Lymphoma in Australian Border Collies: pedigree and genetic investigations

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Declaration

I certify that the intellectual content of this thesis, and experimental work described, is the product of my own work and that all the assistance received in preparing this thesis, experimental design and analysis and sources have been acknowledged. The work presented in this thesis has not been submitted in any form for a degree at any other university or institutions.

Yee Ka Katrina Cheng

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Abstract

Lymphoma is a common and often aggressive cancer in dogs. In Australia, Border Collies have higher incidence of lymphoma compared to other breeds. However, the clinical characteristics of lymphoma in Australian Border Collies (ABCs) have not been investigated. The aetiology of lymphoma is complex and likely involves both environmental and genetic risk factors. A heritable component has been suggested in canine lymphoma due to the presence of familial clustering, and breed predisposition. However, the genetic mechanisms contributing to the disease are not yet understood.

The primary aims of this thesis are to describe clinical characteristics in ABCs, as well as to investigate evidence of heritability and genomic regions associated with the disease. A detailed survey collating clinical and pedigree information was conducted among breeders and owners of ABCs. The survey received 246 respondents, identifying 57 ABCs diagnosed with lymphoma (Chapter two). Multicentric, high grade B-cell was identified as the most common form of lymphoma in ABCs. The mean age of diagnosis was 9.16 years (standard deviation ± 3.43), with female dogs being twice as likely to develop lymphoma compared to males. Pedigree analyses revealed clusters of affected individuals: 21 cases descended from two sires and 28 cases shared a common female ancestor, supporting a heritable component of the disease in the breed.

Genome-wide association studies (GWAS) have previously identified genomic variants associated with lymphoma risk in other dog breeds, such as Bullmastiffs and Golden Retrievers. This study examined whether the previously identified 35 at-risk loci in these breeds are also associated with lymphoma risk in ABCs (Chapter three). Analysis of genotypes in 23 cases and 223 controls ABCs revealed no statistically significant associations at these loci, suggesting that germline risk factors for lymphoma differ among breeds.

To further explore genetic variants in lymphoma, genotypes of these at-risk loci were investigated in an affected ABC and six of its close family members. None of the genotypes of the at-risk SNPs were present exclusively in the affected dog. Therefore, the previously described at-risk loci showed no indication of segregation with development of lymphoma in this family.

GWAS was subsequently performed using these seven cases (an affected ABC and its closely related family) and 216 control cases. The most prominent association signal was located on chromosome 4 (CFA4), spanning a 2.5Mb region. Two cancer related genes, *GNG4* and *ARID4B* were found in the region. *GNG4* is linked to CXCR4 signalling pathway, which has been implicated in aggressive B-cell lymphoma in people.

This thesis contributes to the knowledge of lymphoma in dogs, particularly in ABCs. Further research is needed to identify the candidate genes linked to lymphoma susceptibility in ABCs and other breeds, enabling advancements in diagnostic and development of specific treatments.

References for published chapter two is cited at the end of the chapter, while references for chapters one, three and four are listed at the end of the thesis.

Table of Contents

Authorship attribution statement

Chapter two of this thesis is published as Cheng KY, Soh P, Bennett PF, Williamson P. Lymphoma in Australian Border Collies: survey results and pedigree analyses. Aust Vet J. 2019 Jan;97(1-2):14-22

I was equal first author for this paper with Dr. Pamela Soh. I co-designed the study with the coauthors. The survey was designed by the coauthors, and survey data was collected by Dr. Pamela Soh and me. I analysed the clinical information of the dataset and wrote the majority of the manuscript, while Dr Pamela Soh conducted pedigree analyses and wrote the part of the manuscript relating to pedigree analyses. All the co-authors contributed to revision of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

No content produced by generative AI tools has been used in the preparation of this thesis.

Yee Ka Katrina Cheng

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Peter Bennett

List of tables

Chapter 1

Chapter 3

List of figures

Chapter 2

Chapter 3

List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABCs	Australian Border Collies
BMD	Bernese Mountain Dog
CFA	Canine Chromosome
CAR-T	Chimeric antigen receptor T cells
CT	Computed tomography
CI	Confidence interval
CFC	Contribution, Inbreeding, Coancestry
CLL	Chronic lymphocytic leukaemia
CNAs	Copy number aberrations
DNA	Deoxyribonucleic acid
DLBCL	Diffuse large B cell lymphoma
EBV	Epstein-Barr virus
EqG	Equivalent complete generations
FBXW7	F-box and WD repeat domain-containing protein 7
GI	Gastrointestinal
GWAS	Genome-wide association studies
GC	Germinal centre
HL	Hodgkin lymphoma
HS	Histiocytic sarcoma
F	Inbreeding coefficients
LD	Linkage disequilibrium
MHC	Major histocompatibility complex
MZL	Marginal zone lymphoma
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
mAB	Monoclonal antibody
WF	National Cancer Institute Working Formulation
NGS	Next-generation sequencing

NHL	Non-Hodgkin’s lymphoma
OR	Odds ratio
PARR	PCR for antigen receptor rearrangements
PTCL-NOS	Peripheral T-cell lymphoma not otherwise specified
PET	Positron emission tomography
POT1	Protection of telomeres 1
SETD2	SET-domain containing 2
SNP	Single nucleotide polymorphism
SD	Standard deviation
SE	Standard error
TZL	T-zone lymphoma
REAL	The Revised European-American Classification of Lymphoid
TRAF	Tumour necrosis factor receptor-associated factor
WHO	World Health Organisation

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Chapter 1: Literature review

1.1 Introduction

Cancer is the leading cause of death in pet dogs worldwide[1-6]. About one in four dogs and half of the dogs older than 10 years old are affected by cancer[7]. Cancer incidence data from population-based cancer registries reported estimated cancer incidence ranging from 99 to 804 per 100,000 dogs at risk[3,8-12]. Cancer is a genetic disease, arising from accumulation of mutations that result in cancer cells' capability to sustain growth, eliminate normal constrains of proliferation and maintenance of genomic stability, promote immortalisation and modify and maintains a supportive environment for survival, invasion and metastasis[13]. Mutations of various critical genes and dysregulation of molecular pathways are often required in the development of cancer[14]. Lymphoma is one of the most common malignant cancers in dogs. It describes a group of neoplasms that originate from lymphocytes. These neoplasms often affect lymphoid tissues such as lymph nodes, spleen, and bone marrow, but also any other tissues in the body. This review aims to provide a summary of the current knowledge on canine lymphoma, with an emphasis on the genetic basis of lymphoma and breed predisposition.

1.2 Canine lymphoma

1.2.1 Overview

Lymphoma is the most common haematopoietic malignancy in dogs and is often fatal despite treatments. Similar to non-Hodgkin's lymphoma (NHL) in people, canine lymphoma is a heterogenous group of lymphoid malignancies classified based on their anatomical origin, specific lymphocyte lineage (cytological, histological and immunological characteristics) and biological behaviour. The true incidence of lymphoma in dogs is difficult to ascertain due to the lack of canine tumour registries. The annual incidence rate of canine lymphoma was estimated at 13 to 114 per 100,000 dogs, reported in various studies[10,11,15,16]. The incidence in both dogs and human seems to be rising without obvious reasons identified[17-21]. Middle-aged to older dogs are primarily affected. A decreased risk has been reported for intact females[17]. Breed predisposition in lymphoma suggests a heritable component of the disease, but the aetiology of lymphoma is likely multifactorial. The potential for canine lymphoma to serve as a model for human cancer, combined with improved pet care associated with the human-animal bond, has led to more recent studies focusing on the pathology/classification, genetic

mechanisms, and treatments in canine lymphoma. These studies have continued to broaden our understanding of the disease.

1.2.2 Aetiology

1.2.2.1 *Infectious factors*

Various viruses have been associated with lymphomagenesis in people, including Epstein-Barr virus (EBV), human T-lymphotropic virus, human immunodeficiency virus, Kaposi sarcoma-associated herpesvirus, and hepatitis C virus[22]. EBV, a Gammaherpesvirus, is associated with a minority of mainly B-cell subtypes of lymphoma in people. These include Hodgkin's lymphoma (HL), Burkitt lymphoma, diffuse large B cell lymphoma (DLBCL) , post-transplant lymphoproliferative disease and nasal T/NK-cell lymphoma[23,24]. The exact pathogenesis of EBV in lymphoma remains unknown. In vitro model has demonstrated EBV-encoded latent genes induce B-cell transformation by altering cellular gene transcription and constitutively activating key cell-signalling pathways, such as NF-κB and *MYC* deregulation[25]. Studies using different serological and molecular techniques have identified EBV-related DNA (Deoxyribonucleic acid) and antibodies for EBV in the peripheral blood and bone marrow of healthy pets[26-28]. Moreover, Huang *et al.* found that the presence of EBV DNA sequences and proteins in malignant lymphocytes of dogs with spontaneous B-cell lymphoma, as well as higher antibody titres in these dogs compared to T-cell lymphoma or controls[28]. These suggested that EBV may contribute to B-cell lymphomagenesis in a subset of infected dogs. In contrast, EBV sequence was not found in lymphoma tissues and there were no differences in seropositivity between dogs with lymphoma and unaffected dogs[27,29].Similarly, a study in cats failed to demonstrate any association between feline gammaherpesvirus and lymphoma[30].

1.2.2.2 *Environmental factors*

Exposure to pesticides, particularly herbicides containing 2,4-dichlorophenoxyacetic acid (2,4-D) has been suggested to be linked to an increased risk for NHL in people[31,32]. Environmental risk has been shown in some studies, but results have been inconsistent. A hospital-based case-control study found a significant 30% increased risk of lymphoma in dogs living in homes exposed to 2,4-D, and the risk of canine lymphoma increased to twofold with four or more yearly owner applications of 2,4-D. However, follow-up re-analysis by another group did not support the finding[33]. A recent US epidemiologic study investigating household exposure to a variety of environmental chemicals demonstrated that the use of pesticides, particularly when

professionally applied, was associated with a significant higher risk of lymphoma[34]. No association between the use of flea and tick control products and lymphoma was found in the same study[34]. In contrast, a European case-control study found no association with pesticide use in canine lymphoma, but residency in industrial areas and use of paints and solvents by owners increased the risk of lymphoma[35]. While exposure to passive tobacco smoke were significantly associated with development of lymphoma in two studies, a study on Golden Retrievers failed to find a significantly link[36,37,38]. Moreover, dogs with high or very high level of exposure to magnetic fields were more likely to develop lymphoma that those with low exposure[39]. Living in areas with illegal landfilling and unauthorised incineration, and those environment with waste incinerators, pollution and radioactive waste were proposed to be associated with canine lymphom[37]. However, in Pastor *et al*. (2009) study, the exact chemicals or pollutants were not studied, and the lack of details of exposure suggested that these were only risk indicators rather than factors[40].

1.2.2.3 *Immunologic factors*

Dysfunctional immune function has been implicated in canine lymphoma. Dogs with immune-mediated thrombocytopenia had a higher risk of subsequent development of lymphoma[41]. Previous diagnosis of atopic dermatitis has been associated with a higher risk of cutaneous lymphoma, *mycosis fungoides* in dogs[42]. However, the effect of chronic immunosuppressive drug therapy on lymphomagenesis cannot be excluded. Organ transplants receiving immunosuppressive medications have been associated with higher risk of lymphoma in cats and in people[43,44].

1.2.2.4 *Genetic predisposition*

Breed predisposition is well documented in canine lymphoma[8,16,17,40,45-50]. Breed predisposition, together with incidence of familial clustering provide evidence of germline susceptibility to lymphoma[51-53]. Selective breeding of certain phenotypic characteristics within breeds has resulted in limited diversity[54]. Deleterious mutations predispose to lymphoma could be enriched in different breeds, as a byproduct of breed formation[54]. Germline variants, candidate genes and molecular pathways contributing to lymphoma risk have been proposed in genetic studies in both dogs and people[55-58]. However, it is most likely that multiple variants with small individual effect sizes contribute to genetic risk in a complex disease, as in

lymphoma[55,58]. Breed predisposition and genetic basis of lymphoma is further discussed in later section (1.2.3.3).

1.2.3 Epidemiology

1.2.3.1 Age

Lymphoma can affect dogs of any age, but middle aged to older dogs are frequently affected. Studies have reported mean ages ranging from 6.6 to 9.1 years[16,40,46,47,59-62], with peak incidences occurring between eight and 10 years[10,12,16,62]. These trends were observed in both male and female dogs[10,47,59]. A study based on the Swiss Canine Cancer Registry, found that the peak incidence of lymphoma in 2466 dogs was 6 years[50]. A German study with 411 dogs with lymphoma found that dogs with T-cell lymphoma were significantly younger compared to the ones with B-cell subtype, but this was not identified in other studies[59,62,63].

1.2.3.2 Sex and neuter status

Male dogs have been reported to be at increased risk of lymphoma[10,40,47,49], while another study did not identify sex as a risk factor[64]. In humans, a reduced risk of NHL has been observed in postmenopausal women using hormone therapy compared to non-users, particularly among current users and those initiated therapy after the age of 50 years[65]. In dogs, hormone exposure is most commonly measured by neuter/spay status. A recent Australian study with 6201 cases showed an increased odds ratio (OR) for lymphoma in neutered animals (OR: 3.2), including both males (OR: 2.8) and females (OR: 4.4)[49]. Similarly, other studies found a protective effect against lymphoma in intact Vizslas[64], Golden Retrievers[66] and all breeds[17], as well as in intact males of Golden Retrievers specifically[66]. In contrast, neutering was not associated with lymphoma risk in Labrador Retrievers[66].

1.2.3.3 Breed

Many reported predisposed breeds are larger dogs, including Boxers, Bullmastiffs, Bulldogs, Basset Hounds, St Bernards, Bouviers des Flandres, Rottweilers, German Shepherds, Dobermans and Vizslas. Smaller breeds, such as Scottish Terriers, Airedale Terriers and Pembroke Welsh Corgis are reported to have increased lymphoma risk[16,32,46,47,49]. This most likely reflects genetic predisposition, instead of the influence of growth hormones[67]. Dachshunds, Yorkshire terriers and Pomeranians were reported to have lower relative risk.

There is also a different incidence of subtypes of lymphoma within breed. Boxers, "Spitz" breeds and Asian "lap" dogs had increased risk of developing T-cell tumours, compared to increased incidence of -tumours in Basset Hounds, Cocker Spaniels and Dobermans[62].

1.2.4 Classification, pathology and prognosis

1.2.4.1 Anatomical form and stage

Multicentric is the most common form of lymphoma, occurring in about 80% of affected dogs[48,61]. Most dogs present with enlargement of a single or multiple peripheral lymph nodes, in addition to other lymphoid tissues, such as liver and spleen. Multicentric lymphoma is staged according to the World Health Organisation (WHO)'s system (Table 1)[68]. This is further defined by substage based on the absence or presence of systemic signs; substage 'a' and 'b' respectively. Dogs presented with advanced stage (stage V)[69] and substage 'b'[70,71] generally have shorter remission duration compared to those with less advanced stage (Stage I or II) and substage 'a' when treated with chemotherapy.

Gastrointestinal (GI), mediastinal and cutaneous forms each account for about five to 10% of canine lymphoma[48,61]. GI lymphoma can be focal or diffuse and is typically of T-cell origin[72]. In the last decade, small cell/low grade GI lymphoma has been better characterised in dogs. This can be hard to distinguish with lymphocytic-plasmacytic inflammatory bowel disease, but both conditions have good prognosis with treatments[73]. Boxers and Shar-peis may be overrepresented in cases of GI lymphoma[72,74].

Mediastinal lymphoma is often of T-cell origin and characterised by enlargement of the cranial mediastinal lymph nodes, thymus or both[75]. Hypercalcaemia may be seen in affected dogs.

Cutaneous lymphoma mostly originates from T-cells, particularly CD8+ phenotype[76]. The epitheliotropic form, which commonly affects older dogs, is more common than the non-epitheliotropic form[76]. In advanced cases, cancerous cells can be found in the lymph nodes, internal organs and blood (known as Sézary syndrome) [77]

Lymphoma can affect any organs, but atypical forms of lymphoma, such as primary hepatosplenic, intravascular (ocular and nervous system), cardiac, nasal, pulmonary and

urogenital are rare[78]. Extranodal lymphomas are generally associated with poor prognosis compared to dogs with multicentric lymphoma[79-83].

Table 1. World Health Organisation's clinical staging system for lymphoma in domestic animals

Stage	
I	Involvement limited to a single node or lymphoid tissue in a single organ (excluding bone marrow)
II	Involvement of many lymph nodes in a regional area
III	Generalised lymph node involvement
IV	Liver and/or spleen involvement (± stage III)
V	Manifestation in blood and involvement of bone marrow and/or other organ systems ((± stage IV)
Substage	
a	Absence of systemic signs
b	Presence of systemic signs

1.2.4.2 Histological classification and grade

The Revised European American Classification of Lymphoid Neoplasms (REAL)/ World Health organisation (WHO) system, adapted from the human system[84], is used in dogs for relatively reproducible diagnosis of different subtypes of lymphoma[85]. Moreover, this classification has been shown to provide prognostic indications[86]. This system classifies each subtypes with distinct biological behaviour (indolent/ aggressive) based on anatomic locations (nodal or extranodal), tissue architecture (diffuse or nodular/follicular), morphology (cell size, cellular and nuclear features, including mitotic rate) and immunophenotype (B- and T- cell)[61,85 87]. The most frequent lymphoma is DLBCL, followed by peripheral T-cell lymphoma not otherwise specified (PTCL-NOS**)**, T-zone lymphoma (TZL), T-lymphoblastic lymphoma and marginal zone lymphoma (MZL)[88].

Other systems that have been used previously include the National Cancer Institute Working Formulation (WF) and the updated Kiel system[89,90]. The WF used criteria, includes pattern (diffuse or follicular), and cell type (such as small-cleaved cell, large cell, immunoblastic), but not immunophenotype. The updated Kiel classification includes pattern, morphology (centroblastic, centrocytic or immunoblastic) and immunophenotype of the cancer cells[75].

