See Fig. 3-5 and Fig. 3-6 for examples of positive staining. One BCL sample did not have results available due to insufficient sample available for staining.

All positive cases were high grade TCL. 2/5 were high grade gastrointestinal lymphomas (making 2/5 of the included high grade gastrointestinal lymphomas positive for c-Kit), and there was one each of cutaneous non-epitheliotropic TCL, epitheliotropic TCL, and nodal PTCL-NOS. No obvious patterns were identified between c-Kit positivity and morphological diagnosis, however the 20% prevalence of c-Kit positive high grade gastrointestinal TCL is interesting and warrants further investigation in a larger cohort of these cases.

The proportion of neoplastic cells staining varied between samples: 3/5 samples showed diffuse staining (ie all neoplastic cells were positive); 1/5 showed interspersed positive cells; and 1/5 showed diffusely positive cells limited to only two areas of the slide. This case (sample 34) involved 23 intestinal pinch biopsy fragments on the slide (see Fig. 3-7a). All fragments had varying numbers of CD3 positive neoplastic lymphocytes, however in only two fragments were the lymphoma cells c-Kit positive. In these fragments the lymphoma cells showed a diffusely positive weak cytoplasmic stain (Fig. 3-7b and Fig. 3-7c).

The staining pattern observed also varied between positive samples, and included membranous (1/5), cytoplasmic (3/5), and golgi (1/5).

The staining intensity was strong in 1/5, moderate in 1/5 and weak in 3/5 samples.

3.1.4 CD30 IHC

Forty-six samples were stained with CD30 antibody and a summary of positive patient and staining characteristics are shown in Table 3-5. See Fig. 3-8 for positive control sample.

3/46 samples were considered positive and all were TCL (3/46; 6.5%). All positive cases were high grade TCL. The positive cases included on each of: nodal PTCL-NOS, high grade subcutaneous TCL, and cutaneous non-epitheliotropic lymphoma. Three cases of TCL did not have results available due to insufficient sample for staining.

Only one sample (case 33 – high grade cutaneous non-epitheliotropic TCL) showed diffuse staining (see Fig. 3-9). The other two positive samples only had interspersed positive cells (<1% of all neoplastic cells). In one sample (subcutaneous TCL) the positive cells were present interspersed throughout the sample however the proportion of positive cells was increased in some specific areas of the slide.

Table 3-1. Summary of patient characteristics, diagnoses and immunohistochemistry results.

	Sample ID*	Age (years)	Sex	Breed	Sample Site (specific site)	Neoplastic lymphocyte size	Nodal lymphoma: Diffuse vs nodular	Stated Diagnosis	Clusterin	C-Kit	CD30
T-cell	1	11.7	M	Retriever	LN (SMLN)	SMALL-INTERMED	Nodular	TZL			
	2	5	FN	Pug	Eye/optic nerve	INTERMED-LARGE	n/a				
	3	13	F	Staffordshire bull terrier	Cutaneous		n/a	Cutaneous T-cell	+		
	4	9.6	MN	Cocker spaniel	LN	INTERMED	Nodular	TZL	+		
	5	11	F	Yorkshire terrier	LN (Popliteal LN)	LARGE	Diffuse	PTCL-NOS			
	6	7.3	FN	Cairn terrier	LN (Popliteal and SMLN)	INTERMED	Diffuse	PTCL-NOS			+
	7	8.3	F	Inuit dog	LN (Popliteal LN)	LARGE	Nodular	TZL			
	8	12	F	Border collie	Cutaneous	LARGE	n/a	Cutaneous (non-epitheliotropic)		+	
	9	11	MN	Golden retriever	Small intestinal (duodenum)	LARGE	n/a	High-grade GI T-cell lymphoma	+		
	10		FN	Cocker spaniel	LN	LARGE	Diffuse	PTCL-NOS			

	11	4	M	English springer spaniel	LN (SMLN)	LARGE	Diffuse	PTCL-NOS			
	12	6.8	MN	Cocker spaniel	LN (Popliteal LN)	INTERMED-LARGE	Diffuse	PTCL-NOS			
	13	8	F	WHWT	Subcutaneous mass	LARGE	n/a				
	14	6.3	M	Cross	LN (SMLN)	SMALL	Nodular	TZL			
	15	8.7	MN	Springer spaniel	LN (PS and popliteal LN)	LARGE	Diffuse	PTCL-NOS			
	16	8	FN	Bullmastiff	LN (PSLN)	LARGE	Diffuse	PTCL-NOS			
	17	5	MN	Labrador	LN (popliteal LN)		Diffuse	PTCL-NOS	+		
	18	7.1	MN	CKCS	LN (popliteal and SMLN)	SMALL-INTERMED	Nodular	TZL			
	19	11	F	Bull dog	LN (mediastinal)	INTERMED	Diffuse	Mediastinal lymphoma			
	20	6.5	FN	Cross	LN (SMLN)	INTERMED-LARGE	Diffuse	PTCL-NOS	+		
	21	11.8	FN	Grey hound	Mesenteric mass	INTERMED-LARGE	n/a				
	22	6	M	Dogue de Bordeaux	Testicular mass	LARGE	n/a				
	23	3	MN	Cocker spaniel	LN (PSLN)	LARGE	Diffuse	PTCL-NOS			
	24		M	Yorkshire terrier	Cutaneous	INTERMED-LARGE	n/a	Cutaneous (non-epitheliotropic)			

	25	8.8	F	Cross	LN (popliteal LN)	INTERMED	Diffuse	PTCL-NOS			
	26	7.3	M	Labradoodle	LN (PSLN)	SMALL-INTERMED	Diffuse	PTCL-NOS	+		
	27	11.3	FN	Labrador	Cutaneous	n/i	n/a	Cutaneous (non-epitheliotropic)			
	28	10.3	M	WHWT	Subcutaneous	INTERMED-LARGE	n/a		+		+
	29	13.5	MN	Cross	LN (SMLN)	LARGE	Diffuse	PTCL-NOS			
	30	10.2	F	Boxer	Mucocutaneous	n/i	n/a	Epitheliotropic T-cell lymphoma		+	
	31	12	M	Cocker spaniel	LN (PSLN)	SMALL	Diffuse	PTCL-NOS			
	32	9.2	F	Boxer	Cranial mediastinal mass	INTERMED	Diffuse	Mediastinal lymphoma			
	33	4.2	F	Dogue de Bordeaux	Skin (pinna)	INTERMED-LARGE	n/a	Cutaneous (non-epitheliotropic) lymphoma			+
	34	11.6	M	Cross	Small intestinal mass (duodenum)	INTERMED-LARGE	n/a	High-grade GI T-cell lymphoma		+	
	36	3	FN	Weimaraner	Muscle	Intermediate-large	n/a				
	37	10.3	MN	Cross	Small intestine (jejunum)	Intermediate-large	n/a	High-grade GI T-cell lymphoma			
	38	11.7	MN	Labrador	LN (PSLN)	INTERMED	Nodular	TZL	+		
	40	11.3	M	Schnauzer	LN	LARGE	Diffuse	PTCL-NOS	+		

	41	7.8	M	Labrador	Large intestinal mass (colon)	LARGE	n/a	High-grade GI T-cell lymphoma		+	
	42	7.8	FN	Labrador	Small intestine	SMALL	n/a	Low grade GI T-cell lymphoma			
	44	7.7	FN	Corgie	Skin mass	LARGE	n/a	Cutaneous (non-epitheliotropic) T-cell lymphoma	+		
	45	9	M	Cocker spaniel	Small intestinal mass (duodenum)	INTERMED-LARGE	n/a	Angiocentric T-cell lymphoma			
	46	9.8	M	Golden retriever	LN (SM and PSLN)	SMALL	Nodular	TZL	+		
	47	12.3	M	Staffordshire bull terrier	LN (SMLN)	LARGE	Diffuse	PTCL-NOS		+	
	48	8.5	M	Labrador	Stomach	INTERMED	n/a	Low-grade GI lymphoma			
	49	10.3	FN	Shih-tzu	Duodenum	LARGE	n/a	Epitheliotropic T-cell lymphoma			
B-cell	51	10	MN	WHWT	LN	LARGE	Diffuse	DLBCL			
	52	4.6	M	Border collie	LN	INTERMED-LARGE	Diffuse	DLBCL			
	54	11	M	Schnauzer	LN (popliteal LN)	LARGE	Diffuse	DLBCL			
	55	11	MN	Cross	LN	INTERMED-LARGE	Diffuse	DLBCL			
	56		F	Belgian shepherd	LN (SMLN)	INTERMED-LARGE	Diffuse	DLBCL			

	57	10	MN	Irish setter	LN	LARGE	Diffuse	DLBCL			
	58	10.7	F	Jack Russel terrier	LN	INTERMED	Diffuse	DLBCL	+		
	59	4.9	F	Miniature schnauzer	LN (PSLN)	LARGE	Diffuse	DLBCL			
	60	12.1	MN	Schnauzer	Stomach mass	LARGE	n/a	High-grade GI B-cell lymphoma			
	61	9.4	MN	Collie	Spleen	LARGE	n/a	Mantle cell lymphoma	+		
	62	11.4	MN	Border collie	LN (PSLN)	INTERMED-LARGE	Diffuse	DLBCL			
	63	2.7	FN	WHWT	Cutaneous mass	INTERMED-LARGE	n/a	Cutaneous (non-epitheliotropic) B-cell lymphoma			
	64	5	FN	Weimaraner	Tonsil	INTERMED-LARGE	Diffuse	DLBCL	+		

*Sample ID numbers assigned before samples excluded - 59/64 samples were used in final study.
n/i: not included in report; n/a: not applicable; M: male; ME: male entire; F: female; FE: female entire; LN: lymph node; SMLN: submandibular lymph node; PSLN: prescapular lymph node; TZL: T-zone lymphoma; PTCL-NOS: peripheral T-cell lymphoma – not otherwise specified; DLBCL: diffuse large B-cell lymphoma; WHWT: West highland white terrier; CKCS: Cavalier Kind Charles spaniel, GI: gastrointestinal.

Table 3-2. Summary of IHC staining patterns in positive samples of canine lymphoma.

