Transcriptomic analysis of canine B and T cell lymphoma

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Abstract

Gene expression profiling in human lymphoma has identified a number of genes and pathways of prognostic value that could also be used as targets for therapy.

The aim of the present study was to compare gene expression in canine B-cell lymphoma, T-cell lymphoma and control lymph node samples using RNA-Seq to identify differentially expressed pathways which may have a role in pathogenesis and could be used as possible treatment targets.

RNA was extracted from lymph node tissue collected from six dogs with B-cell lymphoma, five dogs with T-cell lymphoma, one dog with B and T-cell lymphoma and eight controls. The RNA was sequenced using an Illumina NextSeq500 platform, reads were aligned to the current canine genome and gene expression profiles were determined for each group. Pathway analysis was also performed to identify networks enriched for differentially expressed genes together with activated and repressed molecular pathways. After stand-alone analysis of the dataset, it was combined with a previous dataset representing eighteen dogs with B-cell lymphoma and five dogs with T-cell lymphoma in order to increase the power of the analysis.

Principal component analysis revealed that the three groups, i.e. B-cell lymphoma, T-cell lymphoma and controls, formed separate clusters with distinct expression signatures. Network analysis highlighted different pathways overrepresented in each type of lymphoma. In B-cell lymphoma tissue, the NF-κB pathway, cell cycle control and DNA repair pathways were over-represented with overexpression of ribosomal and minichromosome maintenance proteins also noted. In contrast for T-cell lymphomas, mRNA synthesis, FGFR and RUNX pathways were highlighted together with those relating to innate immunity.

This project identified several aberrant pathways in B-cell and T-cell lymphoma that have not previously been reported and which can be considered candidate targets for developing novel treatment, thereby informing future studies.

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Declaration

I, Mariana Lopes, declare that the work in this thesis is original, and was carried out by myself except the RNA-Sequencing which was performed at Glasgow Polyomics. Paul Capewell assisted with the preliminary data processing prior to the Reactome analysis, and the gene enrichment analysis. It has not been submitted in any form for another degree or professional qualification.

Some of the work was presented as an abstract at the 2022 ECVIM Congress in Gothenburg.

List of abbreviations:

ABC - Activated B-Cell Like DLBCL

AKT - Protein Kinase B

BAFFR - B cell activation factor receptor

BCL2 - B-cell lymphoma 2

B-CLL -B-cell chronic lymphocytic leukaemia

BCR – B-cell receptor

bp – base pairs

BLIMP1 - B lymphocyte–induced maturation protein 1

BMD - Bernese Mountain Dog

BTK - Bruton Tyrosine Kinase

CCND3 - Cyclin-D3

CD40 - Cluster of differentiation 40

CDC25A - Cell Division Cycle 25A

CDC45 - Cell division control protein 45

CDC7 - Cell Division Cycle 7

CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A

CDK4 - Cyclin-Dependent Kinase 4

CDK6 - Cyclin-Dependent Kinase 6

cDNA - complementary DNA

CHEK1 - Checkpoint kinase 1

DAP12 - DNAX-activation protein 12

DLBCL – Diffuse Large B-Cell Lymphoma

DNA - Deoxyribonucleic Acid

ERK - extracellular signal-regulated kinase

FBXW7 - F-box/WD repeat-containing protein 7

FE - Female Entire

FGFR - Fibroblast Growth Factor Receptor

FLT3 - Fms Related Receptor Tyrosine Kinase 3

FN - Female Neutered

GATA3 - GATA binding protein 3

GCB - Germinal Centre B-Like DLBCL

GINS1 - GINS Complex Subunit 1

GSD - German Shepherd Dog

GWAS - Genome-Wide Association Studies

HPF – High power field

IGK - Immunoglobulin Light Chain Kappa Locus

IκB – Inhibitor of κB

IκK - IκB kinase

IL – Interleukin

IRF4 - Interferon Regulatory Factor 4

ITGAL - Integrin Subunit Alpha L

JRT - Jack Russell Terrier

KIT – CD117

LSA - Lymphoma

LTβR - Lymphotoxin β-Receptor

MAP - Mitogen-activated protein

MAP3K14 - Mitogen-activated protein kinase kinase kinase 14

MCM - Minichromosome Maintenance

ME - Male Entire

MN - Male Neutered

mTOR - mammalian Target Of Rapamycin

MUO - Meningoencephalitis of Unknown Origin

NF-κB - Nuclear Factor Kappa B

NGS – Next Generation Sequencing

NHL – Non Hodgkin's lymphoma

NIK - NF- κB inducing kinase

NK – Natural Killer

ORC - Origin recognition complex

PARR- PCR for antigen receptor rearrangements

PCA – Principal Component Analysis

PCR - Polymerase chain reaction

PI3K - Phosphatidyl-Inositol 3-Kinase

POT1 - Protection Of Telomeres 1

PRDM1 - PR domain containing 1 with zinc finger domain

PTCL-NOS – Peripheral T-cell lymphoma – not otherwise specified

PTEN - Phosphatase and Tensin Homolog

RANK - Nuclear Factor Kappa B

Rb – Retinoblastoma protein

REAL - Revised European American Lymphoma

RFC4 - Replication Factor C Subunit 4

RIN - RNA integrity number

RNA - Ribonucleic acid

RORC - RAR-related orphan receptor C

RPA2 - Replication Protein A2

RPL4 - Ribosomal Protein L4

RPS7 - Ribosomal Protein S7

RT-PCR - reverse transcription polymerase chain reaction

RUNX - Runt-related transcription factor

SATB1 - Special AT-rich Sequence Binding Protein 1

SINE - Selective Inhibitors of Nuclear Transport

SMLN - submandibular lymph nodes

SSP1 - Osteopontin

STX8 – Syntaxin 8

TBX21 - T-Box Transcription Factor 21

TLR – Toll Like Receptor

TNF - Tumour Necrosis Factor

TRAF3 - TNF receptor-associated factor 3

TRPC6 - Transient receptor potential cation channel, subfamily C, member 6

VEGFR-1 - Vascular Endothelial Growth Factor Receptor-1

VTM- Viral Transport Medium

WHO - World Health Organization system

XBP1 - X-Box Binding Protein

1. Introduction

This thesis focuses on the classification of canine lymphoma by molecular techniques, using gene expression measured at the ribonucleic acid (RNA) level. Historically, canine lymphoma was classed as a single disease with minimal subclassification other than anatomic localisation. In contrast, classification of human lymphoid neoplasms is much more complex, with over 80 subtypes being recognised (Alaggio et al., 2022) and in recent years it has been acknowledged that canine lymphoma is equally complex. To understand the rationale for the project, it is important both to understand the current methods of lymphoma classification and to appreciate the existing knowledge of the underlying genetic pathways altered in the disease.

1.1. Canine lymphoma

Canine lymphoma is a group of malignancies that arise from the lymphoreticular cells, mostly from lymphoid tissues such as lymph nodes, spleen and bone marrow, although it can be found in almost any location (Vail & Young, 2019). Lymphoma is the most common haematopoietic neoplasia in dogs, accounting for 80-90% of this class of tumour (Dobson et al., 2002) and 7-24% of all canine neoplasias (Vail & Young, 2019). While lymphoma can occur in dogs of any age, middle-aged to older dogs are most commonly affected (Turek et al., 2008). There is no gender predisposition and middle-sized to large breeds are over-represented (Comazzi et al., 2018). This is most likely related to an element of genetic predisposition in specific large breeds rather than related to growth hormone levels, which has been suggested not to play an important role in lymphoma pathogenesis (Lantinga van Leeuwen et al., 2000).

1.1.1. Aetiology

The aetiology of canine lymphoma is not yet well established and several contributing factors have been suggested including environmental, hereditary and immunological causes.

1.1.1.1. Environmental factors

Since the prevalence of both canine lymphoma and its human equivalent, Non-Hodgkin's lymphoma (NHL), has been increasing, it has been suggested that environmental factors may play a role in lymphoma pathogenesis in both species, given the proximity of living conditions between dogs and humans (Cartwright et al., 1999; Zandvliet, 2016; Howlader, 2011). Some of these factors may include proximity to industrial areas, exposure to chemicals, living near waste incinerators, radioactive or polluted sites and exposure to magnetic fields, either due to carcinogenic or immunosuppressive properties of these factors (Teske, 1994; Reif, Lower & Ogilvie, 1995; Gavazza et al., 2001; Pastor et al., 2009). However, a clear cause-effect relationship has not yet been identified.

1.1.1.2. Hereditary factors

It has also been suggested that a genetic predisposition may contribute to the aetiology of lymphoma (Teske et al., 1994). The limited genetic variation within specific canine breeds together with a high number of offspring compared to humans provide a good opportunity for the identification of specific genetic risk factors. Specific canine breeds appear to be predisposed to the development of lymphoma, such as the Boxer, Bulldog, Bull Mastiff, Bernese Mountain Dog, Rottweiler, Scottish Terrier, Basset Hound, Airedale Terrier, Golden Retriever, Australian Cattle Dog and Doberman, amongst others. Familial clustering is also reported in some of these breeds (Edwards et al., 2003, Onions, 1984, Teske, 1994, Teske et al., 1994, Yau et al., 2017, Modiano et al., 2005), further supporting the theory that underlying genetic factors may play a role in the pathogenesis of lymphoma.

In a study of canine lymphoma which included 87 dog breeds, 15 showed a significantly increased predisposition for a specific lymphoma subtype, further reinforcing the supposition that a hereditary characteristic may represent a risk factor for the development of lymphoma (Modiano et al., 2005). In particular, increased breed risk in T-cell lymphoproliferative diseases seems to have been acquired ancestrally and has been retained between related breed groups (Modiano et al., 2005). In the study, boxers were found to be predisposed to T-cell lymphoma, while German Shepherd Dogs, Rottweilers, Dobermans and Cocker Spaniels appeared predisposed to B-cell lymphoma (Pastor et al., 2009; Modiano

et al., 2005) and Golden Retrievers appeared predisposed to both B-cell and T-cell lymphoma (Modiano et al., 2005). These findings have led to increased interest and research into the specific somatic and germline mutations implicated in the pathogenesis of canine lymphoma, which will be discussed further in a later section (1.2. Gene expression in canine lymphoma).

1.1.1.3. Immunological factors

Immune dysfunction may also contribute to the genesis of lymphoproliferative diseases. Impaired humoral and cellular function have already been identified in dogs with lymphoma compared with dogs with non-haematopoietic neoplasia (Weiden et al., 1974); dogs with immune-mediated thrombocytopenia are more likely to develop lymphoma than dogs without the disease (Keller, 1992), although it has yet to be established if the immune dysfunction is caused by the lymphoma or if it precedes its development. This link has already been established in humans, where there is an increased risk of developing lymphoreticular neoplasia after spontaneous or iatrogenic immunosuppression (Penn & Starzl, 1973; Filipovich et al., 1987). A correlation was also found between auto-immune diseases and increased incidence of lymphoma (Symmons, 1985). However, secondary immune dysfunction has also been identified in patients with NHL, with reduced B and T-cell levels noted regardless of how advanced the disease was (*naïve* lymphoma, in remission or under treatment) and without any significant differences in different degrees of malignancy (low, intermediate or high-grade) (Janowska-Wieczorek et al., 1987).

1.1.2. Classification

Canine lymphoma can currently be classified in four main ways, based on cytology, histology, immunophenotype or anatomic location.

1.1.2.1. Cytological classification

Lymphomas can be classified according to their cytological features using the human Kiel classification system adapted to the canine species (Lennert & Feller, 1991). Classification is based morphologically on cellular and nuclear size, nuclear shape, the density of the chromatin, the number, size and distribution of the nucleoli, the extension and basophilia of the cytoplasm, and the mitotic index. Using these criteria, low grade lymphomas represented only 26% of cases, with

the remainder being classified as high grade lymphomas (Fournel-Fleury et al., 1997).

1.1.2.2. Histological classification

A variety of morphological and immunophenotypic features determined using histopathology can be used to classify subtypes of lymphoma. Several attempts have been made to correlate canine lymphoma histotype to prognosis, using numerous histological classification schemes, mostly adapted from those used for human NHL as most canine disease is equivalent to this subtype (Marconato, Gelain & Comazzi, 2013). Figure 1 represents a simplified histological approach to canine lymphoma classification based on architecture, cell size and immunophenotype (Seelig et al, 2016). Large-cell lymphoma typically has an aggressive disease course while small-cell lymphoma has an indolent disease course (Flood-Knapik et al., 2013).

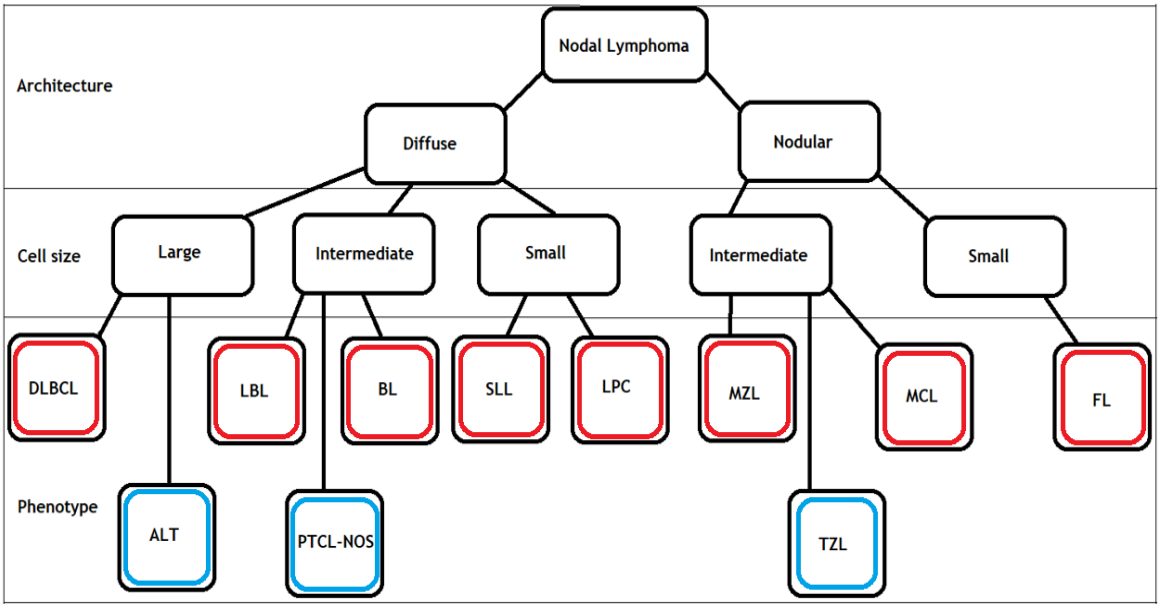

Figure 1. The histologic approach toward the classification of canine nodal lymphoma. Adapted from Seelig et al., 2016. ALT - Anaplastic large cell T-cell lymphoma; BL - Burkitt lymphoma; DLBCL - diffuse large B-cell lymphoma; FL - follicular lymphoma; LBL - lymphoblastic lymphoma; LPC - lymphoplasmacytoid lymphoma; MCL - mantle cell lymphoma; MZL - marginal zone lymphoma; PTCL-NOS - peripheral T-cell lymphoma, not otherwise specified; SLL - small lymphocytic lymphoma; TZL - T-zone lymphoma. Red boxes – B-cell lymphomas; Blue boxes – T-cell lymphomas.

Canine lymphomas can also be classified according to grade, with the most common grading scheme used being that of World Health Organization (Alaggio et al., 2022) which has been applied to multiple large cohorts of dogs with lymphoma (Valli et al., 2013; Valli et al., 2011). Grading for most types of lymphoma is mainly determined by the mitotic activity on histopathology. Lymphomas with a mitotic count of <5 per high power field (HPF) are considered "low-grade", those with a mitotic count between 6-10/HPF are considered "medium-grade" and lymphomas with a mitotic count >10/HPF are considered "high-grade". There is some confusion about the use of this terminology, as clinically the terms "high-grade" and "large cell" are used in an interchangeable way due to the expected aggressive clinical course that most large-cell lymphomas will have. In the same way, small cell lymphomas are often called "low grade lymphomas", which is incorrect and can lead to confusion in classification, as large cell lymphomas can be classified as low grade if their mitotic index is <5/HPF. For that reason, it has been recommended that the terms "indolent", "intermediate" and "aggressive" behaviour are used to describe the clinical course of specific lymphoma subtype, and that the terms "low-", "medium-" and "high-grade" are used only to stratify lymphomas into their histopathological grading categories (Avallone et al., 2021).

Multiple human classification systems have been applied to canine lymphoma over the years, with varying success. The *National Cancer Institute Working Formulation* (NCI sponsored study, 1982) and the updated Kiel schemes (Lennert & Feller, 1990) were adapted to canine lymphoma and appear to have some prognostic significance. The Working Formulation classifies lymphoma according to cellular pattern (diffuse or follicular) and cell type (small cleaved cell, large cell or immunoblastic) without including information regarding immunophenotype. The updated Kiel scheme includes architecture pattern, morphology (centroblastic, centrocytic or immunoblastic) and immunophenotype of the tumour cells. In both systems tumours can also be classified as low-, intermediate- or high-grade.

One of the criticisms of these early classification systems for canine lymphoma was that they did not incorporate extranodal lymphomas as a separate category. More recently, the Revised European American Lymphoma/World Health Organization system (REAL/WHO) has been proposed, which has been adapted

from the human lymphoma classification (Harris et al. 1999). It incorporates anatomical, morphological and immunophenotypic criteria, interpreting different types of lymphoma not just as different cell types, but as different diseases as well. This is the classification that is currently used in canine lymphoma, since it correlates better with prognosis, assists in the development of more specific treatments, and has better comparative capabilities. Three histopathological studies have assessed its applicability to the dog (Table 1) and agree that B-cell tumours predominate, that diffuse large B-cell lymphoma (DLBCL) is the predominant B-cell subtype and peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) is the predominant T-cell subtype.

Table 1a- World Health Organization Classification System for Canine B-cell lymphoma

	Percentage of all lymphomas		
Category	Seelig et al., 2016	Vezzali et al., 2010	University of Wisconsin-Madison
B-cell Neoplasms	69	78.9	59
Precursor B lymphoblastic leukaemia/lymphoma	-	2.4	8.2
B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma	-	2.4	0.8
Lymphocytic lymphoma – intermediate type	-	0.8	-
Lymphoplasmacytic lymphoma	-	3.3	0.8
Mantle Cell lymphoma	2	1.6	-
Follicular centre cell lymphomas	-	2.4	-
Marginal zone lymphoma (splenic, nodal, mucosa-associated lymphoid tissue)	8	3.3	2.5
Plasma cell myeloma/plasmacytoma	-	16.3	9.8
Diffuse large cell lymphoma	52	33.3	24.6
T-cell rich, B-cell lymphoma	-	0.8	-
Large cell immunoblastic lymphoma	-	10.6	10.7
Mediastinal (thymic) large B-cell lymphoma	-	0.8	-
Burkitt's lymphoma/leukaemia	-	0.8	1.6
Other B cell	8	-	-

Adapted from Vail & Young, 2019

Table 1b: World Health Organization Classification System for Canine T-cell lymphoma

	Percentage of all lymphomas		
Category	Seelig et al., 2016	Vezzali et al., 2010	University of Wisconsin-Madison
T-cell and Natural Killer (NK) Cell Lymphomas	31	21.1	41
Precursor T lymphoblastic lymphoma/leukaemia	3	6.5	9.8
T-cell chronic lymphocytic leukaemia	-	3.3	0.8
Intestinal T-cell lymphoma	-	4.1	4.1
Mycosis fungoides/Sezary syndrome	-	1.6	11.5
Cutaneous nonepitheliotropic lymphoma	-	3.3	-
Anaplastic large cell lymphoma	-	-	0.8
Peripheral T-cell lymphoma-not otherwise specified	15	2.4	13.1
T-zone lymphoma	4		
Other T cell	7	-	-
Other	3		

Adapted from Vail & Young, 2019

1.1.2.3. Immunophenotypic classification

Lymphoma usually arises from either B-cells or T-cells, but on rare occasions can also arise from natural killer (NK) cells. Canine B-cell lymphomas are more common than T-cell lymphomas which in general only represent between 10-38% of all lymphomas (21-41% in the three studies above) (Vail & Young, 2019). Mixed B-cell and T-cell lymphoma occurs at a frequency as high as 22%, and null cell tumours represent less than 5% of all lymphomas (Greenlee et al., 1990; Vail & Young, 2019; Ernst et al., 2016). There are no significant sex or age differences between B or T cell lymphomas (Ponce et al., 2010), but as discussed previously, some breeds are predisposed to particular immunophenotypes, such as Boxers

being predisposed to T-cell lymphomas and Cocker Spaniels, Dobermans, German Shepherd Dogs and Rottweilers being predisposed to B-cell lymphoma (Modiano et al., 2005).