Tumours can be categorised as low-grade, intermediate-grade or high-grade based on size and/or mitotic rate of cancer cells in the WHO classification systems[48,85]. Lymphomas with zero to five, six to 10 and greater than 10 mitoses /400X field were grade as low, medium and high grade respectively[85]. Majority of canine lymphomas are considered intermediate or high-

grade (70-95%) with poorer prognosis compared to dogs with indolent or low-grade tumours[86,91 92].

1.2.4.3 Immunophenotype and common classifications of lymphoma

Lymphomas are characterised into B-cell or T-cell in origin, as determined by the expression of specific makers expressed by the tumour cells. In both people and dogs, B-cell lymphoma comprised 85-90% and 62%-75% of all human and canine lymphoma respectively[48,84,93]. Some of the common classifications of lymphoma are discussed below.

DLBCL

Within the B-cell lymphomas, DLBCL is the most common subtype, comprises 37% and 47-73% of human and canine B-cell lymphoma respectively[48,84,93]. However, some dog breeds have a higher incidence of certain immunophenotype of lymphoma (discussed in section 1.3.2). DLBCL is characterised by diffuse arrangement of sheets of neoplastic B cells (determined by identification of specific markers on tumour cells, including CD79a, CD21, CD20 and Pax5 by various methods) and the uniformly large nuclei (larger than two red cells in diameter) and scant cytoplasm of the neoplastic cells. Nuclei are usually round or rarely cleaved or indented. Thinning of lymph node capsule, compression of peripheral and medullary sinuses, destruction of normal nodal structures and filling of the medullary cords are seen[85].

Using gene expression profile, DLBCL can be further divided into molecular subtypes, including those that arise from cells within the germinal centre (GC) or from cells post-germinal centre activated (activated B-cell) in people[94]. Although similar subtypes are found in dogs using "dog-specific" genes, DLBCL in dogs is suggested to be more closely resembles the activated B-cell form in people[95,96]. Similar activating pathways, including B-cell receptor and NF-κB pathway associated with activated B-cell lymphoma are found in both species[96]. A variety of immunohistochemical algorithms have been developed to separate DLBCL into the two molecular subtypes with different prognosis; activated B-cell is associated with poor survival [97-99]. However, these algorithms were not useful in dogs[95] and the prognostic factors will be discussed in a later section (section 1.2.7).

PTCL-NOS

PTCL-NOS is the most common type of T-cell lymphoma in dogs, comprising 15% of all canine lymphoma cases[85]. PTCL is characterised by diffuse proliferation of neoplastic T-cells (identified by specific markers on tumour cells, including CD3, CD4, CD5, CD8 by various methods)[100]. The NOS category of peripheral (extrathymic) T-cell lymphomas include all T-cell lymphomas that are not well categorised and cannot be further differentiated based on morphology and cell size[85]. Neoplastic cells are often large but may be variable in cell size, nucleoli number and size and mitotic rates [61]. They are often associated with features of vascular proliferation and necrosis [85]. This is often an aggressive form of lymphoma with poor prognosis even with treatment[100].

TZL

TZL is a type of canine nodal lymphoma in which the neoplastic cells expand the paracortex and medullary cords but do not efface the nodal architecture[85]. Small or intermediate sized neoplastic cells predominate, with rare mitoses seen[85]. Clinical behaviour is often indolent, with less aggressive behaviour compared to other subtypes.

MZL

MZL is an indolent form of canine B-cell lymphoma, involving most commonly spleen or lymph nodes [85]. It is characterised by multinodular infiltrate of neoplastic cells surround fading remnants of germinal centres, resembling the marginal zone of the lymph node follicle[85]. Cytological features include small sized mature cells, nuclei of intermediate size with prominent single central nucleoli and abundant lightly stained cytoplasm[85]. Mitotic rate is often low except in advanced cases[85]. Dogs with advanced nodal MZL may have poor survival outcomes[101].

1.2.5 Clinical features, diagnosis and treatments

1.2.5.1 Clinical features

Dogs affected with multicentric lymphoma often present with generalised lymphadenopathy[102]. Some dogs present without systemic signs (substage ‘a’), while some may have non-specific signs, such as anorexia, weight loss, lethargy, vomiting, diarrhoea, emaciation, breathing difficulty and fever (substage ‘b’)[103]. Dogs with liver, spleen or bone marrow involvement may be presented with hepatosplenomegaly, anaemia, fever associated with neutropenia, and bleeding associated with thrombocytopenia[102]. Other clinical presentations are dependent on

the anatomical location of disease, and can include findings such as: dyspnoea due to thoracic lymphadenopathy, pulmonary infiltration or pleural effusion[75], oedema of the heck and neck from cranial vena cava syndrome[78], variable skin lesions[104], ocular signs, neurological signs[75]. This list is not exhaustive. Paraneoplastic immune-mediated anaemia and thrombocytopenia has been reported in dogs with lymphoma[71].

1.2.5.2 *Clinical pathology*

Non-specific abnormalities in minimal database blood and urine tests are found in most affected dogs. The most common ones include mild, non-regenerative anaemia associated with anaemia of chronic disease, neutrophilia associated with inflammatory response, mild increase in liver enzymes activities secondary to organ involvement or reactive hepatopathy and mild proteinuria[105]. Regenerative anaemia may be seen in dogs with gastrointestinal blood loss or immune mediated haemolytic anaemia. Dogs with bone marrow involvement may have moderate to severe anaemia, neutropenia, thrombocytopenia and lymphocytosis[106]. Bone marrow evaluation, with aspirates or core biopsy, can be performed for complete staging and prognostication. Increased kidney parameters may be associated with dehydration, hypercalcaemia or secondary to renal infiltration[106]. Hypoproteinaemia is observed in dogs with alimentary lymphoma. Monoclonal gammopathies occurred uncommonly[107]. Paraneoplastic hypercalcaemia is seen more often in dogs with T-cell lymphoma than those with B-cell lymphoma[108].

1.2.5.3 *Diagnostic imaging*

Thoracic radiographs, abdominal radiographs or ultrasonography are indicated to assess the locations of lymphoma and extent of internal involvement (i.e., staging). These may show pulmonary infiltrates, thoracic lymphadenopathy, cranial mediastinal mass, pleural effusion, hepatosplenomegaly and liver and spleen infiltration[109,110]. Advanced imaging modalities, including computed tomography (CT), magnetic resonance imaging, positron emission tomography (PET) or a combination of these are performed occasionally and are limited to equipment availability[111-113]. PET/CT imaging is the current standard of care for predicting durability of treatment response, and guiding treatment regimes in people with lymphoma[114].

1.2.5.4 Cytology, histopathology and immunophenotyping

Examination of cellular morphology from neoplastic tissues (lymph nodes or other organs) from fine needle aspirates cytology is often adequate in diagnose dogs with high grade lymphoma[115]. An increased monoclonal population of intermediate to large sized lymphocytes is suggestive of lymphoma. Histopathology, the assessment of tissue architecture, immunophenotype, cellular morphology and mitotic count by biopsy of tissues is required in some cases to confirm the diagnosis, especially in low grade lymphomas[115]. Histopathology and immunophenotyping are also required for further classification of lymphoma[85]. Immunophenotyping can be performed on cytological, histological samples or individual cells in a fluid medium (flow cytometry) and is determined by the expression of molecules specific for B cells and T cells[116,117].

1.2.5.5 Molecular diagnostic tests

In additional to immunophenotyping, flow cytometry also identifies subtypes of lymphoma with better prognosis, such as indolent T-zone with predominantly CD45- neoplastic cells[92].
PCR for antigen receptor rearrangements (PARR) is a clonality assay, which amplifies the variable regions of the immunoglobulin genes and the T-cell receptors. This assists the differentiation of lymphoma from reactive lymphoid processes, detecting minimal residual disease and immunophenotyping. PARR has shown to be inferior in immunophenotyping compared to immunohistochemistry and flow cytometry. MicroRNA (miRNA) expressions have been investigated in dogs with lymphoma in limited studies. The potential in using miRNA for immunophenotyping and prognostic indicator was demonstrated in a recent study[118].

1.2.6 Treatment

1.2.6.1 Chemotherapy

Chemotherapy is the standard of care treatment for canine lymphoma, as most affected dogs have systemic involvement. In rare cases of localised lymphoma, local modality such as surgery or radiation therapy may be considered. Multidrug combination chemotherapy is the treatment of choice for intermediate and high-grade lymphoma. Vincristine, cyclophosphamide, doxorubicin-based protocols, are the most used drugs for dogs with B-cell lymphoma. This induces remission in about 80-95% of dogs, with median progression free

survival and overall medial survival time of six to eight months and 10 to 12 months respectively[119]. Approximately a quarter of treated dogs will be alive two years after initiation of these protocol. Long term maintenance chemotherapy has not been shown to be superior in both dogs and people. Dogs with aggressive T-cell lymphoma may be treated with doxorubicin-based protocols, or differently with alternative lomustine or mechlorethamine-based protocols, depending on the clinicians' preference. The response rate and duration of response to chemotherapy may be inferior in dogs with aggressive T-cell lymphoma. Single agent chemotherapy, such as doxorubicin and lomustine, is used as induction therapy due to owners' preference. Rescue protocols are used when animals fail to respond to first-line protocols or when their lymphoma relapses, but the duration of response is often short. Prednisolone is used as palliative medication for dogs with lymphoma, often result in short-lived remission of one to two months, but occasionally longer. Indolent or low-grade lymphoma is treated with less intense chemotherapy, such as prednisolone and chlorambucil, or treatment is not required until the disease is advanced.

1.2.6.2 *Radiation therapy*

Radiation therapy can be used in dogs with localised lymphoma for curative or palliative intent, or used together or after chemotherapy as consolidation therapy in dogs with multicentric lymphoma[75,120]. For the latter case, radiation is often delivered after induction of remission with chemotherapy to the cranial and subsequent caudal half of the body in a staged fashion. Median duration of remission and median survival of 311-455 days and 486-684 days was reported in dogs treated with combination of half body irradiation and multiagent chemotherapy protocol[121,122]. Myeloablative total body irradiation carries the risks of severe myelosuppression and bone marrow, or peripheral blood stem cell transplantation, is required[123-125]. This treatment approach is in the early stages of investigation, with lack of information showing enhanced efficacy and safety compared to traditional treatments.

1.2.6.3 *Immunotherapy*

In addition to multi-agent chemotherapy, the use of monoclonal antibody (mAb) targeting CD20 receptor, such as rituximab has significantly improved survival in people with B-cell non-Hodgkin lymphoma (NHL)[126]. However, rituximab failed to bind to canine lymphoma cells [127]. Canine-specific mAb are currently being developed throughout the world[128-131]. Various anti-tumour vaccine strategies have been preliminarily investigated, including using

autologous cancer cells combined with Freund's/other adjuvants[132-134], and genetic vaccine targeting telomerase reversed transcriptase[135]. Notably, the addition of an active immunotherapy consisting of hydroxylapatite ceramic powder and heat shock proteins purified from canine tumours (APAVAC®) to a standard CHOP based protocol has been shown to improve survival in dogs with B-cell lymphoma[136]. Moreover, studies on chimeric antigen receptor T cells (CAR-T) and immune checkpoint blockade are currently underway following some success in humans[137,138].

1.2.6.4 Other therapies

Recently, two small molecules, Rabacfosadine succinate (Tanovea-CA1) and Verdinexor (Laverdia-CA1) have been approved fully and conditionally respectively by the U.S. Food and Drug Administration for the treatment of canine lymphoma respectively. Rabacfosadine succinate is a potent inhibitor of the major DNA polymerases, resulting in induction of apoptosis in tumour cells. Response rates of 50 to 85% have been reported as sole treatment, and treatment with Doxorubicin resulted in survival outcomes similar to those treated with conventional CHOP based therapy[139,140]. Verdinexor is an oral selective inhibitor of nuclear export, responsible for binding and nuclear export of a large variety of important tumour suppressor genes. Overall response rate of 37% for a median time to progression of 29 days, has been reported as a single agent treatment, but higher response (71%) was seen in dogs with T-cell lymphoma[141]. Another kinase inhibitor of KIT, PDGFR and LYN, Mastinib (Kinavet) is used in dogs with epitheliotropic lymphoma, where 70% response rate was shown in a study [142]. In phase I and II clinical RV1001, an orally bioavailable PI3K inhibitor, an injective response of 62% to77% was reported in dogs with B-cell and T-cell lymphoma[143].

1.2.7 Prognosis

The prognosis of dogs with lymphoma is generally poor but is highly variable depending on various factors, including histopathological classification and grade[61,91-93,115,144], immunophenotype[69,145,146], WHO clinical stage[47,70] and substage[70,71], anatomic location[79-83], proliferative assays[147,148], molecular expressions, clinical pathology and biomarkers (e.g. presence of anaemia, various peripheral blood cells ratio, hypercalcaemia, lactate dehydrogenase activity, thymidine kinase activity and serum albumin)[69,147-153] and others (sex, steroid pre-treatment and cranial mediastinal lymphadenopathy, post-chemotherapy neutropenia and geological location)[71,154-156]. The strongest prognostic factors include

histopathological classifications and grade, immunophenotype, WHO substage and anatomic locations [61,69-71,79-83,91-93,115,144,157].

Canine lymphomas can be categorised into three distinct molecular subgroups with distinct prognoses using quantitative real-time RT-PCR assays[86]. These include 1) low-grade T-cell lymphoma, including TZLs, 2) high-grade T-cell lymphoma, including lymphoblastic T-cell lymphoma and PTCL-NOS, and 3) B-cell lymphoma, including MZL, DLBCL and Burkitt lymphoma. This classification is prognostically significant, with the longest times in dogs with low-grade T-cell lymphomas, intermediate for dogs with B-cell lymphoma, and shortest for dogs with high-grade T-cell lymphomas [86]. Canine lymphomas with trisomy of chromosome 13, high-class II Major Histocompatibility Complex (MHC) expression, VH1-44 have been associated with a better prognosis [158-161], while p53, p16 messenger ribonucleic acid (mRNA) expression and gain of chromosome 31 were associated with worse survival [162,163,164]. Canine DLBCL was not separable into GCB and activated B-cell molecular subgroups by using human immunohistochemical algorithms, however two distinct molecular groups with significant difference in progression-free survival were identified using a canine-specific gene signature[95].

1.3 Genetic basis of lymphoma

Lymphoma is a complex disease, and the accumulation of multiple genetic lesions is most likely to contribute to lymphomagenesis. Our understanding of the genetic basis of canine lymphoma is relatively poor compared to human oncology, in which many genes and cellular pathways contributing to lymphoma have been identified. Genetic lesions can be resulted from somatic changes within tumours and/or germline variants with potential driver mutation functions.

1.3.1 Somatic mutations

Somatic changes in canine lymphoma, including chromosomal aberrations, gene mutations, epigenetic remodelling and dysregulated signalling pathways have been investigated. Chromosome aberrations were found in canine lymphoma using comparative genomic hybridisation. Thomas *et al.* (2011) found primarily recurrent copy number aberrations (CNAs): gains along CFA13 and 31 and partial loss of CFA26 in canine B-cell lymphoma, compared to a more frequent and wider distribution of recurrent CNAs in T-cell lymphoma[165]. When combining with human data, the region on CFA13 was narrowed to the syntenic region

on human chromosome 8 containing the oncogenes *MYC* and *c-KIT*, amplified in B-cell lymphomas from both species[165]. Similarly, copy number gains along CFA13 and CFA31 were again commonly observed in canine DLBCL[164]. In these segments, oncogenes commonly associated with cancers, such as *MYC*, *LDHB*, *KIT* and *PDGFRα* are annotated. In contrast, deletion of the *CDKN2A/B* tumour suppressor gene and inactivation of p16/Rb pathway were more commonly found in canine high grade T-cell lymphoma.

Recent advances in gene sequencing technologies, including next-generation sequencing (NGS) have led to identification of genetic mutations in canine lymphoma. This has contributed to the understanding of pathogenesis of cancer, disease characteristics (different molecular subtypes, response to therapy, prognosis and prognostic indicators), as well as potential treatment targets. Commonly found candidate genes implicated in canine B-cell lymphoma include *TRAF3*, *POT1*, *FBXW7*, *TP53*, *DDX3X*, *SETD2* and *MYC*[166-171]. One study examined breed-specific somatic mutations with exome sequencing in three breeds: Boxers with T-cell, Cocker Spaniels with B-cell, and Golden Retrievers with both T-cell and B-cell lymphoma[166]. Focusing on individual breeds allows the discovery of somatic mutations reflecting a relatively homogenous genetic background specific to the breeds. Expectedly, the significant mutated genes are different in the two immunophenotype groups. The most common recurrent mutations found in dogs with B-cell lymphoma were in *TRAF3*, *MAP3KI4*, *FBXW7* and *POT1*[166]. In contrast, the most significantly mutated genes in T-cell lymphoma, included *SATB1*, *TBC1D26*, *PSMA*, *COX8A* and *PTEN*[166]. The study found large overlaps of mutated genes and pathways in B-cell lymphoma in Cocker Spaniels and Golden Retrievers, whereas T-cell lymphomas from Golden Retrievers and Boxers are very different. This may be the result of somatic mutations directly reflecting genetic background but also reflects the different subtypes of T-cell lymphomas, (i.e. different genetic features) occurred in Boxers, and Golden Retrievers, aggressive forms of T-cell lymphoma and indolent form of T-cell lymphoma respectively. In addition to shared mutated genes found in people, the authors also found close similarities of altered genetic pathways to human DLBCL, including mutations affecting NF-kB signalling and histone modifiers.

1.3.1.1 *TRAF3 and NF-kB signalling pathways*

TRAF3 is a member of the tumour necrosis factor receptor-associated factor (TRAF) family of cytoplasmic adaptor proteins. It is a tumour suppressor gene. Loss of gene function results in

induction of transcription of anti-apoptotic Bcl2 family proteins, and upregulation of NF-kB activity, leading to B cell survival. *TRAF3* mutation is one of the most common somatic mutations in canine B-cell lymphoma, found in 30-53%[166,168,169,171-173]. No association with survival has been identified in dogs with the mutation[173]. Germ-line mutations affecting *TRAF3* were also identified in 17.5% of cases, and the majority of these lacked somatic mutation[169]. *TRAF3* mutations have been seen in people with classical HL, and multiple myeloma, as well as other malignancies. Although no point mutations have been found in DLBCL in people, *TRAF3* is recurrently deleted and concomitantly downregulated at the mRNA level in a subset of cases[169]. Upregulation of NF-kB pathway is involved in activated B-cell subtype of DLBCL in people and is associated with poorer survival than the GCB subtype[94]. Similarly, gene expression profiling also separates canine B-cell lymphoma into the two molecular subtypes, with higher expression of NF-kB pathways genes and poorer prognosis seen in the canine activated B-cell subtype[95].