Antibody	Disease	Number positive	Staining patterns
c-Kit	TCL (n=46)	5/46	3 diffuse (1 golgi, 1 cytoplasmic, 1 membranous), 1 geographically diffuse cytoplasmic, 1 interspersed cytoplasmic
	BCL (n=13)	0/12 (1 not available)	
CD30	TCL (n=43)	3/43 (3 not available)	1 rare interspersed, 1 majority negative with a focal geographic area of neoplastic cells with 100% positivity, 1 diffuse
	BCL (n=13)	0/13	
Clusterin	TCL (n=43)	11/43 (3 not available)	4 Mild, 7 rare
	BCL (n=13)	3/13	1 Mild, 2 rare

TCL: T-cell lymphoma; BCL: B-cell lymphoma.

Table 3-3. Clusterin IHC: Summary of positive samples.

Lymphoma type	Sample number	Lymphoma details	Staining characteristics
TCL	3	Cutaneous	Rare moderate golgi
	4	TZL	Rare moderate golgi
	9	High grade GI (duodenal)	Mild strong golgi
	17	Nodal PTCL-NOS	Rare moderate golgi
	20	Nodal PTCL-NOS	Mild moderate golgi
	26	Nodal PTCL-NOS	Rare moderate golgi
	28	Intermediate-large cell, subcutaneous	Mild moderate golgi
	38	TZL	Rare moderate golgi
	40	Nodal PTCL-NOS	Rare moderate golgi
	44	Large cell cutaneous non-epitheliotropic	Mild moderate golgi
	46	TZL	Rare moderate golgi
BCL	58	Nodal DLBCL	Rare moderate golgi
	61	Splenic mantle cell lymphoma	Rare moderate golgi
	64	Tonsilar DLBCL	Mild strong golgi

TCL: T-cell lymphoma; BCL: B-cell lymphoma; TZL: T-zone lymphoma; GI: gastrointestinal; PTCL-NOS: peripheral T-cell lymphoma, not otherwise specified.

Table 3-4. C-Kit IHC: Summary of positive samples.

Sample number	Lymphoma details	Staining characteristics
8	Large cell cutaneous non-epitheliotropic TCL	Diffuse moderate golgi staining
30	Mucocutaneous epitheliotropic TCL	Diffuse weak cytoplasmic
34	High grade GI TCL	2/21 biopsies with diffuse weak cytoplasmic (see picture)
41	High grade GI TCL	Diffuse strong membranous
47	Nodal PTCL-NOS	Interspersed ~30% of cells weak cytoplasmic

TCL: T-cell lymphoma; GI: gastrointestinal.

Table 3-5. CD30 IHC: Summary of positive samples.

Sample number	Lymphoma details	Staining characteristics
6	Nodal PTCL-NOS	Interspersed (<1%) population with strong cytoplasmic
28	Intermediate-large cell, subcutaneous TCL	Interspersed (<1%) population with weak-moderate membranous-cytoplasmic staining. Most positive cells within a single geographic region (up to 10% of neoplastic cells positive in densest region).
33	Intermediate-large cell, cutaneous non-epitheliotropic TCL	Diffuse moderate membranous

PTCL-NOS: peripheral T-cell lymphoma, not otherwise specified; TCL: T-cell lymphoma.

Fig. 3-1. Clusterin IHC control sample: canine spinal cord (40x magnification) with clusterin immunostaining. The neurons (arrows) show punctate cytoplasmic staining as described in Shafie *et al*(Shafie et al., 2014).

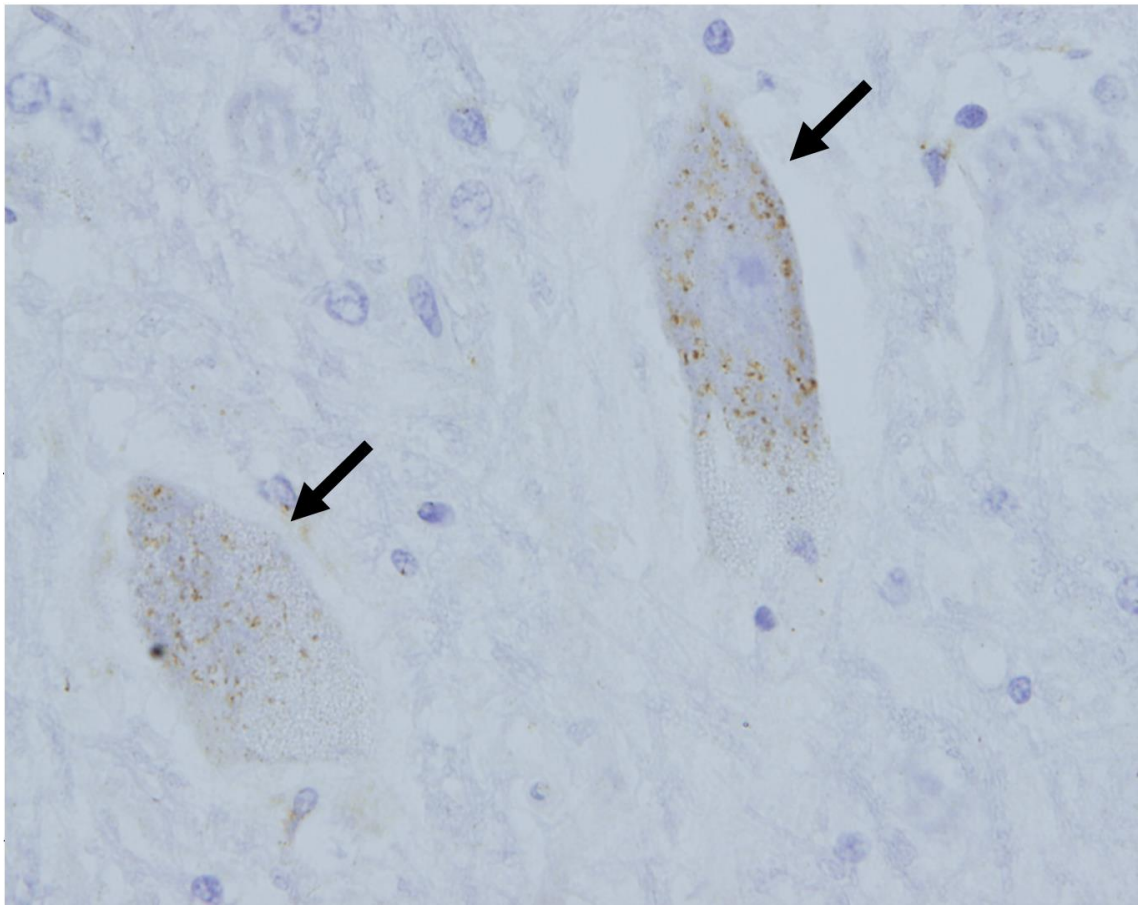


Fig. 3-2. Clusterin (40x magnification): Large cell nodal T-cell lymphoma (case 40). Region of highest number of positive cells showing moderate golgi pattern staining (arrows).

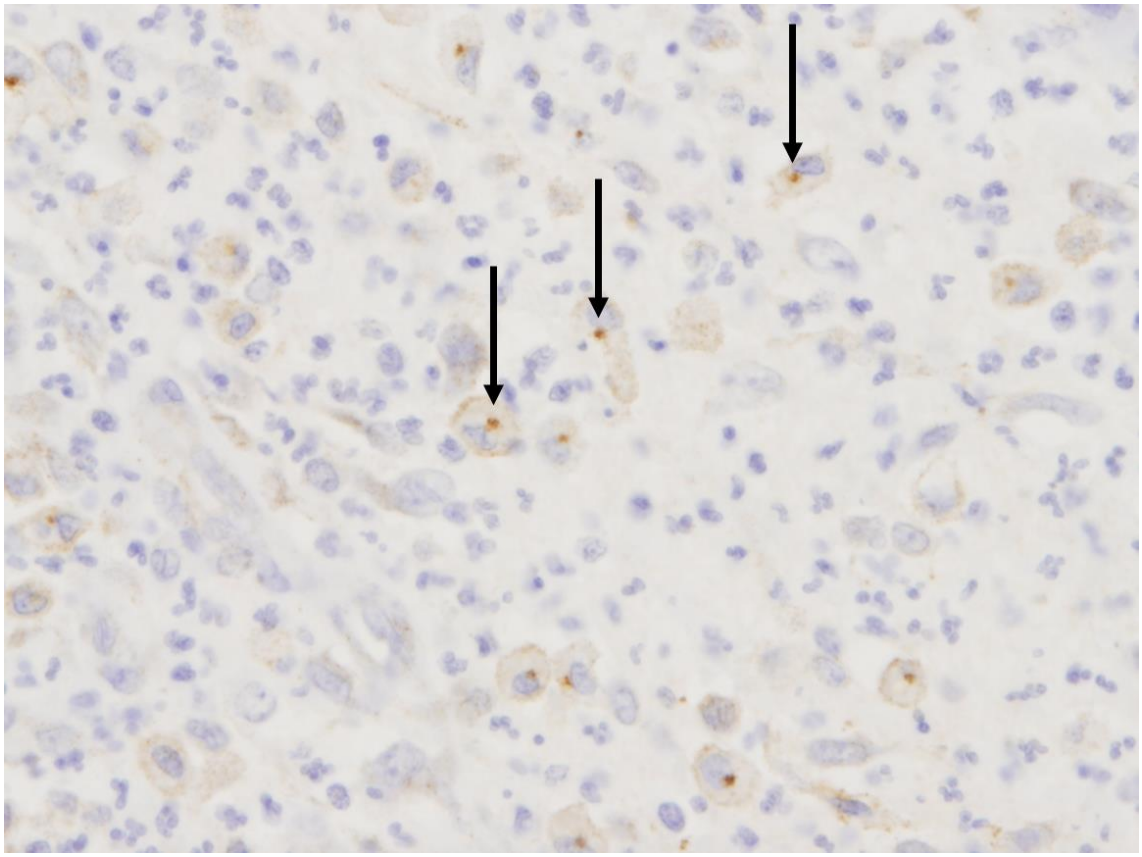


Fig. 3-3. Clusterin (40x magnification): Non-specific fine granular staining seen in almost all cases.