1.1.2.4. Anatomical classification

Canine lymphoma has a wide diversity of clinical presentations that carry different prognoses and respond to different treatments. Anatomically, canine lymphomas can be grouped according to the WHO classification (Owen, 1980), and every group has a distinct clinical presentation.

1.1.2.4.1. Multicentric lymphoma

Multicentric lymphoma is the most common presentation of lymphoma, accounting for 75-80% of all cases of lymphoma. It is characterised by generalised and non-painful lymphadenomegaly, usually affecting initially the submandibular or prescapular lymph nodes (Vail & Young, 2019; Zandvliet, 2016). Most dogs are asymptomatic on first presentation, with 20-40% of cases progressing to develop systemic signs, such as lethargy, anorexia, weight loss and pyrexia. Thoracic and abdominal lymphadenomegaly, hepatosplenic and bone marrow involvement may also be present (Teske, 1994). It can be further classified as stage I, II, III, IV or V according to the WHO criteria depending on which lymph nodes and other organs are affected, and substage a or b depending on the absence or presence of systemic clinical signs (Owen, 1980).

1.1.2.4.2. Alimentary lymphoma

Alimentary lymphoma is the most common type of lymphoma in cats but only accounts for 5-7% of lymphomas in dogs (Vail & Young, 2019). While it can present as a single isolated lesion, it is more frequently detected as multifocal or diffuse lesions, with loss of wall layering, infiltration of the mucosa and submucosal layers, mucosal ulceration and occasionally transmural serosal infiltration. Mesenteric lymphadenomegaly is commonly present. Clinical signs commonly include weight loss, anorexia, vomiting, diarrhoea and malabsorption signs such as hypoproteinaemia (Rassnick et al., 2009, Frances, Lane & Lenard 2013). Differentiation between alimentary lymphoma and lymphoplasmacytic enteritis can be difficult, and it has been proposed that it may be a gastrointestinal

inflammatory change that precedes malignant transformation into lymphoma (Carrasco et al., 2015).

1.1.2.4.3. Mediastinal lymphoma

Mediastinal lymphoma is characterised by craniomediastinal lymphadenomegaly, thymic involvement or both (Day, 1997) in the absence of peripheral lymphadenomegaly. In one study, a mediastinal mass was present in 20% of all cases of canine T-cell lymphoma (Fournel-Fleury et al., 2002). It has almost exclusively a T-cell immunophenotype and clinical signs may include cough, dyspnoea, tachypnoea or polyuria and polydipsia. These latter signs usually result from hypercalcaemia, a common paraneoplastic syndrome secondary to mediastinal lymphoma (Moore et al., 2018).

1.1.2.4.4. Cutaneous lymphoma

Cutaneous lymphoma is most commonly characterised by diffuse or multifocal lesions on the skin or mucocutaneous areas and it is typically of T-cell subtype. It can be epitheliotropic or non-epitheliotropic, the former being more common, and representing 3-8% of all lymphoma cases (Chan, Frimberger & Moore 2018). Skin lesions are variable in appearance and include diffuse erythema, scaling, focal hypopigmentation, plaques and nodules (Fontaine, Heimann & Day 2010). WHO classification recognises three forms of cutaneous epitheliotropic T-cell lymphoma: *mycosis fungoides* (named after the mushroom like appearance of the lesions), Sézary syndrome and pagetoid reticulosis, which are similar diseases to their human counterparts (Rook, 2019).

1.1.2.4.5. Other lymphoma presentations

When lymphoma presents in specific organs, clinical signs are varied depending on location. Primary central nervous system lymphomas can present with weakness, proprioceptive deficits and ataxia (LaRue et al., 2018). Ocular lymphoma can present with uveitis or conjunctivitis (Wiggans et al., 2014), while hepatosplenic lymphoma presents with non-specific signs, such as lethargy and anorexia (Keller et al., 2013).

Although distinct separate localisations can occur, it is not uncommon for there to be overlap of presentations, for example alimentary lymphoma with peripheral lymph node enlargement, or mediastinal lymphoma with alimentary involvement.

1.2. Gene expression in canine lymphoma

Genomic analysis has confirmed the presence of several mutated genes and aberrant pathways in the pathogenesis of canine lymphoma, as suggested by the breed dispositions to the disease, already discussed above. Although there has been some attempt to link genetic/transcriptomic changes to immunophenotype, with more information available on B-cell lymphoma vs T-cell lymphoma (Avery, 2020), there is not much detail on changes within specific histologic classifications other than for DLBCL. Table 2 summarises the aberrantly expressed pathways and mutated genes that have already been identified in canine B-cell and T-cell lymphoma and that are discussed in the next sections.

Human classification of lymphoma has started to incorporate transcriptomic information in order to improve diagnosis and to facilitate the use of more targeted treatments (Young et al., 2019). For example, a molecular classification assay named Lymph3Cx, which is based on the expression of 58 genes, has been validated for differentiating between primary mediastinal large B-cell lymphoma and DLCBCL (Mottok et al., 2018, Ramsower et al., 2019; Roschewski, Phelan & Wilson, 2020). Given the similarities between the human and canine disease, it may be hypothesised that a transcriptional biomarker approach may also have value in the field of canine lymphoma. Over the past 20 years, numerous research groups have started to investigate this field (Avery, 2020).

Table 2: Genomic aberrations identified in canine B-cell and T-cell lymphoma

B-cell LSA	Reference	T-cell LSA	Reference
- Increased expression/ enrichment			
NF-κB signalling	(Richards et al., 2013; Aresu et al., 2019; Mudaliar et al., 2013)	PI3K/AKT pathway	(Harris et al., 2019)
MAPK signalling	(Giannuzzi et al., 2019)	MTOR	(Harris et al., 2019)
JAK-STAT signalling	(Giannuzzi et al., 2019)	KIT	(Giantin et al., 2013)
BCL2 (and c-FLIP and XIAP)	(Gaurnier-Hausser et al., 2011; Curran et al., 2017)	P53	(Sokolowska, Cywinska & Malicka 2005)
MYC	(Curran et al., 2017)		
MAP3K14	(Elvers et al., 2015)		
- Decreased expression/ loss of function			
TRAF3	(Smith et al., 2020; Bushell et al., 2015; Elvers et al., 2015)	PTEN	(Harris et al., 2019; Roode et al., 2016)
POT1	(Smith et al., 2020; Elvers et al., 2015)	CDKN2	(Roode et al., 2016; Fosmire et al., 2007)
FBXW7	(Elvers et al., 2015)	SATB1	(Harris et al., 2019; Elvers et al., 2015)

LSA – Lymphoma

1.2.1. Chromosome abnormalities

Major structural chromosomal abnormalities have been identified in canine multicentric lymphoma using the technique of comparative genomic hybridisation to study over and under-represented regions of chromosomes. Gains of chromosome 13 and 31 and loss of chromosome 14 are common karyotypic abnormalities (Thomas et al., 2003), with gain of chromosome 13 being a positive prognostic indicator for the duration of first remission and overall survival time (Hahn et al., 1994). Chromosome 13 carries the oncogene *Myc*, and the gain of part or all of this chromosome may lead to *Myc* overexpression in canine lymphoma (Thomas et al., 2011). Chromosomal anomalies in non-protein coding areas can also have an important role in pathogenesis, such as deletions in chromosome 13q14, which encodes miR-15a/miR16-1 and is found in 12% of B-cell chronic lymphocytic leukaemia (B-CLL) canine cases. These microRNAs are thought to control expression of the B-cell lymphoma 2 gene (*BCL2*), with loss of their expression resulting in *BCL2* upregulation (Spina & Rossi, 2019).

A range of chromosomal abnormalities have also been reported in canine T-cell lymphoma, including loss of chromosomes 11, 17, 22, 28 and 38 (Thomas et al., 2011), with the tumour suppressor gene cyclin-dependent kinase inhibitor 2A (*CDNK2)* being present on chromosome 11. Loss of *CDKN2* has been identified in canine T-cell lymphoma, and it is specific to a more aggressive subtype of T-cell lymphoma, with loss of *CDKN2* not being a feature of indolent T-cell lymphoma or B-cell lymphoma (Fosmire et al., 2007).

1.2.2. Genome-Wide Association Studies

Genome-wide association studies (GWAS) screen the entire genome of large numbers of individuals (both affected by a disease and controls) to find associations between genetic variants within those individuals and their underlying disease (Sud et al., 2017). In Golden Retrievers in the United States, GWAS have shown that two loci on chromosome 5 account for 20% of the risk of this breed developing both B-cell lymphoma and haemangiosarcoma. Interestingly, changes in gene expression at these loci, such as Transient receptor potential cation channel, subfamily C, member 6 (*TRPC6)* and Syntaxin 8 (*STX8)*, were associated with both down-regulation of nearby genes implicated in pathways involved in immune cell activation and enrichment for differentially expressed genes in pathways associated with T-cell mediated responses. However, these were not accompanied by coding mutations suggesting that germ-line regulatory mutations play a role in tumorigenesis in these diseases (Tonomura et al., 2015). Golden Retrievers with lymphoma appear to be highly sensitive to induction of chromosome damage by mutagens, suggesting alterations in Deoxyribonucleic Acid (DNA) repair mechanisms may also be involved in tumourigenesis (Thamm et al., 2013).

Analysis of different dog breeds predisposed to B-cell (Cocker Spaniels and Golden Retrievers) and T-cell lymphoma (Boxers and Golden Retrievers) revealed a large overlap in commonly mutated genes and aberrantly expressed pathways in the two breeds predisposed to B-cell lymphoma. In particular, frequent mutations were identified in the TNF receptor-associated factor 3 (*TRAF3)* gene, which affects nuclear factor kappa B (*NF-κB*) signalling*; FBXW7* (F-box with 7 tandem WD40), which is involved in the regulation of cell cycle progression, cellular differentiation, and survival by targeting cyclin E for degradation and controlling the stability of MYC; and *POT1* (protection of telomeres 1) which has a role in

telomere maintenance. In contrast, there was little overlap in the two breeds predisposed to T-cell lymphoma, with mutations affecting the genes in the phosphatidyl-inositol 3-kinase (PI3K)/protein kinase B (AKT)-mammalian target of rapamycin (mTOR) pathway (PI3K-AKT-mTOR pathway) being present in almost half of Boxers with lymphoma, such as in the tumour suppressor gene phosphatase and tensin homologue (*PTEN)*. The finding that this genetic change was much less prevalent in other breeds predisposed to T-cell or B-cell lymphoma suggests genetic background may play a significant role in the pathogenesis of T-cell lymphoma (Elvers et al., 2015).

Several other genes are implicated in lymphoma pathogenesis. The tumour suppressor gene *TP53* can be mutated in canine lymphoma, with both germline and somatic mutations identified (Veldhoen et al., 1998; Setoguchi et al., 2001). The presence of such mutations is associated with a poor response to treatment and reduction in the overall survival time (Koshino et al., 2016). Overexpression of p53 protein (mutated protein is not cleared from the cell) has also been associated with higher grade and older age in dogs with lymphoma (Sueiro, Alessi & Vassallo, 2004), with p53 protein expression being higher in T-cell lymphomas compared to B-cell lymphomas (Sokolowska, Cywinska & Malicka, 2005). p16 (also known as CDKN2), a regulator of the retinoblastoma (Rb) pathway of cell proliferation and inhibitor of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) is very commonly deleted or inactivated almost exclusively in high grade, T-cell lymphoma. However, alternative pathways that lead to Rb deficiency are also very prevalent in high grade B-cell lymphoma, particularly c-Myc overexpression, which increases CDK4 expression. Both p16 and Rb inactivation were associated with shorter survival times, which is concordant with the fact that Rb inactivation is much less common in dogs with low grade lymphoma (Fosmire et al., 2007).

1.2.3. Epigenetic changes

Epigenetic factors, such as DNA methylation and chromosome stability can also contribute to lymphoma development. Genomic hypomethylation is a feature of the majority of canine lymphoma cases and around one third of canine leukaemia cases, suggesting that deregulation of the normal DNA methylation mechanisms may be implicated in the pathogenesis of lymphoma (Pelham, Irwin & Kay 2003).

1.2.4. Telomerase activity

Telomerase activity is also increased in the lymph nodes of dogs with lymphoma compared to lymph nodes of control dogs (Renwick et al., 2006). Telomerase is an important enzyme in the maintenance of telomere length, which plays a key role in genomic stability. Telomeres are compound structures at the end of a chromosome which prevent chromosome ends from being recognized as sites of DNA damage. Shortening of the telomeres results in DNA damage responses and triggers cell senescence. Maintenance of telomere length by telomerase is crucial for the survival of cancer cells (Jafri et al., 2016), and POT1, which plays a role in both telomere maintenance and protection, has been found to be mutated in several cancer types, including lymphoma, in both humans and dogs (McMaster et al., 2018; Smith et al., 2020).

1.3. Genetic and transcriptomic similarities between human and canine lymphoma

Conventional diagnosis of canine lymphoma is based on cytology or histopathology for assessment of cell morphology and architecture, combined with immunocytochemistry, immunohistochemistry and flow cytometry to assess immunophenotype, and PCR for antigen receptor rearrangements (PARR) to assess clonality. However, in humans, molecular diagnostic techniques have been applied which allow further characterisation of lymphomas based on unique genetic expression patterns (or "signatures") and which can be predictive of prognosis and treatment outcome (Alizadeh et al., 2000). An example of this is the Lymph3Cx molecular assay that is currently being used in clinical trials for the treatment of different subtypes of lymphoma (Roschewski, Phelan & Wilson, 2020).

There has been an increasing interest in identifying molecular similarities and differences between human and canine lymphoma, not only to reinforce the role of canine lymphoma as an appropriate model for human lymphoma, but also to investigate possible therapeutics that may benefit both species (Bushell et al., 2015). For example, a fusion between the immunoglobulin light chain kappa locus (IGK) and Cyclin-D3 (CCND3) has been identified in one case of canine DLBCL, and it has been hypothesised that this could account for the CCND3 overexpression found in both species, even though this specific fusion has not yet been identified in human DLBCL (Ulve et al., 2017).

In humans, microarray analysis of B-cell lymphomas has identified two distinct gene expression patterns characteristic of different stages of B-cell differentiation: germinal centre B-like DLBCL (GCB) and activated B-cell like DLBCL (ABC). These transcriptional patterns are prognostically significant, with patients showing an activated B-like DLBCL expression profile having a much poorer overall survival (Alizadeh et al., 2000). Different B-cell lymphoid malignancies arise from cells at different stages of lymphocyte maturation and neoplastic lymphocytes seem to retain the genetic "signature" of the cell from which they originate (Lossos et al., 2000) but oncogenic mutations subvert their normal programme. For example, normal germinal-centre B cells lack the antiapoptotic activities of B-cell lymphoma 2 (BCL2) and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway and therefore will eventually undergo apoptosis. After malignant transformation, they avoid cell death by acquiring activating translocations of *BCL2* or by constitutively activating *NF-κB* (Lenz & Staudt 2010).

Canine B-cell lymphomas can also be separated into germinal and post-germinal centre subtypes using gene expression data, with similar expression profiles to the subtypes of human DLBCL (Richards et al., 2013). However, it appears that the relative frequencies of these subtypes differs significantly between humans and dogs, with the majority of human DLBCLs being classified as GCB and the majority of canine DLBCLs having genomic features suggestive of the human ABC subtype, namely the common enrichment of differentially expressed genes in the non-canonical *NF-κB* signalling pathway, *TRAF3* mutations and double expression of both *MYC* and *BCL2*. Other significant differences can be found between human and canine lymphoma in other lymphoma subtypes, namely in the expression of the anti-apoptotic BCL2 in follicular lymphomas. In 85% of human follicular lymphomas, BCL2 expression is typically enhanced by a chromosome translocation. Canine follicular lymphoma is a much less common subtype than in humans. Chromosome translocation is not a feature of this condition and BCL2 expression is very heterogenous despite other marked similarities between the human and canine disease. The changes in dogs may shed some light on the pathophysiology of the human cases which do not show the classic chromosome translocation (Thomas et al., 2017).

1.3.1. *NF-κB* signalling in human and canine lymphoma

The *NF-κB* signalling pathway is reported to be one of the most commonly perturbed pathways in human B-cell lymphoma (Grondona et al., 2018), and it has also been reported to be aberrant in canine B-cell lymphoma (Gaurnier-Hausser & Mason, 2015). It is also a good example of how gene expression profiling and better characterisation of specific lymphoma subtypes can lead to the discovery of new treatment targets and for this reason, it is discussed in more detail below.

1.3.1.1. Normal *NF-κB* signalling

NF-κB is a transcription factor family that mediates the cellular response to stimuli such as cytokines, free radicals, DNA damage, and bacterial or viral antigens, and is induced as a response to cellular stress (Mowla, Perkins & Jat, 2013). *NF-κB* has a regulatory role in inflammatory responses and other pathological processes, including cell survival, proliferation, differentiation, cell adhesion and the cellular microenvironment (Balaji et al., 2018; Perkins, 2012).

NF-κB also plays an important role in haematopoiesis, promoting survival and differentiation in all stages of cellular development of the lymphoid and myeloid cell lineages (Gerondakis et al., 2012), allowing activation of both the innate and adaptive immune system and initiating inflammatory responses to pro-inflammatory signals (Mowla, Perkins & Jat, 2013). When activated in these settings, *NF-κB* inhibits programmed cell death by transactivating the expression of antiapoptotic genes (Perkins & Gilmore, 2006), however it can also promote programmed cell death in response to apoptotic stimuli in certain cell types and thus exhibits pleiotropic behaviour (Kucharczak et al., 2003).

The two main pathways of *NF-κB* activation are the canonical (classical) and non-canonical (alternative) pathways. The canonical pathway is triggered by toll-like microbial pattern recognition receptors (TLRs) (Figure 2) and pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), with downstream effects leading to inflammatory responses, immune regulation, and cell proliferation (Lawrence et al., 2001). The non-canonical pathway is activated by initiation of B cell activation factor (BAFFR), CD40, lymphotoxin ß-receptor (LTßR), or receptor activator for nuclear factor kappa B (RANK) signalling with downstream effects leading to B cell maturation and lymphoid organogenesis (Balaji et al., 2018).