1.3.1.2 *POT1*

POT1 (Protection of Telomeres 1) has a prominent role in telomerase activity[174]. Loss of function has been shown to increase chromosomal instability, leading to tumourigenesis[175]. *POT1* mutation has been found in 14-34% of cases of B-cell lymphoma in dogs[166,172,173,176,177]. An association between *POT1* mutations and reduced survival has been found in dogs undergoing treatments in one study[172], but not in other studies[173,177]. Dysfunction of telomerase activities are common in human cancers and mutations of *POT1* are frequently found in human chronic lymphocytic leukaemia (CLL)[178]. Germline mutations in *POT1* have been identified in various familial cases of malignancies in people, including HL and CLL[177,179,180].

1.3.1.3 *FBXW7*

FBXW7 (F-box and WD repeat domain-containing protein 7) is an important tumour suppressor gene, playing a key role in the ubiquitin-proteasome system (UPS)[181]. It controls proteasome-mediated degradation by ubiquitinating oncoproteins, such as c-Myc, Mcl-1, cyclin E and Notch[181]. *FBXW7* mediates apoptosis through targeting STAT3 for ubiquitylation and degradation in DLBCL in people[182]. In canine B-cell lymphoma, *FBXW7* mutation was identified in 13-25%[166,172,173,183]. Furthermore, dogs with *FBXW7* have been shown to have significantly shorter overall survival compared to dogs without the mutation[173]. It was found that 41% of *FBXW7* mutations in canine B cell lymphoma occur at the codon corresponding to

one of the two commonly mutated codons in people[166]. *FBXW7* is commonly mutated in many cancers, including haematopoietic cancers in people.

1.3.1.4 *TP53*

TP53 is a tumour suppressor gene, and the most frequently mutated gene in human cancers[184]. It is often referred to as the "guardian of the genome" due to its role in responding to various external and internal stresses, such as DNA damage, activation of oncogenes, nutrient deprivation and hypoxia[185]. *TP53* induces apoptosis by direct transcriptional activation of the pro-apoptotic BH3 pathway, which is activated by stress conditions[186]. *TP53* mutation was found in 8-25%of dogs with B-cell lymphoma[166,172,173,183,170.] Poorer survival outcome and treatment response have been identified in dogs with *TP53* mutations undergoing chemotherapy[170,172]. Similarly, *TP53* mutation is correlated with poor prognosis in people with NHL[187]. Germline mutations result in Li-Fraumeni syndrome, which affected patients are at risk of a variety of early-onset cancers. Germline *TP53* mutation has been reported in dogs diagnosed with lymphoma[188,189].

1.3.1.5 *SETD2*

SETD2 (SET-domain containing 2) mutation was found to be the second most common altered genes, found in 31% of dogs with DLBCL[172]. Another two studies found 11% and 13% of dogs harbouring the mutation[173,183]. SETD2 is a methyltransferase with various substrates, including histone and other transcription factors[172]. Loss of its tumour suppressor rule results in loss of genomic stability, disruption of the p53 apoptosis response, chromatin remodelling, and altered recruitment of proteins in DNA damage response[190]. In people, *SETD4* mutations are more commonly seen in T-cell lymphoma, but only less than 10% of DLBCL[191].

1.3.1.6 *MYC*

In additional to CNAs of CFA13 (a region encompassing *MYC*), somatic mutations of *MYC* were also found in 13-21%of dogs with B cell lymphoma[166,172,183]. The proto-oncogene *c-MYC* is an essential global transcription factor, involving in 10-15% of all human genes[192]. Dysregulation of *MYC* results in impaired functions in normal cell cycle, cell growth, survival, cellular metabolism and biosynthesis, adhesion and mitochondrial function[193]. *MYC* plays an important role in lymphomagenesis in people, and *c-MYC* rearrangement with one of the immunoglobulin gene loci is the hallmark of Burkitt's lymphoma[194]. On the other hand, *c-MYC*

rearrangement is identified in five to 15% of DLBCL in people, while increased c-MYC protein expression is seen in approximately 30-50% of cases[194]. In DLBCL, rearrangement of *c-MYC*, *BCL2* or *BCL6* represented a complex karyotype[168]. These are termed double-hit or triple-hit lymphoma and is associated with inferior survival and are refractory to conventional treatment with R-CHOP[195,196]. Similarly, affected dogs carrying *MYC* mutations had shorter survival when treated with chemotherapy[172].

1.3.2 Germline variants

Hereditary cancer syndromes account for nearly 10% of cancers in people[197]. These cancers arise from germline mutation in specific genes that increase an individual's susceptibility to cancer development. Notable examples include BRCA-associated breast cancer and Li-Fraumeni syndrome, which is associated with germline mutations in the tumour suppressor gene *TP53*[198]. In veterinary medicine, very few known hereditary cancer syndromes are recognised. One example is the rare inherited condition, known as canine hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis in German Shepherd Dogs, caused by mutations in the *BHD* gene[199]. Most of these mentioned hereditary cancer syndromes exhibit a simple autosomal dominant pattern of inheritance and are associated with high penetrance cancer susceptibility genes[198].

In contrast, we know that the cause of most cancers is likely multi-factorial, including a combination of environmental and genetic factors[200]. Studying germline variants may allow us to identify the risk genotypes for a disease suggested genetic predisposition. The advantages of identifying susceptible individuals include the potential for early detection, preventative cancer measures and targeted pharmacologic treatments[198]. It also helps identifying specific pathways associated with variants of genes, providing new insights into cancer biology. Dogs, as a model for studying cancer genetics, offer unique advantages due to their more limited genomic diversity within breeds, compared to humans, who have higher level of phenotypic and genetic variation[201]. This can aid in mapping cancer susceptibility genes in complex disease like cancer, offering valuable insights for translational research.

1.3.2.1 *Evidence of heritability in lymphoma*

In dogs, familial clustering, breed predisposition and suspected germline variants strongly suggest a heritable component contributing to cancer pathogenesis. Breed predisposition in

canine lymphoma is well-documented (discussed in section 1.2.2), although variations can occur based on geographical location. Furthermore, breed predisposition to specific lymphoma subtypes further supports the role of inherited germline variants. For example, Cocker Spaniels exhibit an increased risk for B-cell lymphoma, while Boxers are more prone to aggressive, and lymphoblastic T-cell lymphoma[40,108,202]. Familial cases of lymphoma have also been reported in families of 59 bullmastiffs from three households died from lymphoma over a three-year period[53]. Similar clustering has been reported in other breeds, such as Rottweilers and Otterhounds [51,52].

In people, evidence for familial predisposition has been demonstrated by studying twins and familial aggregation. There was a 100-fold higher risk of HL in monozygotic twins of patients with HL compared to background incidence rate, whereas there was no excess risk in dizygotic twins[203]. There was also increased risk of developing NHL for those with first degree relative with diagnosed with NHL (1.8-fold) and HL (1.5-fold)[204]. The risk of developing DLBCL was found to be 10-fold higher in people with first-degree relatives of DLBCL[205].

1.3.2.2 *GWAS*

Linkage studies identify chromosomal regions that show excessive sharing of inherited alleles among affected individuals in generations of families. However, significant linkage has not been identified in DLBCL patients, suggesting that multiple, low-to-moderate risk variants may be more relevant than highly penetrant variants in lymphomagenesis[206]. It is also recognised that much of the genetic architecture of cancer susceptibility is explained by polygenic inheritance. In contrast to linkage studies, case-control association studies of sequence variation in germline DNA are much more efficient in identifying low-penetrance alleles with the use of genotyping technologies[207]. Single nucleotide polymorphism (SNP), a single base-pair change in the DNA sequence is the most common type of genetic variation in the human genome. GWAS compare the frequency of SNPs in a large series of unrelated patients with cancers with those in matched healthy individuals to identify genetic variants associated with cancer risk. Commercially available genome platforms, “chips” are used to genotype the DNA from both groups. GWAS of most of the common cancers have been performed in people, and some of these have also been performed in dogs in recent years. It is recognised that most cancer risk loci identified through GWAS are located in non-coding regions of the genome, resulting in altered expression or regulation of a gene.

1.3.2.3 GWAS in canine lymphoma

A GWAS investigating genetic risks for B-cell lymphoma and haemangiosarcoma in Golden Retrievers in the United States suggested that regulatory mutations affecting T-cell mediated immune response contribute to the developing of both cancers[55].

Two shared predisposing loci, located on chromosome 5 between 29.6Mb and 34.1Mb, were identified in the study. Together they contribute to about 20% of the risk of developing these cancers. Within these regions, several partially overlapping haplotypes, predispose somewhat differently to the two cancers. The predisposing germ-line mutations seem to be regulatory as no exon coding changes were found. Gene expression study of the lymphomas found that these risk haplotypes are associated with down-regulation of several nearby genes, including *TRPC6*, *STX8*, *BIRC3*, *ANGPTL5* and *KIAA1377*. TRPC6 is a transient receptor calcium channel involved in T-cell activation[208,209], while *BIRC3* encodes an anti-apoptotic protein associated with B-cell and other cancers [210].

Another GWAS investigated over 300 cases of lymphoma across multiple breeds ,but failed to detect significant associations[211]. However, when only cases in Golden Retrieves were used in the analysis, a quantitative trait locus on Chromosome 4 was found. Severe candidate genes were identified, including *MCC*, *MXD3* and *FGFR4*. *MCC* is a candidate tumour suppressor gene that is thought to negatively regulate cell cycle progression[212]. *MXD3* encodes a member of the *MYC* superfamily[213]. MYC is an important transcriptional regulator, affecting cell proliferation, differentiation and apoptosis. Its role in cancer development has been reported in a few cancers, such as B-cell acute lymphoblastic leukaemia in people[214]. The authors believed that the genetic basis for most canine complex disease will be shared across breeds, based on human disease studies. The failure in identifying genetic risks was due to small sample size and variable tagging of causal variants across breed. However, it is unknown whether the genetic risk for lymphoma would be breed specific or not.

Labadie *et al*. studied TZL in Golden Retrievers and found associated regions on chromosome 8 and 14[215]. Non-synonymous mutations in three hyaluronidase genes (*SPAM1*, *HYAL4* and *HYALP1*) on chromosome 14 were identified. The authors suggested that TZL pathogenesis could be related to hyaluronan breakdown and subsequent production of pro-inflammatory and

pro-oncogenic byproducts. These mutations in hyaluronidase genes were also found to be associated with mast cell tumours in another study[216], but not in dogs in the Labedie *el al*, dataset. Variants affecting genes with regulatory function were identified, including *DIO2*, *CEP128*, *GTF2A1*, *STON2* and *SEL1L*. It was interesting that Golden Retrievers in other continents, such as Europe or Australia are not known to be predisposed to TZL, suggesting the possibility of unique genetic risk within breeds that may have segregated with breed line.

A recent combined GWAS investigated genetic risk of histiocytic sarcoma (HS) in four closely related breeds, Burmese Mountain Dogs (BMD), Rottweilers, Flat-coated retrievers and Golden Retrievers[217]. HS risk locus was refined on the *CDKN2A* locus on 11, but other new loci on chromosomes 2,5,14 and 20 were also identified. When combining cases of HS with lymphoma in BMD, variants at loci within CFA5 was identified. *SPNS3* was a candidate gene, which its role in cancer is unknown, but its paralogous gene (*SPNS2*) is important in immunological development. These SNVs are located in one of the two lymphoma predisposing peaks found in the Golden Retrievers by Tonomura *el al*[55]. When another analysis was performed with the HS, LSA in BMD combined with LSA in Golden Retrievers, increased signal on CFA5 was found. These suggested that BMDs, and Golden Retrievers share common risk loci on CFA5 that are involved in haematopoietic cancers.

Bullmastiff is a known predisposed breed for lymphoma, and a GWAS study identified risk loci on CFA13 and CFA33 [58]. Four potential function candidate genes including *MYC*, *PVT1*, *SENP7* and *NFKBIZ* were identified. These genes have functional links to *MYC* regulation.

At the time of writing the current literature review, our research group has published a paper on GWAS analysis in Border Collies, in which significant SNPs in regions on chromosomes 18 and 27 were found[218]. Candidate genes identified include *DLA-79*, *WNT10B*, *LMBR1L*, *KMT2D* and *CCNT1*.

1.3.2.4 *GWAS studies of NHL in people*

GWAS have successfully identified more than 60 susceptibility loci for specific NHL subtypes. One study found that none of the genetic loci identified were associated with all four NHL subtypes, including CLL, follicular lymphoma, DLBCL and MZL[219].

A GWAS conducted in in the Chinese population identified a susceptibility locus rs6773854, located between *BCL6* and *LPP* on oncogene-rich chromosome 3q27 that was significantly associated with increased risk of DLBCL[56]. Recently, the first large-scale GWAS of DLBCL with over 4000 cases conducted among individuals of European ancestry identified five novel loci achieving genome-wide significance, including 6p25.3(*EXOC2*), 6p21.33 (*HLA-B*), 2p23.3 (*NCOA1*), and 8q24.21 (near *PVT1* and *MYC*)[220]. The strongest associations after imputing *HLA* alleles were with the *HLA-B* SNP rs2523607 and the *HLA-B**08:01 allele. These results strongly suggest *HLA-B**08:01 as the primary MHC association with DLBCL risk[220]. HLA molecules present pathogen and tumour-derived peptides to cytotoxic T-lymphocytes thereby initiating the adaptive immune response[221]. Class I molecules have been linked to a variety of immune-mediated diseases and cancers, including HL, follicular lymphoma and DLBCL[222-225]. Three of the five GWAS genome-wide significant SNPs from the European study were also associated with DLBCL in an East Asian population[226]. These included *EXOC2*, *PVT1* and *HLA-B*. *PVT1* is a non-coding RNA affecting *MYC* activation, an important oncogene in lymphomas. *EXOC2* has roles in cell death regulation and host defers. It was estimated that common SNPs, including but not limited to the GWAS-discovered loci, explained about 16% of the variance in DLBCL risk overall[226]. GWAS may also provide potential prognostic or therapeutic targets in lymphoma. A prospective GWAS study also identified 10 SNPs as biomarkers to predict R-CHOP efficacy in DLBCL patients[227]. Wild-type patients showed a prolonged progression-free survival or overall survival compared with patients carrying deleterious alleles[227]. Similarly, GWAS studies of other subtypes of NHL have been performed and readers are directly to this recent review for findings in other subtypes[228].

Most SNP associations identified have been cancer-specific, however at-risk genomic loci associated with multiple cancers have been identified both in people and in dogs. This comprises one-third of SNPs found in people and are termed pleiotropic loci. In people, SNP rs2736100 at 5p15.33 (*TERT*) is associated with risk of many cancers, including glioma, bladder and lung cancers. Another example is the locus at 9p21.3 (*CDKN2A- CDKN2B*) was involved in many cancers, including glioma, melanoma, acute lymphocytic leukaemia and lung cancer. Similarly, studies in dogs have found shared loci associated with multiple cancer types, some are organ-specific, such as haematologic cancers in dogs with HS, haemangiosarcoma and lymphoma.

1.4 ABCs

1.4.1 Origin of the breed

The Border Collies originated in the border regions of Scotland over 350 years ago. It is believed that they came from a mixed ancestry of larger and more robust herding dogs, including the Bob-tailed sheepdogs, Bearded Collies and the Harlequin Collies. They were developed to herd livestock in harsh conditions of the mountainous terrain, especially during winters. They were introduced to other countries, including Australia in early 1900s due to their reputation of intelligence, strong desire to work, and calm approach in herding livestock. Since then, they have been selectively bred to have a smooth coat to suit the drier and hotter climates in Australia. Border Collies are relatively healthy breed, with median estimates longevity of 13.5 years[6]. Ceroid Lipofuscinosis, Trapped Neutrophil Syndrome and Collie Eye Anomaly are some of the hereditary diseases known in the breed. These diseases are seen less commonly nowadays due to the adoption of genetic tests by breeders. Border Collies are not known to be a breed with higher risk of cancers compared to other breeds. In a survey study of mortality in purebred dogs in the UK, 23% of Border Collies died from cancer related causes compared to some breeds with close to 50% mortality from cancer [2]. These include Flat Coated Retrievers, Irish Water Spaniels, BMD and Rottweilers[2].

1.4.2 Evidence of lymphoma predisposition in ABCs

In the UK study investigating breed incidence of lymphoma in insured dogs, Border Collies may have increased risk of developing lymphoma compared to other breeds, but it did not reach statistically significance (OR 1.32; 95% CI 0.4-4.35)[16]. Two studies have investigated breed risk in lymphomas in ABCs. Border Collies were shown to have increased risk in developing lymphoma in the referral population (OR 3.38, CI 1.52-7.53)[60]. Similarly, in another study with >6000 affected dogs in a broader population, there was an increased risk of lymphoma in Border Collies (OR 1.8, 95% CI 1.6-2.0)[49].

1.5 Conclusion

Lymphoma is a very common disease in dogs, yet the aetiologies remain largely unknown. ABCs have increased risk in development of lymphoma, suggesting germline mutations may contribute to the disease. The current thesis aims to advance the understanding of the genetics

of lymphoma in the breed. This research could lead to early detection of disease, targeted therapeutic options and breeding strategies that may improve the welfare of affected animals.

1.6 Aims

The primary aim of the thesis was to investigate the epidemiology of lymphoma in ABCs, as well as to explore the heritability and genetic risk factors in the breed.

In Chapter two, epidemiology and disease characteristics of lymphoma in ABCs was studied through a general health survey. Pedigree analyses revealed 21 affected dogs that descended from two sires and 28 cases with a common female ancestor, suggesting a heritable component of the disease.