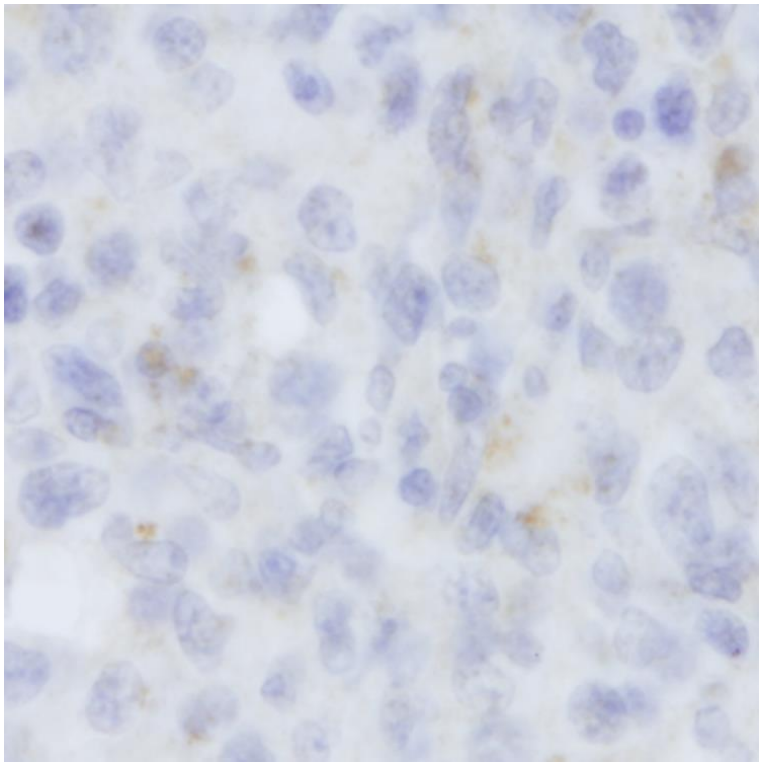


Fig. 3-4. C-Kit control sample: canine mast cell tumour (20x magnification) showing membranous staining.

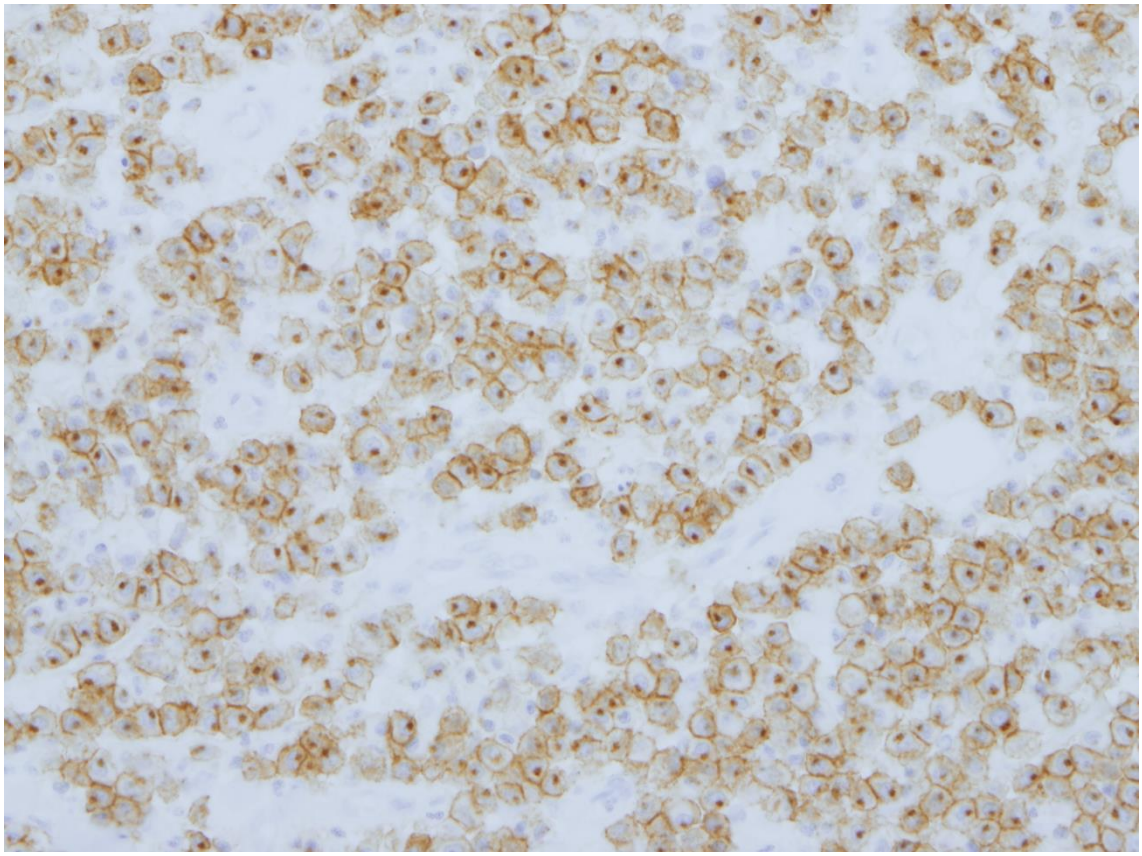


Fig. 3-5. C-Kit (20x magnification): Strong membranous staining (case 41).

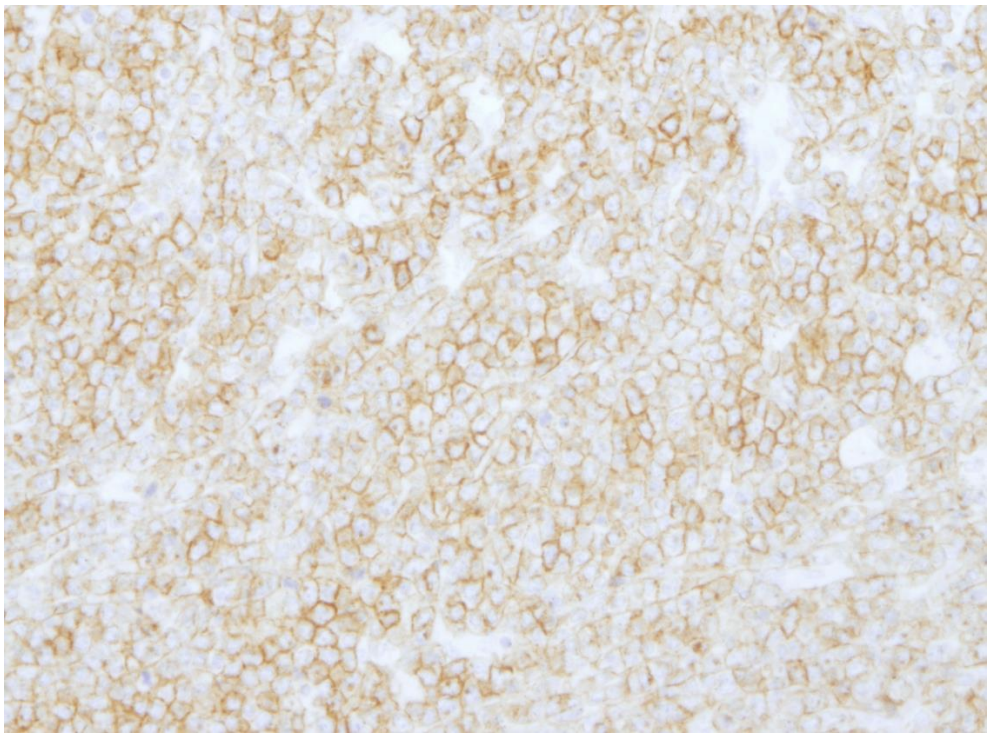


Fig. 3-6. C-Kit (40x magnification): Case 47, weak interspersed (~30% neoplastic cells) cytoplasmic staining (three examples shown by arrows).

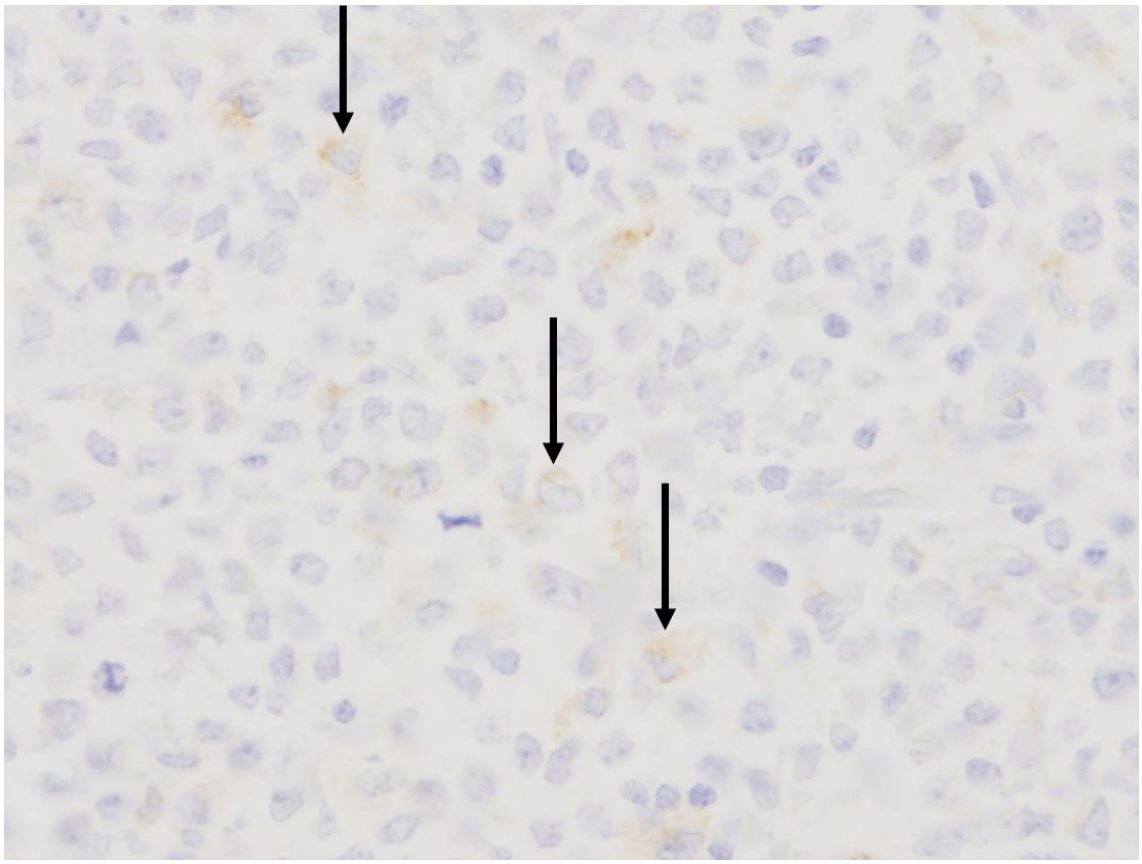


Fig. 3-7. C-Kit staining on intestinal pinch biopsies (case 34): a) unmagnified view of slide showing only 2 pinch biopsies with positive stain (arrowheads), b) non-staining area with negative staining in all neoplastic cells and only scattered positive mast cells (arrows) (10x magnification), c) positive area of slide showing mild diffuse cytoplasmic staining (40x magnification).