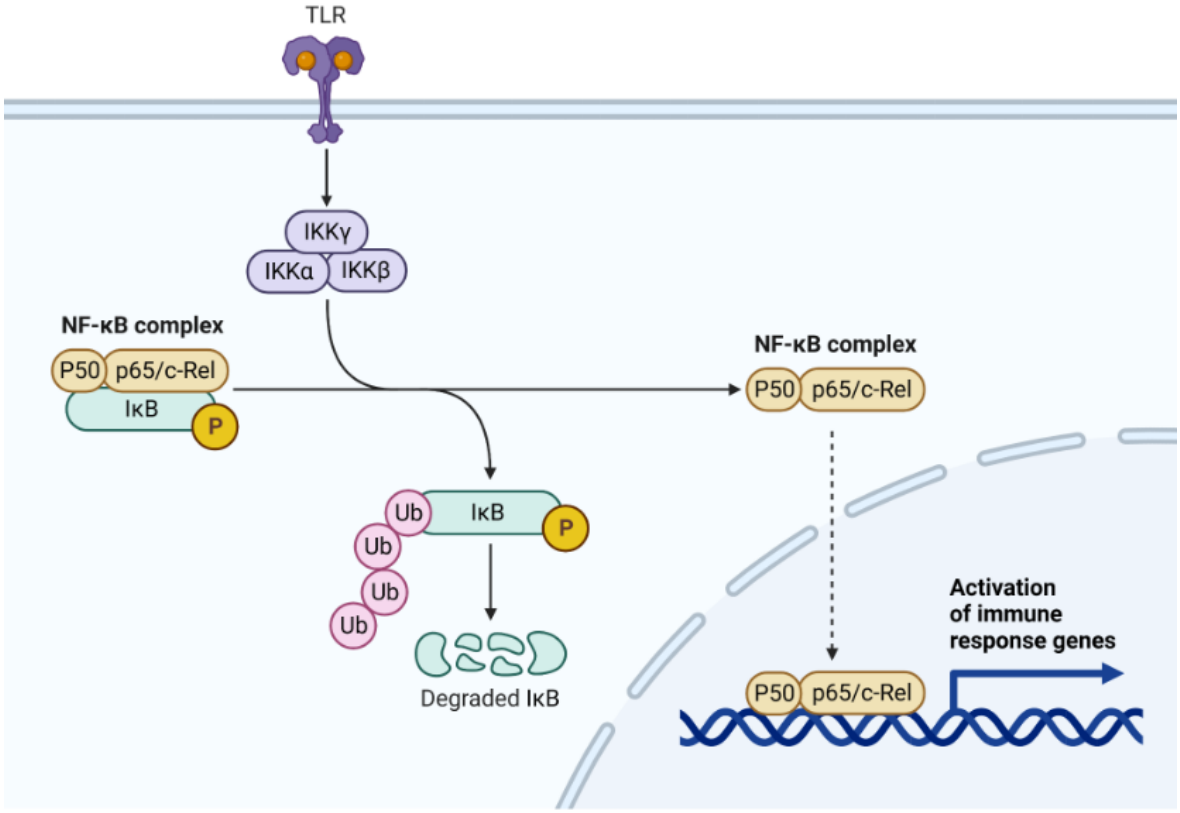

Figure 2. Example of activation of the canonical *NF-κB* pathway – adapted from "BioRender: Scientific Image and Illustration Software".

1.3.1.2. *NF-κB* signalling in B-cell lymphoma

NF-κB pathways play an important role in normal B-cell maturation and activation, which likely explains its key role in B-cell lymphoid malignancies (Staudt 2010). Constitutive activation of the *NF-κB* pathways is a common molecular event in both human and canine lymphoma, particularly in DLBCL (Seelig et al., 2017), with different patterns of transcriptional changes identified in the different subtypes, as mentioned previously. The human ABC subgroup of DLBCL expresses genes associated with plasma cells such X-Box Binding Protein 1 (*XBP1*), a regulator of immunoglobulin secretion, and Interferon Regulatory Factor 4 (*IRF4*), a key transcription factor in B-cell differentiation and activation, among many others (Mudaliar et al., 2013). Full differentiation into plasma cells is blocked in the ABC DLBCL subgroup, as they also have inactivating mutations in PR domain containing 1 with zinc finger domain (*PRDM1*)/B lymphocyte-induced maturation protein 1

(*BLIMP1*), which plays an important role in the terminal differentiation of plasma cells (Pasqualucci et al., 2006). In contrast, the GCB subgroup of DLBCL constitutively expresses B-cell lymphoma 6 (*BCL6*), which causes downregulation of genes involved in plasma cell differentiation, cell-cycle progression, responses to DNA damage, and apoptosis (Lenz & Staudt, 2010; Ci et al., 2009) and therefore stops differentiation into activated B-cells/plasma cells. *BCL6* expression is restricted to germinal centre B-cells. When antigens activate B-cell receptors, Mitogen-activated protein (MAP) kinase-mediated phosphorylation of BCL6 protein occurs, which leads to its degradation, and allows the cell to differentiate (Basso & Dalla-Favera, 2010). *NF-κB* activity is also repressed by BCL6 in human DLBCL cell lines, and this has been confirmed by a negative correlation between *NF-κB* associated genes in human cases of DLBCL and the levels of BCL6. This mutual negative regulation between *BCL6* and *NF-κB* further supports the GCB- and ABC-like DLBCL subclassification (Perez-Rosado et al., 2008). There is also similar direct inhibition between *BCL6* and *IRF4*. *IRF4* is silenced in centroblasts but is induced in B-cells exiting the germinal centre, and it can also directly repress BCL6, thus promoting terminal differentiation of B cells (Ci et al., 2009).

The canonical *NF-κB* pathway is the one most commonly activated in both human and canine DLBCL (Mudaliar et al., 2013), but increased expression of genes in the *NF-κB* non-canonical pathway has also been identified in a subset of canine DLBCL. This mirrors the situation in human ABC-like type DLBCL, although in dogs different mutated genes are implicated in the activation of the same pathway. The *TRAF3* gene, which encodes a negative regulator of the noncanonical *NF-κB* pathway, is inactivated in around 40% of canine B-cell lymphomas. Both germline and somatic mutations have been identified, suggesting both inherited and acquired mutations of this gene could play a role in the pathogenesis of canine B-cell lymphoma. TRAF3 mutations are also a feature of a subset of human DLBCL (Bushell et al., 2015), and are more prevalent in the ABC subtype (Schmitz et al., 2018).

Although changes in *NF-κB* pathway are mainly associated with B cell lymphoma, in a subset of human peripheral T-cell lymphomas, *NF-κB* pathways are also activated and are associated with an overall poor survival rate (Odqvist et al., 2013). When canine T-cell lymphomas have been investigated for the prevalence of known human T-cell lymphoma characteristics, significant overlap of gene

expression changes has been identified both at the transcriptional and protein level, with increased expression in the genes implicated in the mitogen-activated protein kinase (MAPK) pathway and *NF-κB* signalling, many of which may represent interesting targets for treatment (McDonald et al., 2018).

Although many studies point to a tumour-promoting function for *NF-κB* subunits, evidence also exists for tumour suppressor functions, particularly through the link between *NF-kB* and the tumour suppressor gene p53 (Perkins, 2004). There is evidence to suggest that *NF-κB* subunits can be recruited to the p53 tumour suppressor pathway, resulting in enhancement of p53 induced cell death, in turn leading to repression of cyclin D1 expression, and sensitisation of cells to TNF-induced apoptosis (Perkins & Gilmore 2006). The different roles of *NF-κB* as a tumour promotor or suppressor are still under investigation.

1.4. Next generation sequencing technology

The way in which genetic and transcriptional changes have been identified in lymphoma has changed over the years as techniques have progressed. Originally, targeted gene expression studies were performed, where the expression of specific genes likely to play a role in cancer were investigated one at a time. This has largely been replaced by global gene expression techniques, such as microarray analysis where the expression of multiple genes at the same time can be investigated to compare different tumours of the same histological subtype. Microarray technology has largely been superseded by next generation sequencing (NGS) and RNA-Seq. These technologies, which rely on the availability of a reference genome, allow the massive parallel sequencing of RNA molecules in biological samples, and this has changed the approach to the individual cancer patient in human medicine. Several canine genomes have also been sequenced, and as the dog is a good model for human diseases given the greater similarity of the canine genome to the human genome compared to the mouse genome (Paoloni & Khanna, 2008), efforts have been made to apply similar techniques to canine diseases (Morris, 2016).

Transcriptome-wide gene expression analysis with RNA isolation and array hybridisation has been used to identify prognostically significant subtypes of lymphoma in dogs, and similar drivers to some human lymphoma subtypes have been identified (Frantz et al., 2013). Briefly, the phases of an array experiment

can be divided into fabrication of the array, RNA extraction from the tumour, RNA preparation and labelling, application of the labelled RNA to the array, measurement of the relative amounts of hybridisation, image acquisition, and data analysis (Clarke et al., 2001). However, there are several limitations to microarray analysis, one of many being that previous knowledge of the sequences being analysed is essential for array design, which affects array effectiveness in the case of incorrect or incomplete genome information. Also, as microarray analysis relies on hybridisation between nucleic acids, analysis of highly related sequences and non-repetitive fractions of genome can be challenging because of cross-hybridisation. It can be also difficult to detect low-abundance genes or quantify changes in highly represented sequences. Additionally, results are not always accurately reproduced between different platforms and laboratories due to the large variety of microarray formats available (Shendure, 2008; Hurd & Nelson, 2009). This means it may difficult, if not impossible, to cross-compare datasets generated from different experiments.

Because of these limitations with microarrays, RNA-Seq has developed as an alternative method of assessing global gene expression. RNA-Seq features massively-parallel short-read DNA sequencing. The extracted RNA is reverse transcribed to cDNA (complementary DNA), adaptors are attached to one or both ends, and fragments are sequenced to give sequence 'reads' of 30–400 base pairs (bp) in length. Reads are either aligned against a reference genome or assembled without the genomic sequence to reconstruct the transcriptome of the sample (Morris, 2016; Hurd & Nelson, 2009). RNA-Seq has several advantages that have taken cancer genomics to a whole new level. For example, it does not carry any of the limitations previously mentioned about microarray analysis, it has a significant lower cost per base compared to earlier techniques, and it can generate as much data in 24 hours as several hundred Sanger-type DNA capillary sequencers, with the advantage of being operated by a single person.

Given the greater output associated with NGS, the knowledge of the genome and associated mutations and aberrantly expressed pathways has also expanded, which can have important practical applications clinically, both for diagnostic and therapeutic purposes. For example, depending on the specific mutations that are identified in a specific tumour from a specific patient, different drugs can be chosen to target a specific aberrant pathway (Khotskaya, Mills & Mills Shaw, 2017).

Although NGS has enabled analysis of tumour genomes to progress dramatically, several challenges are still associated with this approach, which the need for bioinformatic support for filtering and interpretation of the results given the complex nature of this analysis. Additionally, as a specific tumour can have multiple aberrant pathways, choosing which pathway should be targeted and with which drugs is not always clear (Khotskaya, Mills & Mills Shaw 2017) and assessing the significance of any genetic mutation detected represents a further challenge, as many mutations may simply be 'bystanders', which do not result in cell transformation (Morris, 2016).

1.5. Targeted therapies in humans

Conventional therapy for lymphoma involves systemic chemotherapy. The standard treatment for canine, clinically high-grade, lymphoma is a CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone)-based chemotherapy protocol, as it achieves the highest response rates and longest response durations, with median survival times reported around 10-12 months for B-cell lymphoma (Zandvliet, 2016; Curran & Thamm, 2016). T-cell lymphomas carry a worse prognosis with the same treatment (Rebhun et al., 2011), and despite multiple other protocols being attempted, survival times have not significantly improved and remain around 6-12 months (Angelo, Cronin & Keys 2019; Brown et al., 2018).

Genomic analysis has changed the treatment of human lymphoma dramatically in recent years, leading to the availability of targeted treatment options for different lymphoma subtypes. The standard treatment for DLBCL in humans is R-CHOP, a combined chemo-immunotherapy treatment with rituximab, an anti-CD20 monoclonal antibody, and a CHOP-based chemotherapy protocol (Susanibar-Adaniya & Barta, 2021). As ABC DLBCL carries a worse prognosis than GCB DLBCL, efforts have been made to target B-cell receptor (BCR) signalling and *NF-κB* pathway activation in this subtype, although with limited success. However, addition of ibrutinib, an inhibitor of Bruton tyrosine kinase (BTK), which is required for the activation of *NF-κB,* has led to an improved outcome in a subset of younger patients and/or patients with overexpression of *BCL-2* and *MYC* (Younes et al. 2019; Susanibar-Adaniya & Barta, 2021). Interestingly, this drug was first shown to be useful in canine B-cell non-Hodgkin lymphoma, further highlighting the crossover between the human and canine species (Honigberg et al., 2010).

Different treatment options are also being developed for 'double-hit' or 'triple-hit' DLBCL (DLBCL with *MYC* and *BCL2* and/or *BCL6* rearrangements), and BCL-2 overexpression, with improved outcomes compared to standard treatments (Morschhauser et al., 2021; Dunleavy et al., 2018).

1.5.1. Possible treatment targets in canine lymphoma

In the same way that the *NF-κB* pathway has been targeted in human lymphoma, there has been some preliminary research in dogs. Interference with the non-canonical *NF-κB* pathway by small interfering RNAs has led to apoptosis in canine DLBCL cells, suggesting this pathway may be a possible treatment target (Seelig et al., 2017). In addition, selective inhibitors of nuclear transport (SINE) have shown activity against canine lymphoma, which may be secondary to the stabilisation of IκB (inhibitor of *κB*), an inhibitory molecule of the *NF-κB* pathway, in the cell nucleus, which may neutralise *NF-κB* activity (Schlein & Thamm, 2022). Some clinical advances are now happening in this field, with verdinexor, a compound with SINE activity, being recently approved for the treatment of canine lymphoma (Sadowski et al., 2018). In a similar way, selective inhibitors of IκK (IκB kinase), a complex responsible for phosphorylation of IκB and its subsequent degradation, have been used in the treatment of dogs with relapsed B-cell lymphoma and have resulted in reduced *NF-κB* target gene expression and reduced tumour burden (Gaurnier-Hausser et al., 2011). Several other strategies have been employed to target the *NF-κB* pathway with some success, emphasising the importance of further characterisation of driver mutations and aberrant pathways in canine lymphoma, which can lead to more varied and targeted treatment options (Guzman et al., 2007; Kojima et al., 2013; Matsuda et al., 2010; Pawlak et al., 2017). In canine acute lymphocytic leukaemia, an internal tandem duplication of FLT3 (Fms Related Receptor Tyrosine Kinase 3) was found in 12% of cases, and treatment with a FLT3 inhibitor resulted in a decrease in cell line growth and inhibition of downstream signalling pathways (Suter et al., 2011). It is hoped that further research will propel forward the possibilities for the treatment of canine lymphoma.

BTK inhibitors have also been trialled for the treatment of canine lymphoma. Use of acalabrutinib, which is more selective and potent than ibrutinib, resulted in reduced proliferation of a canine lymphoma cell line and also led to a modest clinical response in dogs with spontaneous lymphoma (Harrington et al., 2016).

1.6. Objectives:

Although transcriptomic analysis has increasingly been applied to canine lymphoma in recent years, overall, the number of studies remains small as does number of cases examined. A previous PhD project conducted at the University of Glasgow (EW PhD) generated a transcriptomic dataset from a small group of B-cell and T-cell lymphomas, but was limited by overall small sample size, a relatively low proportion of T-cell subtypes and a lack of normal control tissue to use as a comparator. Consequently, the aim of this thesis was to investigate gene expression using RNA-Seq in a larger sample of both B and T-cell canine lymphomas as well as in control material from lymph nodes from dogs without lymphoma. The objectives of this work were to (1) improve subclassification of each lymphoma immunophenotype and (2) identify unique gene pathways which may have a role in pathogenesis and could be targeted for treatment. Although this project was designed as a stand-alone study, there also existed the opportunity to include the previously generated EW PhD dataset to maximise the sample size and therefore data validity.

2. Materials and Methods

2.1. Initial tissue sample selection

Tissue samples (both lymphoma and controls) were collected from dogs referred to the Small Animal Hospital of the University of Glasgow for investigation of their condition between March 2016 and April 2021, with informed consent obtained from owners prior to sampling. Collection of samples was approved by the School of Veterinary Medicine Ethics committee (Ref EA40/19).

2.1.1. Dogs with lymphoma

2.1.1.1. Inclusion criteria

Dogs in the lymphoma group required a confirmed cytological or histopathological diagnosis of lymphoma and immunophenotyping through either immunohistochemistry, PARR or flow cytometry. Immunophenotype as part of the diagnostic work up of the case was not required for sample inclusion, provided it was determined retrospectively before NGS was performed.

2.1.1.2. Exclusion criteria

Samples with a low RNA concentration (ie samples with less than 1µg of RNA available for NGS) or quality (RNA integrity number (RIN) < 5) after initial sample quality assessment were excluded from NGS.

2.1.2. Control Group

2.1.2.1. Inclusion criteria

Dogs in the control group were client owned dogs that presented for a variety of neoplastic and non-neoplastic reasons. These dogs required a presumptive or definitive diagnosis for the complaint that brought them to the hospital and no evidence of lymphoma on investigations performed during their period of hospitalisation. If they had neoplasia, it was either a non-haemopoietic neoplasia or the lymph node extracted was distant from the site of neoplasia, which was not a part of the usual metastatic drainage pathway and therefore unlikely to contain metastatic cells. For example, this may include a normal sized popliteal lymph node in a dog diagnosed with hepatocellular carcinoma.

2.1.2.2. Exclusion criteria

Dogs were excluded from the control group if they had a diagnosis of (non-nodal) lymphoma or lacked a definitive diagnosis but lymphoma was one of the main

differential diagnoses. Samples with a low RNA concentration or quality score after initial sample quality assessment were also excluded from NGS.

2.2. Sample collection

For dogs with lymphoma, lymph node samples (either biopsy samples or fine needle aspirate samples) were collected as part of the diagnostic management of the case or at the time of euthanasia, either before treatment (*naïve* lymphoma) or at the time of relapse (i.e. from gross disease). Surplus diagnostic material was retained for the study. All samples (biopsy and fine needle aspirates samples) were stored in viral transport medium (VTM) at -80°C until processing. The majority of the lymph node samples from the control dog group (7/8) were collected purely for the research project, shortly after euthanasia (less than 6 hours) through incisional or excisional biopsy, although one sample was obtained *ante-mortem* during a planned surgical procedure (amputation of a thoracic limb).

2.3. Sample processing prior to NGS

2.3.1. Immunophenotyping

For lymphoma samples which were not immunophenotyped as part of the diagnostic work up for the case, DNA was extracted from the tissue samples using the All prep DNA/RNA mini kit (Qiagen) at the same time as RNA extraction to obtain samples for NGS. The DNA samples were subsequently sent to VPG Exeter (Unit 1b Exeter Science Park, Babbage Way Clyst Honiton, Exeter EX5 2FN) for PARR. The immunophenotype of the lymphoma, defined in terms of cell clonality, was determined using B-cell and T-cell primers.

2.3.2. RNA and DNA extraction

The All prep DNA/RNA mini kit (Qiagen) was initially used to extract RNA from both lymphoma and control samples, together with DNA from the lymphoma samples that had not been immunophenotyped during the diagnostic work up. Before DNA/RNA extraction, all biopsy samples were sliced into smaller specimens (<30 g) according to the manufacturer's instructions. Both the smaller biopsy specimens and fine needle aspirate specimens were transferred to RNAlater® (Thermo Fisher Scientific, Waltham, MA USA). The rest of the DNA/RNA extraction process was conducted according to the manufacturer's instructions. The goal was to have between 1 – 4 µg of RNA per sample for RNA-

Seq, so depending on each sample concentration a final volume of 10 – 68 µl was obtained per sample for NGS. Residual DNA was removed from each RNA sample using the DNase Max kit (Qiagen). All samples were stored at -80 °C until it was time for further analysis.