In Chapter three, genotype data analyses were performed in the ABCs. The previously reported at-risk loci in other breeds were investigated in the affected ABCs. These at-risk loci were also investigated in the family of an affected ABC. GWAS was subsequently performed using these seven cases (an affected ABC and its closely related family) and 216 control cases.

In Chapter four, the findings of chapter two and three and its implications in lymphoma research are discussed.

Chapter 2 Published paper: Lymphoma in Australian Border Collies: survey results and pedigree Analyses

A health survey was completed by owners and breeders of Australian Border Collies and results were analysed. The aims of this chapter were to investigate the clinical characteristics (including age, gender and the most common subtype) of lymphoma in Border Collies in Australia, and to identify the relationship between affected cases through pedigrees analyses. Results of the study identified the occurrence of familial clustering, suggesting a heritable component of lymphoma in the breed.

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SMALL ANIMALS

Lymphoma in Australian Border Collies: survey results and pedigree analyses

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Objectives The aims of this study were to (1) describe the results of a survey on the clinical features of lymphoma in Australian Border Collies and (2) investigate familial clustering of lymphoma-affected dogs by means of pedigree analyses.

Methods Clinical and pedigree information was collected from surveys completed by owners or breeders of Australian Border Collies. Relationships between dogs were derived from pedigree data and kinship was analysed by network and cluster-based algorithms.

Results A total of 246 respondents completed the survey and 57 lymphoma-affected Australian Border Collies were identified. The mean age of diagnosis was 9.16 (SD ± 3.43) years and the median was 9.7 years (range 2–15 years). The odds of female dogs affected with lymphoma were twice those of males in the reported data (OR = 2.06; 95% CI = 1.13–3.73; P = 0.02). Multicentric, high-grade B-cell lymphoma was the most common form in these dogs. Pedigree analyses identified 21 affected dogs that descended from two sires and 28 cases with a common female ancestor. Average inbreeding between both affected and unaffected dogs was similar (0.16, SD ± 0.06 and 0.15, SD ± 0.06, respectively).

Conclusion The survey confirmed the presence of a relatively large number of cases of lymphoma in Australian Border Collies, consistent with our previous report of increased risk in this breed. Some dogs were diagnosed at a very young age, but the age ranged over the normal lifespan. Pedigree analyses identified multiple cases within family groups, suggesting a heritable component of the disease in this breed.

Keywords Australia; Border Collies; lymphoma; lymphosarcoma; pedigree

Abbreviations ABC, Australian Border Collie; CFC, Contribution Inbreeding Coancestry; CI, confidence interval; EqG, equivalent complete generations; F, inbreeding coefficients; OR, odds ratio; SD, standard deviation; SE, standard error

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Lymphoma is one of the most common canine cancers, but its aetiology remains largely unknown. Development of lymphoma is assumed to be the result of complex interactions between genetics and environment. Genetic factors may be broadly categorised into germline variants with potential driver mutation function and somatic changes within tumours. Somatic hypermutation, whole chromosomal aberrations, altered gene expression and epigenetic changes have all been documented in dogs with lymphoma.[1–8] Other factors related to environmental effects have also been implicated, including exposure to herbicides and other chemicals,[9–12] residing in an industrial area[9,13,14] and exposure to strong magnetic fields.[15] There is further evidence for increased risk associated with a history of immune-mediated thrombocytopenia,[16] atopic dermatitis[17] and gammaherpesvirus infections.[18]

Breed predisposition indicates a potential genetic component for the risk of developing disease. Previous studies have identified breeds with a consistent increased risk of lymphoma despite different geographic locations,[13,19–24] but reported at-risk breeds may also vary between geographic regions.[19,21,25] Although this may be explained by breed popularity or environmental factors in different regions, selective breeding based on genetically restricted subpopulations will also contribute.

Our recent study of the demographics of more than 6000 dogs with lymphoma in Australia demonstrated an increased breed risk (odds ratio (OR) = 1.8; 95% confidence interval (CI) 1.6–2.0) in Border Collies compared with the general population.[25] However, in that study pedigree information was not available for individually affected dogs, preventing a more detailed investigation of any potential genetic aetiology. Collection and analysis of pedigree data has the potential to identify modes of inheritance and carriers of disease in a population. Pedigree analysis has been shown to be useful in several disease studies in dogs, including identifying a common ancestor for neuronal ceroid lipofuscinosis in a Japanese population of Border Collies[26] and segregation of a canine hereditary renal cancer syndrome in German Shepherd Dogs.[27]

To the best of our knowledge, pedigree analysis of dogs with cancer has never been performed in an Australian setting. The goals of the current survey were to identify lymphoma cases among Australian Border Collies (ABCs) and to collect associated pedigree data. We report the clinical features of lymphoma in these dogs, as well as an analysis of kinship within the affected subpopulation and compare it with unaffected dogs and a broader registered Border Collie pedigree.

Materials and methods

Approval to conduct the general health survey was obtained from the Human Research Ethics Committee at the University of Sydney (Project no. 2016/559). The survey was conducted between June 2016 and December 2017.

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An electronic link for a web-based or hardcopy survey was distributed to owners or breeders of ABCs. The study was promoted in the newsletter of the Border Collie Club of NSW Inc., on the University of Sydney's webpage and by direct communications with practising veterinarians in Australia.

The survey was divided into three sections (Supplementary File 1). The first section of the survey sought contact details of respondents and primary veterinarians, as well as details of the dogs (registered name and number, microchip number, date of birth, sex, neutered status, age when neutered and country of birth). Respondents were asked for permission for the investigators to contact the primary veterinarian for more information. Medical records and pathology results were requested if consent was granted by the respondents. Review of available medical records, and any required amendments to survey data, was performed by a board-certified veterinary oncologist (PB) and oncology resident (KYC).

The second section asked the respondents about the current health status of their dogs, including 'healthy', 'has an illness' or 'deceased', details of illness and cause of death if applicable. Respondents who had dogs with lymphoma were asked additional questions specific to lymphoma, including age at diagnosis, concurrent illness, clinical features, treatment and outcomes, as well as details of known related dogs diagnosed with lymphoma. The final section requested pedigree information on affected dogs. Respondents were invited to give additional comments at the end of the survey.

Dogs included for analysis in the study were purebred Border Collies with an Australian lineage. Dogs were considered purebred if supported by pedigree documentation or identified as such by a veterinarian or the owner if unregistered. Dogs were considered Australian if registered in Australia or had at least one great-grandparent from an Australian lineage based on pedigree details or were born in Australia. Dogs were excluded if these criteria were not met. Dogs were also excluded if health status was not stated on the survey. Repeated entries were removed. Upon review of available medical records, dogs were excluded if clinical assessment by a veterinarian was inconsistent with the diagnosis of lymphoma.

Statistical analysis

Chi-squared test was used to calculate the sex predisposition to lymphoma. A P-value < 0.05 was considered statistically significant. Pedigree information was obtained from the survey and cross-checked with an extensive pedigree database with over 80,000 dogs collated from collected data and publically available data. Dogs were included in the pedigree analyses if there was complete pedigree information for at least three generations. The program Contribution, Inbreeding, Coancestry (CFC)[28] was used to fetch all generations preceding surveyed dogs available in the database and to calculate inbreeding values. Inbreeding was calculated in CFC using the method by Sargolzaei et al.[29] The number of equivalent complete generations (EqG), a measure of the depth of the pedigree and defined as the sum of the proportion of known ancestors in each generation, was calculated using optiSel (implemented in R).[30,31]

A kinship matrix based on all generations of pedigree information was created using the software packages pedigree and kinship2 (implemented in R).[32–34] A group of 10 unaffected individuals was selected to compare kinship values to a dam of interest that occurred frequently in the pedigrees of affected dogs. NetView (implemented in R) was used to generate a high-definition relationship network between lymphoma-affected and -unaffected dogs, based on the kinship matrix.[34–36] NetView clusters based on mutual *k*-nearest neighbours were created to visualise the relatedness between individuals using pedigree data.[34,36] A *k*-value of 11 was used to cluster dogs included in the survey. To focus on the relationship between affected dogs, a cluster analysis and heatmap display based on the kinship matrix of these dogs was generated, using the package gplots and the Manhattan distance algorithm.[37] Pedigree charts were created using the web-based PhenoTips Playground (playground phenotips.org).[38] Inkscape software version 0.92 (Inkscape Software, USA, www.inkscape.org) was used to annotate the pedigree. A heritability estimate for lymphoma was generated using the software package ASReml-R.[39]

Results

A total of 339 responses were received, 246 of which met the inclusion criteria. Of these, 117 (48%) responders were from New South Wales. The others were from Victoria (n = 40; 16%), Queensland (n = 37; 15%), Western Australia (n = 25; 10%), South Australia (n = 12; 5%), Australian Capital Territory (n = 7; 3%), Tasmania (n = 5; 2%), countries other than Australia (n = 2; 1%) and Northern Territory (n = 1; < 1%).

In total, 113 ABCs (46%) were female (23 entire; 83 neutered; 7 unreported) and 133 (54%) were male (36 entire; 91 neutered; 6 unreported). Of these, 138 (56%) were healthy dogs with no history of lymphoma; 51 (21%) were dogs diagnosed with diseases other than lymphoma.

A total of 57 (23%) dogs had been diagnosed with lymphoma. Age of diagnosis was known for 56 dogs, with a mean age of 9.16 (SD ± 3.43) years and a median of 9.7 years (range 2–15 years) (Figure 1). The median age of unaffected dogs was 12 years (range 2–15 years). Of the 57 lymphoma-affected dogs, 34 (60%; 25 neutered; 5 entire; 4 unknown) were female and 23 (40%; 17 neutered; 6 entire) were male. Among those reported, the odds of female dogs developing lymphoma were more than twice those of male dogs (OR = 2.06; 95%CI = 1.13–3.73; P = 0.02).

Medical records from primary veterinarians were available for review for 24 (42%) dogs and amendments were made if applicable. The anatomical form of lymphoma was reported in 51 (89%) dogs. The multicentric form was the most common and found in 43 (84%) dogs. There were 4 (8%) dogs with alimentary, 3 (6%) with cutaneous, and 1 (2%) with an extranodal form. The immunophenotype was available in 19 of 54 (35%) dogs with lymphoma: 16 (84%) were B-cell and 3 (16%) were T-cell; 21 (37%) dogs had the grade of lymphoma recorded, including 18 (86%) with high-grade, 2 (9%) with intermediate and 1 (5%) with low-grade lymphoma. Staging was reported in 34 (60%) dogs. As most dogs did not have complete diagnostic tests for staging, the results were grouped as Stage I, II–IV and suspected V or leukaemia, which were identified in 1 (3%), 25 (73%), and 8 (24%) dogs, respectively.

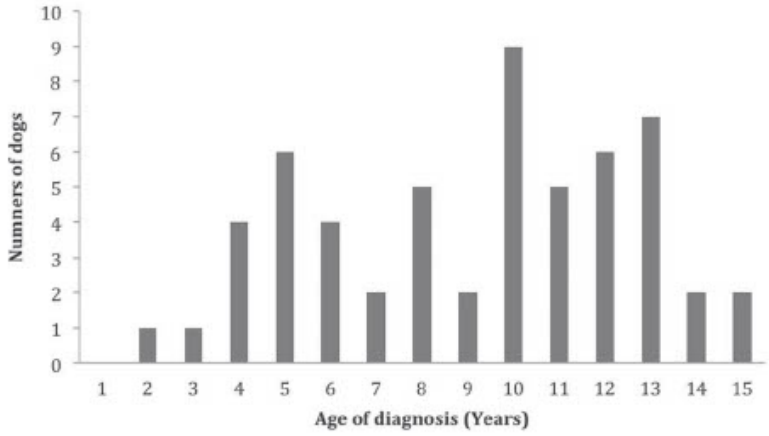

Figure 1. Age of diagnosis in lymphoma-affected Australian Border Collies (n = 103).

As for medical histories, 5 dogs had previously had other tumours, including 3 with masses of unknown origin and 1 each with lipoma and ameloblastoma; 9 dogs had a history of infections, including 6 with skin or ear infections and 3 with urinary tract infections. None of the dogs had a history of immune-mediated thrombocytopenia. Of the other illnesses recorded, 16 dogs included 4 with arthritis, 3 with skin allergy, 2 with renal disease and 1 each with liver disease, epilepsy, vestibular disease, laryngeal paralysis, urinary incontinence, pancreatitis and discoid lupus erythematosus.

The questions on treatments for lymphoma were answered by 50 (88%) responders: 13 (26%) dogs did not receive any treatment and 37 (74%) received treatment, including 20 (54%) with chemotherapy, 8 (22%) with prednisolone alone and 6 (16%) with 'other'. Three dogs (8%) had surgery, with two cases of surgery alone and one of surgery and chemotherapy.

At least three generations of pedigree information were available for 103 of the 246 (40%) dogs included in the study; 39 of the 57 (68%) dogs with lymphoma had pedigree information for analysis. Three dogs were born in the USA, but had at least one Australian grandparent or great-grandparent and were therefore included in the analysis.

Between 9 and 17 generations were recovered for lymphoma-affected dogs (2645 dogs in total, including generation 0 dogs) and between 10 and 18 generations for unaffected dogs (3634 dogs in total, including generation 0 dogs). After removing 14 dogs with only one known parent and keeping only unique dogs between both datasets, 3933 dogs remained. With this data, a kinship matrix was created and the lymphoma-affected and -unaffected dogs were subset for analyses and to generate the NetView and heatmap images. In NetView, nodes represent individual dogs and lines (edges) represent relationships between dogs, where proximal nodes with short lines indicate closely related dogs. Two sub-networks of dogs were evident in the NetView analysis, with a spread of affected dogs throughout both subgroups (Figure 2). In the first major cluster (Figure 2 top left), there were a greater number of closely related affected dogs than in the second cluster (Figure 2 lower right), which had fewer unaffected dogs. Subsequently, kinship cluster analysis and pedigree mapping were performed to investigate the relatedness of affected dogs only (Figure 2).

Similar to the population structure shown in Figure 2, lymphoma-affected dogs were divided into two major groups in the kinship cluster analysis (Figure 3). The upper right quadrant contained 30 dogs further divided into two clusters, one with 14 closely related dogs (ϕ = 0.17–0.39), and the other cluster consisting of 16 dogs with a wider range of kinship (ϕ = 0.09–0.36). Of the 14 closely related dogs, 5 descended from dog–50624 (IV-2) and 9 descended from dog–50681 (V-1) in the pedigree, both of which shared a common ancestor, dog–65895 (II-4) (Figures 3, 4).

Pedigree mapping revealed the direct ancestry of affected dogs. In some cases the animals appeared in the same line or family group. These included two instances of affected dam and daughter, a pair of affected littermates, two instances of affected parental sibling and two instances of affected half-siblings. A subtotal of 28 cases were mapped to one common ancestor, dog–67066 (I-2) (Figure 4), which was the second most frequently occurring dam in the pedigrees of affected dogs (14 times). Dog–65895 (II-4) from this pedigree was related to 23 cases, predominantly through two heavily used sires that descended from this dog: dog–50624 (IV-2) and dog–50681 (V-1). These two dogs were each related to 14 and 12 cases, respectively (5 cases were shared between these dogs). Dog–50624 (IV-2) appeared in the pedigrees of affected dogs 17 times and 25 times in unaffected dogs. Dog–50681 (V-1) appeared 8 times in both affected and unaffected dogs. A total of 11 (39%) of the 28 affected dogs related to the common dam (dog–67066) were diagnosed at 11 years or older. The remaining 17 dogs were diagnosed under 10 years of age (3.5–9.9 years) In the 4 cases of dogs descended from dog–44286 (VI-2) there was a mean age of diagnosis of 4.5 years (range 3.5–6.1 years) The littermate of this dog, dog–44277 (VI-1) was related to 6 other cases, with a mean age of diagnosis of 8.6 years (range 3.5–15.25 years). Among the 10 ABCs randomly selected from the extensive database to compare kinship values to the common dam in the pedigree, there were 13–15 generations of ancestry available and the average kinship value to the common dam was 0.14 (SD $\pm$ 0.03) (Figure 4).

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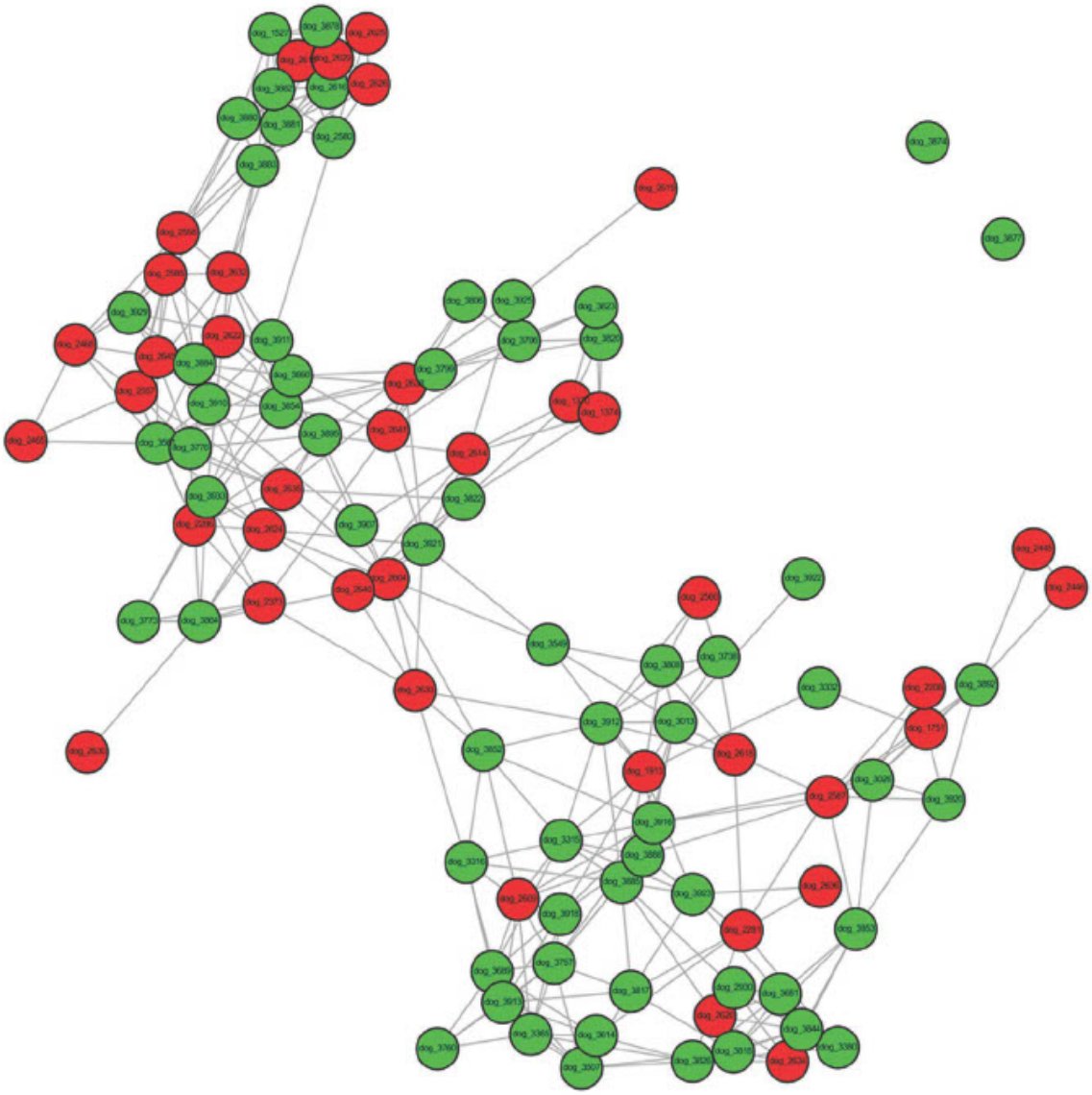

Figure 2. Relationship network of lymphoma-affected (red nodes) and –unaffected (green nodes) Australian Border Collies. The network captured 39 lymphoma cases in the sample population of 103 dogs.