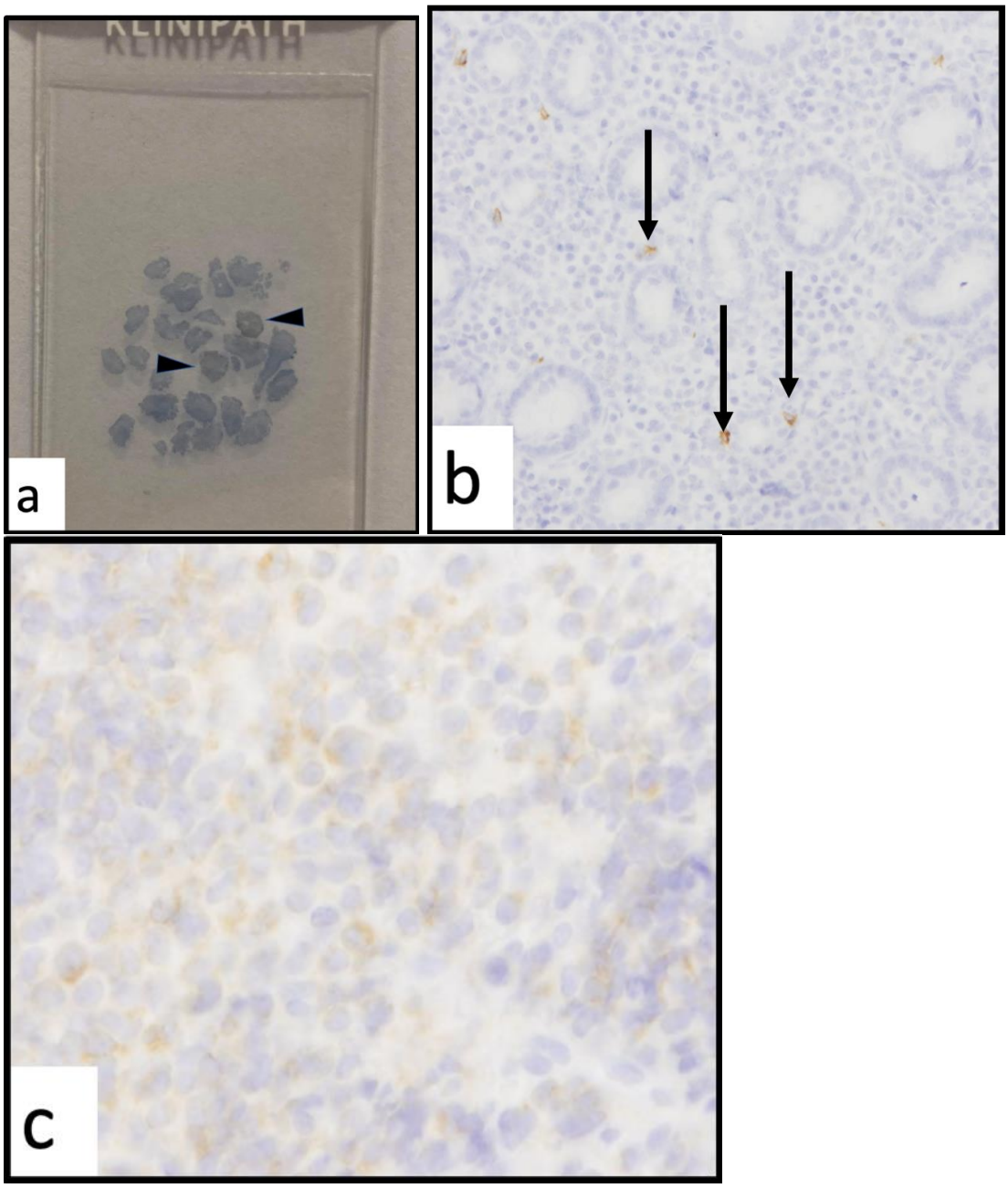


Fig. 3-8. CD30 control sample: canine mast cell tumour (40x magnification) showing granular cytoplasmic staining.

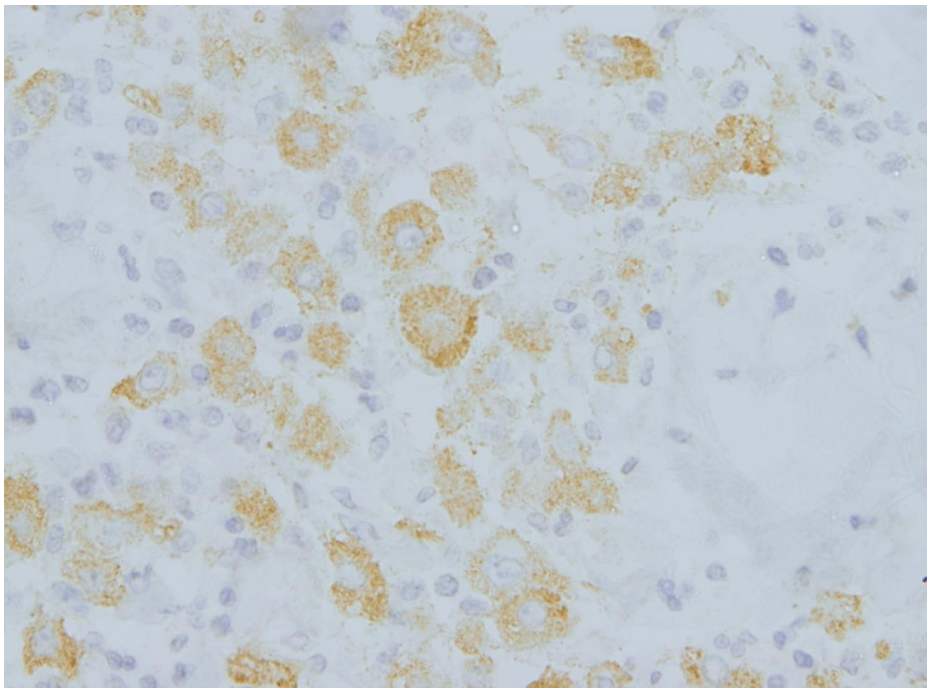
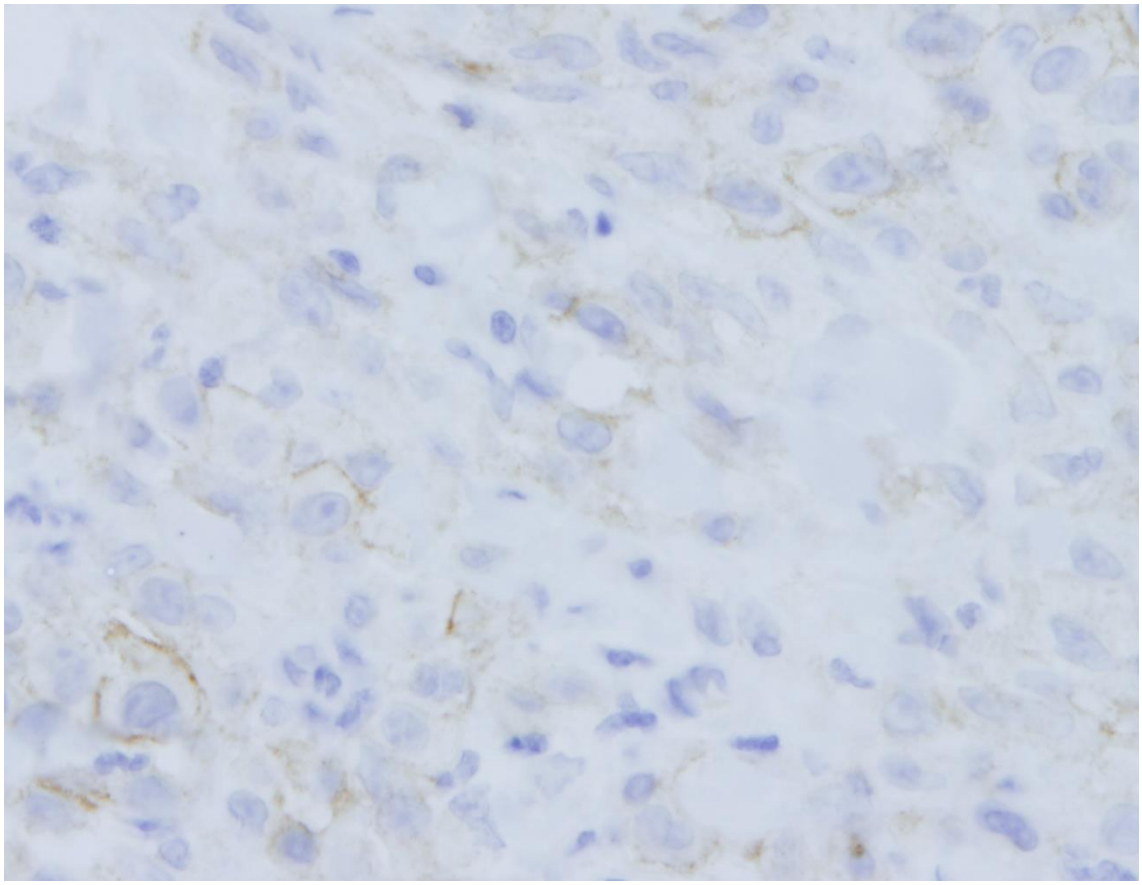


Fig. 3-9. CD30 (40x magnification): Moderate diffuse membranous staining (case 33).



3.2 TRAF3

In this part of the study, the *TRAF3* gene was assessed in a cohort of dogs with B-cell lymphoma to confirm the high prevalence of *TRAF3* mutations found in previous studies (Bushell et al., 2015, Waugh, 2015) and characterise these mutations. We also assessed a cohort of dogs with non-BCL diseases (including four with TCL) to see if *TRAF3* mutations were specific to BCL, or if they occurred with other conditions. Unfortunately, the BCL cases had varied treatments and incomplete records so it was not possible to correlate *TRAF3* analysis with patient outcomes or specific BCL subtypes.

3.2.1 Patients

Forty-nine patients were included in the study; these comprised 28 cases with lymphoma (24 cBCL, four cTCL) and 21 cases with non-lymphoma diseases (see Table 3-6 and Table 3-7). Four cBCL samples did not have sufficient sample available for TRAF exon 9 sequencing (all other sequencing was performed), so an additional four cBCL cases were recruited for TRAF3 exon 9 sequencing alone, giving a total of 53 cases for *TRAF3* exon 9 (28 cBCL).