2.3.3. Sample quality assessment

The quality and concentration of the extracted RNA was assessed using the Agilent Bioanalyzer 2100 with the Agilent RNA 6000 Nano kit (Agilent Technologies LDA UK Ltd, Stockport, UK) according to the manufacturer's instructions (Table 3).

Table 3: RNA quality results from 26 samples extracted with the All prep DNA/RNA mini kit (Qiagen)

Sample	Concentration (ng/µl)	Group	RIN
A01	76.94	Control	5.9
B01	45.72	Control	8.3
C01	90.70	Control	6.4
D01	295.75	Control	5.0
E01	200.52	Control	6.2
F01	136.03	Control	4.0
G01	39.55	T-cell LSA	5.8
H01	82.03	B-cell LSA	8.3
A02	139.42	B-cell LSA	8.6
B02	156.13	B-cell LSA	8.3
C02	159.92	B and T-cell LSA	7.6
D02	65.88	T-cell LSA	2.6
E02	79.89	B-cell LSA	4.8
F02	69.02	T-cell LSA	8.5
G02	1.71	T-cell LSA	NA
H02	5.63	T-cell LSA	[6.5]
A03	314.08	B-cell LSA	8.6
B03	58.98	Control	7.3
C03	49.20	Control	7.3
D03	103.50	Control	6.7
E03	14.81	T-cell LSA	8.3
F03	40.18	T-cell LSA	8.0
G03	391.94	B-cell LSA	8.0
H03	100.76	B-cell LSA	8.6
A04	66.00	T-cell LSA	7.5
B04	228.87	T-cell LSA	9.2

LSA: lymphoma; RIN: RNA quality score

2.3.4. Additional RNA extractions and quality assessment

The samples G02 and H02 did not have sufficient RNA concentration for further processing so they were excluded at this stage.

Given the recommended minimum RIN>8 for RNA samples sent for next generation sequencing, a different method of extraction with TRIzol (Invitrogen™) was attempted for 11 of the 13 samples with RIN <8 and the RNA quality was again assessed (Table 4). For these 11 samples, the tissue was lysed by adding 1ml of TRIzol to 50-100mg of tissue samples and homogenising. For RNA isolation, the lysate and TRIzol were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform per millilitre of TRIzol was added and the tube was shaken vigorously for 15 second, followed by incubation at room temperature for 2-3 minutes. The sample was centrifuged at 12,000 g for 15 minutes at 4 °C. After centrifugation, 400 µl of the upper aqueous phase which contained the RNA were transferred to a new RNase-free tube, and 400 µl of 70 % ethanol were also added. The mixture was vortexed to mix well. Binding, washing and elution were the final stages of the protocol. Here, 700 µl of the mixture were transferred to a Spin Cartridge (Qiagen) with a collection tube, which was then centrifuged at 12,000 g for 15 seconds at room temperature. The flow-through was discarded and the Spin Cartridge was reinserted into the same Collection Tube. These steps were repeated until the entire sample was processed. Then, 700 µl of Wash Buffer I were added to the Spin Cartridge, and then the mixture was centrifuged at 12,000 g for 15 seconds at room temperature. After this, the flow-through and the Collection Tube were discarded, the Spin Cartridge was inserted into a new Collection Tube and 500 µl of Wash Buffer II with ethanol were added to the Spin Cartridge, and the mixture was centrifuged at 12,000 for 15 seconds at room temperature. The flow-through was discarded and the Spin Cartridge was reinserted into the same Collection Tube. This step was repeated once more. Afterwards, the mixture was again centrifuged at 12 000 g for one minute at room temperature to dry the membrane. The Collection Tube was discarded and the Spin Cartridge was inserted into a Recovery Tube. Then, 30-100 µl of RNase-Free Water were added to the centre of the Spin Cartridge and the sample was incubated at room temperature for one minute. The Spin Cartridge with the Recovery Tube were centrifuged one last time for 2 minutes at > 12,000 g at room temperature to obtain the purified RNA, which was

then stored at -80°C. The quality and concentration of the extracted RNA was again assessed using the Agilent Bioanalyzer 2100 with the Agilent RNA 6000 Nano kit (Agilent Technologies LDA UK Ltd, Stockport, UK) according to the manufacturer's instructions (Table 4).

Table 4: RNA quality results from 11 samples re-extracted with the TRIzol method compared with the All prep DNA/RNA mini kit (Qiagen)

Sample	Group	RIN TRIzol	RIN All prep DNA/RNA mini kit
D02	T-cell LSA	2.1	2.6
A01	Control	3.5	5.9
C01	Control	5.8	6.4
D01	Control	2.5	5.0
E01	Control	2.5	6.2
F01	Control	2.6	4.0
G01	T-cell LSA	3.0	5.8
C02	B and T-cell LSA	9.6	7.6
B03	Control	6.9	7.3
C03	Control	7.4	7.3
D03	Control	6.4	6.7

LSA: lymphoma; RIN: RNA quality score

2.3.5. Final sample selection for NGS

As the quality of the RNA was worse with the TRIzol extraction method when compared to the All prep DNA/RNA mini kit (Qiagen) for 8 of the 11 samples, ongoing sample analysis was performed on the samples extracted with the All prep DNA/RNA mini kit (Qiagen). As the budget allowed for only 20 samples to undergo next generation sequencing, and a total of 26 samples had been extracted, the six samples with the worst RIN and/or RNA concentration were excluded (Table 5).

Table 5: Samples that were excluded after RNA quality analysis

Sample	Concentration (ng/µl)	Group	RIN
A01	76.94	Control	5.9
B01	45.72	Control	8.3
C01	90.70	Control	6.4
D01	295.75	Control	5.0
E01	200.52	Control	6.2
~~F01~~	~~136.03~~	~~Control~~	~~4.0~~
~~G01~~	~~39.55~~	~~T-cell LSA~~	~~5.8~~
H01	82.03	B-cell LSA	8.3
A02	139.42	B-cell LSA	8.6
B02	156.13	B-cell LSA	8.3
C02	159.92	B and T-cell LSA	7.6
~~D02~~	~~65.88~~	~~T-cell LSA~~	~~2.6~~
~~E02~~	~~79.89~~	~~B-cell LSA~~	~~4.8~~
F02	69.02	T-cell LSA	8.5
~~G02~~	~~1.71~~	~~T-cell LSA~~	~~NA~~
~~H02~~	~~5.63~~	~~T-cell LSA~~	~~[6.5]~~
A03	314.08	B-cell LSA	8.6
B03	58.98	Control	7.3
C03	49.20	Control	7.3
D03	103.50	Control	6.7
E03	14.81	T-cell LSA	8.3
F03	40.18	T-cell LSA	8.0
G03	391.94	B-cell LSA	8.0
H03	100.76	B-cell LSA	8.6
A04	66.00	T-cell LSA	7.5
B04	228.87	T-cell LSA	9.2

LSA: lymphoma; RIN: RNA quality score

2.4. Library preparation and next generation sequencing

The final 20 purified RNA samples were sent for library preparation and sequencing at Glasgow Polyomics, University of Glasgow. Total RNA was rRNA depleted using the Qiagen Fast Select HMR kit, and the libraries prepared using the TruSeq® Stranded mRNA kit (Illumina), according to the manufacturer's instructions. This kit recommended using input RNA with a RIN ≥8 and between 0.1-4 ug of RNA per sample. After purification of the RNA, paired-end sequencing was performed on an Illumina NextSeq 500 sequencer. Unlike single-end sequencing, paired-end sequencing allows sequencing of both ends of a fragment, leading twice the number of reads and more accurate read alignment (Bashir et al., 2008).

2.4.1. Next Generation sequencing technique

Figure 3 shows a simplified representation of the next generation sequencing technique. The first step before sequencing was the conversion of RNA into cDNA (complementary DNA) via reverse transcription. Then, the cDNA was fragmented, and the sample was prepared for sequencing, which included the addition of adapters to the ends of the cDNA fragments. Additional motifs were also introduced, such as sequencing binding sites and regions complementary to the flow cell oligonucleotides. The flow cell is a glass slide with lanes where the amplification takes place. Each lane is a channel coated with a lawn composed of two types of oligonucleotides. Hybridisation was enabled by the first of the two oligonucleotides on the surface, which was complementary to the adapter region on one of the fragment strands. A polymerase created a strand complementary to the hybridised fragment and this new double stranded molecule was denatured, and the original template was washed away. The strands were clonally amplified through bridge amplification. In this process, the strand folded over and the adapter region hybridised to the second species of oligonucleotide on the flow cell; a polymerase again generated a complementary strand, creating a double stranded bridge. When the bridge was denatured, it created two single stranded copies of the molecule that were tethered to the flow cell. This process was repeated over and over again, and occurred simultaneously for millions of clusters, resulting in clonal amplification of all the fragments. After bridge amplification, the reverse strands were cleaved and washed off, leaving only the forward strands for sequencing. The 3' ends were blocked to prevent unwanted priming. Sequencing then started with the extension of the first sequencing primer to

produce the first read. With each cycle, fluorescently tagged nucleotides competed for addition to the growing chain, and only one was incorporated based on the sequence of the template. After the addition of each nucleotide, the clusters were excited by a light source and a characteristic fluorescent signal was emitted. The numbers of cycles determine the length of the read and the emission wavelength, along with the signal intensity, determined the base call. Millions of clusters were sequenced in a massively parallel process. After completion of the first read, the read product was washed away. In this step, the index 1 read primer was introduced and hybridised to the template, and the read was generated in the same way as the first read. After completion of the index read, the read product was washed off, and the 3' ends of the template were deprotected. The template then folded over and bound to the second oligo on the flow cell. Index 2 was read in the same manner as index 1, and polymerases extended the second flow cell oligo, forming a double stranded bridge, which was then linearised and the 3' ends were blocked. The original forward strand was cleaved off and washed away, leaving only the reverse strand. Read 2 began with the introduction of the read 2 sequencing primer, and in the same way as for read 1, the sequencing steps were repeated until the desired read length was achieved, and the read 2 product was then washed away. In the end of this process, for each sample, reads with similar stretches of base calls were locally clustered, and forward and reverse reads were paired, creating contiguous sequences, which were later aligned to the reference genome (see below).

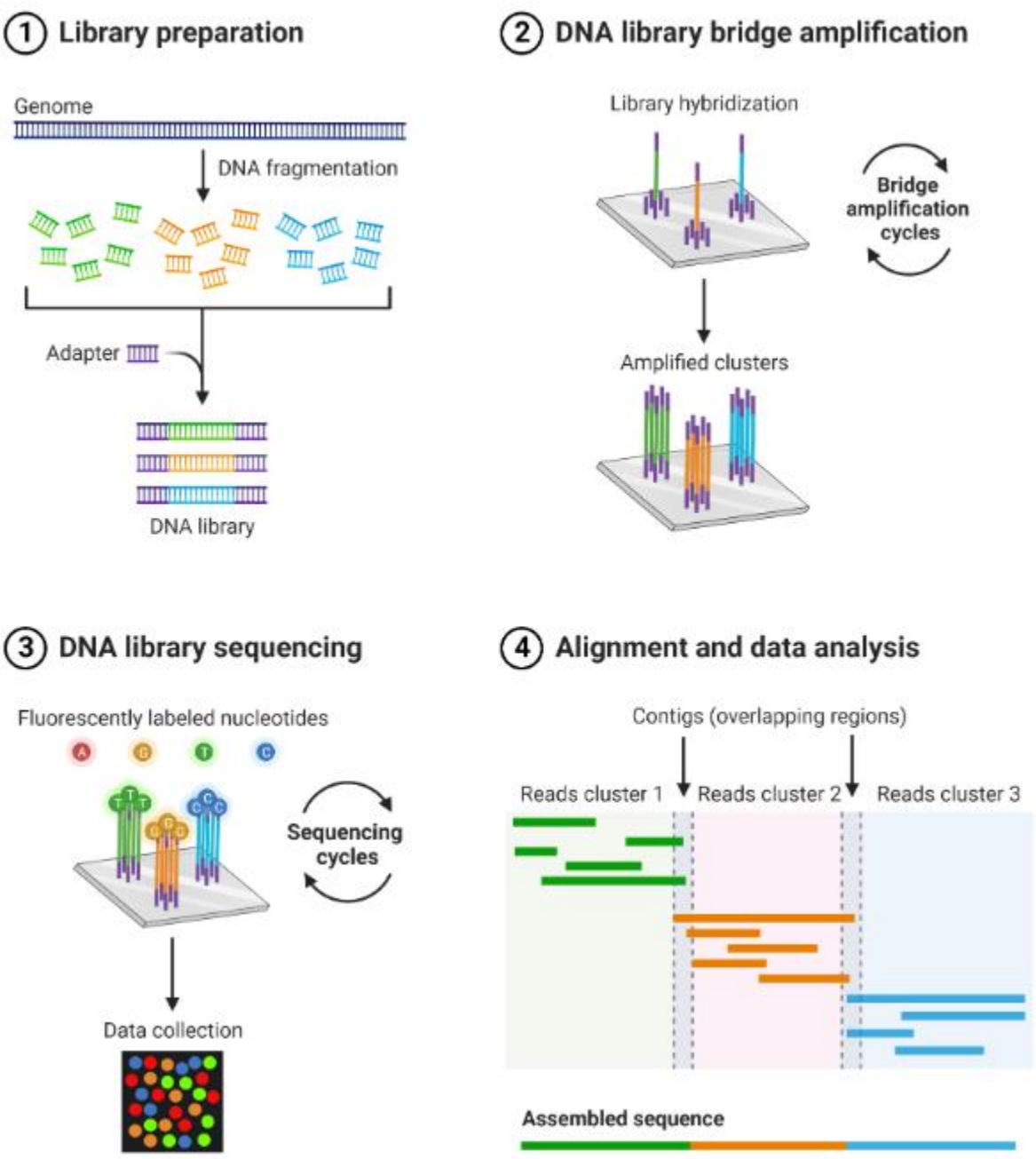

Figure 3: Next generation sequencing technique – adapted from "BioRender: Scientific Image and Illustration Software".

2.5. Preliminary data analysis pipeline

A summary of the initial data processing of each sample is shown in Figure 4. Following sequencing, the data was first quality assessed by FastQC. This indicated there were no issues with any of the quality metrics. Adapters were then removed using Trimmomatic 0.39 with the default paired end settings (Bolger, Lohse & Usadel, 2014) and aligned to the reference genome (CanFam6) (non-predicted transcripts) using the default parameters of STAR 2.7.1.a (Dobin et al., 2013), and not the predicted transcriptome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000002285.5/). CanFam6 has a 100-fold coverage, and the mean sequencing depth for exons was approximately 11x across all samples, although as depth is related to expression level there was a large range of depth within each sample. The number of reads mapping to each gene were quantified using the quantmode function of STAR. Raw count data for all samples were imported into the DESeq2 1.8.1 package (Love et al., 2014) of R 3.2.1 (R Core Team, 2017) and used to identify differential gene expression and perform principal component analysis (PCA). Intergroup comparisons of differential expression (B-cell lymphoma vs T-cell lymphoma, T-cell lymphoma vs controls and B-cell lymphoma versus controls) were performed using the contrast function of DESeq2 and likelihood ratio tests (LRTs). For pathway analysis, all genes with an adjusted p value < 0.05 were introduced into Reactome and mapped against human data. To account for the high false discovery rates inherent to transcriptomic studies, p values were adjusted to account for false discovery using the Benjamini and Hochberg approach implemented in DESeq2 (Love et al., 2014). Finally, the 1,000 most significant differentially expressed transcripts in each comparison were used for pathway enrichment analysis using the Reactome 3.7 database (Fabregat et al., 2018), mapping canine gene IDs to human where possible. Interactors were not included.

After initial analysis, the initial dataset was combined with a previous dataset (EW PhD), which will be detailed in section 3.2. For this step, batch correction was performed to correct expression differences potentially arising due to batch effect using an empirical Bayes approach with the ComBat function of sva 3.48 (Zhang, Parmigiani & Johnson, 2020).

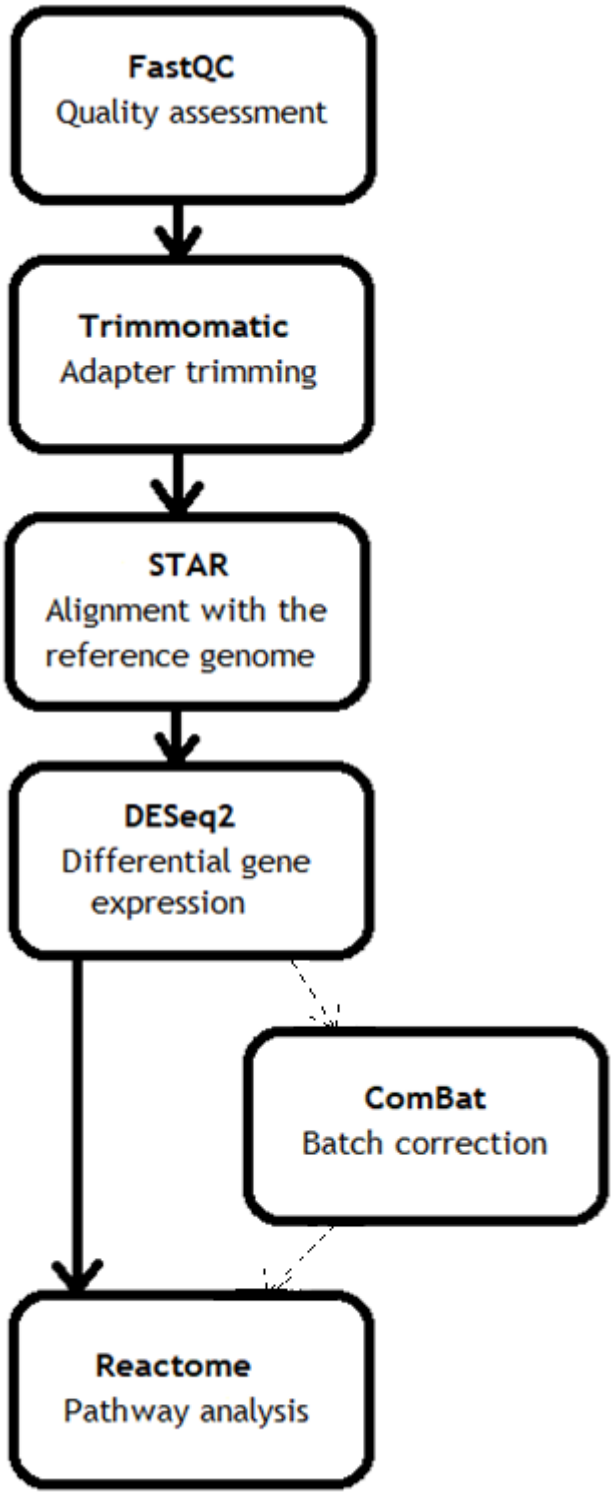

Figure 4: Preliminary data analysis pipeline. Thick arrows represent the main pipeline and dotted thin arrows represent the alternative pipeline that was followed when the initial dataset was combined with an additional dataset

3. Results

3.1. Initial dataset analysis

3.1.1. Patient characteristics

At the end of the sample collection process, tissue samples from six dogs with B-cell lymphoma, five dogs with T-cell lymphoma and one dog with B and T-cell lymphoma were recruited for the lymphoma group (Table 6). The majority of samples (8/12) were collected at the time of diagnosis prior to any treatment, although four out of the twelve dogs had received either steroids (two dogs, sample ID B02 and E03) or steroids and chemotherapy (two dogs, sample ID F03 and G03). Lymph nodes from a control group of eight dogs that had been seen at the Small Animal Hospital of the University of Glasgow for a variety of other conditions were also collected (Table 6). Samples from these 20 dogs were sent for NGS.