The inbreeding coefficient (F), a value used to describe the relatedness between an individual's parents, was calculated for each dog based on all preceding pedigree data available from the pedigree database containing 80,000 dogs. The mean inbreeding coefficient for the 103 surveyed pedigree dogs was 0.15 (SD ± 0.06, range 0.01–0.32), while for lymphoma-affected and -unaffected dogs, the values were 0.16 (SD ± 0.06, range 0.04–0.27) and 0.15 (SD ± 0.06, range 0.01–0.32), respectively. When frequency distributions of F among lymphoma-affected and -unaffected dogs were examined, they followed a similar pattern (Figure 5). Inbreeding values for the two heavily used sires, dog–50624 (IV-2) and dog–50681 (V-1) (Figure 4), were 0.18 and 0.22, respectively. The most frequently occurring dam (15 times) was the dam of dog–77563 (I-1) and occurred frequently because of its relationship to two affected dogs. This included dog–5596 (F = 0.157), whose grandparent was dog–77563 (I-1) and the fifth most frequently occurring dam (10 times, not shown in pedigree) was both its grandparent and great-grandparent. This dog was not related to the common dam of interest and

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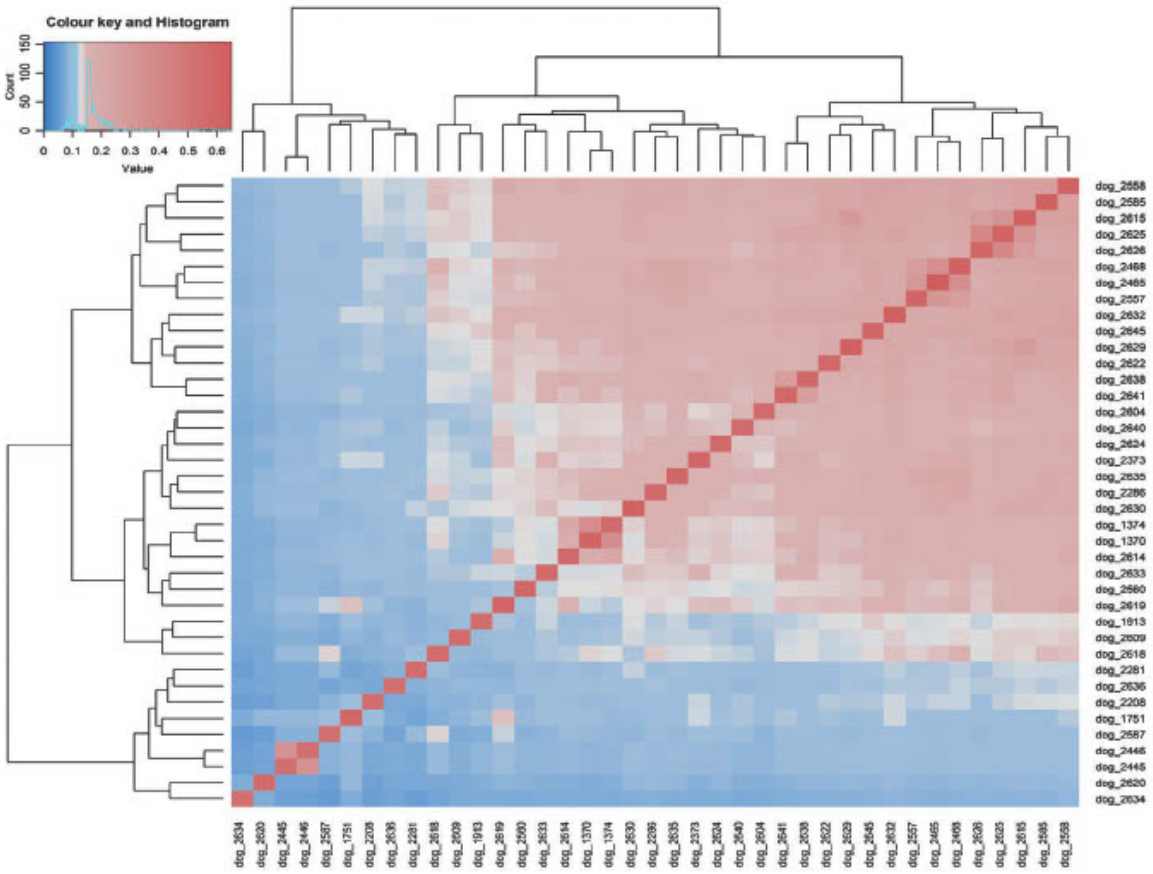

Figure 3. Heatmap analysis of lymphoma-affected Australian Border Collies (n = 39) based on a kinship matrix using pedigree data. Each row and its corresponding column is a dog. Manhattan clustering was used to create the dendrograms. Blue cells indicate two individuals are less related, while red cells indicate two individuals are more related. Frequency of kinship values is represented in the colour key and histogram.

was not included in the count of 28 related cases. The second affected dog (dog–37478) had a lower F-value of 0.049; however, its dam had an F-value of 0.17 because of cross-breeding of descendants from the fourth most popular dam (11 times). The third most frequently appearing dam (13 times) was the offspring of the suspect dam and was included in the pedigree (dog–65911 (II-2)).

Pedigree mapping assisted in identifying relationships of antecedents for lymphoma-affected dogs. Some notable examples included one dog (F = 0.23; EqG = 11.76) with three half-sibling grandparents, another dog (F = 0.25; EqG = 13.49) whose paternal grandsire, dam and maternal grand-dam's sire were half-siblings and another dog (F = 0.27; EqG = 12.89) whose dam was a half-sibling of the paternal grand-dam (common dam) and also a half-sibling of the paternal grandsire (common sire). Heritability for lymphoma was calculated using the 103 pedigree dogs with their 3831 ancestors and was estimated to be 0.04 (SE ± 0.19). This estimate should be viewed with caution, given the limited numbers of dogs in the lymphoma-affected group relative to the extended pedigree and incomplete disease data, all of which would contribute to the accuracy of this estimate (Figure 5).

Discussion

Based on an exhaustive search of published studies, this is the first breed-specific study describing the clinical features and pedigree analyses for Border Collies affected with lymphoma in Australia. The survey identified the largest group of lymphoma-affected ABCs with accompanying pedigree data that has been reported to date. The analyses performed provided evidence that there is familial clustering in affected ABCs, consistent with conclusions from epidemiological studies that a genetic predisposition for canine lymphoma is present in ABCs.[22,25]

The mean age of diagnosis in this study was 9 years and was comparable to that of previous non-breed-specific studies, which reported a mean range of diagnosis from 6.7 to 8.3 years.[13,19,20,40,41] The peak age for developing lymphoma in this study was 7–11 years, which is

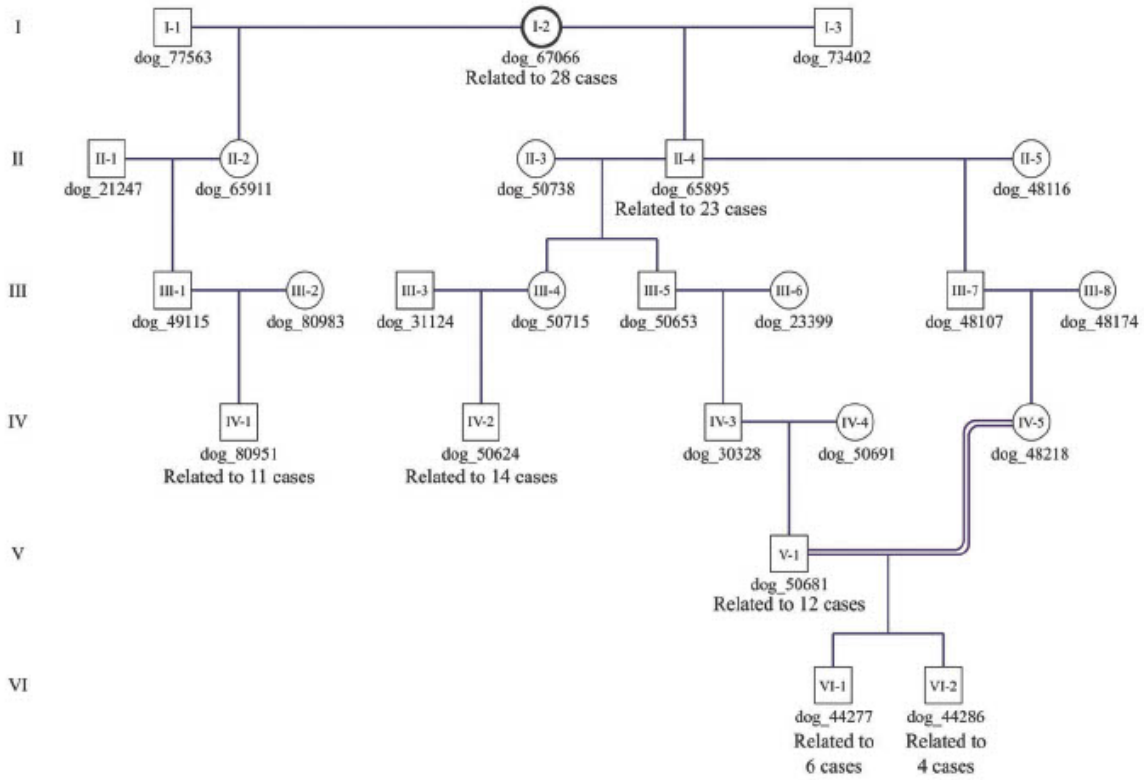

Figure 4. Pedigree of a family of Australian Border Collies related to 28 lymphoma cases, sharing a common female ancestor, dog–67066 (I-2). Other antecedents are annotated with the number of known cases related to them.

consistent with previous reports.[1,19,42] However, there was a very wide range of ages for disease onset and there were a significant proportion of dogs (29%) that were diagnosed between the ages of 1 and 6 years. This earlier onset of lymphoma has also been observed in some other predisposed breeds. For example, Bullmastiffs have been shown to have a substantial number of cases of lymphoma between 4 and 6 years of age in both the UK[19] and Australia.[21] In people, familial risk of non-Hodgkin's lymphoma is not limited to early onset cases,[43] but it is apparent in other haematological malignancies. Cancers diagnosed at an earlier age of onset tend to have a more pronounced hereditary component than late-onset cancers.[44] It may be useful to investigate potential differences in aetiology between age groups of dogs and to identify contributing genetic risk factors.

Female dogs had twice the odds of being reported as affected by lymphoma than male dogs in this study, which was in contrast to previous findings across multiple breeds that females,[25] and particularly intact females, had a decreased risk[21], although there may be some breed variation for sex predisposition.[25] Furthermore, the common practice of desexing in Australia most likely explains the small number of entire dogs in this study, making interpretation of this information difficult. In people, being female and the use of hormone therapy with oestrogen and/or progestin have been associated with a decreased risk of non-Hodgkin's lymphoma,[45,46] although another study did not show the same relationship.[47]

In total, 84% of ABCs in this study had B-cell lymphoma, which is consistent with previous studies.[1,20,40,41,48] The most common type of lymphoma reported in this study was multicentric, high-grade B-cell lymphoma, with the most common clinical stages being II–IV. A previous study reported that 66% of all affected dogs had B-cell lymphoma and in some breeds B-cell lymphoma represented more than 80%.[1] The prevalence of different immunophenotypes has not been reported in Border Collies, but 10 of 11 Border Collies included in a previous report were diagnosed with B-cell lymphoma.[1]

Immunophenotyping information in this study was only provided by 19 responders, which may be an indicator that there is little motivation to perform the tests if dogs were being treated with palliative prednisolone or that there is lack of awareness of the benefits of performing such tests in general veterinary practice.

A history of having another malignancy or infection was not common in the ABCs in this study. It has been reported that dogs with immune-mediated thrombocytopenia have a 5-fold elevated risk of developing lymphoma,[16] but none of the dogs in the present study had a reported history of this. Most of the treated dogs in the study

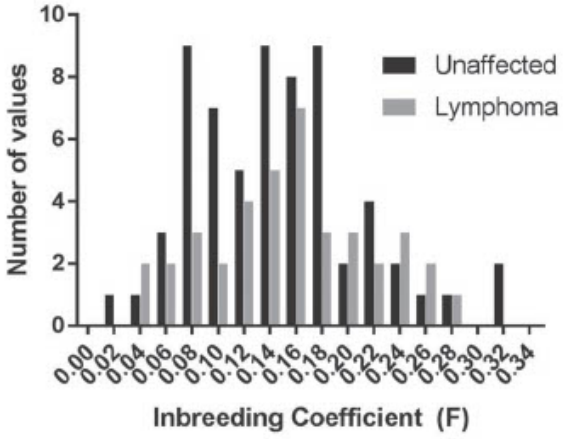

Figure 5. Distribution of inbreeding coefficients between lymphoma-affected (n = 39) and -unaffected (n = 64) Australian Border Collies.

had been administered chemotherapy or palliative prednisolone and the rest had undergone surgery or other treatments not specified by the respondents. More detailed information on treatment was not requested.

Breed predisposition,[13,19–24,41,49,50] a high incidence of specific immunophenotypes in some breeds[1,13,51] and familial clustering[52–54] support a role for a contribution of genetic background to the risk of developing lymphoma. The most commonly reported breeds include Boxers, Bulldogs and Bullmastiffs.[19,21,52] Boxers have a significantly higher prevalence of developing the T-cell subtype, compared with a higher prevalence of the B-cell subtype in Cocker Spaniels, Doberman Pinschers, German Shepherd Dogs and Rottweilers.[1,13,51] Moreover, increased prevalence of T-cell lymphoma has been demonstrated in genetically related dog breeds, such as 'Spitz' breeds and Asian 'lap' dogs.[1] This suggests that a susceptibility to developing T-cell lymphoma may have arisen prior to breed formation.[1]

Affected and unaffected dogs were dispersed throughout the two subgroups identified in the NetView analysis. Hence, the extent of relationships and familial clustering between affected dogs was more closely investigated. Familial clustering for lymphoma has previously been reported in Bullmastiffs, Rottweilers and Otterhounds.[52–54] Familial clustering in the ABCs of this study was apparent in both the network distribution and cluster analysis. For example, the pedigrees of 14 related (ϕ = 0.17–0.39) lymphoma-affected dogs included 2 frequently used sires. Examining the pedigrees for these sires showed that they were related to 21 cases in total. Mapping of pedigrees also revealed a common female ancestor for 28 cases. It is possible that the most frequently occurring dam contributed to the genetic susceptibility of lymphoma in its descendants; however, it did not account for the other line of descendants from dog–65895 (II-4), which was related to many other cases. Thus, we considered that the common dam (dog–67066 (I-2)) was the origin of a potential genetic risk in this pedigree because it bridged the relationships between both major lines of descendants, which was not seen in the other popular dams. Further studies would be required to confirm the genetic contributions towards disease susceptibility for individuals in this pedigree.

Average inbreeding estimates for affected and unaffected dogs were similar and did not distinguish any direct effect of inbreeding on the occurrence of lymphoma. However, the estimates from this study are much higher than previously reported in ABCs (0.041, n = 20,173; EqG = 7.6)[55] and may reflect lower genetic heterozygosity in this subpopulation of dogs. The identification of frequently used sires in the pedigree of some of these dogs is a common source of increased inbreeding values in purebreds. Further investigation using molecular data would provide more details on the differences of this subpopulation and the effects on lymphoma occurrence. An accurate estimation of heritability was not possible with the data used in this study, given the relatively small sample size, as well as the missing data from the ABC population and lack of known disease status of ancestors. Although familial predisposition may also indicate influences of environmental or infectious factors, these are considered to be unlikely because the majority of dogs in the present study did not share the same environmental exposure.

Similarly, familial predisposition has been demonstrated in people with different subtypes of lymphoma.[56–58] A 1.7-fold increased risk of non-Hodgkin lymphoma was reported for those with a first-degree relative with the same disease.[56,57] Moreover, a large cancer registry study has shown that first-degree relatives of diffuse large B-cell lymphoma cases had an almost 10-fold increased risk of developing it.[59] Understanding of the mechanisms in lymphoma predisposition is limited in people. Using techniques such as targeted sequencing[60] or whole exome/genome sequencing,[61–63] germline variants in 49 genes have been associated with diffuse large B-cell lymphoma.[64] Most of these genes are involved in DNA repair or immune function[60,64] and are suspected to contribute to the pathogenesis of lymphoma. Similarly, knowledge of germline mutation in dogs is limited. One study of Golden Retrievers in North America identified two shared predisposing loci contributing to a 20% increased risk of developing lymphoma and haemangiosarcoma.[65] The present study demonstrated that breed-specific genetic studies have the potential to clarify genetic risks without the confounding effects of inter-breed diversity.