Eleven lymphoma samples (10 cBCL, one cTCL) had matched non-tumour samples for comparison to determine whether mutations were somatic or germline. Nineteen lymphoma samples (15 cBCL, four cTCL) had RNA-Seq data available (Waugh, 2015) for comparison. The age of patients ranged from 1.1 years to 14.5 years (median 7.5 years). Twenty-two breeds and eight crossbreed dogs were included, and no breeds were overrepresented.

Fig. 3-10 shows an example of PCR product on an agarose gel to confirm the product was of the expected size. Fig. 3-11 shows chromatograms and three examples of mutations that were identified.

3.2.2 Sequencing/Mutation analysis

In total three exons from *TRAF3* were sequenced based on the location of mutations in previous RNA-seq data (Waugh, 2015) and in previous publications (Bushell et al., 2015, Elvers et al., 2015). In six individual cases unsatisfactory DNA amplification and/or DNA sequencing data was obtained for one of the three exons sequenced (five *TRAF3* exon 9, one TRAF3 exon 11). Since *TRAF3* exon 9 was predominantly affected by this, an additional four cBCL cases were recruited for *TRAF3* exon 9 sequencing alone, giving a total of 28 cBCL cases which had at least partial *TRAF3* sequencing.

Eleven deleterious mutations of *TRAF3* were found in 10 of 24 cBCL patients, a prevalence of 36%. No deleterious mutations were identified in cTCL or non-lymphoma cases. The mutations identified are described in Table 3-8. One patient had two deleterious mutations identified.

Most (9 of 11) deleterious mutations were frameshift mutations, leading to a change in the downstream amino acid sequence and premature termination of the translation, causing truncation of the protein. One further mutation was a nonsense mutation causing immediate termination of translation and truncation of the protein. The final mutation was an in-frame deletion of 12 base pairs (four amino acids). Protein modelling was not performed, however this mutation was assumed to be deleterious. Fig. 3-12 shows the location of the mutations on the TRAF3 protein, and the protein domains impacted.

Five of 10 dogs with a deleterious *TRAF3* mutation had matched non-tumour DNA. In four patients no mutation was identified in the matched tissue, indicating the mutations were somatic. In one dog with a frameshift mutation the wild-type allele was not detected (loss of heterozygosity), and an identical mutation was present in the matched tissue, suggestive of a germline mutation.

Of the 10 patients with deleterious mutations, RNA-Seq data was available for six for comparison. In two of the six patients, Sanger sequencing detected mutations which had not been identified by RNA-Seq. In the remaining four patients, the mutations identified in RNA-seq and Sanger sequencing were identical.

In addition to the above mutations, 39 instances of non-deleterious (synonymous) variants were identified in 29 patients. These were spread across disease types, with a mutation identified in 15/28, 3/4, and 13/21 cBCL, cTCL and non-lymphoma cases respectively. Ten patients had two separate synonymous variants. Of the ten patients with a deleterious mutation, five also had synonymous variants. Four separate synonymous variants were detected, of which three had been identified previously (see Table 3-9). Since these variants were found in a variety of disease types including non-lymphoma cases, they likely represent polymorphisms. This is supported by the finding that the same synonymous variant was present in the non-tumour DNA (germline) in six of seven cases with matched DNA samples. In the final case, the synonymous variant was only identified in the lymphoma sample. In this case the wild-type allele was not present (loss of heterozygosity).

Table 3-6. Canine B-cell lymphoma (cBCL) and canine T-cell lymphoma (cTCL) cases included in the *TRAF3* study.

Case group	Diagnosis	Tests for lymphoma diagnosis and immunophenotyping	Samples for DNA extraction
cBCL (*n*=28)	cBCL (*n*= 28)	Cytology, flow cytometry and PARR (*n*= 20) Histology, IHC, PARR and flow cytometry (*n*= 4) Cytology and PARR (*n*= 2) Histology, IHC and PARR (*n*= 1) Cytology and flow cytometry (*n*= 1)	Lymph node aspirates (*n*= 28)
cTCL (*n*=4)	High-grade (*n*=3)	Cytology, flow cytometry and PARR (*n*=2) Histology, IHC and PARR (*n*=1)	Lymph node aspirates (*n*=4)
	TZL (*n*=1)	Histology, IHC, flow cytometry and PARR (*n*=1)	

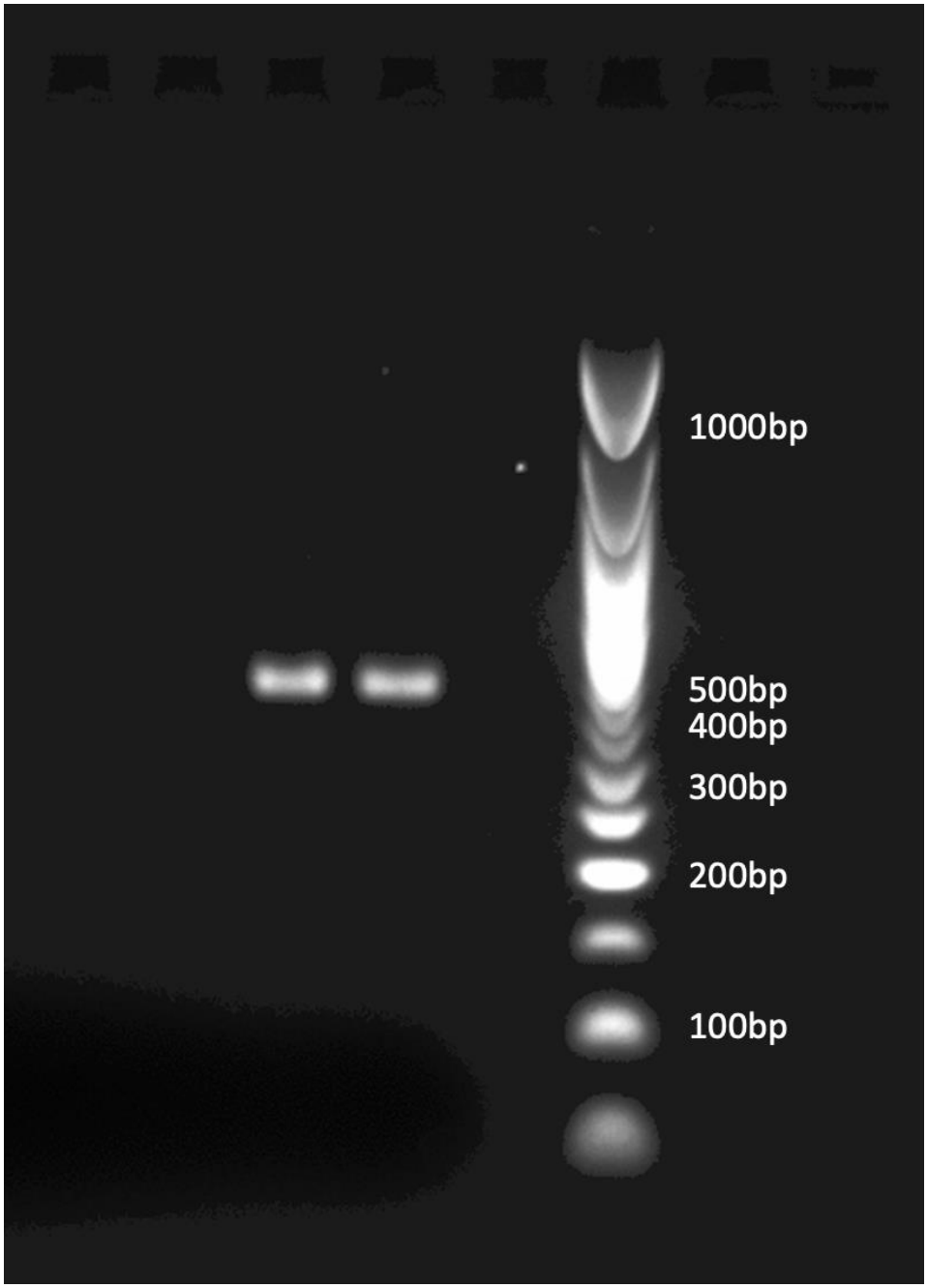
IHC, immunohistochemistry; PARR, PCR for antigen rearrangement; TZL, T-zone lymphoma.

Table 3-7. Non-lymphoma cases included in the *TRAF3* study.

Diagnosis (n=21)	Samples for DNA extraction
Lysosomal storage disease (*n*=1)	FFPE lymph node
Soft tissue sarcoma (*n*=2)	FFPE lymph node (*n*=2)
Idiopathic immune-mediated thrombocytopenia (*n*=1)	Peripheral blood
Idiopathic immune-mediated neutropenia (*n*=1)	Bone marrow aspirate
Glaucoma (*n*=2)	Peripheral blood (*n*=2)
Idiopathic epilepsy (*n*=1)	Lymph node aspirate
Idiopathic immune-mediated polyarthritis (*n*=2)	Lymph node aspirate (*n*=2)
Allergic skin disease (*n*=1)	Peripheral blood
Portosystemic shunt (*n*=1)	Peripheral blood
Idiopathic/infectious lymphadenopathy which resolved with antibiotics (*n*=1)	Lymph node aspirate
Arrhythmogenic right ventricular cardiomyopathy (*n*=1)	Lymph node aspirate
Inflammatory bowel disease (*n*=1)	FFPE intestinal biopsy
Polyneuropathy (*n*=1)	Lymph node aspirate
Lymph node lipomatosis (*n*=1)	FFPE lymph node
Non-neoplastic reactive lymphadenopathy (diagnosis based on: histopathology and PARR [*n*=1], histopathology and IHC [*n*=1], histopathology, IHC, and PARR [*n*=1], cytology and PARR [*n*=1])	FFPE lymph node (*n*=3) Lymph node aspirate (*n*=1)

FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; PARR, PCR for antigen receptor rearrangement.

Fig. 3-10. Two exon 11 samples visualised under UV light on one percent agarose gel. The size of the two positive samples is estimated at ~550 base pairs based on the DNA ladder on the right. This is consistent with exon 11 sequencing.