Table 6: Characteristics of 20 patients from which samples were submitted for NGS

Sample ID	LSA Lineage	Presentation	Age	Sex	Breed
H01	B-cell	Multicentric	3 years	MN	Shih-tzu
A02	B-cell	Multicentric	10 years	FN	Collie cross
B02	B-cell	Multicentric	10 years	MN	Crossbreed
A03	B-cell	Multicentric	13 years	MN	Bichon Frise
G03	B-cell	Multicentric	11 years	FN	JRT
H03	B-cell	Multicentric	6 years	FE	GSD
F02	T-cell	Multicentric	7 years	ME	Border Collie
E03	T-cell	Muscular	5 years	FN	Beagle
F03	T-cell	Multicentric	6 years	MN	Cockapoo
B04	T-cell	Multicentric	11 years	MN	Bearded Collie
A04	T-cell	Multicentric	10 years	MN	Boxer
C02	B and T-cell	Mediastinal	1 year	FN	Tibetan Terrier

Sample ID	Type of control	Diagnosis	Age	Sex	Breed
B01	Non-neoplastic	Diabetes mellitus	12 years	FN	Dachshund
B03	Non-neoplastic	Fore limb fracture	1 year	ME	Border Collie
C01	Non-neoplastic	Pulmonary oedema	14 years	MN	Chihuahua
A01	Non-neoplastic	MUO	3 years	ME	Boxer
D01	Non-neoplastic	Myelomalacia	5 years	FE	Dachshund
C03	Neoplastic	Osteosarcoma	7 years	ME	BMD
D03	Neoplastic	Haemophagocytic histiocytic sarcoma	3 years	ME	BMD
E01	Neoplastic	Hepatocellular carcinoma	6 years	MN	Crossbreed

LSA: lymphoma; MN: Male Neutered; ME: Male Entire; FN: Female Neutered; FE: Female Entire; MUO: Meningoencephalitis of unknown origin; BMD: Bernese Mountain Dog; JRT: Jack Russell Terrier; GSD: German Shepherd Dog

3.1.2. NGS Data Quality

The mean number of reads generated per sample was 53,365,780 (range 40,528,626 to 95,496,546). The mean number of reads discarded after adapter trimming and quality filtering was 3,625,905 (range 1,988,128 to 5,661,158), leaving a mean of 49,739,874 reads (range 38,540,498 to 90,184,652) with a mean PHRED score > Q30 of 93%, indicating very good base call accuracy. The paired end reads (mean 24,869,937, range 19,270,249 to 45,092,326) were then mapped to the reference dog genome (CanFam6) with a mean mapped percentage of 92% (range 82 to 95%) (Table 7).

Table 7: Sequencing data statistics: mapping of paired end reads to reference dog genome

Sample ID	Total Paired Reads	Mapping %	Mean Exon Depth
H01	21,993,894	93%	10.17867878
A02	21,181,383	94%	9.660058432
B02	24,027,496	95%	9.310058191
A03	24,804,929	92%	16.46927651
G03	25,301,557	94%	14.23504017
H03	24,079,018	94%	11.39274029
F02	45,092,326	82%	32.46635416
E03	32,688,050	92%	3.54240606
F03	22,261,288	94%	10.16807059
B04	22,619,098	91%	13.91405686
A04	24,174,292	88%	11.35099784
C02	24,804,929	92%	16.46927651
B01	28,998,647	95%	10.60702109
B03	22,387,974	93%	10.86504878
C01	24,212,334	93%	11.00862881
A01	19,270,249	92%	7.483261653
D01	19,575,773	91%	7.395730852
C03	24,313,186	85%	13.31561243
D03	21,517,782	90%	7.917583811
E01	24,094,543	94%	8.419305474

3.1.3. Principal component analysis

Principal component analysis was performed to visually analyse the difference in gene expression between the three groups (B-cell lymphoma, T-cell lymphoma and controls). This was obtained using DESeq2 VST (Variance stabilised transformation). The three groups formed three distinct clusters with a single B and T-cell lymphoma sample (sample 12) sitting in between the B-cell and T-cell lymphoma clusters (Figure 5). PC1 accounted for 32% of the variance in the dataset and could discriminate B-cell lymphomas and controls from T-cell lymphomas. PC2 accounted for 16% of the variance and discriminated the controls and T-cell lymphomas from the B-cell lymphomas. There was a single T-cell outlier (sample 8), which was a dog with muscular T-cell lymphoma. The tissue collected from this dog was a normal sized lymph node, which may account for difference in its gene expression compared to the other T-cell samples.

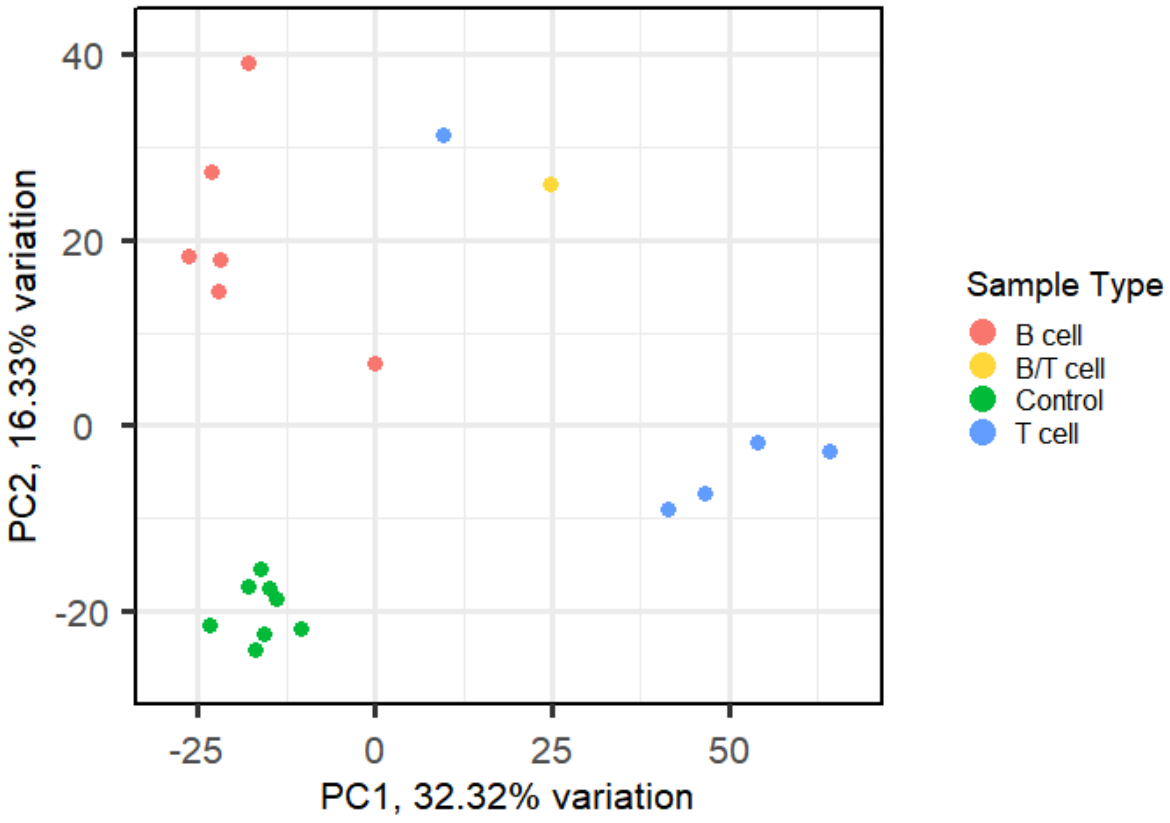

Figure 5: PCA plot for all 20 samples

3.1.4. Gene expression

Intergroup comparisons were performed for gene expression analysis. The B and T-cell lymphoma sample (sample C02, number 12 on the PCA plot) was excluded, as it did not belong to any of the groups. The T-cell outlier (sample E03, number 8 on the PCA plot) had a clearly distinct gene expression profile from the rest of the group and, unusually, originated from a grossly normal lymph node. It was decided, therefore, that this sample was anomalous and un-representative of the T-cell lymphoma group and so was also excluded from the intergroup analysis. Thus only 18 dogs were included in the final analysis.

Initial comparisons between samples examined differential expression of genes on a global scale. There were 491 differentially expressed genes (adjusted p value < 0.05) when comparing B-cell lymphomas and controls. Appendix 1 shows the top 50 differentially expressed genes between B-cell lymphoma and controls. The gene which was most differentially overexpressed in B-cell lymphoma was FLT-1, which encodes Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1), with an adjusted p value of 3.73×10^{-12} and a log2fold decrease of 4.71. When looking at genes previously reported to be aberrantly expressed in canine B-cell lymphoma, MAP3K14 (Mitogen-activated protein kinase kinase kinase 14) was overexpressed in B-cell lymphomas when compared to controls with a log2fold decrease of 2.33, although this did not achieve significance (adjusted p value 0.055). There was no significant difference in the expression of MYC, BCL2, TRAF3 or POT1.

There were 1,017 differentially expressed genes (adjusted p value < 0.05) when comparing T-cell lymphomas and controls. Appendix 2 shows the top 50 differentially expressed genes between T-cell lymphoma and controls. KIT (CD117) was significantly overexpressed in T-cell lymphomas when compared to controls, with a log2fold change of -5.45 and an adjusted p value of 0.002. There was no significant difference in the expression of PTEN, GATA3 (GATA binding protein 3), TBX21 (T-Box Transcription Factor 21), MTOR or SATB1 (Special AT-rich Sequence Binding Protein 1).

There were 623 differentially expressed genes (adjusted p value < 0.05) when comparing B-cell lymphomas and T-cell lymphomas. Appendix 3 shows the top 50 differentially expressed genes between B-cell lymphoma and T-cell lymphoma.

and controls. KIT and GATA3 were significantly overexpressed in T-cell lymphomas when compared to B-cell lymphoma, with a log2fold change of -4.11 and -6.42, respectively, and an adjusted p value of 0.04 and 0.0003, respectively. There was no significant difference in the expression of MYC, BCL2, TRAF3, POT1, PTEN, TBX21, MTOR or SATB1.

3.1.5. Pathway analysis

After individual gene expression comparisons, the distribution of differentially expressed genes among signalling pathways was compared, taking all genes with an adjusted p value <0.05 into consideration. Several differentially expressed pathways were identified using Reactome software based on gene expression levels and mapping gene symbols to human data to predict the potential effects on pathway.

When comparing B-cell lymphomas with controls, 58 pathways were found to be differentially expressed, with 48 being upregulated and 10 being downregulated. This was calculated using Reactome Z score which is provided based on available literature. Some pathways were found in more than one category, which in this context are broader pathway families, such as cell cycle control, immune system, metabolism, amongst many others. Of the pathways found to be upregulated (Table 8a), 14 were related to cell cycle control, 11 were related to gene expression and transcription, seven were related to the immune system, six were related to metabolism, three were related to disease, two were related to DNA replication, two were related to metabolism of proteins, two were related to signal transduction, two were related to transport of small molecules, one was related to vesicle-mediated transport, and one was related to cellular responses to stimuli.

Table 8a. Pathways up-regulated in B-cell lymphomas when compared with controls

Pathway name	**FDR**	**Fold change**
Proton/oligopeptide cotransporters	0.00001	2.16840592286992
Events associated with phagocytolytic activity of PMN cells	0.00001	2.06621982148459
APC/C-mediated degradation of cell cycle proteins	0.00001	0.427536678642833
Regulation of mitotic cell cycle	0.00001	0.427536678642833
APC/C:Cdc20 mediated degradation of mitotic proteins	0.00001	0.422754261745307
Cdc20:Phospho-APC/C mediated degradation of Cyclin A	0.00001	0.410620714235054
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfaction of the cell cycle checkpoint	0.00001	0.40866056061599
Regulation of APC/C activators between G1/S and early anaphase	0.00001	0.371299892443741
Asymmetric localisation of PCP proteins	0.00001	0.31029998823412
TCR signalling	0.00001	0.276595051953903
Downstream TCR signalling	0.00001	0.261884990733519
Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins	0.001	0.42740751126707
TICAM1, RIP1-mediated IKK complex recruitment	0.001	0.305039740658034
RNA Polymerase III Transcription Initiation From Type 1 Promoter	0.002	0.341789184100382
RNA Polymerase III Transcription Initiation From Type 2 Promoter	0.002	0.339389943094457
IKK complex recruitment mediated by RIP1	0.002	0.307768063207777
G2/M DNA replication checkpoint	0.003	0.664227481343376
Phosphorylation of proteins involved in the G2/M transition by Cyclin A:Cdc2 complexes	0.003	0.492128666254587

CDK-mediated phosphorylation and removal of Cdc6	0.003	0.329980908162638
Defective SLC39A4 causes acrodermatitis enteropathica, zinc-deficiency type (AEZ)	0.004	3.69493668280221
Alpha-linolenic (omega3) and linoleic (omega6) acid metabolism	0.004	0.742204066266152
Alpha-linolenic acid (ALA) metabolism	0.004	0.742204066266152
SCF-beta-TrCP mediated degradation of Emi1	0.004	0.308543542947825
Citric acid cycle (TCA cycle)	0.005	0.531296713340416
HIV Transcription Initiation	0.005	0.509403986800358
RNA Polymerase II HIV Promoter Escape	0.005	0.509403986800358
RNA Polymerase II Promoter Escape	0.005	0.509403986800358
RNA Polymerase II Transcription Pre-Initiation And Promoter Opening	0.005	0.509403986800358
RNA Polymerase II Transcription Initiation	0.005	0.509403986800358
RNA Polymerase II Transcription Initiation And Promoter Clearance	0.005	0.509403986800358
Neurophilin interactions with VEGF and VEGFR	0.005	0.42328770489126
CLEC7A (Dectin-1) signalling	0.005	0.304617282960658
FCERI mediated NF-kB activation	0.005	0.250768814947497
Post-chaperonin tubulin folding pathway	0.006	0.607414951500202
HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand	0.006	0.388993809993292
RNA Polymerase III Transcription Initiation From Type 3 Promoter	0.006	0.179163894648629
Switching of origins to a post-replicative state	0.007	0.399929617615042
RNA Polymerase III Chain Elongation	0.007	0.263967920815557
RNA Polymerase III Transcription Initiation	0.007	0.190423613620322
Iron uptake and transport	0.009	0.414052716259761
SCF(Skp2)-mediated degradation of p27/p21	0.009	0.363087367648801
Cargo concentration in the ER	0.009	0.210916557669309
RNA Polymerase III Transcription	0.009	0.17312412755856
RNA Polymerase III Abortive And Retractive Initiation	0.009	0.17312412755856

E2F-enabled inhibition of pre-replication complex formation	0.01	0.836857538963984
Pyruvate metabolism and Citric Acid (TCA) cycle	0.01	0.464805276875044
Tryptophan catabolism	0.01	0.297717977333391
Beta-oxidation of pristanoyl-CoA	0.01	0.237407027419151

Of the pathways found to be downregulated (Table 8b), five were related to metabolism, two were related to the neuronal system, two were related to disease, one was related to haemostasis, and one was related to signal transduction. Interestingly, at least three pathways involved in NF-κB signalling were found to be upregulated in B-cell lymphomas when compared to controls: TICAM1, RIP1-mediated IκK complex recruitment; IκK complex recruitment mediated by RIP1; and FCERI mediated NF-κB activation. The pathways "TCR signalling" and "Downstream TCR signalling" were also found to be upregulated; the significance of this finding is unknown, as we would expect these pathways to be upregulated in the T-cell lymphoma group, rather than in the B-cell lymphoma group.

Table 8b. Pathways down-regulated in B-cell lymphomas when compared with controls

Pathway name	**FDR**	**Fold change**
Defective SLC16A1 causes symptomatic deficiency in lactate transport (SDLT)	0.00001	-1.058647430398
Defective CYP4F22 causes ARCI5	0.001	-3.10412865294475
Beta oxidation of palmitoyl-CoA to myristoyl-CoA	0.002	-0.651423865368872
Arachidonate production from DAG	0.004	-0.461736625573833
Ionotropic activity of kainate receptors	0.006	-0.0535803034992848
Activation of Ca-permeable Kainate Receptor	0.006	-0.0535803034992848
Beta oxidation of octanoyl-CoA to hexanoyl-CoA	0.008	-0.821338983594867
Beta oxidation of hexanoyl-CoA to butanoyl-CoA	0.008	-0.821338983594867
The fatty acid cycling model	0.008	-1.60315866226709
The proton buffering model	0.008	-1.60315866226709

When comparing T-cell lymphomas with controls, 42 pathways were found to be differentially expressed, with 39 being upregulated and 3 being downregulated. Of the pathways found to be upregulated (Table 9a), 12 were related to disease, 11 were related to signal transduction, three were related to gene expression and transcription, six were related to the immune system, three were related to metabolism, three were related to vesicle-mediated transport, one was related to the transport of small molecules, and one was related to haemostasis.

Table 9a. Pathways up-regulated in T-cell lymphomas when compared with controls

Pathway name	**FDR**	**Fold change**
Events associated with phagocytolytic activity of PMN cells	0.00002	3.14511545183882
Defective MAT1A causes MATD	0.00002	2.54263403778059
Defective ABCG8 causes GBD4 and sitosterolemia	0.00002	2.23099515510606
Defective ABCG5 causes sitosterolemia	0.00002	2.23099515510606
Sodium/Proton exchangers	0.00002	1.15158008783468
Lectin pathway of complement activation	0.00002	0.729012728475499
Arachidonate production from DAG	0.00002	0.580893978480014
Inhibition of membrane repair	0.00002	0.528732126053389
Prevention of phagosomal-lysosomal fusion	0.00002	0.474457791094609
InlB-mediated entry of Listeria monocytogenes into host cell	0.00002	0.471133512272941
Suppression of phagosomal maturation	0.00002	0.463956736050727
FCERI mediated Ca+2 mobilisation	0.00002	0.371851975535774
Synthesis of PI	0.002	0.717167548865554
Signaling by FGFR2 in disease	0.002	0.531337634800575
Cargo recognition for clathrin-mediated endocytosis	0.002	0.347363148470198
DAP12 signalling	0.002	0.302400839988943
DAP12 interactions	0.002	0.284434555406708
Downstream signalling of activated FGFR3	0.002	0.22370803551709
Downstream signalling of activated FGFR2	0.002	0.214760980769655
Downstream signalling of activated FGFR1	0.002	0.210961203453645
Creation of C4 and C2 activators	0.004	0.544005793160529
RNA Polymerase III Transcription Termination	0.004	0.511572588192754
Response of Mtb to phagocytosis	0.004	0.497306790374233
Negative regulation of MET activity	0.004	0.49208720496358
Signalling by NTRK3 (TRKC)	0.004	0.314538277997406
Uptake and function of diphtheria toxin	0.004	0.266100186183374
Downstream signalling of activated FGFR4	0.004	0.213273908211824

Signalling by FGFR4 in disease	0.004	0.197571966835728
Scavenging by Class F Receptors	0.004	0.140619546773321
Deletions in the AXIN genes in hepatocellular carcinoma result in elevated WNT signalling	0.006	4.96566880191348
Signalling by FGFR3	0.006	0.426620779264721
Signalling by FGFR4	0.006	0.421095543082754
RNA Polymerase III Transcription	0.006	0.377563267665241
RNA Polymerase III Abortive And Retractive Initiation	0.006	0.377563267665241
Acyl chain remodelling of PG	0.008	0.609964250470239
Endosomal Sorting Complex Required For Transport (ESCRT)	0.008	0.581580438084811
Signalling by FGFR1	0.008	0.401770877099474
Synthesis of PS	0.008	0.195482687260387
RHOD GTPase cycle	0.008	0.179574680823641

Of the pathways that were found to be downregulated (Table 9b), two were related to disease and one was related to signal transduction. There were nine pathways related to Fibroblast Growth Factor Receptor (FGFR) signalling upregulated in T-cell lymphoma compared to controls: signalling by FGFR2 in disease; downstream signalling of activated FGFR3; downstream signalling of activated FGFR2; downstream signalling of activated FGFR1; downstream signalling of activated FGFR4; signalling by FGFR4 in disease; signalling by FGFR3; signalling by FGFR4; and signalling by FGFR1.