Study limitations

The current survey avoided potential referral bias from a single institution, but inaccurate information provided by respondents was a potential limitation. Misclassification bias of lymphoma was minimised by the investigators' clinical assessment of medical records and confirmation from respondents when clarification was required. Moreover, the extent of the information on ABC pedigrees was improved by utilising a collated pedigree database. Further limitations of the current study involved potential selection bias towards owners who had experience of dogs with lymphoma, despite our attempts to negate this effect in the survey's design and by advertising it as a general health survey. Breeders with multiple affected dogs would be more motivated to complete the survey. However, many owners of individual ABCs also participated in the study and the relatedness with other affected dogs was only found after examination of pedigree information. Several breeders completed the survey

for multiple dogs in their kennels, including unaffected dogs, which may have affected the distribution seen in NetView and likely influenced the estimates for inbreeding and heritability in the population. Analysis of 10 randomly selected dogs revealed moderate kinship to the common dam, which reflected the significant genetic contribution from individuals in this pedigree to the ABC population. The genetic contribution of these individuals to population structure should be incorporated into any future molecular genetic analyses. This may have also contributed to the appearance of two sires multiple times in the pedigrees of both affected and unaffected dogs. It is important to note that dogs that were grouped as unaffected at the time of surveying may have the potential to develop lymphoma later in life. It is plausible that the two sires have passed down a genetic risk to their 21 affected descendants; however, genetic investigations will be needed to pursue the molecular mechanism associated with disease heritability, because there was no simple pattern of segregation of disease in these dogs.

In conclusion, this study provided evidence of a heritable component of canine lymphoma and is the first to report this relationship in the ABC. Results from the survey indicated that a proportion of dogs are susceptible at a young age, but also showed a similar median age of diagnosis with other breeds. Further genetic studies on ABCs should focus on the risk in young dogs and incorporate genotypic data to identify genomic-risk regions. Ultimately, breeding strategies may be modified to reduce the incidence of lymphoma in this breed.

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Conflicts of interest and sources of funding

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Chapter 3: Genetic investigations on the risk of lymphoma in Australian Border Collies

Author contributions

Associate Professor Peter Williamson, Dr Pamela Soh and I were involved in the conception of the chapter and design of experimental analysis. I performed the data analysis, interpreted the results, wrote the chapter and prepared the figures and tables. Dr Pamela Soh assisted in data analysis and preparation of figures. Associate Professor Peter Williamson and Associate Professor Peter Bennett reviewed the chapter.

3.1 Introduction

Canine lymphoma is a very common and often fatal cancer despite aggressive treatment with chemotherapy. Breed predisposition suggests a genetic component in the development of disease[16,40,49,59], however the genetic basis of predisposition remains largely unknown[54,166].

Selective breeding for certain phenotypic characteristics results in extensive linkage disequilibrium (LD) within dog breeds[20]. This limited genetic diversity within the breed populations has allowed efficient identification of candidate genes associated with germline mutations in simple and complex diseases[211,229-231]. Previous genome-wide association studies (GWAS) have suggested implicated genetic regions associated with risk in multiple canine cancers, including lymphoma[55,211], histiocytic sarcoma[232], mast cell tumour[211,233,216], squamous cell carcinoma[234], haemangiosarcoma[55], osteosarcoma[235], and mammary tumour[236].

A GWAS in Golden Retrievers identified two shared predisposing loci on chromosome (CFA) 5 that contributed 20% of the risk of developing B-cell lymphoma and haemangiosarcoma[55]. Hayward *et al*. found a quantitative trait locus on CFA4 associated with lymphoma in Golden Retrievers containing several candidate genes, including *MCC*, *MXD3* and *FGFR4*[211]. In contrast, our group identified a different candidate region on CFA13, containing the proto-oncogene *MYC*, that was associated with B-cell lymphoma in Australian Bullmastiffs[58]. These suggest that germline variants associated with lymphoma predisposition may differ among breeds.

Border Collies have been shown to have increased risk in the development of lymphoma, particular the B-cell subtype in Australia[49]. The primary aim of the study was to investigate if the reported at-risk loci in other breeds are associated with lymphoma in ABCs. The second aim of the study was to investigate if the closely related family of an affected ABC has the same previously reported at-risk loci of other breeds. The third aim was to investigate any potential at-risk loci associated with risk of lymphoma in the family.

3.2 Materials and methods

3.2.1 Case selection and sample collection

Privately owned purebred Border Collies with an Australian lineage with cytological and/or histological diagnosis of lymphoma were included as cases in the study. Twenty-three dogs diagnosed with lymphoma and 223 unaffected dogs were included in the analyses. Medical records were reviewed and confirmed by veterinary oncology specialists at the University Veterinary Teaching Hospital Sydney when available. Immunophenotype of lymphoma was recorded if results were available. Dogs with T-cell lymphoma were excluded. Dogs with no reported history of lymphoma were included in the control group. Blood samples were collected for genotyping with the owner's informed consent. A survey was invited to be completed by dog owners and breeders, providing details of the dog's current health status, details of lymphoma (when applicable) and pedigree information. The project was approved by the University of Sydney Animal Ethics Committee under protocol numbers 2016/550 and 2018/1391.

3.2.2 Genotyping

DNA extraction was performed with fast spin-column procedure, following standard protocols and manufacturer's recommendations (QIAamp DNA Blood Kit Quigen, Melbourne, Victoria). Genotyping was performed for 230,000 single nucleotide polymorphisms (SNPs) using the CanineHD BeadChip (Illumina, San Diego, CA, USA) at Gene-Seek Inc (Lincoln, NE).

3.2.3 Genetic investigation of ABCs on previously reported at-risk SNPs in other breeds

Thirty-five SNPs were selected from previously reported at-risk SNPs ("at-risk SNPs") associated with lymphoma in other breeds. These included 21 genome-wide significant SNPs located in the B-cell and combined B-cell and haemangiosarcoma risk haplotypes in Golden Retrievers; 13 selected chromosome-wise associated SNPs from the top 30 at risk SNPs associated with B-cell lymphoma in Bullmastiffs; and one near genome-wide significant SNP in another Golden Retrievers study of lymphoma (Table 1). Fine scale examination of individual genotypes in ABCs for these 35 "at-risk SNPs" was performed using alignment and genotype colour coding in Microsoft Excel 2021. The frequency of each genotype in cases and controls was calculated using the "COUNTIF" function in Excel. Statistical analyses were performed to compare the frequency of each homozygous genotype, and secondly the risk

allele frequency in cases and controls using Fishers exact test implemented in Graph Pad Prism version 9.0.1, (GraphPad Software, San Diego California USA). P value of <0.05 was considered significant. An odds ratio (OR) of greater than 1 was interpreted as indicating a potential risk factor.

Table 1. The 35 "at-risk SNPs" and frequencies previously reported in the Golden Retrievers and Bullmastiffs. There were 34 cases and 48 controls, 41 cases and 172 controls, 29 cases and 194 controls in the studies of Hayward *et al*., Tonomura *et al*. and Mortlock *et al*. respectively. Data was extracted from the original studies.

Chr	SNP Position	Freq. (controls)	Freq. (cases)	OR	P-Value
5	33422865	0.04	0.24	1.36	6.48E- 05
5	33845636	0.04	0.23	1.39	2.34E- 05
5	33888351	0.04	0.22	1.37	6.39E- 05
5	33851492	0.09	0.22	1.27	9.02E- 06
5	33854327	0.09	0.23	1.28	2.66E- 06
5	34088493	0.1	0.22	1.25	2.07E- 05
5	34106119	0.1	0.24	1.26	6.07E- 06
5	34117726	0.1	0.25	1.27	3.05E- 05
5	29613573	0.48	0.32	1.18	3.2E- 05
5	29623349	0.48	0.32	1.18	3.2E- 05
5	29699676	0.49	0.32	1.19	5.89E- 06
5	29716926	0.49	0.32	1.19	5.44E- 06
5	29748609	0.47	0.3	1.19	1.03E- 05
5	29748871	0.47	0.3	1.19	1.03E- 05
5	29762601	0.47	0.3	1.19	1.03E- 05
5	29778962	0.48	0.3	1.2	3.23E- 06
5	29795750	0.48	0.3	1.2	3.23E- 06
5	29867304	0.47	0.28	1.21	1.09E- 06
5	29870177	0.47	0.28	1.22	8.74E- 07
5	29892306	0.48	0.29	1.22	4.63E- 07
5	29893423	0.47	0.29	1.21	1.62E- 06
13	25208544	0.621	0.139	0.099	4.97E- 05
13	25341980	0.621	0.139	0.099	4.97E- 06
13	25423168	0.621	0.139	0.099	4.97E- 07
13	25423547	0.621	0.139	0.099	4.97E- 08
13	27637942	0.293	0.778	8.456	1.35E- 05
13	26343261	0.621	0.139	0.099	4.97E- 05
13	26354649	0.621	0.139	0.099	4.97E- 05
13	25008163	0.517	0.139	0.151	4.02E- 05
13	28029410	0.293	0.778	8.456	1.35E- 05
13	25423080	0.304	0.806	9.512	2.52E- 05
13	25519392	0.31	0.806	9.247	2.52E- 05
13	29743592	0.552	0.139	0.131	1.47E- 05
13	27865959	0.517	0.167	0.187	4.22E- 05
4	35564350	0.646	0.279	Not reported	4 E- 07

3.2.4 Genetic investigation of the family of an affected ABC

A pedigree chart was generated using the web-based pedigree tool, "Progeny Clinical" from Progency Genetics[237]. Genotype data of the "at-risk SNPs" in an affected three-year-old ABC "BC2", and its six closely related, unaffected family members (Parents, half-sibling, three Grandparents) was tabulated in Excel (Figure 1). To analyse the relatedness of this family with other ABCs, a high-definition relationship network was constructed using NetView (implemented in R). The relationship network was generated between "BC2", its "Relatives", other cases of lymphoma and control dogs. This was done by using a kinship matrix[238-240] derived from all generations of pedigree information, employing the software packages "pedigree" and " Kindship2" implemented in R[239].

GWAS analysis was performed in this family of the affected ABC as cases and 216 controls. Initially, 223 controls were included, but seven were excluded due to failing quality control. Although underpowered, the analysis was considered useful for highlighting differences in SNP frequencies. PLINK v1.9 software was used to perform the analysis with 10,000 permutations using 229,411 SNPs for seven family members as "cases" and 216 controls [241]. The R package "qqman" was used to generate Manhattan plot, and q-values were calculated using the R package "qvalue" for a false discovery rate cut-off of 0.001 and 0.05[242]. A kinship matrix was created in PLINK (using –distance-matrix function) and used as input [243]. The SNPs of the chromosome with the highest number of top 100 SNPs were further investigated. Protein coding genes associated with these SNPs were identified using BioMart and NCBI viewer (National Center for Biotechnology Information, Bethesda MD, USA).

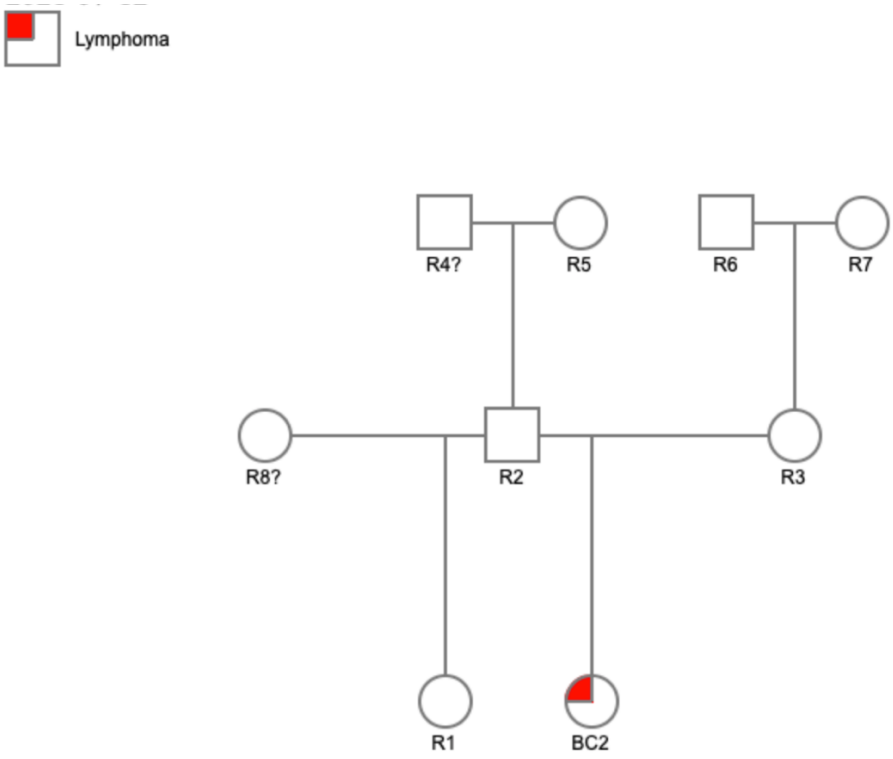

Figure 1. Pedigree showing the affected dog "BC2" and its six closely related family members ("R1" to "R8"). "?" denotes dogs that were not genotyped.

3.3 Results

3.3.1 Animals

Twenty-three dogs diagnosed with lymphoma and 223 unaffected dogs were included in the analyses. The median age of diagnosis was nine years old (95% CI 7.5-10.5), with a range of 2.9 to 15.3 years old. Fifteen dogs had B-cell lymphoma, while the immunophenotype was unknown in 8 dogs. Twelve dogs had multicentric lymphoma, while the rest had unknown forms. Dogs with primary alimentary or cutaneous lymphoma were excluded, as T-cell subtype is more common in these forms.

3.3.2 Genotype data analyses of all Border Collies

A total of 35 "at-risk SNPs" loci were analysed to assess homozygosity in the ABCs data (Table 2). The frequency of each of the homozygous genotypes were compared in the cases and controls. None of the homozygous genotypes showed statistical significance.

The frequency of all homozygous genotypes at risk allele positions was compared in the cases and controls (Table 3). Two SNPs showed statistical significance. These were located at CFA5:29,699,676bp (P=0.04, OR 0.5, 95% CI 0.2664 to 0.9842) and CFA5:29,716,926bp (P=0.04, OR 0.5, 95% CI 0.2721 to 0.99). These SNPs were contained in the coding sequence for the *CEP126* gene and the *ANGPTL5* gene, respectively. However, these SNPs were at a lower frequency in the affected dogs and neither of these genes has been reported to have any association with lymphoma or cancer in people or dogs.

3.3.3 Genotype data analyses of specific affected family

Genotypes of the "at-risk SNPs" of an affected ABC and its close family members were examined. None of the genotypes of the "at-risk SNPs" were found exclusively in the affected dog, but not in its family members (Table 4). In NetView (Figure 2), the affected ABC and its close family members were located in a cluster of cases, where 12 other cases of lymphoma were identified. Three of the dogs with lymphoma were located in close proximity to the family of the ABC with lymphoma. GWAS analysis included seven family members and 216 controls. Of the top 100 SNPs, 70 SNPs reached genome-wide significance (P- value <5x10-8). The most prominent association signal was located on CFA4 with 14 of the top 100 SNPs (Figure 3 and Table 5). Most of these spanned a 2.5Mb region on CFA4 (2,247,303bp to 4,730,173bp),

containing two cancer-related genes, *GNG4* and *ARID4B*. None of the genotypes of these 14 top SNPs CFA4 were present exclusively in the affected dog within the family (Table 6).