Bp: base pairs

Fig. 3-11. Example mutations as depicted on sequencing chromatograms (CLC Workbench). A) Heterozygous C deletion (asterisk) resulting in frameshift, B) heterozygous C-T nonsense mutation (arrow), C) homozygous G-A synonymous variant (arrowhead). The reference sequence is depicted at the top of each example. The coloured lines represent the fluororescent signal detected for each nucleotide (red: T, blue: C, black: G, green: A). The height of each coloured spike represents the relative intensity of the signal. By visually inspecting the chromatogram, it is possible to determine what abnormality (mutation) has occurred in the sequence.

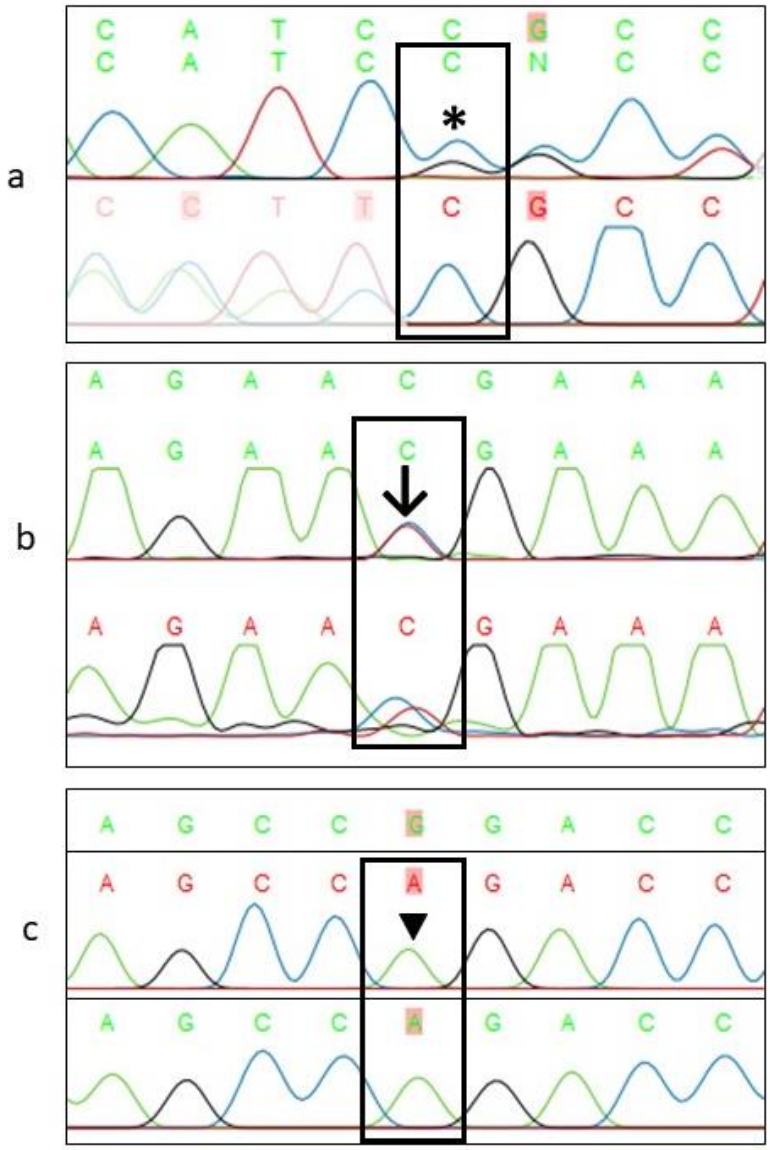


Table 3-8. Deleterious *TRAF3* mutations in canine B-cell lymphoma.

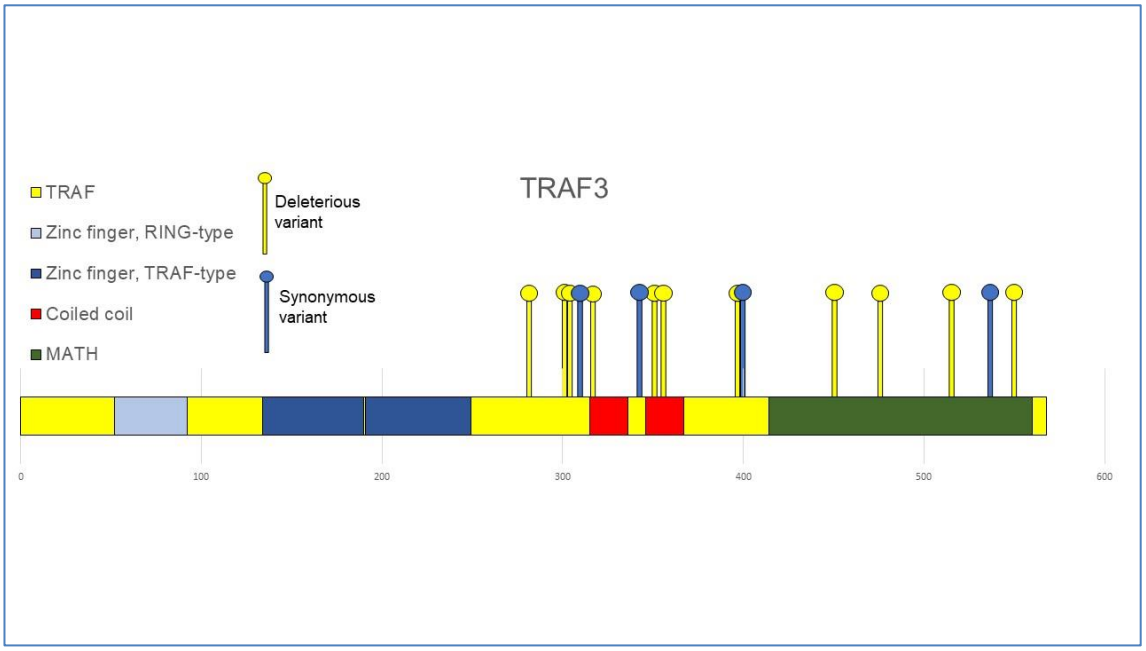
Exon	Position	Mutation	Type	Allele frequency	Amino acid change	Protein domains affected	Present in matched sample (if tested)	Present in RNA-seq	Rs# (if previously reported)
9	8:70782999	T-deletion	Frameshift	Heterozygous	Ile302MetfsTer20	TRAF		Yes	
9	8:70782945	A-T point	Nonsense	Heterozygous	Lys286Ter	TRAF	N	Yes	rs851689319
9a	8:70783037	CCAAAATA insertion (duplication)	Frameshift	Heterozygous	Leu317ProfsTer9	TRAF	N	NT	
9	8:70783003	A-insertion	Frameshift	Wild-type allele ND	Glu303GlufsTer9	CC		NT	
10	8:70788018	ACAG-deletion	Frameshift	Heterozygous	Asp324AlafsTer6	CC		No	
10	8:70788012-70788026	AGTAATAGACAGCC-deletion	Frameshift	Wild-type allele ND	Arg321ArgfsTer102	CC	Y	No	
11	8:70789277	A-deletion	Frameshift	Heterozygous	Tyr446SerfsTer7	TRAF		Yes	
11	8:70789371-70789383	ATGCGTGGAGAG deletion	In-frame deletion	Heterozygous	Val477_His480del	MATH		NT	
11	8:70789530	TC-insertion	Frameshift	Heterozygous	Ala510AlafsTer14	MATH	N	NT	
11	8:70789589	A-deletion	Frameshift	Wild-type allele ND	Lys549LysfsTer9	MATH		Yes	

11a	8:70789131	C-deletion	Frameshift	Heterozygous	Arg397AlafsTer21	MATH	N	NT

Y, yes; N, no; NT, not tested; CC, coiled-coil domain; MATH, meprin and TRAF homology domain; NGS, next generation sequencing; NT, not tested; ND, not detected.
a Same dog.
b Mutation detected in a homologous location in human melanoma.

RS#: Accession number used by genomic databases to identify single nucleotide polymorphisms.

Fig. 3-12. Position of *TRAF3* mutations in relation to protein domains. An overview of the variants identified, showing the amino acid position and predicted protein domain of each variant.



TRAF: tumour necrosis factor activating factor; RING: really interesting new gene; MATH: meprin and TRAF homology.

Table 3-9. *TRAF3* synonymous variants identified in the present study.

Gene	Exon	Position	Mutation	Type	Total (n)	Allele Frequency (n)	Disease (cBCL, non-cBCL)	Present in matched sample	Rs# (if previously reported)
TRAF3	9	8:70783013	A-G	Synonymous	24	Heterozygous 13 Homozygous 11	cBCL 5 Non-cBCL 8 cBCL 6 Non-cBCL 5	Present in 4/5 matched samples	rs24528193
	10	8:70788076	C-T	Synonymous	1	Heterozygous 1	Non-cBCL 1	No matched samples.	
	11	8:70789480	G-A	Synonymous	11	Heterozygous 7 Homozygous 4	cBCL 4 Non-cBCL 3 cBCL 1 Non-cBCL 3	Present in 1/1 matched samples	rs853019186
	11	8:70789213	C-T	Synonymous	3	Heterozygous 2 Homozygous 1	cBCL 1 Non-cBCL 1 cBCL 1	Present in 1/1 matched samples	rs851492892

cBCL, canine B-cell lymphoma; cTCL, canine T-cell lymphoma.

4 Discussion

This thesis investigated new immunohistochemical and genetic markers which could be useful in subclassifying canine lymphomas.

C-kit, CD30, and clusterin were all able to be used in canine lymphoma, and nearly 40% (18/46) of TCLs assessed in this study were positive for at least one of the immunohistochemical markers investigated. RNA-seq and Sanger sequencing were both able to detect mutations in the *TRAF3* gene, and 36% of BCLs had a deleterious *TRAF3* mutation detected.

4.1 Assessment of novel immunohistochemical markers to improve canine T-cell lymphoma subclassfication

Currently, due to a lack of validated diagnostic methods, the majority of canine high-grade TCL are classified as PTCL-NOS. As more diagnostic techniques become available, it is almost certain that this classification will be further refined, being subdivided into different groups with significant differences in prognosis and treatment. This study aimed to assess several IHC markers that are routinely used for human lymphoma subclassification in a population of canine lymphoma samples. The population we assessed was predominantly TCL, as there is currently a significant lack in the ability of veterinary oncologists to subclassify these cases. The markers initially selected included markers of ALCL (CD30, ALK), and Tfh (PD-1, CXCR5). After difficulties were encountered optimising these antibodies for canine samples, clusterin and c-Kit were also added. Clusterin has a possible role as a marker of ALCL in human lymphoma. C-Kit has no defined role in human lymphoma diagnosis, however it has not been assessed in canine lymphoma. We aimed to characterise c-Kit staining in a variety of canine lymphomas and determine whether this marker might have a role in canine lymphoma subclassification.