Table 9b. Pathways down-regulated in T-cell lymphomas when compared with controls

Pathway name	**FDR**	**Fold change**
PI3K events in ERBB4 signalling	0.006	-0.218285466218311
TLR3 deficiency - HSE	0.008	-2.52478867493731
TICAM1 deficiency - HSE	0.008	-2.52478867493731

When comparing T-cell lymphomas with B-cell lymphomas, there were 44 pathways that were differentially expressed, with 27 upregulated in T-cell lymphoma (Table 10a) and 17 downregulated in T-cell lymphoma (Table 10b). There were seven pathways related to transport of small molecules, six related to signal transduction, four related to immune system, three related to disease, three related to metabolism, two related to cellular responses to stimuli, one related to haemostasis and one related to developmental biology that were upregulated in T-cell lymphoma compared to B-cell lymphoma, and seven pathways related to immune system, three related to cell cycle, two related to vesicle-mediated transport, two related to metabolism, two related to disease, and one related to gene expression and transcription that were downregulated in T-cell lymphoma compared to B-cell lymphoma.

Table 10a. Pathways up-regulated in T-cell lymphomas when compared with B-cell lymphomas

Pathway name	**FDR**	**Fold change**
Lectin pathway of complement activation	0.0000478	3.859865640542
Initial triggering of complement	0.0000478	1.403186163988
Creation of C4 and C2 activators	0.0000478	1.170897305234
Plasma lipoprotein assembly, remodelling, and clearance	0.0000478	0.4152219089755
ATF6 (ATF6-alpha) activates chaperones	0.0000478	1.324066691361
Signal amplification	0.0000478	0.9717277321964
P2Y receptors	0.0000478	3.941450093121
Nucleotide-like (purinergic) receptors	0.0000478	2.686933688541
Sodium/Proton exchangers	0.0000478	1.57722098927
Defective MAT1A causes MATD	0.0000478	4.402724057256
Reelin signalling pathway	0.0000478	2.081857641051
CREB3 factors activate genes	0.0000478	2.146795251795
Plasma lipoprotein remodelling	0.0000478	0.9531719958963
Stimuli-sensing channels	0.0000478	0.2597320112211
Class A/1 (Rhodopsin-like receptors)	0.0095	0.05767663340807
Serotonin receptors	0.00478	1.181284691190
Relaxin receptors	0.00478	0.6573496453655
Beta oxidation of decanoyl-CoA to octanoyl-CoA-CoA	0.00478	3.009742867579
Assembly of active LPL and LIPC lipase complexes	0.00478	1.328242742916
ABC transporters in lipid homeostasis	0.00478	0.5567084317623
Glycerophospholipid biosynthesis	0.0095	0.2493492622294
G alpha (i) signalling events	0.00478	0.1342701528997
Beta oxidation of butanoyl-CoA to acetyl-CoA	0.0095	3.214456414921
Translation of Replicase and Assembly of the Replication Transcription Complex	0.00478	0.6969303159425
Ion channel transport	0.0095	0.2987963570226
Loss of phosphorylation of MECP2 at T308	0.0095	3.313740469279
Prolactin receptor signalling	0.0095	0.7212794700178

Table 10b. Pathways up-regulated in B-cell lymphomas when compared with T-cell lymphomas

Pathway name	**FDR**	**Fold change**
Insulin effects increased synthesis of Xylulose-5-Phosphate	0.0000478	-2.88801273437
Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane	0.0000478	-1.12924049048
Transport of connexons to the plasma membrane	0.0000478	-1.12924049048
Defective SLC22A5 causes systemic primary carnitine deficiency (CDSP)	0.0000478	-3.39906028654
MECP2 regulates transcription factors	0.00478	-0.0178082213053
Sealing of the nuclear envelope (NE) by ESCRT-III	0.00478	-0.0335692341606
Beta defensins	0.00478	-2.27157472086
Defensins	0.00478	-2.27157472086
Nicotinamide salvaging	0.0095	-0.508149901468
DAP12 interactions	0.00478	-0.281768092420
DAP12 signalling	0.00478	-0.281768092420
Role of LAT2/NTAL/LAB on calcium mobilization	0.00478	-0.973593466706
Defective B4GALT7 causes EDS, progeroid type	0.0095	-0.54223084382
FLT3 signalling through SRC family kinases	0.0095	-0.406488522590
Fc epsilon receptor (FCERI) signaling	0.0095	-0.193413509086
Phosphorylation of proteins involved in G1/S transition by active Cyclin E:Cdk2 complexes	0.0095	-1.68860784627
The role of GTSE1 in G2/M progression after G2 checkpoint	0.00478	-0.20488037913

3.1.6. Gene enrichment analysis

As current pathway analysis tools are biased towards human and mouse experimental data, a second pathway comparison that does not depend on predicting pathway effects was performed (gene enrichment analysis). To avoid size biases in the lists of genes used to assess pathway enrichment, the 1,000 most differentially expressed genes (based on absolute foldchange) identified by DESeq2 were used. This analysis identifies pathways which are enriched, i.e. they contain the most differentially expressed genes, although individual genes within the pathway may be upregulated or downregulated. When comparing B-cell lymphomas with controls, there were five enriched pathways, all related to the synthesis/translation and metabolism of proteins. Some of the most frequently identified genes in the enriched pathways between B-cell lymphoma and controls included multiple significantly overexpressed ribosomal proteins, such as RPL4 (Ribosomal Protein L4), RPS7 (Ribosomal Protein S7), RPS5 (Ribosomal Protein S5), RPS3 (Ribosomal Protein S3), RPL8 (Ribosomal Protein L8), RPL18 (Ribosomal Protein L18), amongst others.

When comparing T-cell lymphomas with controls, there was one enriched pathway related to gene expression and transcription ("RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function"), one enriched pathway related to signal transduction ("STAT3 nuclear events downstream of ALK signalling") and one pathway related to disease ("Loss of MECP2 binding ability to 5mC-DNA") (Figure 6). There were no significant differences identified between B-cell lymphoma and T-cell lymphoma.

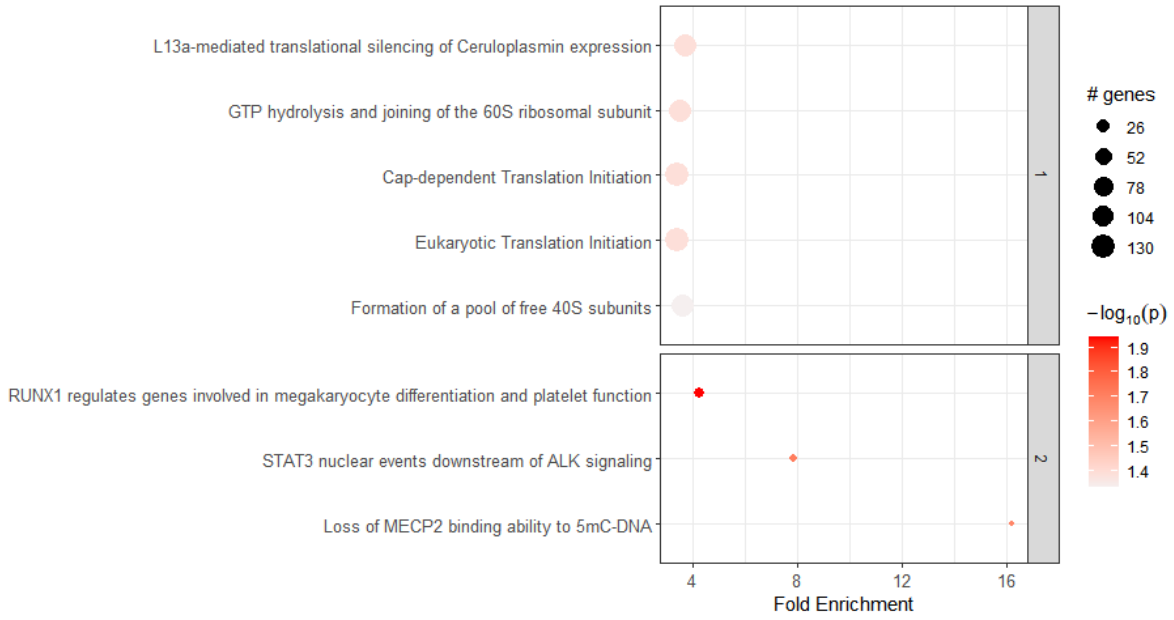

Figure 6: Bubble plot showing the pathways most enriched for differentially expressed genes as identified by gene enrichment analysis. Top plot represents B-cell lymphoma vs controls, and bottom plot represents T-cell lymphoma vs controls. Size of bubble reflects the number of genes in the pathway, intensity of colour reflects the level of significance and position of bubble in the plot reflects the fold enrichment.

3.2. Combined dataset analysis

Since the comparisons above were still based on a relatively small number of samples (18, after exclusion of 2 samples), the analysis could have been prone to bias and not necessarily representative of all dogs with B-cell and T-cell lymphoma. Therefore, to increase sample numbers and lead to a more robust analysis, the dataset was combined with a previous dataset of lymph node and other neoplastic tissue samples from 18 dogs with B-cell lymphoma and five dogs with T-cell lymphoma that had been used in a previous NGS study in Glasgow (Table 11). As such, RNA-Seq expression data was already available for these samples. From now on, the data from the previous NGS study will be referred to as the "previous dataset", the data from the current study as the "new dataset", and the merged data from both studies as the "combined dataset".

Table 11: Patient characteristics from previous NGS dataset

Sample	Lineage	Presentation	Age	Sex	Breed
1	B-cell	Multicentric	6y	M	Golden Retriever
2	B-cell	Multicentric	6y	MN	Labrador
3	B-cell	SMLN	9y	FN	Retriever
4	B-cell	Multicentric, ocular	7y	M	Bullmastiff
5	B-cell	Multicentric, ocular	7y	MN	Labrador
6	B-cell	Multicentric	7y	MN	Lurcher
7	B-cell	Multicentric	5y	M	Lurcher
8	B-cell	Multicentric	4y	M	German Shepherd Dog
9	B-cell	Multicentric	8y	M	Labrador
10	B-cell	Multicentric	3y	FN	Dogue de Bordeaux
11	B-cell	Multicentric	12y	M	Terrier
12	B-cell	Multicentric, ocular	7y	FN	Crossbreed
13	B-cell	Multicentric	4y	M	German Shepherd Dog
14	B-cell	Multicentric	12y	F	Crossbreed
15	B-cell	Multicentric	7y	MN	Lhasa Apso
16	B-cell	Multicentric, ocular	7y	M	Bernese Mountain Dog
17	B-cell	Multicentric	8y	FN	Dachshund
18	B-cell	Multicentric	4y	FN	Yorkshire Terrier
19	T-cell	Multicentric	8y	MN	Crossbreed
20	T-cell	Multicentric	4y	M	Labrador
21	T-cell	Multicentric	6y	M	Boxer
22	T-cell	SMLN	8y	MN	Golden Retriever
23	T-cell	Multicentric, thymic	8y	MN	Airedale

LSA: lymphoma; MN: Male Neutered; M: Male Entire; FN: Female Neutered; F: Female Entire, SMLN: submandibular lymph nodes

The final combined dataset comprised 33 dogs with lymphoma (24 B-cell lymphoma and 9 T-cell lymphoma) and eight controls. From this group of dogs, six dogs with B-cell lymphoma and four dogs with T-cell lymphoma belonged to the new dataset, and 18 dogs with B-cell lymphoma and five dogs with T-cell lymphoma belonged to the previous dataset. All controls were from the new data set.

3.2.1. Principal Component Analysis

Principal component analysis was repeated using the NGS data from both sample populations. When combining datasets, a significant batch effect was observed between the two individual datasets, possibly due to different RNA extraction methods and different storage (Figure 7). While there was some discrete clustering of the different experimental groups, particularly the T-cell lymphomas and control samples, the B-cell lymphomas from the different datasets formed two distinct clusters. PC1 accounted for 63% of the variance, which largely separated the previous and new datasets. PC2 accounted for 14% of the variance.

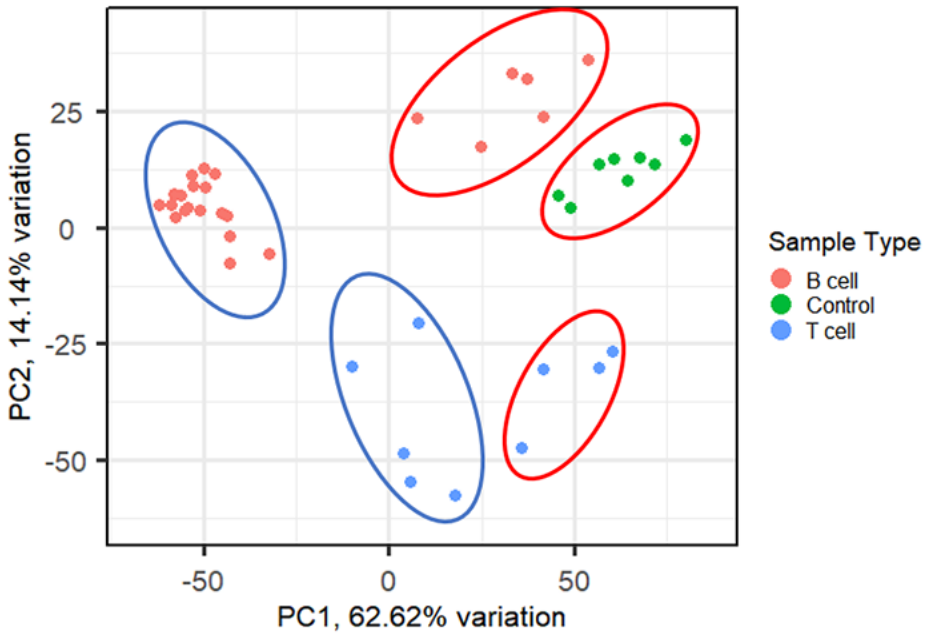

Figure 7: PCA plot for combined dataset before batch correction. Samples circled in red belonged to the new dataset, and samples circled in blue belonged to the previous dataset

In order to account for this batch effect, expression levels were adjusted using an empirical Bayes approach with the ComBat function of sva 3.48 (Zhang, Parmigiani & Johnson, 2020). A new PCA was obtained after adjustment with ComBat (Figure 8), showing improved clustering of the three groups (B-cell lymphomas, T-cell lymphomas and controls) regardless of the dataset from which they originated. PC1 accounted for 38% of the variance, and discriminated the three groups, PC2 accounted for 17% of the variance and partitioned the B-cell lymphomas and T-cell lymphomas from the controls.

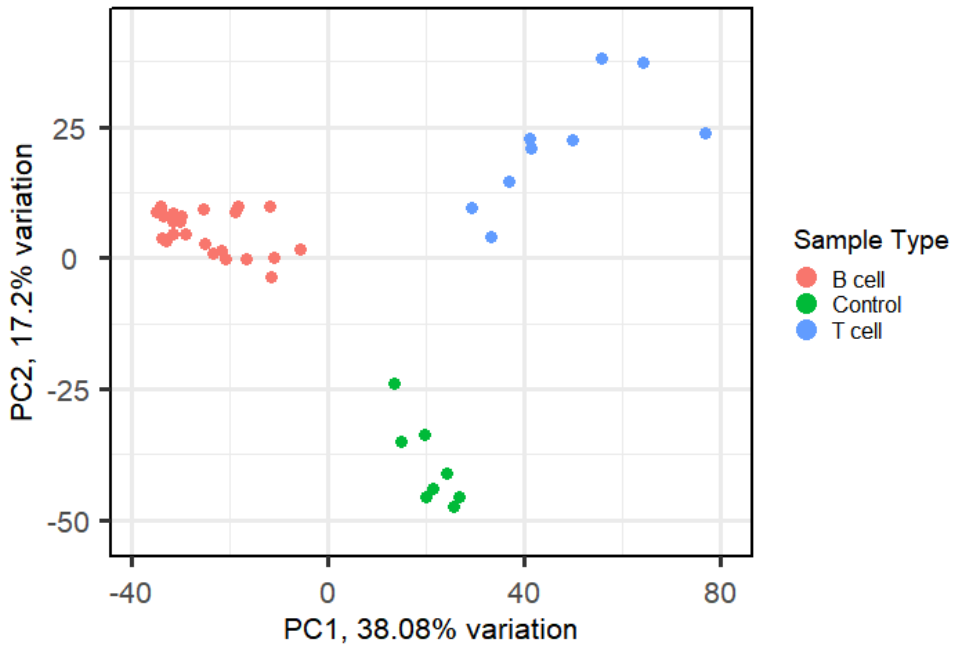

Figure 8: PCA plot for combined dataset following batch correction

3.2.2. Gene expression

Analysis of the batch-corrected combined dataset identified 7,483 differentially expressed genes (adjusted p value < 0.05) when comparing B-cell lymphomas and controls (Figure 9). MAP3K14 was not found to be overexpressed in B-cell lymphomas when compared to controls. There was also no significant difference in the expression of MYC, BCL2, TRAF3 or POT1.

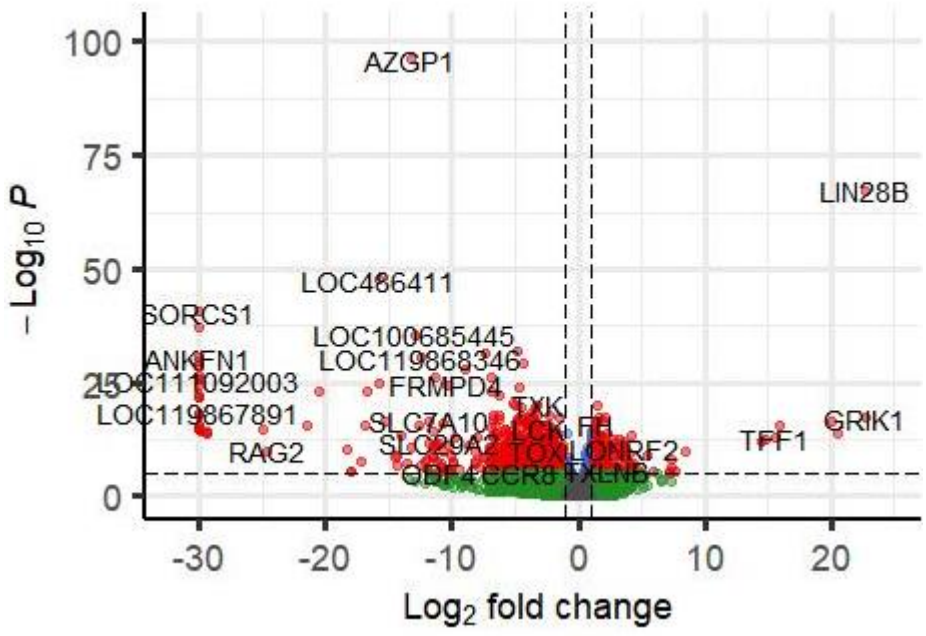

Figure 9: Volcano plot showing differentially expressed genes (adjusted p value < 0.05) between B-cell lymphoma and controls for the combined dataset. The green dots have a large fold change (>2), the blue dots have a small p value (10 x 10^-6) and the red dots have a large fold change and small p value.

There were 6,095 differentially expressed genes (adjusted p value < 0.05) when comparing T-cell lymphomas and controls (Figure 10). KIT was not significantly overexpressed in T-cell lymphomas when compared to controls, which contrasts with the findings from the new dataset analysis alone. On the other hand, GATA3, MTOR and SATB1 were significantly overexpressed in T-cell lymphoma compared to controls.