Table 2. Frequency of homozygous genotypes of "at-risk SNPs" in ABCs

Chr	Position	Genotype	Cases (Genotype freq)	Control (Genotype freq)	OR	P-Value	95% CI
5	33422865	G G	0.91	0.91	1.038	>0.9999	0.2535 to 4.786
5	33845636	G G	0.57	0.61	0.8264	0.6605	0.3390 to 1.973
5	33851492	A A	0.35	0.48	0.5726	0.2722	0.2408 to 1.402
5	33851492	G G	0.13	0.11	1.193	0.7317	0.3508 to 4.265
5	33854327	A A	0.35	0.48	0.5895	0.2765	0.2481 to 1.441
5	33854327	G G	0.13	0.11	1.214	0.729	0.3569 to 4.336
5	34088493	A A	0.39	0.48	0.6953	0.5107	0.2895 to 1.683
5	34088493	G G	0.09	0.11	0.7495	>0.9999	0.1648 to 2.931
5	34106119	A A	0.48	0.56	0.7094	0.509	0.3120 to 1.637
5	34117726	C C	0.48	0.56	0.7254	0.512	0.3193 to 1.676
5	29613573	T T	0.39	0.33	1.277	0.645	0.5317 to 3.112
5	29623349	A A	0.43	0.33	1.527	0.3608	0.6353 to 3.747
5	29699676	A A	0.43	0.62	0.4748	0.1158	0.1989 to 1.162
5	29716926	A A	0.43	0.62	0.4748	0.1158	0.1989 to 1.162
5	29748609	C C	0.43	0.61	0.5003	0.1234	0.2095 to 1.226
5	29748609	A A	0.13	0.06	2.375	0.1874	0.6664 to 8.776
5	29748871	A A	0.43	0.59	0.5256	0.1817	0.2201 to 1.287
5	29748871	C C	0.13	0.06	2.375	0.1874	0.6664 to 8.776
5	29762601	A A	0.43	0.58	0.5559	0.1914	0.2332 to 1.358
5	29762601	G G	0.13	0.06	2.215	0.2096	0.6265 to 7.939
5	29778962	G G	0.43	0.59	0.5422	0.1865	0.2277 to 1.323
5	29778962	A A	0.13	0.06	2.25	0.2039	0.6363 to 8.061
5	29795750	A A	0.43	0.59	0.5316	0.1835	0.2232 to 1.298
5	29795750	G G	0.13	0.05	2.686	0.1514	0.7471 to 10.30
5	29867304	A A	0.83	0.84	0.9124	0.7734	0.3142 to 2.608
5	29870177	A A	0.83	0.85	0.8444	0.7618	0.2887 to 2.420
5	29892306	A A	0.35	0.24	1.707	0.3082	0.7004 to 4.282
5	29892306	G G	0.13	0.23	0.4929	0.4274	0.1497 to 1.588
5	29893423	A A	0.35	0.24	1.728	0.3066	0.7094 to 4.334
5	29893423	G G	0.13	0.28	0.38	0.1414	0.1160 to 1.209
13	25208544	C C	0.83	0.79	1.293	0.7915	0.4550 to 3.662
13	25341980	A A	0.7	0.64	1.294	0.6532	0.5175 to 3.322
13	25423168	G G	0.52	0.55	0.8995	0.8287	0.3904 to 2.044
13	25423168	A A	0.09	0.08	1.143	0.6963	0.2461 to 4.811
13	25423547	T T	0.52	0.52	0.9935	>0.9999	0.4293 to 2.250
13	25423547	A A	0.09	0.1	0.8312	>0.9999	0.1824 to 3.270
13	27637942	G G	0.96	0.9	3.043	0.4868	0.4957 to 32.72
13	26343261	T T	0.17	0.27	0.5692	0.4526	0.2023 to 1.762

13	26343261	A A	0.22	0.31	0.633	0.4748	0.2482 to 1.774
13	26354649	A A	0.3	0.26	1.246	0.6248	0.4869 to 3.188
13	26354649	G G	0.22	0.3	0.6512	0.4778	0.2553 to 1.823
13	25008163	G G	0.57	0.49	1.3	0.6617	0.5341 to 3.094
13	25008163	A A	0.09	0.11	0.8528	>0.9999	0.1871 to 3.354
13	28029410	G G	0.7	0.61	1.455	0.5031	0.5837 to 3.737
13	25423080	G G	0.52	0.54	0.9238	>0.9999	0.4013 to 2.101
13	25423080	C C	0.09	0.07	1.206	0.6838	0.2586 to 5.170
13	25519392	A A	0.35	0.33	1.105	0.8186	0.4621 to 2.711
13	25519392	G G	0.35	0.18	2.484	0.0902	0.9986 to 6.398
13	29743592	G G	0.52	0.43	1.471	0.3861	0.6360 to 3.339
13	29743592	A A	0.09	0.14	0.5683	0.7492	0.1264 to 2.427
13	27865959	A A	0.83	0.75	1.604	0.6089	0.5174 to 4.517
13	27865959	G G	0.09	0.01	6.444	0.0801	1.080 to 32.52
4	35564350	G G	0.91	0.91	1.068	>0.9999	0.2605 to 4.921

Table 3. Frequency of risk alleles in ABCs

Chr	Position	Genotype	Risk Alleles freq (cases)	Risk Alleles freq (controls)	P-Value	OR	95%CI
5	33422865	G G	0.96	0.95	>0.9999	1.10	0.2654 to 4.900
5	33845636	G G	0.76	0.77	0.86	0.95	0.4712 to 1.959
5	33851492	A A	0.61	0.69	0.32	0.71	0.3904 to 1.318
5	33851492	G G	0.39	0.31	0.32	1.40	0.7589 to 2.561
5	33854327	A A	0.61	0.68	0.32	0.72	0.3938 to 1.333
5	33854327	G G	0.39	0.32	0.32	1.38	0.7501 to 2.540
5	34088493	A A	0.65	0.68	0.74	0.87	0.4491 to 1.679
5	34088493	G G	0.35	0.32	0.74	1.15	0.5954 to 2.227
5	34106119	A A	0.72	0.74	0.73	0.89	0.4629 to 1.804
5	34117726	C C	0.72	0.74	0.72	0.88	0.4570 to 1.780
5	29613573	T T	0.70	0.59	0.16	1.61	0.8251 to 3.071
5	29623349	A A	0.72	0.58	0.08	1.81	0.9563 to 3.609
5	29699676	A A	0.65	0.79	0.04	0.50	0.2664 to 0.9842
5	29716926	A A	0.65	0.79	0.04	0.50	0.2664 to 0.9842
5	29748609	C C	0.65	0.77	0.10	0.55	0.2942 to 1.081
5	29748609	A A	0.35	0.23	0.10	1.76	0.8950 to 3.280
5	29748871	A A	0.65	0.77	0.10	0.57	0.3048 to 1.117
5	29748871	C C	0.35	0.23	0.10	0.57	0.3048 to 1.117
5	29762601	A A	0.65	0.76	0.15	0.60	0.3211 to 1.169
5	29762601	G G	0.35	0.24	0.15	1.68	0.8551 to 3.114
5	29778962	G G	0.65	0.76	0.11	0.59	0.3151 to 1.147
5	29778962	A A	0.35	0.24	0.11	1.71	0.8719 to 3.173
5	29795750	A A	0.65	0.77	0.10	0.56	0.3022 to 1.103
5	29795750	G G	0.35	0.23	0.10	1.78	0.9062 to 3.309
5	29867304	A A	0.91	0.91	>0.9999	1.01	0.3715 to 2.752
5	29870177	A A	0.91	0.92	0.78	0.94	0.3453 to 2.582
5	29892306	A A	0.61	0.51	0.22	1.48	0.7861 to 2.700
5	29892306	G G	0.39	0.51	0.16	0.62	0.3433 to 1.179
5	29893423	A A	0.61	0.48	0.12	1.71	0.9064 to 3.110
5	29893423	G G	0.39	0.52	0.12	0.58	0.3216 to 1.103
13	25208544	C C	0.91	0.88	0.63	1.42	0.4996 to 3.848
13	25341980	A A	0.83	0.79	0.70	1.25	0.5898 to 2.668
13	25423168	G G	0.72	0.74	0.86	0.91	0.4752 to 1.844
13	25423168	A A	0.28	0.26	0.86	1.10	0.5422 to 2.104
13	25423547	T T	0.72	0.71	>0.9999	1.04	0.5424 to 2.082
13	25423547	A A	0.28	0.29	>0.9999	0.97	0.4804 to 1.844
13	27637942	G G	0.98	0.93	0.34	3.36	0.6083 to 35.23
13	26343261	T T	0.41	0.48	0.44	0.75	0.4086 to 1.425

13	26343261	A A	0.46	0.52	0.44	0.78	0.4236 to 1.408
13	26354649	A A	0.54	0.48	0.44	1.29	0.7172 to 2.378
13	26354649	G G	0.46	0.52	0.44	0.78	0.4205 to 1.394
13	25008163	G G	0.74	0.69	0.51	1.29	0.6655 to 2.484
13	25008163	A A	0.26	0.30	0.73	0.83	0.4306 to 1.610
13	28029410	G G	0.83	0.77	0.46	1.39	0.6588 to 2.961
13	25423080	G G	0.72	0.73	0.86	0.92	0.4783 to 1.859
13	25423080	C C	0.28	0.27	0.86	1.09	0.5379 to 2.091
13	25519392	A A	0.50	0.57	0.35	0.74	0.4099 to 1.340
13	25519392	G G	0.50	0.43	0.44	1.30	0.7152 to 2.344
13	29743592	G G	0.72	0.64	0.33	1.42	0.7483 to 2.841
13	29743592	A A	0.28	0.36	0.33	0.70	0.3519 to 1.336
13	27865959	A A	0.87	0.87	>0.9999	1.03	0.4144 to 2.395
13	27865959	G G	0.13	0.13	>0.9999	0.97	0.4176 to 2.413
4	35564350	G G	0.96	0.94	>0.9999	1.32	0.3289 to 5.828

Table 4. Genotypes of the "at-risk SNPs" of an affected ABC and its close family members

Chr	Position / Dogs#	BC2	R1	R2	R3	R5	R6	R7
5	33422865	GG	GG	GG	GG	GG	GG	AG
5	33845636	GG	AG	AG	GG	AG	GG	GG
5	33845636	GG	GG	GG	GG	GG	GG	GG
5	33851492	AA	AG	AG	AA	AG	AA	AA
5	33854327	AA	AG	AG	AA	AG	AA	AA
5	34088493	AG	AG	AA	AG	AG	GG	AG
5	34106119	AG	AG	AA	AG	AA	AG	AG
5	34117726	AC	AC	CC	AC	CC	AC	AC
5	29613573	AT	TT	TT	AA	TT	AT	AT
5	29623349	AG	AA	AA	GG	AA	AG	AG
5	29699676	AA	AA	AA	AA	AG	AG	AG
5	29716926	AA	AA	AA	AA	AG	AG	AG
5	29748609	CC	CC	CC	CC	AC	AC	AC
5	29748871	AA	AA	AA	AA	AC	AC	AC
5	29762601	AA	AA	AA	AA	AC	AC	AC
5	29778962	GG	GG	GG	GG	AG	AG	AG
5	29795750	AA	AA	AA	AA	AG	AG	AG
5	29867304	AA	AA	AA	AA	AA	AA	AC
5	29870177	AA	AA	AA	AA	AA	AA	AC
5	29892306	GG	AA	AG	GG	AA	AG	GG
5	29893423	GG	AA	AG	GG	AA	AG	GG
13	25208544	CC	CC	CC	AC	CC	CC	AC
13	25341980	AA	GG	AG	AG	AA	AA	AG
13	25423168	AG	GG	GG	AG	GG	AG	AG
13	25423547	AT	TT	TT	AT	TT	AT	AT
13	27637942	GG	GG	GG	GG	GG	GG	GG
13	26343261	AT	TT	TT	AT	AT	AA	TT
13	26354649	AG	AA	AA	AG	AG	GG	AA
13	25008163	AG	GG	GG	AG	AG	GG	GG
13	28029410	GG	GG	GG	AG	GG	AG	AG
13	25423080	CG	GG	GG	CG	GG	CG	CG
13	25519392	AG	GG	GG	AA	AG	AG	AA
13	29743592	GG	GG	GG	GG	AG	AG	GG
13	27865959	AA	AA	AA	AA	AA	AA	AA
4	35564350	AG	GG	AG	GG	GG	GG	GG

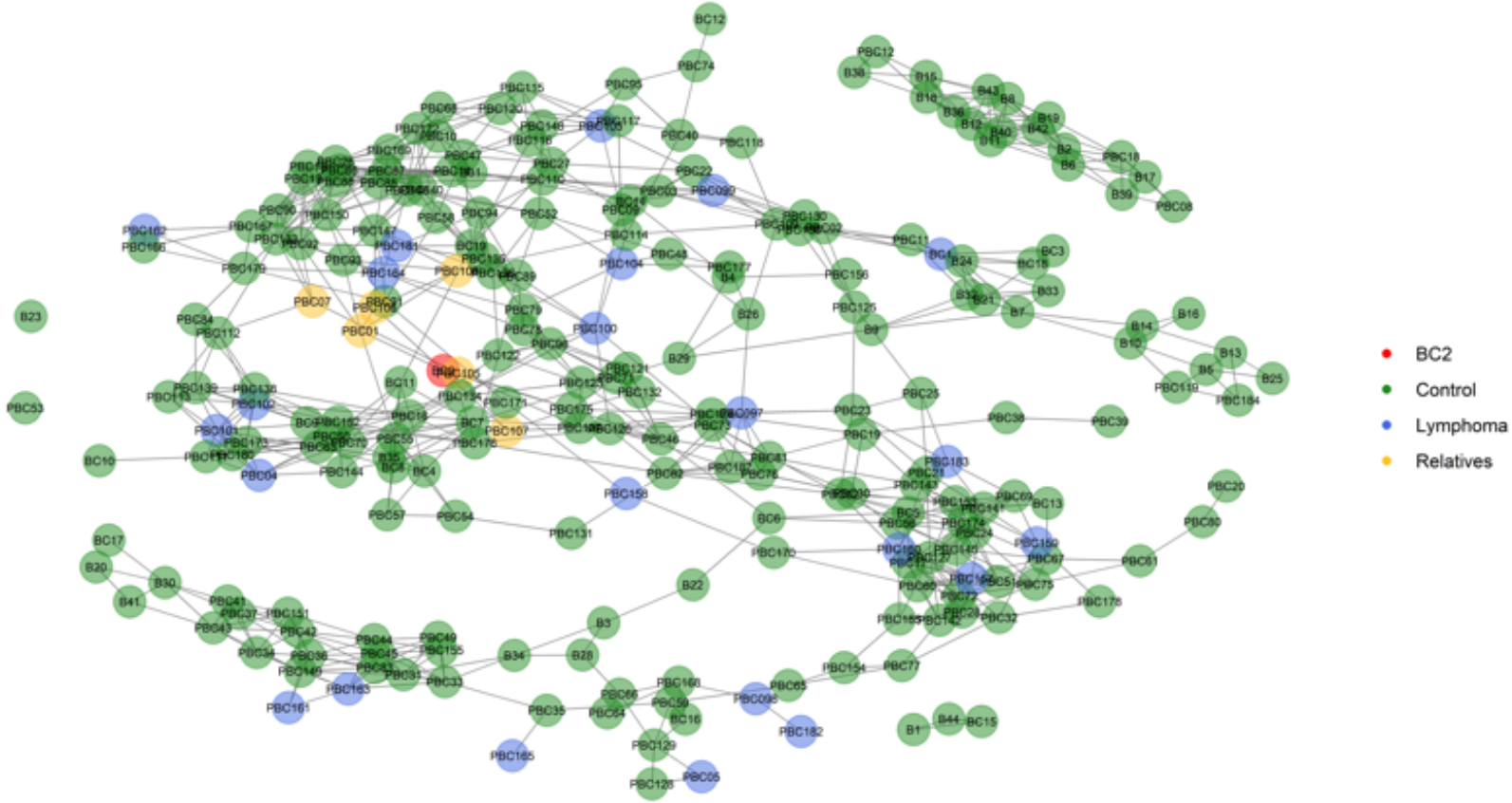

Figure 2. High-definition network visualisation of ABCs population structure. The network shows the distribution of lymphoma affected dog "BC2", its close relatives "Relatives", other affected ABCs and controls. Twelve dogs with lymphoma and the family are located in the sub-network on the left top corner. The network captured 24 dogs with lymphoma (including BC2), 228 dogs without lymphoma (including 6 relatives of BC2).

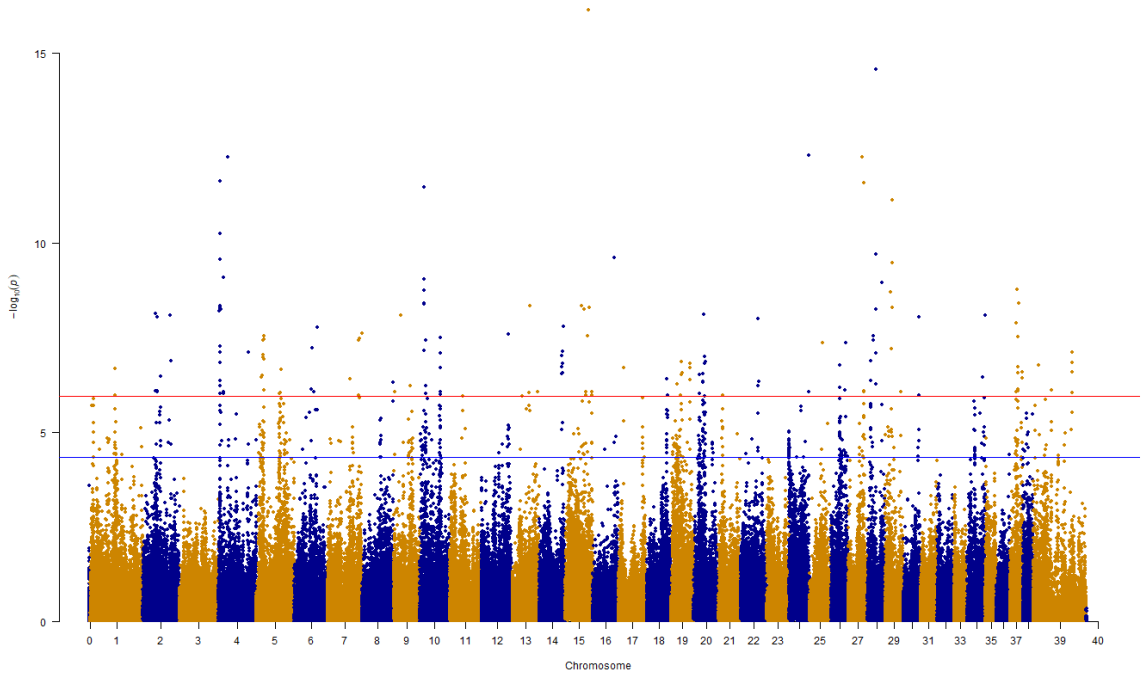

Figure 3. The Manhattan plot based on GWAS for seven family members and 216 controls. Family members included ABCs family with one affected dog and six closely related relatives. Alternating colours denote different chromosomes. CFA4 showed the strongest signal. Cut-off is a false discovery rate (FDR) of 0.001 (red line) and 0.05 (blue line)

Table 5. Fourteen SNPs on CFA4 amongst the top 100 genome-wide significantly SNPs

Chr	BP	Freq. in cases	Freq. in controls	OR	P-Value	Q-Value	r2 value with top SNP on CFA4 (4.2589621)
4	2589621	0.2143	0.005051	53.73	2.38E-12	4.38E-09	N/A
4	4368490	0.2143	0.005051	53.73	2.38E-12	4.38E-09	1
4	2763832	0.2143	0.007109	38.09	5.90E-11	7.23E-08	1
4	2939382	0.2143	0.007614	35.55	2.74E-10	2.52E-07	1
4	12434113	0.2143	0.008021	33.73	8.21E-10	6.03E-07	0
4	4353724	0.2143	0.009901	27.27	4.66E-09	2.27E-06	1
4	4312269	0.2143	0.00995	27.14	5.12E-09	2.27E-06	1
4	2690054	0.2143	0.01	27	5.63E-09	2.27E-06	1
4	4730173	0.2143	0.01	27	5.63E-09	2.27E-06	1
4	2247303	0.2143	0.01005	26.86	6.18E-09	2.27E-06	1
4	2667832	0.2143	0.01232	21.87	5.46E-08	1.83E-05	1
4	2652513	0.2143	0.01256	21.44	7.61E-08	2.15E-05	1
4	70391271	0.2143	0.01256	21.44	7.61E-08	2.15E-05	0
4	2726033	0.2143	0.01309	20.56	1.48E-07	3.88E-05	1

Table 6. Genotypes of the 14 top SNPs on CFA4 of an affected ABC and its close family members

Chr	Position / Dogs#	BC2	R1	R2	R3	R5	R6	R7
4	2589621	A G	G G	G G	A G	A G	G G	A G
4	4368490	A C	A A	A A	A C	A C	A A	A C
4	2763832	A G	G G	G G	A G	A G	G G	A G
4	2939382	A G	A A	A A	A G	A G	A A	A G
4	12434113	A G	A A	A A	A G	A G	A A	A G
4	4353724	A G	G G	G G	A G	A G	G G	A G
4	4312269	A G	G G	G G	A G	A G	G G	A G
4	2690054	A G	G G	G G	A G	A G	G G	A G
4	4730173	A G	G G	G G	A G	A G	G G	A G
4	2247303	A G	G G	G G	A G	A G	G G	A G
5	2667832	A G	G G	G G	A G	A G	G G	A G
4	2652513	A G	G G	G G	A G	A G	G G	A G
4	70391271	A G	G G	G G	A G	A G	A G	G G
4	2726033	A G	A A	A A	A G	A G	A A	A G

3.4. Discussion

The breed predisposition in lymphoma suggests that genetic risk plays a role in lymphomagenesis. Mapping a complex trait, particularly focusing on the relative genetic homogeneity within a breed, has allowed identification of at-risk regions in canine lymphoma[55,58,211]. These include studies of commonly affected breeds, including Golden Retrievers and Bullmastiffs. However, it is unclear that if these predisposing loci and mechanisms of tumorigenesis are the same among breeds. To compare the germline risks in lymphoma among different breeds, this study investigated the 35 previously reported at-risk loci and failed to find similar risk in affected ABCs. Moreover, the investigation of an affected family suggested that a candidate locus (CFA4) may be involved in lymphoma predisposition in that family.