This study identified several B-cell and T-cell lymphoma cases with positivity for the IHC markers tested (c-Kit, CD30, clusterin). Most cases (17/18 TCL and 3/3 BCL) were positive for only one of the assessed markers. One case (case 28 – high-grade subcutaneous TCL) was positive for both CD30 and clusterin, however the pattern of staining was different for the different stains, indicating that the populations of positive cells were not identical in both samples (although there might have been overlap).

C-Kit IHC has been extensively reported in canine mast cell tumours with either membranous or cytoplasmic staining, however it has been infrequently reported in canine lymphoma. The largest study assessed 25 cases of canine lymphoma (14 BCL, 15 TCL) using ICC, and found 6/14 BCL and 7/11TCL were positive respectively, with all cases showing cytoplasmic staining of varying intensity (Giantin et al., 2013). This study provided no information on the subclassification of the lymphomas beyond BCL or TCL. A second study investigating the use of masitinib in the treatment of epitheliotropic T-cell lymphoma found no expression of c-Kit when 8 tumours were assessed using immunohistochemistry (Holtermann et al., 2016). A case report of a dog with epitheliotropic lymphoma identified moderate cytoplasmic staining (Shiomitsu et al., 2012). A golgi staining pattern for c-Kit in canine samples has not previously been described, however it has been reported in some human tumours (Hughes et al., 2004, Jaramillo et al., 2012a). The significance of the golgi staining pattern is unknown, however in gastrointestinal stromal tumours golgi c-Kit staining pattern has been associated with c-Kit mutations (Jaramillo et al., 2012b).

C-Kit IHC studies in human lymphoma have provided contradictory and inconsistent results. Regarding CD30 positive lymphoma subtypes, different studies have found either frequent (Pinto et al., 1994) or very infrequent (Rassidakis et al., 2003, Rassidakis et al., 2004) c-Kit positivity, with no known reason for this discrepancy. Similarly, a study of human DLBCL found 37% of cases showed some c-Kit positivity (Vakiani et al., 2005), however a second study of c-Kit expression in 1166 cases of lymphoma, which included 385 cases of DLBCL, found only two cases positive for c-Kit, neither of which was DLBCL. These inconsistencies have meant that c-Kit IHC has not found a role in the diagnosis or management of human lymphoma.

In our study we found that all c-Kit positive cases were high grade TCL. 2/5 of the positive cases were high grade gastrointestinal lymphoma, which represented 20% (2/5) of all high grade gastrointestinal lymphoma cases assessed in the study. Also 2/5 positive cases were cutaneous lymphoma (one non-epitheliotropic, one epitheliotropic). These results could indicate that TCL, particularly high grade gastrointestinal and possibly cutaneous TCL, is more likely to be c-Kit positive. However, frequent c-Kit positivity in cases of cutaneous lymphoma is not supported by the previously mentioned study assessing c-Kit IHC in 8 dogs with epitheliotropic lymphoma, which found no positive cases. It would have been interesting to assess our samples for c-Kit mutations to determine whether IHC positivity, or staining pattern, correlated with mutation status. It would also be interesting to assess if c-Kit positive cases have a different prognosis or respond better to c-Kit inhibitors (such as toceranib and masitinib), compared to c-Kit negative cases. Unfortunately this was beyond the scope of the current study.

CD30 IHC is essential for the subclassification of human lymphoma, as CD30 positivity is obligatory in the diagnosis of ALCL. CD30 may also be prognostic in some lymphoma subtypes such as DLBCL (Hao et al., 2015, Hu et al., 2013). Currently there is no defined use for CD30 in canine lymphoma, however it has been used in a previous case report (Pittaway et al., 2018) and case series (Stranahan et al., 2019) to diagnose cases of ALCL. In our study, three cases were positive for CD30, all of which were TCL. Only one case showed diffuse staining, with the other two cases having <1% of neoplastic cells positive. In humans ALCL makes up ~16% of all TCL (Swerdlow et al., 2008). The prevalence of ALCL in the canine TCL population is unknown, partly due to the lack of validated diagnostics (eg CD30 IHC) required to definitively differentiate ALCL from other TCL subtypes. Seven percent (3/46) of TCL cases were positive in our study. It is unknown whether these cases were truly ALCL. Human ALCL classically show atypical lymphoid cells with pleomorphic nuclei and membranous-golgi CD30 positivity in the majority of neoplastic cells (Swerdlow et al., 2008). Previous reported canine cases of ALCL have shown membranous to cytoplasmic CD30 positivity in the majority (50-100%) of neoplastic cells. In our study, 2/3 positive cases showed only infrequent (<1%) staining of neoplastic cells, and in one of these cases the neoplastic population comprised small-intermediate lymphocytes, therefore it would appear to be unlikely that these two cases truly represented ALCL.

ALCL in humans can be further subclassified into four subtypes: ALK negative primary systemic ALCL, ALK positive primary systemic ALCL, primary cutaneous ALCL, and breast implant associated ALCL. The third positive case reported in our study, which was intermediate-large cell non-epitheliotropic cutaneous TCL and showed diffuse moderate CD30 positivity, could have been a true case of primary cutaneous ALCL. Future studies assessing CD30 positivity, particularly to diagnose ALCL cases, are needed to further assess if this subclassification has prognostic significance in canine lymphoma. Furthermore, it would be interesting to trial treatment with CD30 immunotherapy (bentuximab vedotin) in these cases to determine whether this is a viable treatment option for canine lymphoma patients, and whether CD30 IHC status predicts response to CD30 immunotherapy.

Clusterin IHC does not have a defined role in the management of human lymphoma. The majority of ALCL cases are positive for clusterin, leading to the suggestion that clusterin could be used as a possible marker of ALCL in cases of poorly differentiated round cell tumours (Nascimento et al., 2004). In our study, only one of the possible ALCL CD30 positive cases (case 28) was also positive for clusterin; however, as previously mentioned, this case is unlikely to be a true example of ALCL. In our study the prevalence of positivity was similar between TCL and BCL, with 11/43 (26%) TCL and 3/13 (23%) BCL cases positive. All cases showed golgi staining which is consistent with clusterin IHC reports in human lymphoma (Nascimento et al., 2004, Olsen et al., 2009), however the intensity and percentage of positive staining cells varied between cases. In human lymphoma, there have been conflicting results regarding the proportion of neoplastic cells that stain positively for clusterin. Nascimento *et al (Nascimento et al., 2004)* found that 22/33 of clusterin-positive ALCL cases had >25% neoplastic cells positive for clusterin, and 15/22 of the positive cases had >75% of neoplastic cells positive. However a separate study by Olsen *et* al (Olsen et al., 2009) found that the majority (63/71) of clusterin-positive lymphoproliferative diseases showed <25% of neoplastic cells being positive, and 42/63 positive cases showed <1% of neoplastic cells being positive. The results of our study are more in line with Olsen *et al*, with all positive cases showing less than 25% of neoplastic cells positive for clusterin. As with the other markers assessed in our study, it would be interesting to assess clusterin in a larger prospective study to determine whether clusterin positivity is of prognostic significance.

Several other T-cell markers had to be abandoned in this study due to an inability to confirm specific positive staining on canine tissue within the timeframe of the project. These included the Tfh-cell markers CXCR5 and PD1, and the ALCL marker ALK. CXCR5 and ALK IHC have never been reported in canine patients.

ALK has previously been assessed in canine pulmonary adenocarcinomas (Mariotti et al., 2014). In this study increased ALK expression was found with RT-PCR, however ALK IHC was negative. As in our study, it was not clear whether the ALK IHC was negative due to lack of the ALK antigen, lack of IHC optimisation for canine tissue, or insufficient homology between the canine and human ALK epitopes to allow anti-human ALK antibodies to bind. A second study (Hocker et al., 2017) assessed *in situ* phosphorylation of ALK in 16 canine nasal carcinomas. This study found 43.7% of cases had a phosphorylated ALK, but did not assess ALK with IHC.

A major hurdle in using ALK in canine IHC is the lack of a reliable positive canine control tissue to confirm specific positive results in the dog. Aside from the two studies mentioned above, no other studies have assessed canine ALK and only one of the above studies attempted ALK IHC, which was unsuccessful. In our study we did not have access to reliable positive human control samples, such as embryonic brain, ALK positive ALCL, or appendix/small intestine. In the human tonsil and Hodgkin's lymphoma samples assessed, no positive staining cells were noted, as expected. In the canine tonsil sample, no positive staining cells were identified, which could have been due to lack of antigen (which is likely based on the localisation of human ALK antigen), insufficient homology between the canine and human ALK epitope, or due to suboptimal IHC protocol (eg insufficient antigen retrieval, loss of antigen due to extended period in formalin etc).

CXCR5 and PD-1 IHC were also unsuccessful in our study. Similar to ALK it is not clear whether this was due to lack of antigen in our samples, lack of homology between the human antigen and the canine epitope, or due to the IHC protocol used. CXCR5 and PD-1 are markers of Tfh. In a study assessing 146 human PTCLs (Rodríguez-Pinilla et al., 2008), 29% were found to have arisen from Tfh on the basis of PD-1 positivity. A previous study successfully used PD-1 IHC in canine samples (Choi et al., 2020). Unfortunately, the antibody used in this study is not commercially available so was not available for our study. The PD-1 and CXCR5 antibodies used in our study have not previously been used in canine IHC. Tonsils are an appropriate control for CXCR5 and PD-1 in human IHC. Tonsil tissue should contain CXCR5 positive lymphocytes in the germinal centres and mantle zones, and PD-1 positive macrophages in the germinal centres and epithelial crypt cells (Smith et al., 2003). Unfortunately our canine tonsil controls were uniformly negative with all attempted IHC protocols. It is not clear why we were unable to identify positive cells in our IHC and we were unfortunately not able to optimise the CXCR5 of PD-1 stains within the timeframe of the project.

Future work to optimise these stains for canine lymphoma samples is warranted in order to identify canine TCLs of Tfh origin, as this subclassification suggests a distinct pathogenesis and could prove to be prognostically or therapeutically significant.