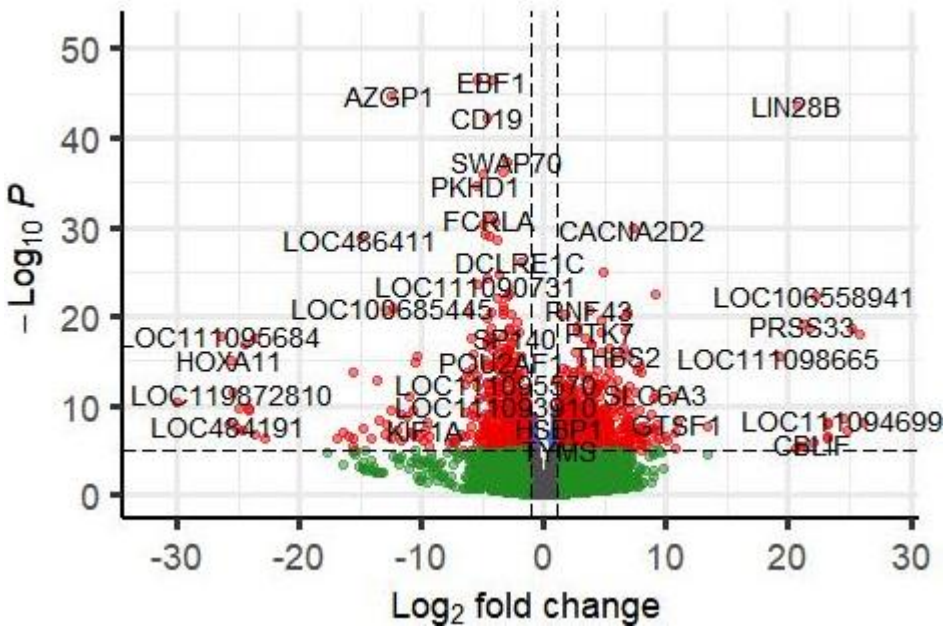

Figure 10: Volcano plot showing differentially expressed genes (adjusted p value < 0.05) between T-cell lymphoma and controls for the combined dataset. The green dots have a large fold change (>2), the blue dots have a small p value (10 x 10^-6) and the red dots have a large fold change and small p value.

There were 8,922 differentially expressed genes (adjusted p value < 0.05) when comparing B-cell lymphomas and T-cell lymphomas (Figure 11). GATA3, SATB1 and KIT were significantly overexpressed in T-cell lymphomas when compared to B-cell lymphomas. There was no significant difference in the expression of PTEN, TBX21, or MTOR.

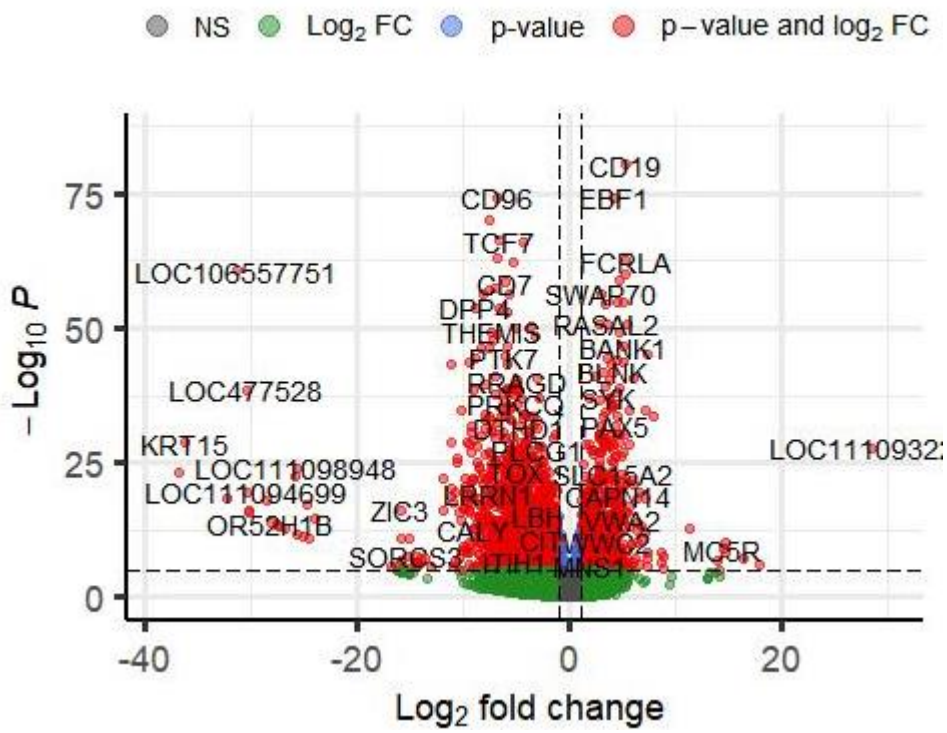

Figure 11: Volcano plot showing differentially expressed genes (adjusted p value < 0.05) between B-cell lymphoma and T-cell lymphoma for the combined dataset. The green dots have a large fold change (>2), the blue dots have a small p value (10 x 10^-6) and the red dots have a large fold change and small p value.

3.2.3. Gene enrichment analysis

Gene enrichment analysis was repeated on the combined dataset, again looking at only the 1,000 most differentially expressed genes. As the initial Reactome based pathway analysis was inherently biased towards human and mouse experimental data, and as it was felt that the gene enrichment analysis on the initial dataset led to more meaningful results, the initial pathway analysis was not repeated on the combined dataset. When comparing B-cell lymphomas with controls, there were 24 enriched pathways: 11 related to cell cycle, seven related to DNA replication, five related to metabolism of proteins and one related to protein localisation. Some of the most frequently identified genes in the differentially expressed pathways between B-cell lymphoma and controls included multiple significantly overexpressed proteins involved in cell cycle regulation, such as RFC4 (Replication Factor C Subunit 4), RPA2 (Replication Protein A2), CDC7 (Cell Division Cycle 7), CDC6 (Cell Division Cycle 6), CDC25A (Cell Division Cycle 25A), Origin recognition complex (ORC) 1 and ORC6, CHEK1 (Checkpoint kinase 1),

and multiple minichromosome maintenance proteins (MCM6, MCM3, MCM2, MCM4, MCM5).

When comparing T-cell lymphomas with controls, there was only one enriched pathway, which was related to gene expression and transcription (RUNX3 Regulates Immune Response and Cell Migration). In this pathway, SSP1 (Osteopontin) and ITGAL (Integrin Subunit Alpha L) were significantly upregulated in T-cell lymphoma compared to controls and RORC (RAR-related orphan receptor C) was significantly downregulated.

When comparing B-cell lymphoma and T-cell lymphoma, there were 15 enriched pathways: 11 related to immune system, one related to haemostasis, one related to neuronal system, one related to muscle contraction and one related to gene expression and transcription. Some of the enriched pathways included "Antigen activates B Cell Receptor (BCR) leading to generation of second messengers", "Signalling by the B Cell Receptor (BCR)" as expected for B-cell lymphoma, and "TCR signalling" as expected for T-cell lymphoma. The pathways "DAP12 interactions" and "DAP12 signalling" were found to be enriched in the comparison between B-cell lymphoma and T-cell lymphoma. In analysis of the new dataset alone, these pathways were upregulated in T-cell lymphoma compared to controls, and upregulated in B-cell lymphoma compared to T-cell lymphoma as well.

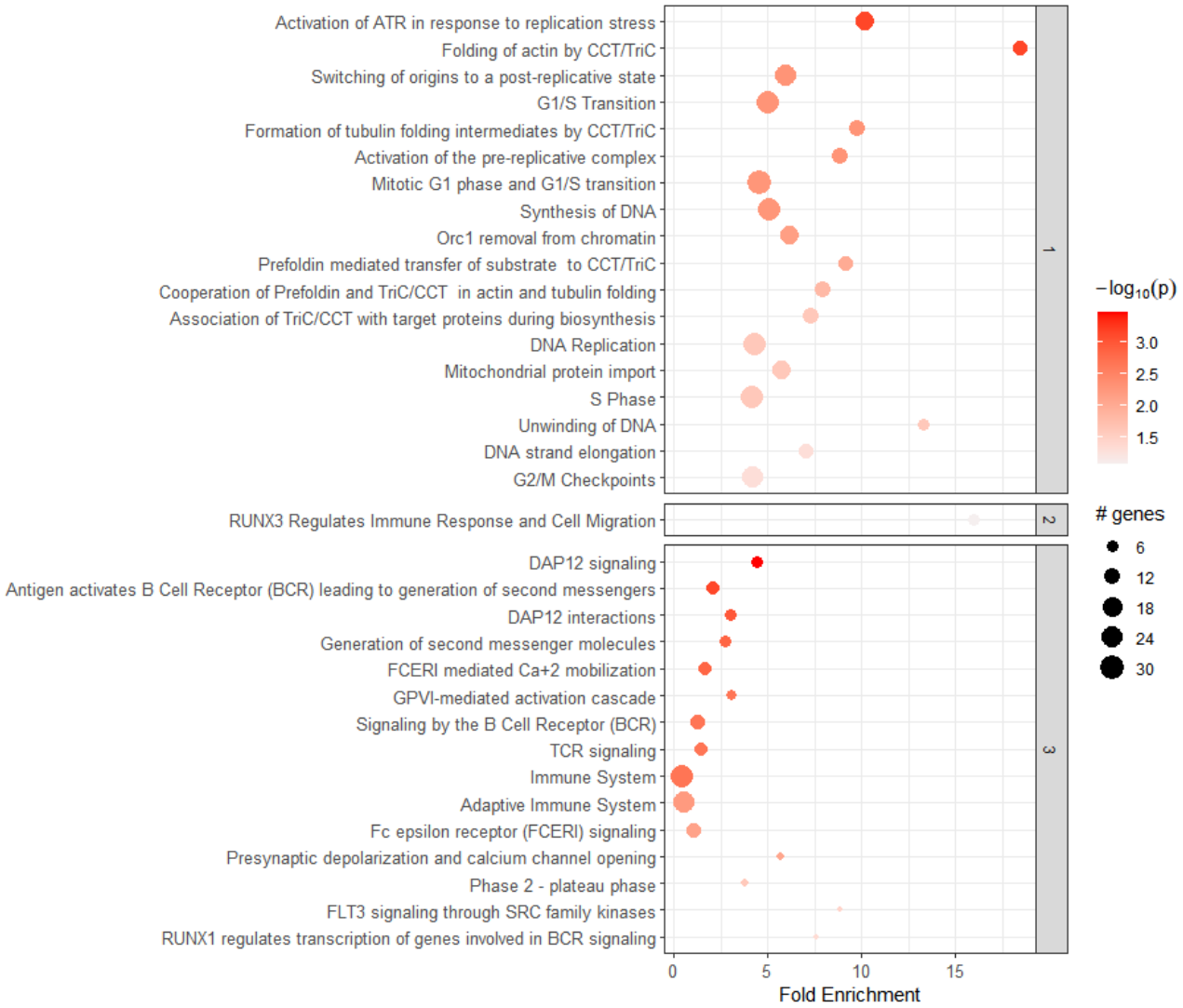

Figure 12: Bubble plot illustrating enriched pathways identified by gene enrichment analysis for combined dataset. Top plot represents B-cell lymphoma vs controls, middle plot represents T-cell lymphoma vs controls and lower plot represents B-cell lymphoma vs T-cell lymphoma. Size of bubble reflects the number of genes in the pathway, intensity of colour reflects the level of significance, and position of bubble in the plot reflects the fold enrichment

4. Discussion:

The aim of this project was to use NGS to characterise the transcriptomic landscape of canine B-cell and T-cell lymphoma and compare it with control samples, to aid in the subclassification of lymphoma and to potentially identify molecular pathways for targeted therapy.

B-cell lymphoma, T-cell lymphoma and control tissue could be differentiated on the basis of their gene expression profiles, as revealed by PCA. Three groups of samples clustered separately, suggesting different pathways may be involved in the pathogenesis of B-cell and T-cell lymphoma, with both having a clearly separate profile from non-neoplastic lymph nodes. A single B and T-cell sample (sample ID C02, sample number 12) was positioned between the B-cell lymphoma and T-cell lymphoma clusters, as anticipated. The diagnosis for this sample was achieved with PARR, which revealed both B and T-cell clonal receptor rearrangements.

The PCA of the new dataset identified a single outlier in the T-cell lymphoma group (sample ID E03, sample number 8). This was from a dog that had been diagnosed with a particularly rare subtype of lymphoma, namely primary muscular lymphoma with cutaneous involvement, and the sample collected was from a lymph node that was not confirmed as being affected by lymphoma. Our prediction was that this sample would either cluster with the T-cell lymphoma group or with the control group, however it displayed a gene expression profile closer to the B-cell lymphoma group. It could be hypothesised that this was a misdiagnosis, and that this patient's true disease was a B-cell lymphoma, however this patient had both PARR and immunohistochemistry performed on a biopsy of an affected muscle confirming its T-cell phenotype, making this unlikely. It therefore remains unclear why this sample had a genetic signature closer to B-cell lymphomas, and perhaps it would have been more appropriate to analyse the affected muscle rather than the lymph node, however at the time of sample collection a muscular sample was not available for this patient.

The main limitation of this study was a small sample size, particularly for the T-cell lymphoma group, which inevitably limited the power of the statistical analysis. The analysis of the combined dataset went some way to addressing this issue. After batch correction, some small differences between batches were lost which

could have led to loss of possibly relevant results (as an example, KIT was significantly overexpressed in T-cell lymphoma compared to controls on the new dataset analysis, but the same was not found on the combined dataset analysis). Additionally, batch correction can add false positive results (Zindler et al., 2020) due to the possible magnification of small and non-significant differences, which may explain the increased number of significantly differentially expressed genes (adjusted p value < 0.05) in the combined dataset analysis compared to the new dataset analysis. However, the increased number of differentially expressed genes in the combined dataset analysis compared to the new dataset analysis alone was felt most likely to be secondary to increased statistical power. As the samples used for analysis was not composed of purified cancer cells and included whole tumour tissue, it is also possible that some of our results also reflect changes in the tumour microenvironment of B-cell and T-cell lymphoma rather than mutations specific to the cancer cells, which may account for some unexpected results.

It would have been interesting to analyse each phenotype (B-cell and T-cell) separately in more detail and see if pathway analysis could identify subclusters that may shed some light into the pathogenesis of specific lymphoma subtypes (for example, low grade vs high grade lymphoma or diffuse vs follicular lymphoma), or which additional aberrant pathways are evident during treatment that may be involved in relapse (naïve lymphomas vs relapsed lymphoma). Unfortunately, the small number of dogs in our study population precluded a meaningful analysis of subgroups as, for example, only 4 of 12 dogs with lymphoma from the new dataset received any treatment.

4.1. Transcriptomic alterations in B-cell lymphoma

Multiple pathways related to the NF-κB signalling pathway were found to be upregulated in canine B-cell lymphoma, as expected and as previously reported, and confirming that this is a central pathway which may be targeted for the development of new therapeutic drugs. MAP3K14 is the main activator of the alternative NF-κB pathway (Haselager & Eldering, 2022), and gain of function of this gene product has been reported in human classical Hodgkin's lymphoma (Otto et al., 2012), splenic marginal zone lymphoma (Rossi et al., 2011) and also canine B-cell lymphoma (Elvers et al., 2015). MAP3K14 was overexpressed in the new dataset (log2foldchange -2.3, adjusted p value 0.055), despite not achieving

significance, possibly due to the small sample size and a type II error. NF-κB-inducing kinase (NIK), the protein encoded by MAP3K14, has been suggested as a possible therapeutic target in B-cell malignancies (Haselager & Eldering, 2022), and our results may support a similar therapeutic strategy in dogs with B-cell lymphoma as well. However, further studies would be required to confirm this, as in our combined dataset analysis, MAP3K14 was not found to be overexpressed in B-cell lymphoma, contrary to our expectations. This may be a real finding or may be secondary to batch correction, which may eliminate small differences between the different groups in the different datasets.

On gene enrichment analysis of B-cell lymphoma compared to controls for the new dataset, multiple pathways involving the metabolism of proteins, mainly ribosomal proteins, were found to be enriched for differentially expressed genes. This is not something that has been extensively reported in human or canine lymphoma to date. Healthy tissues and cancers are known to have distinct ribosomal protein transcripts (Dolezal, Dash, & Prochownik, 2018), and tumour cells are often characterised by higher expression of ribosomes in order to sustain accelerated rates of cell growth and division (Pecoraro et al., 2021). In our dataset, the majority of ribosomal proteins in B-cell lymphoma appeared to be overexpressed, which may be a reflection of the higher rate of cell turnover compared to normal tissue. Interestingly, patients with missense mutations in ribosome proteins and associated ribosomopathies are also at increased risk of developing cancer (Kampen et al., 2020), however the mechanism for this is poorly understood, as ribosomopathies are characterised by a hypo-proliferative phenotype.

On gene enrichment analysis of B-cell lymphoma compared to controls for the combined dataset, the top enriched pathway was “Activation of ATR in response to replication stress”. The relationship between DNA repair mechanisms and cancer is not a simple one, as defective DNA repair leads to genetic instability and carcinogenesis, although suppression of DNA repair mechanism can be particularly detrimental to cancer cells (Lecona & Fernandez-Capetillo, 2014). Some of the significantly overexpressed genes involved in this pathway from our combined dataset included several MCM proteins, ORC1 and ORC6, RFC4, RPA2, CDC7, CDC6, CDC25A and CHEK1.

In the new dataset analysis, the expression of MCM proteins was not significantly different between B-cell lymphoma and controls, however in the combined dataset analysis MCM6, MCM3, MCM2, MCM4, MCM5 were found to be overexpressed in B-cell lymphoma, suggesting the results of the initial analysis were secondary to small sample size and type II error. MCMs play essential roles in DNA replication initiation and have been found to be overexpressed in a number of cancers (Yu et al., 2020). In humans, MCM2 has been reported to be a marker of proliferation and poor prognosis in DLBCL (Obermann et al., 2005), while MCM3 was found to be overexpressed in human cutaneous T-cell lymphoma and predictive of disease severity and prognosis (Jankowska-Konsur et al., 2015). The MCM2-7, CDC45 protein (Cell division control protein 45) and the GINS complexes together compose the CMG complex, the human replicative helicase, which has been investigated as a possible therapeutic target in multiple cancer types in humans (Seo & Kang, 2018). Interestingly, although they were not a part of the 1000 more relevant genes in our data, the CDC45 protein and three out of four of the genes encoding the GINS complex subunits (GINS1, GINS3 and GINS4) were overexpressed in B-cell lymphoma compared to controls on analysis of the combined dataset, suggesting that targeting the CMG may also be a valid treatment strategy in dogs with B-cell lymphoma.

4.2. Transcriptomic alterations in T-cell lymphoma

On analysis of the new dataset, multiple pathways involved in FGFR signalling were found to be upregulated in T-cell lymphoma, despite a lack of significant differences in the expression of the genes FGFR1, FGFR2, FGFR3 and FGFR4 between T-cell lymphoma and controls, suggesting that other downstream effector genes may be affected. Similarly, FGFR has been implicated in human lymphoma but more associated with B cell subtypes. In human relapsed or refractory DLBCL, FGFR1 was found to be a possible novel therapeutic target despite lack of expression of FGFR1 from the lymphoma cells (Siervi et al., 2022). That study suggested that the anti-neoplastic effect of FGFR1 inhibitors was dependent on tumour-associated macrophages in the microenvironment. Additionally, interleukin-10 was found to block VEGF- and FBF-2-induced proliferation of microvascular endothelial cells *in vitro* and consequently angiogenesis in Burkitt lymphoma cell lines (Cervenak et al., 2000). These findings highlight how targeting the tumour microenvironment can lead to advances in

treatment, and it may be hypothesised that canine patients may benefit from similar treatment strategies.