Lymphoma risk loci have been reported in regions on CFA13 in the Bullmastiffs from a GWAS[58]. A region about 1.2Mb between CFA13:25.2-36.4Mb accounted for over 23% of phenotypic variance in lymphoma risk in Bullmastiffs. This region contained potential candidate genes associated with cell cycle progression and cell proliferation in cancer in people, including *MYC* and *PVTI*[58]. In contrast, the study in Golden Retrievers found two associated loci on CFA5 contributed nearly 20% of the risk for haemangiosarcoma and B-cell lymphoma[55]. The authors further investigated gene expression in tumours and found that the identified risk haplotypes were associated with impaired T-cell mediated immune response[55]. Similarly, another study in Golden Retrievers found a different locus on CFA4 with several suggested candidate genes, *MCC*, *MXD3* and *FGFR4*[211]. The current study showed that the previously reported predisposing loci were not associated in the ABCs. Although the frequency of risk alleles was significantly higher in cases at two loci located on CFA5, no cancer associated genes were identified in this area, suggesting that predisposing loci could be different among breeds. However, LD is extensive within dog breeds, so the involvement of linked candidate genes cannot be excluded.

When investigating the family of an affected ABC, the previously described at-risk loci showed no indication of segregation with development of lymphoma. In the NetView analysis, this family was closely associated with other cases of lymphoma, suggesting familial clustering, which has been previously reported in Bullmastiffs, Rottweilers and Otterhounds[51-53]. This family may have an increased risk of developing lymphoma, but the majority were not affected

reflecting weak heritability or possibly the lack of other concurrent genetic risks or environmental factors contributing to the development of lymphoma. To test for other potential loci this family was included as "cases" and in a GWAS. As expected with this comparison, a large number of significant loci were detected, reflecting the small sample size of the "cases" and restricted genotypes within the subgroup. However, a region in CFA4 showing strongest signals was identified and contained two cancer-related genes, *GNG4* and *ARID4B*.

GNG4 is a member of the G-protein γ family, which typically transduces signals from upstream G-protein-coupled receptors[244,245]. This results in activation of PI3K/Akt/MAPK signalling pathways[246]. Functional assays in people revealed the roles of *GNG4*, which include promoting cell adhesion, cell survival and cell proliferation[247,248]. *GNG4* has been reported as a tumour suppressor gene in glioblastoma and renal cell carcinoma, as well as a candidate driver gene of liver metastasis of gastric cancer in people[247-249]. The actions of *GNG4* are linked at least in part, to SDF1a/CXCR4 signalling. CXCR4 is a chemokine receptor and is known to be involved in aggressive B-cell lymphoma in humans[248,250]. Moreover, MAPK pathway mutations have been implicated in different forms of lymphoma, such as cutaneous T-cell lymphoma, CLL and paediatric-type nodal lymphoma [251,252]. The function of ARID- containing proteins is regulation of gene expression in diverse cellular processes including proliferation, differentiation, apoptosis and oncogenesis[253]. Studies in people showed *ARID4B* promotes invasion and metastasis in breast cancer[254,255], initiation and progression in prostate cancer[256] and its expression is a negative prognostic factor for liver and brain cancers and its expression is a negative prognostic factors for liver and brain cancers[257,258]. Conversely, it was demonstrated that the *ARID4B* gene may inhibit acute leukaemia transformation in a mouse model [259].

The frequency of deleterious alleles may increase in subpopulations during the process of selective breeding[260]. Breeds can share an underlying genetic predisposition to particular cancers especially if they share a common founder during breed development. However, the current study supports the view that genetic predisposition for complex diseases like lymphoma can be different in each breed. Border Collies, Golden Retrievers and Bullmastiffs belong to the herding, retrievers and mastiff-like dog clades respectively, suggesting more distant relationship in their ancestors[261]. This may explain the difference in predisposition loci found in the studies. In contrast, a recent GWAS study of haematopoietic cancers, including histiocytic sarcoma, lymphoma and mast cell tumours in Bernese Mountain Dogs, Golden

Retrievers, Rottweilers and Flat-Coated Retrievers showed that these breeds share common risk loci for lymphoma, mast cell tumour and histiocytic sarcoma[217]. This study suggested that the loci linked to cancer in dogs can have pleiotropic effects associated with the risk of several cancers in several breeds. However, as with all complex traits, development of certain cancers can also be due to additive effects of multiple risk loci.

Moreover, the median age of diagnosis is lower (4-6 years) in Bullmastiffs[16,58] compared to ABCs (9.7 years), suggesting that environmental factors may contribute to the disease more opportunistically in ABCs compared to Bullmastiffs. Environmental factors, such as exposure to herbicides and other chemicals, residing in an industrial area, and exposure to strong magnetic fields have previously been implicated in canine lymphoma[34,35,48,262]. Alternatively, Bullmastiffs may have unique genetic risks with larger effect, resulting in disease in younger animals.

One limitation of this study was the potential for underpowering in the GWAS due to small number of cases used. This may increase the risk of false negatives and restricts the ability to identify loci with weaker contributions to disease risk. However, the primary aim was to identify allelic variation associated with lymphoma predisposition, employing a focused and targeted strategy to investigate genetic predispositions in a breed with limited sample availability. Future studies with larger sample sizes are required to validate these findings and discover additional at-risk loci.

3.5. Conclusion

The results of the present study suggested that the germline risk of lymphoma is different among breeds. This study is consistent with ABCs having an increased risk in developing lymphoma, as a relatively large number of affected ABCs was identified but notably within certain lines of the breed. A candidate region in CFA4 could be associated with lymphoma in ABCs, and follow up DNA sequencing studies on related dogs are warranted.

Chapter 4: General discussion

The aim of this thesis was to expand the current knowledge of heritability and genetic risk factors in canine lymphoma, through focusing on the Australian Border Collies (ABCs). Border Collies have been shown previously to have an increased risk of developing lymphoma in UK and Australia[49,60,16]. However, there is a lack of understanding of the disease characteristics, and the mechanisms of inheritance in the breed.

The epidemiology of lymphoma in ABCs was investigated through a general health survey completed by owners and breeders of ABCs (Chapter two). Pedigree analyses were performed on affected dogs from the survey and the results identified many affected cases descended from common ancestries. Genotype data of affected ABCs, and their closely related family was investigated to assess if the previously reported at-risk loci in other breeds also presented in ABCs (Chapter three). Furthermore, genome-wide association analysis was performed between a family of affected ABCs and controls. An at-risk region in CFA4 containing two cancer-related genes was identified. In this Chapter, the overall findings of each chapter and their contributions to lymphoma research are discussed.

Although the epidemiology of canine lymphoma is well known, the disease characteristics in Border Collies were largely unknown. One study investigated prevalence of B- and T-cell lymphoma among breeds and found 10 of 11 Border Collies had B-cell lymphoma[202]. There are reported breed differences in clinical features in lymphoma. There is also known breed predisposition in certain immunophenotype and subtypes of lymphoma, which were discussed in Chapter One. Moreover, some dog breeds, such as Bullmastiff also appear to develop lymphoma at a younger age compared to other breeds[263]. The survey identified 57 lymphoma-affected ABCs, out of 246 responders. Review of medical records of affected dogs revealed that high grade B-cell lymphoma was the most common subtype of lymphoma in dogs. Most dogs have multicentric form, with the most common stage being II-IV. These findings are comparable to other non-breed specific studies. The mean and median age of diagnosis was 9.16 (SD ± 3.43) and 9.7 years (range 2 to 15 years) respectively. This was similar to the common findings in other breeds that middle to older aged dogs are affected, but the disease can occur at any age. This result of this chapter contributes to the current knowledge on clinical features of lymphoma in ABCs, potentially assisting veterinarians and owners of ABCs in the diagnosis of lymphoma.

Pedigree analyses revealed 21 affected dogs descended from two sires and 28 cases with a common female ancestor. In addition, pedigree mapping identified closely related family members affected by the disease, such as first and second-degree relatives. Although environmental factors could contribute to familial predisposition, this is considered unlikely as most dogs in the study were not exposed to the same environmental risk factors. However, several confounding factors, such as selection bias, breed popularity and referral bias may have limited the ability to accurately assess the true influence of breed predisposition. Nevertheless, the observed familial clustering in this breed supports the possibility of heritability in canine lymphoma. Familial clustering has been identified in other breeds, as well as in people. Despite the evidence of heritability in lymphoma, the understanding of exact mechanisms in lymphoma predisposition is limited in both dogs and in people. To further explore the genetic risk factors in lymphoma, analyses of genotypes data of affected and unaffected ABCs were performed in the second component of the research.

Previous genome-wide association studies (GWAS) have identified at-risk loci, and the proposed predisposing germline mutations in lymphoma in several dog breeds. We investigated these at-risk regions in ABCs affected with lymphoma, and did not identify similar risks in affected ABCs. Moreover, the investigation of an affected family suggested that a candidate locus on CFA4 may be involved in lymphoma predisposition in ABCs. Most of these spanned a 2.5Mb region on CFA4 (2,247,303bp to 4,730,173bp), containing two cancer-related genes, *GNG4* and *ARID4B*. These genes have important functions in cell survival and proliferation. They may represent plausible candidate genes of canine lymphoma given their implications in different human cancers. However, given the limited sample size and inherent limitations of family-based analysis, the possibility of a false-positive association cannot be excluded. A comprehensive prospective follow up study is recommended.

Although breed predisposition in different cancer types is well known, neither the genetic mechanisms, nor the similarities or differences between dog breeds are completely understood. The current study suggests the view that genetic predisposition for complex diseases such as lymphoma can be different in each breed, although we cannot rule out the possibility that risk variants of modest effect were present but fell below the threshold of detection in our cohort. This contrasts with another recent GWAS study showing a few different breeds shared risk loci

for lymphoma, mast cell tumour and haemangiosarcoma, suggesting the pleiotropic effects of these risk regions[217].

Future directions

Our research group has further advanced the genetic investigations of ABCs affected with lymphoma with an expanded pedigree analysis and GWAS on a larger number of dogs. These identified a common ancestor in 54 affected dogs, and GWAS revealed significant at-risk regions on chromosomes 18 and 27[218]. Although the results from both studies are not definitive, they offer complementary insights: the larger case-control GWAS was designed to detect common risk variants at the population level, whereas the family-based design aimed to detect lineage-specific variants that might not be captured in the broader population-based analyses.

A limitation in studying complex diseases in ABCs is identifying enough cases to provide power to the analyses. Future studies are encouraged to expand the number of defined lymphoma cases for further validation of these identified at-risk regions, and to further investigate putative mutated gene loci identified in both studies. Additionally, investigation of somatic mutations in these genes and associated pathways may contribute to understanding these genetic risk factors. Eventually, this may enable the development of screening programs for cancer surveillance in at-risk individuals. Ultimately, the incidence of lymphoma may be reduced using breeding programs aiming to eliminate the detrimental genetic risk factors contributing to lymphoma.

Metabolite analysis was also performed by our research group and identified eight metabolites that were prevalent in affected dogs compared to controls[264]. This may potentially identify key biomarkers in lymphoma, providing prognostic value and contributing to novel therapeutic strategies.

One of the challenges encountered by the current research was clinical data and sample collection. Australia's first national registry cancer in pets, the Australian Companion Animal Registry of Cancers was recently established. This organisation aims to facilitate data and sample collection for animal cancer research in Australia. Effort should be made to raise awareness of the registry among pet owners, breeders, animal researchers and veterinarians to expand the source population and support further genetic-based research. If any pedigree lines with a higher incidence of lymphoma were identified from the registry or otherwise, genetic

analyses of these affected dogs and their family members may assist in identification of inheritance patterns.

Conclusion

This thesis has contributed to the knowledge of phenotypes and genetic risk factors in lymphoma, particularly in ABCs. The disease characteristics in affected ABCs were described. Evidence of heritability in lymphoma was suggested by the presence of familial clustering in ABCs. Finally, a candidate locus on CFA4 may be involved in lymphoma predisposition in ABCs. Overall, these findings contribute to the growing body of literature on heritable cancer risk in dogs. Further validation in larger, multi-breed, or specific breed datasets will be required to establish whether specific loci predispose to lymphoma. Such rigorous investigation will ultimately be necessary to inform genetic screening, cancer surveillance, and responsible breeding strategies.

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Appendix

The following pages consist of supplementary data (copy of survey) for the published paper in Chapter two.

By ticking this box, I hereby agree to participate in the survey on a voluntary basis and have read and understood the information provided. ☐

Section 1 General Information

Your details:
Note: All personal information is strictly confidential and will not be provided to any other parties.

Your Name: _____

Your Address: _____

Your Contact number: _____

Your Email: _____

Can we contact you to resolve any further questions we have regarding your dog?

Yes ◯ No ◯

Can we follow up to resolve any questions further with your vet?

Yes ◯ No ◯

Your vet's contact details:

Vet's Name: _____

Vet Practice: _____

Contact number: _____

Email: _____

Your dog's details:

Your dog's call name: _____

Your dog's registered name: _____

Your dog's ANKC registration number: _____

Your dog's date of birth (DD/MM/YYYY): _____

Your dog's sex (M / F) _____

Was your dog desexed (neutered)? _____

If your dog was desexed (neutered), at what age was it desexed? _____

Your dog's country of birth: _____

Section 2 Veterinary Information (Your vet can help provide this information)

What is the current health status of your dog?

Healthy ◯ Has an illness ◯ Deceased ◯

If your dog has an illness please provide details:

_____

_____

(If applicable) **Dog's age at death (to the nearest month)**: _____

Cause of death: _____

The following questions are applicable to dogs that have or had lymphoma if this does not apply to your dog please go to Section 3:

At what age was your dog diagnosed with lymphoma (to the nearest month)?

_____

Is your dog related to another animal that has or had lymphoma?

Yes ◯ No ◯ Unknown ◯

If yes, how are they related, and if known what is the related dog's registered name and ANKC registration number?

_____

_____

Has or did your dog have any other health problems as diagnosed by a veterinarian? (tick all that apply)

Other Tumours Small ☐ Medium ☐ Large ☐ N/A ☐

Tumour type:_____

Infections Minor ☐ Moderate ☐ Severe ☐ Re-occurring ☐ N/A ☐

Infection location:_____

Immune mediated thrombocytopenia Yes ◯ No ◯

Other health problems:

_____

_____

_____

Which type of lymphoma does or did your dog have? (Tick one only)

B-Cell ◯ T-Cell ◯ Unknown ◯

What stage was the lymphoma first diagnosed with?
(Tick one only)

Stage 1 ◯ Stage II ◯ Stage III ◯ Stage IV ◯

Stage V ◯ Unknown ◯

What form of presentation was the lymphoma upon initial diagnosis? (Tick one only)

Multicentric (Generalised) ◯ Mediastinal (Chest) ◯ Alimentary (Digestive Tract) ◯ Extranodal (Eg. skin, eyes, nervous system, kidneys or lungs) ◯

Cutaneous (Skin) ◯ Unknown ◯

What grade was it? (Tick one only)

Low ◯ Intermediate ◯ High ◯ Unknown ◯

What treatment was or has been commenced or completed? (Tick as many boxes as apply):

Surgery ☐ Chemotherapy (Medication) ☐ Other ☐ N/A ☐

What was the outcome from commencing treatment until the present? (Tick one only)

Complete Remission ◯ Partial Remission ◯ Recurrence ◯ Death ◯

Section 3 Pedigree details (fill in or attach pedigree)
If attaching a pedigree please fill out the age at death below if known.

Paternal Grandsire: _____

(If applicable) Age at death: _____

Sire: _____ Reg No: _____

(If applicable) Age at death: _____

Paternal Granddam: _____

(If applicable) Age at death: _____

Maternal Grandsire: _____

(If applicable) Age at death: _____

Dam: _____ Reg No: _____

(If applicable) Age at death: _____

Maternal Granddam: _____

(If applicable) Age at death: _____

THE END
THANK YOU VERY MUCH FOR YOUR TIME