Our study had several limitations. First and foremost, we were not able to, with the help of an experienced veterinary haematopathologist, systematically review the original histopathology and immunophenotyping to assign each case, to the extent currently possible within veterinary medicine, a WHO lymphoma subtype. We designated cases as high grade or low grade and assigned their anatomic location based on review of the animal history (where available) and histology/immunophenotyping reports. The next major limitation was our inability to optimise several of our intended IHC markers for canine tissue, resulting in us having to abandon our investigation into these markers (CXCR5, ALK, PD-1). The optimisations were performed in a commercial laboratory by an experienced technician; however, due to time constraints, there was difficulty co-ordinating the staining and review of the slides by the author and a pathologist. Had time and resources allowed, the author ideally would have performed the optimisation and assessed all attempts with the guidance of a pathologist. Another difficulty, particularly with the ALK antibody, was the lack of a suitable canine control tissue. No validated/confirmed ALK positive canine tissue has been identified. Confirmed canine ALCL (CD30 positive) cases might have been promising candidates for ALK positive control tissues. In humans, ALK positive ALCL is more common that ALK negative lymphoma (ALK positive cases comprise ~70% of all ALCL cases). If this is similar in canine ALCL then it is likely that ALK positive cases would be found in a group of confirmed ALCL cases (for example in the group of 13 ALCL cases described by Stranahan *et al (Stranahan et al., 2019)*). This difficulty highlights the need for more validated IHC markers in canine lymphoma, as if we were able to easily identify CD30 positive cases, we would be better able to identify the population of cases for which ALK IHC may be most useful.

Overall the proportion of TCL cases staining positive for the markers investigated was low, with only 11%, 7%, and 26% of cases considered positive for C-kit, CD30 and clusterin respectively. This low proportion of positive cases results in low absolute numbers of positive cases to assess for trends in the characteristics of positive cases. Assessing a larger group of TCL cases would be interesting to see if the results obtained in this study are repeatable in a larger population, and to identify more positive cases that can be further assessed to determine the significance of positivity with these markers. The low proportion of positive cases could also limit the clinical usefulness of these markers and the benefit of the markers in providing prognostic or therapeutic information will need to be established to justify the cost and sampling requirements to the owner and patient.

Our study has confirmed that canine lymphoma has variable c-Kit, CD30 and clusterin IHC characteristics, and has detailed the staining characteristics in a variety of canine lymphoma samples. Future work is needed to assess whether these characteristics can be used to subclassify canine lymphoma into prognostically different groups, or if they can be used to direct therapy. Ideally future studies would prospectively enrol dogs with TCL, and apply these markers at initial diagnosis, then follow the cases to test whether these markers correlate with outcome, or correlate with specific morphological subtypes. Additionally it is likely that human anti-neoplastic therapies will become available for use in dogs in future, since these markers could be useful in predicting response to these medications (for example using anti-CD30 brentuximab vedotin in CD30 positive cases).

Currently there are few methods of subclassifying canine TCL beyond basic morphology and T-cell immunophenotype. This results in a group termed PTCL-NOS which, when investigated, show variable treatment responses and survival times. This is most likely due to this group containing several distinct TCL diseases which we are currently unable to distinguish. The three stains assessed in this study exclusively (c-Kit, CD30) or predominantly (clusterin) stained TCL cases, and so may be useful in subclassifying TCL cases.

4.2 Investigation of *TRAF3* mutations in dogs with B-cell lymphoma

Our study aimed to assess a cohort of dogs with BCL to confirm and add to the findings of previous studies. The results of our study support previous findings that *TRAF3* mutations are frequent in cases of cBCL. In our study *TRAF3* mutations were present in 36% of cBCL patients. This is similar to previous studies, which found mutations in 30-44% of cBCL cases (Bushell et al., 2015, Elvers et al., 2015). We also assessed a cohort of dogs with non-BCL diseases (including four dogs with TCL) to determine whether *TRAF3* mutations are specific for BCL, or if they occur with other diseases. Deleterious mutations of *TRAF3* were only identified in patients with cBCL, which suggests that this gene is important in the pathogenesis of cBCL.

The cBCL and cTCL cases included in this study were not further subtyped (apart from one case with a confirmed diagnosis of TZL), and therefore it is likely that different cBCL and cTCL subtypes were included. Similar to human BCL, cBCL comprises a diverse group of distinct diseases. The most common BCL subtype in humans and dogs is DLBCL (Valli et al., 2011), and it is likely that the majority of cBCL included in this study were DLBCL. Histology and IHC on all cases would be required to confirm this. As cBCL comprise a heterogeneous group of diseases, it is likely that different subtypes have different mutational characteristics, and future studies to assess how *TRAF3* mutations relate to different cBCL subtypes are required.

TRAF3 is a member of the tumour necrosis factor receptor-associated factor (TRAF) family of cytoplasmic adaptor proteins (Moore et al., 2015) and part of the CD40 signalling cascade which regulates proliferation, immunoglobulin class switching and apoptosis (Elvers et al., 2015). It is considered a tumour suppressor gene, and loss of TRAF3 function has been implicated in human tumours including several types of lymphoma, and multiple myeloma as well as other malignancies (Bushell et al., 2015). Pro-survival signalling (including B-cell activating factor stimulation) results in trimerization of TRAF3 with other proteins, inhibiting its function and ultimately inducing the transcription of anti-apoptotic Bcl2 family proteins, leading to B-cell survival (Moore et al., 2015). TRAF3 also negatively regulates NFkB by targeting NFkB-inducing kinase for ubiquitination and degradation (Bushell et al., 2015, Sun, 2011). Therefore, reduced TRAF3 activity leads to an upregulation of NFkB activity.

The TRAF3 protein has several domains with specific functions (see Fig. 3-12). The coiled coil domains are responsible for hetero-oligomerisation among TRAF family members, which is crucial for interactions between the TRAF proteins and their substrates (Hacker et al., 2011, Qian Yin, 2009). The meprin and TRAF homology (MATH) domain is necessary for receptor interaction and interactions with adaptor proteins (Qian Yin, 2009, Hacker et al., 2011). All mutations identified in the current study would have resulted in premature truncation or change of the TRAF3 protein before or within these domains, and therefore would be expected to disrupt normal TRAF3 function.

In general, most mutations in neoplasms are suspected to be somatic, developing within the tumour as part of tumour initiation and progression. In the present study the majority of detected mutations were suspected to be somatic. In the five dogs with *TRAF3* mutations and matched tissue, only one had the mutation identified in the matched tissue, suggesting a germline variant. However, the matched DNA samples were collected from peripheral blood, so detection of the mutation in circulating neoplastic cells, or free tumour DNA in the blood, cannot be completely excluded. While it is possible that this variant is a germline polymorphism, we did not find this variant in dogs without cBCL, as was seen with the synonymous variants. This suggests that the change may be a genuine germline mutation. A previous study found 17.5% of cBCL *TRAF3* mutations were germline (Bushell et al., 2015), which is similar to the current study (1/5 dogs with matched tissue). Germline mutations in *TRAF3* could result in a genetic predisposition to developing cBCL. Genome wide association studies (GWAS) are needed to confirm this.

The prevalence of *TRAF3* mutations in our study was similar to previous studies (36% vs 30-44%). This is despite sequencing only exons 9, 10 and 11 of the TRAF3 gene. This decision was made on the basis of RNA-Seq results (Waugh, 2015) which only identified variants in these exons. These three exons constitute 51% of the entire TRAF3 sequence and a previous study of TRAF3 mutations in cBCL (Bushell et al., 2015) found that the majority of mutations occurred within exons 9, 10 and 11; however, some mutations were identified in the other exons. As we did not sequence the entire gene, it is possible that our results might have underestimated the true prevalence of mutations in our population.

Most of the identified mutations (9/11) were heterozygous, meaning these patients also had a wild-type allele detected. However, TRAF3 can have a dominant-negative effect, allowing heterozygous mutations to result in phenotypic changes. Mutations to RING-type domain or zinc finger domain (not assessed in this study) can competitively displace normal TRAF3 if the C-terminal TRAF3 domains on the mutated protein are normal, and mutations to the C-terminal TRAF3 domains (identified in this study) can prevent normal TRAF3 recruitment (Force et al., 1997).

This study has several limitations. Similar to many veterinary studies, the sample size was small. Given the prevalence of the mutations, this leads to identification of only a small overall number of mutations. Bulk Sanger sequencing was used, which is insensitive to mutations occurring at ~<20% allele frequency (MacConaill, 2013), so mutations might have been missed if they occurred in only a small proportion of the tumour cells, or if there was a significant number of non-tumour cells within the samples tested. This means the true prevalence of *TRAF3* mutations could have been underestimated. Also, the majority of samples did not have matched non-tumour tissue to assess whether the variants were germline or somatic. Despite the small numbers of matched samples, the proportion of germline and somatic mutations found in this study was similar to previous studies. Only a portion of the genes were sequenced in this study, which may have led to some mutations being missed, and an underestimation of the total number of mutations. Sequenced exons were selected on the basis of RNA-Seq data (Waugh, 2015) and previous studies (Elvers et al., 2015, Bushell et al., 2015) and while these methodologies generally showed good correlation, in 4/12 instances discrepancies were identified (two variants identified only on RNA-Seq and two identified only on Sanger sequencing), possibly as a result of sequencing error in the RNA-Seq dataset. Ideally, sequencing of the full genes would have been performed. Despite this, it is likely that we were able to identify the majority of mutations present.

This study adds further support to the suggestion that *TRAF3* mutations are important in the pathogenesis of cBCL. Future studies looking at potential prognostic and therapeutic implications of these mutations are needed. Unfortunately, due to the heterogeneity in treatment modalities and clinical information available for the included cases in this study, correlation between TRAF3 status and BCL morphological subtype or prognosis was not possible. Studies assessing *TRAF3* germline mutations and predisposition to neoplasia would also add to our understanding of lymphomagenesis in the dog.

4.3 Conclusion

Precise subclassification of lymphoma is crucial to accurately prognosticate and optimise therapies. IHC and molecular techniques are routinely used to subclassify human lymphoma. Canine lymphoma subclassification remains far behind human lymphoma, partly due to the difficulty in performing large scale studies to validate new diagnostic techniques. This study has detailed the IHC characteristics of a variety of canine lymphoma samples using novel IHC stains, some of which have defined roles in human lymphoma TCL subclassification, and has confirmed the high prevalence of *TRAF3* mutations in canine BCL and described their characteristics. This study also indicates that *TRAF3* mutations are specific for BCL, as they were not identified in any other diseases. These preliminary investigations, however, will hopefully provide a basis for future studies to assess these characteristics for their prognostic and therapeutic significance. If significance is found, then these factors might be adopted in future for routine use to subclassify canine lymphomas.

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