There is also a possible link between the FGFR pathways and the runt-related transcription factor (RUNX) proteins in the pathogenesis of some human lymphoid neoplasms. The RUNX family members are known as regulators in important developmental pathways (Ito, Bae & Chuang, 2015) and are essential for haematopoiesis in vertebrates (Sood, Kamikubo & Liu, 2017). A study of human patients diagnosed with "myeloid/lymphoid neoplasm with FGFR1 rearrangement", as per WHO classification, revealed that 78% of patients had RUNX1 mutations (Strati et al., 2018), and this has some similarities with our findings; RUNX1 was overexpressed in the combined dataset in T-cell lymphomas when compared to controls (log2fold change 1.015, adjusted p value 0.015). RUNX1 expression is known also to correlate with immune infiltrates of cancer-associated fibroblasts in several other human tumour types (Tuo et al., 2022), and RUNX1 related pathways may involve interactions between fibroblasts and cancer cells (Kapusta et al., 2018). The pathway "RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function" was differentially expressed in the gene enrichment analysis between T-cell lymphoma and controls for the new dataset, and the pathway "RUNX3 regulates immune response and cell migration" was differentially expressed in the combined dataset, suggesting a role of the RUNX proteins and associated downstream pathways in the pathogenesis of canine T-cell lymphoma.

RUNX1 mutations have been reported in human acute myeloid leukaemia, acute lymphoblastic leukaemia, chronic myelomonocytic leukaemia and myelodysplastic syndrome, and in acute myeloid leukaemia were found to be poor prognostic indicators (Gaidzik et al., 2016). RUNX3 has been clearly established as a tumour suppressor gene in several solid tumours (Ito, 2008), it has been found to be important to NK and CD8 T-cell development (Kohu et al., 2005; Ohno et al., 2008), is oncogenic in NK/T-cell lymphoma in humans (Selvarajan et al., 2017), and collaborates with *Myc* in the oncogenesis of thymic lymphoma, which is usually a T-cell disease (Stewart et al., 2002). A similar collaboration between RUNX2 and *Myc* has been demonstrated in lymphoma murine models (Hay et al., 2019).

In the pathway "RUNX3 regulates immune response and cell migration", the genes ITGAL and SPP1 were found to be overexpressed. The RUNX3 pathway regulates

the transcription of ITGAL, which is a leukocyte integrin involved in transendothelial migration of leukocytes during immune and inflammatory responses, as well as co-stimulation of T-cells. Similarly, the transcription of SPP1 increases in response to increased RUNX3 levels. SPP1 is a multi-functional matricellular phosphoglycoprotein and is involved in many physiological processes such as inflammation, cell adhesion and migration, differentiation, cell survival and apoptosis, as well as regulation of bone matrix mineralisation (Gimba, Brum & Nestal De Moraes, 2019). It has been found to be overexpressed in multiple human cancers, with different prognostic value depending on the type of cancer (Zeng et al., 2022), and is, in addition, involved in the mechanisms of chemo and radioresistance in several human cancers (Gimba, Brum & Nestal De Moraes, 2019). SPP1 is also one of the most upregulated genes in B-cell lymphoma of the central nervous system when compared with nodal and extra-nodal DLBCL (Tun et al., 2008). Dai *et al* have shown that SPP1 induces angiogenesis through activation of PI3K/AKT and ERK1 (extracellular signal-regulated kinase 1)/2, leading to increased VEGF expression (Dai et al., 2009). In a positive feedback loop, VEGF activates PI3K/AKT and the ERK1/2 pathway, and blockage of this pathway could be a possible therapeutic target for some of SPP1's oncogenic effects.

The gene RORC, also involved in the RUNX3 pathway and a regulator of the proinflammatory Th17/IL-17 axis, was found to be downregulated in the combined dataset. The role of the Th17 response in carcinogenesis seems to be context dependent, with pro and anti-tumorigenic roles reported depending on tumour type (Marques et al., 2021). In patients with B-cell NHL, Th17 cells and IL-17 levels seem to be decreased at diagnosis, and increase with response to treatment, suggesting an anti-tumorigenic role (Lu et al., 2016). RORC seems to have a similar tumour suppressive role, since mice with embryonic and adult knockout of RORC developed lymphoblastic lymphoma (Liljevald et al., 2016). Moreover, treatment with RORC inhibitors led to thymic aberrations previously found in RORC-deficient mice preceding thymic lymphoma (Guntermann et al., 2017). Decreased RORC expression has also been implicated in the pathogenesis of human adult T-cell leukaemia/lymphoma, and it was hypothesised that immunotherapy with Th17 cells or RORC agonists could be beneficial in these cases (Subramanian et al., 2019). In the combined dataset, IL-17A, IL-17F and IL-23R (a key gene in Th17 differentiation) (Bystrom et al., 2019) were lower in patients with T-cell

lymphoma compared to controls, which is in line with previous findings from human lymphoma (Yang et al., 2009; Subramanian et al., 2019), and suggests canine patients may benefit from similar immunotherapy strategies. However, IL-17 may also have a pleiotropic role, as it has also been reported to be increased in human patients with cutaneous T-cell lymphoma (Cirée et al., 2004) and NK/T-cell lymphoma (Qi et al., 2023). Additionally, in the same population of canine lymphoma patients, IL-17 was reported to be increased in the tumour microenvironment and decreased in peripheral blood mononuclear cells (Dias et al., 2019).

Gene expression analysis of the combined dataset revealed several transcriptomic abnormalities in canine T-cell lymphoma compared to controls which have been previously reported in human T-cell lymphoma. Similar to the division of human DLBCL into GCB and ABC, human peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) has been divided by gene expression profile into two main subtypes: one characterised by upregulation of GATA3 (PTCL-GATA3) and one characterised by upregulation of TBX21 (PTCL-TBX21) (Heavican et al., 2019). In the combined dataset analysis, GATA3 was significantly overexpressed in T-cell lymphoma compared to controls, suggesting that canine T-cell lymphoma may have similarities with human PTCL-GATA3. Increased MTOR expression in canine T-cell lymphoma has been previously reported (Harris et al., 2019), which is consistent with our findings. In contrast, SATB1, a transcriptional repressor that plays a role in T-cell development (Alvarez et al., 2000), has been found to be mutated with consequent loss of function in human T-cell lymphoma (Harris et al., 2019). In our data, SATB1 was found to be overexpressed in T-cell lymphoma compared to controls, and the reason for this is currently unclear.

DAP12 (DNAX-activation protein 12) is a tyrosine-based immunoreceptor involved in innate immunity mediated by NK cells and myeloid cells, and has been reported to inhibit human B-cell proliferation (Nakano-Yokomizo., 2011). However, our results suggest DAP12 associated pathways are upregulated in B-cell lymphomas compared to T-cell lymphoma, and T-cell lymphoma compared to controls. The significance of these findings is unclear as they seem to contradict its physiological function. Little is known about the role of these pathways in cancer and more specifically in lymphoma, so further work would be required to establish this, given DAP12 may have a pleotropic role (Turnbull & Colonna., 2007).

5. Conclusions and future work:

This work has identified a number of different pathways that may be implicated in the pathogenesis of B-cell and T-cell lymphoma. For B-cell lymphoma, the NF-κB pathway and DNA replication and repair pathways were highlighted, with overexpression of ribosomal and minichromosome maintenance proteins noted. In contrast for T-cell lymphoma, FGFR pathways, RUNX pathways, and pathways relating to innate immunity were identified. Some of these pathways have not been reported previously and may represent valid therapeutic targets.

Future work is required to confirm the expression of key genes and pathways using reverse transcription polymerase chain reaction (RT-PCR) to check validity of NGS results with a focus on the aforementioned pathways. Validation of these results in a separate and larger cohort of patients is recommended to establish the validity of the pathways identified in this work as possible treatment targets.

Study of a larger cohort would also allow analysis of each phenotype (B-cell and T-cell) separately and in more detail, which may allow identification of differentially expressed pathways among subclusters. This form of analysis may shed light into the pathogenesis of specific lymphoma subtypes, for example, low grade vs high grade lymphoma or diffuse vs follicular lymphoma. It may also reveal pathways develop aberrant activity during treatment that may be responsible for relapse, comparing naïve lymphomas with relapsed lymphoma cases.

Appendix

1. Top 50 differentially expressed genes between B-cell lymphoma and controls in the initial dataset

GeneID	Gene Name	log2FoldChange	padj
ENSCAFG00000006701	FLT1	-4.7173	3.73E-12
ENSCAFG00000005766	SYT1	6.878164	2.05E-11
ENSCAFG00000004514	SIAH3	4.522519	7.41E-11
ENSCAFG00000014889	AZGP1	7.106436	1.12E-10
ENSCAFG00000011216	HADH	4.708498	3.05E-10
ENSCAFG00000029990	GLIPR1L2	3.854087	3.05E-10
ENSCAFG00000005309	LBH	6.478143	1.50E-09
ENSCAFG00000003333	GRIK3	7.96533	3.97E-09
ENSCAFG00000028591	POPDC3	-8.86343	1.80E-08
ENSCAFG00000012511	CRYBG2	4.900084	9.99E-08
ENSCAFG00000016940	TBC1D10B	3.393115	2.45E-07
ENSCAFG00000031521	CROCC2	4.197563	2.45E-07
ENSCAFG00000001514	MPST	2.121533	9.76E-07
ENSCAFG00000011770	TEX2	-2.76078	9.76E-07
ENSCAFG00000004523	ERICH6B	2.227928	1.41E-06
ENSCAFG00000007765	TTC29	5.356748	1.41E-06
ENSCAFG00000011421	LMNTD1	4.149354	1.41E-06
ENSCAFG00000010431	CACNA2D2	2.619466	1.64E-06
ENSCAFG00000031767	TUBA3D	-2.73775	1.72E-06
ENSCAFG00000003045	ZNF292	-4.78615	1.87E-06
ENSCAFG00000013191	NRP2	4.896846	2.51E-06
ENSCAFG00000001792	LIMCH1	4.733843	2.77E-06
ENSCAFG00000011790	CASR	4.168409	3.05E-06
ENSCAFG00000015013	LGALS3	-5.33163	3.05E-06
ENSCAFG00000024533	LPO	-5.41501	5.11E-06
ENSCAFG0000004833		3.923194	6.08E-06
ENSCAFG00000028933	IGSF8	4.092043	7.91E-06
ENSCAFG00000000373	TAB2	3.822082	7.92E-06
ENSCAFG00000000764	PRRT1	3.539565	1.03E-05

ENSCAFG00000015922	CCDC22	4.361497	1.98E-05
ENSCAFG00000000994	SKP1	4.212692	2.55E-05
ENSCAFG00000013775	TMC3	2.565688	3.12E-05
ENSCAFG00000012946	DLX2	-7.43216	5.56E-05
ENSCAFG00000049321	C5orf58	2.626106	8.23E-05
ENSCAFG00000032070	SAP18	-2.51324	8.87E-05
ENSCAFG00000045167		-1.63247	0.000102
ENSCAFG00000016463	MAD1L1	3.133626	0.000119
ENSCAFG00000016672	LCORL	-3.04516	0.000136
ENSCAFG00000004263	LRRC41	-1.86205	0.000142
ENSCAFG00000005692	RPTOR	2.697004	0.000162
ENSCAFG00000008312	HPS3	3.303251	0.000162
ENSCAFG00000009433	BAZ2B	-3.20383	0.000162
ENSCAFG00000001314	CACNA1I	5.095009	0.000167
ENSCAFG00000009243	SLC38A4	4.582308	0.000167
ENSCAFG00000008720	POLR2C	2.085741	0.000178
ENSCAFG00000017840	ELP2	-2.39625	0.000178
ENSCAFG00000018970	GRIN2A	-2.8526	0.000178
ENSCAFG00000006818	MTREX	1.954003	0.000204
ENSCAFG00000015898	RBM47	-3.84441	0.000228

2. Top 50 differentially expressed genes between T-cell lymphoma and controls in the initial dataset

GeneID	Gene Name	log2FoldChange	padj
ENSCAFG00000048902		-10.05376577	7.23E-21
ENSCAFG00000028591	POPDC3	18.08349038	7.64E-20
ENSCAFG00000046001		-9.702820922	4.54E-17
ENSCAFG00000013272	MBTPS2	-8.298221642	2.09E-15
ENSCAFG00000004523	ERICH6B	-3.672879782	5.62E-15
ENSCAFG00000030927	NFE2	-22.58784455	1.59E-13
ENSCAFG00000010704	SP140	4.659823865	2.30E-13
ENSCAFG00000006600	LRRC18	3.975746272	6.68E-13
ENSCAFG00000014163	UBE2E3	-4.18280252	3.55E-12
ENSCAFG00000016197	TKFC	3.181662195	3.55E-12
ENSCAFG00000003624	INPP4B	-4.348312887	4.33E-12
ENSCAFG00000008373	IL7	4.744022414	6.97E-12
ENSCAFG00000005732	BAHCC1	-8.083731559	2.63E-11
ENSCAFG00000015810	ZBTB18	3.5879414	3.75E-11
ENSCAFG00000007481		3.541713506	7.73E-11
ENSCAFG00000000373	TAB2	6.08765365	3.48E-10
ENSCAFG00000019208	CACTIN	-7.072230937	5.43E-10
ENSCAFG00000024533	LPO	-7.205729417	5.99E-10
ENSCAFG00000013053	RPS17	2.539884698	1.08E-09
ENSCAFG00000032520	PTPRN2	-6.491219247	2.22E-09
ENSCAFG00000045539		5.572234293	2.45E-09
ENSCAFG00000001358	AGTPBP1	-8.640154269	2.60E-09
ENSCAFG00000001700	SND1	-4.430506794	6.63E-09
ENSCAFG00000017351	SOX30	5.178766416	6.65E-09
ENSCAFG00000041751		7.373602799	6.65E-09
ENSCAFG00000014889	AZGP1	8.616361056	6.91E-09
ENSCAFG00000024672	TMEM211	-6.645694762	7.03E-09
ENSCAFG00000003435	NOL10	3.824443767	7.56E-09
ENSCAFG00000005468	PFKP	2.988442027	7.56E-09
ENSCAFG00000011731	CADM3	5.738280743	8.35E-09

ENSCAFG00000008491	DLGAP4	2.328501455	8.89E-09
ENSCAFG00000007983	MCTP1	-5.043391135	9.44E-09
ENSCAFG00000010923		-2.442390038	1.25E-08
ENSCAFG00000016179	IQSEC2	7.387726056	2.85E-08
ENSCAFG00000012430	BCL9L	4.225115657	2.97E-08
ENSCAFG00000015545	SUV39H1	3.659653337	2.97E-08
ENSCAFG00000017733	POLR3E	-5.071875353	3.14E-08
ENSCAFG00000031918	TMEM26	-5.716771574	3.71E-08
ENSCAFG00000004152	DNAJC1	-9.787191324	3.96E-08
ENSCAFG00000025174		6.9012695	4.19E-08
ENSCAFG00000001106	LAMA2	-4.500243807	4.71E-08
ENSCAFG00000016526	INTS1	-5.818475033	4.71E-08
ENSCAFG00000013770		-4.804320862	5.40E-08
ENSCAFG00000005576	SUPT16H	-3.349681594	7.50E-08
ENSCAFG00000017133	MAST1	3.494669415	7.70E-08
ENSCAFG00000010536	CARMIL1	-5.885191683	1.26E-07
ENSCAFG00000032469	DDIT4	-3.775701093	1.46E-07
ENSCAFG00000013844	NCAM1	-7.247362253	1.71E-07
ENSCAFG00000002765	UBA6	3.472638721	1.75E-07

3. Top 50 differentially expressed genes between B-cell lymphoma and T-cell lymphoma and controls in the initial dataset

GeneID	Gene Name	log2FoldChange	padj
ENSCAFG00000028591	POPDC3	26.94692004	8.97E-44
ENSCAFG00000004523	ERICH6B	-5.900807574	9.30E-36
ENSCAFG00000004514	SIAH3	-7.262797755	9.28E-22
ENSCAFG00000048902		-10.61062596	2.48E-19
ENSCAFG00000007481		4.753227909	5.92E-18
ENSCAFG00000018970	GRIN2A	6.938262342	6.73E-18
ENSCAFG00000015810	ZBTB18	4.658224611	1.97E-17
ENSCAFG00000012511	CRYBG2	-8.124937402	9.56E-17
ENSCAFG00000015135	LDB2	-7.776223409	3.57E-16
ENSCAFG00000011773	C6H7orf50	-3.878721257	6.34E-16
ENSCAFG00000013272	MBTPS2	-9.424370191	6.38E-16
ENSCAFG00000046001		-9.899007757	5.07E-15
ENSCAFG00000008720	POLR2C	-4.314068832	1.91E-14
ENSCAFG00000005732	BAHCC1	-10.91760012	1.46E-13
ENSCAFG00000016197	TKFC	3.414571825	5.52E-13
ENSCAFG00000032520	PTPRN2	-7.982372514	1.45E-12
ENSCAFG00000031918	TMEM26	-7.60666716	2.03E-12
ENSCAFG00000017987	CCDC178	8.999667722	2.46E-12
ENSCAFG00000011216	HADH	-5.986946515	2.69E-12
ENSCAFG00000019208	CACTIN	-8.124251155	8.91E-12
ENSCAFG00000045539		6.363895714	2.26E-11
ENSCAFG00000010247	PRL	-8.692370502	6.45E-11
ENSCAFG00000004422	NSUN6	9.096910771	7.65E-11
ENSCAFG00000013053	RPS17	2.798258272	7.65E-11
ENSCAFG00000041808		4.282354577	7.74E-11
ENSCAFG00000008528	GPR87	-6.439909972	1.41E-10
ENSCAFG00000010525	CDC73	-7.101707318	1.88E-10
ENSCAFG00000014163	UBE2E3	-4.005338043	2.19E-10
ENSCAFG00000001700	SND1	-4.980633763	2.24E-10
ENSCAFG00000023401	CYP4F22	-7.012172892	2.81E-10
ENSCAFG00000041751		7.799704245	4.80E-10
ENSCAFG00000000994	SKP1	-6.579782391	6.25E-10
ENSCAFG00000013008	GMEB2	-3.989345589	6.25E-10
ENSCAFG00000024672	TMEM211	-8.839853743	6.49E-10
ENSCAFG00000010704	SP140	4.119610671	7.67E-10
ENSCAFG00000016463	MAD1L1	-5.112089118	9.82E-10
ENSCAFG00000006600	LRRC18	3.543084124	1.33E-09
ENSCAFG00000016179	IQSEC2	8.06698577	1.62E-09

ENSCAFG00000009472	PTDSS1	4.507688565	2.55E-09
ENSCAFG00000008647	TKT	2.770536443	3.50E-09
ENSCAFG00000016086	MMRN2	-6.874681789	1.08E-08
ENSCAFG00000009354	TOX2	-7.72741154	1.62E-08
ENSCAFG00000046356		5.140374606	1.69E-08
ENSCAFG00000014843	FERMT2	3.981781557	1.99E-08
ENSCAFG00000016940	TBC1D10B	-4.068675423	2.04E-08
ENSCAFG00000016447	CD5L	6.196671315	2.16E-08
ENSCAFG00000025465	TRPV6	-11.21461618	2.16E-08
ENSCAFG00000010431	CACNA2D2	-3.374891257	2.56E-08
ENSCAFG00000002380	ACTR1B	-2.409025339	2.69E-08
ENSCAFG00000008373	IL7	4.05449513	2.85E-08

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