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INVESTIGATIONS OF A NOVEL LYMPHOPROLIFERATIVE DISEASE IN BRITISH SHORTHAIR KITTENS

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy
at Massey University, Palmerston North, New Zealand

Danielle Aberdein
2013
Abstract

In 2009, three sibling British shorthair (BSH) kittens presented with lymphoproliferative disease (LPD) causing massive enlargement of multiple lymph nodes, a presentation that suggested an inherited predisposition to the disease. While aspects of the disease presentation suggested a diagnosis of lymphoma, other features were inconsistent with lymphoid neoplasia. In particular, the consistently young age of affected kittens, the pattern of disease affecting multiple littermates, and the presence of such marked generalised lymphadenopathy, were all atypical for feline lymphoma. This unusual constellation of clinical and pathologic features in affected BSH kittens had not been previously reported in cats but had several similarities to the human disease autoimmune lymphoproliferative syndrome (ALPS), a rare inherited disorder causing persistent LPD, increased numbers of CD3+/CD4-/CD8- double negative T-cells (DNT cells) and variable manifestations of autoimmunity. The majority of human ALPS patients have inherited Fas gene mutations causing defective T-cell apoptosis, although in some patients the cause of disease is still unknown.

The thesis further describes and investigates this novel LPD in BSH kittens. The results of breeding trials, pedigree information and reviews of historical records support an inherited basis for the disease, most likely with either a simple autosomal recessive or modified autosomal dominant mode of inheritance. The typical clinical presentation is the development of a massive multicentric lymphadenopathy, splenomegaly and probable haemolytic anaemia in previously healthy kittens between 5 to 7 weeks of age. Microscopic pathology and immunophenotypic studies are suggestive of multicentric T-cell lymphoma affecting the lymph nodes, spleen, and sometimes other organs, but clonality assays confirm a non-clonal and likely non-neoplastic T-cell LPD. Where tested, the proliferating T-cells show a DNT cell immunophenotype and reduced apoptosis on in situ methods. Qualitative Fas gene abnormalities were not identified in affected kittens using reverse-transcriptase polymerase chain reaction techniques.

The studies described in the thesis therefore confirm a novel and likely non-neoplastic T-cell LPD in BSH kittens with a probable inherited basis. Results support defective T-cell
apoptosis as a possible factor in disease development, although causative genetic abnormalities have not yet been identified. The disease in kittens has several similarities to ALPS in people, although the apparent absence of Fas gene abnormalities in affected kittens may limit the use of the feline disease as a disease model for ALPS.
ACKNOWLEDGMENTS

At times this project has felt like a Sisyphean task, and getting the rock to the top of the hill would not have been possible without the help of an enormous number of people. I have been extremely fortunate to have such a fantastic supervisory team. John Munday has guided the project from start to finish, provided excellent support, advice and humour throughout, and rescued me from the depths of despondency on many occasions (sarcastic red pen notwithstanding). Keith Thompson and Fran Wolber have also provided invaluable advice, encouragement and practical assistance, and I am extremely grateful for their input. The generous financial support provided by the Vice-Chancellor’s Doctoral Scholarship, the Palmerston North Medical Research Fund and the Institute of Veterinary, Animal and Biomedical Sciences Postgraduate Fund has funded many aspects of the research, and has been essential to its completion.

Huge thanks must also go to Rob Fairley at Gribbles Veterinary Pathology for his initial identification of the disease and assistance in uncovering further cases. Thanks also to Rae Pearson and her staff at New Zealand Veterinary Pathology for help processing samples and arranging their overseas transport, as well as allowing me free rein of their laboratory. The help and advice of Bill Vernau, Leslie Lyons and Niels Pedersen at UC Davis, particularly with regard to the immunophenotyping and clonality studies, has been immensely important to the thesis and very much appreciated. I am grateful to George Davis and Clive Felix at Capital & Coast District Health Board for allowing me to spend time in their genetics laboratory and taking the time taken to teach me cell culture and karyotyping techniques. Numerous people have assisted with housing, feeding, cleaning, socialising and drawing blood from small, uncooperative and often rather sharp-clawed kittens and cats, and I thank Dave Thomas and his staff at the Centre for Feline Nutrition, Debbie Chesterfield and Margaret Brown at the Small Animal Production Unit, and Emma Bermingham and Mike Kelly, who allowed their home to be turned into a kitten farm on two occasions. The contributions of several cat breeders to the project have been vital, particularly those of Iris Baker and Daniel Wheeler, whose immense generosity in loaning Luci (Q1) and progeny to me for the duration of the project I still find slightly unbelievable. Thanks also to the many British shorthair breeders who contributed advice...
and information but who do not wish to be acknowledged by name, and who at times must have felt that assisting me with this project ran counter to their own interests.

The support of my pathology colleagues throughout the project has been incredible, and I could not have completed the thesis without their help. I am forever in debt to Keren Dittmer, unofficial supervisor, unstinting provider of advice both technical and practical, rocker of the free world, and perpetually optimistic officemate and friend. Thanks must also go to Eloise Jillings, Mark Collett, Hayley Dann and Keren (again), for relieving me of various teaching commitments to allow me to complete the project. I also thank Laryssa Howe, Matthew Perrott, Evelyn Lupton and Mike Hogan for help with molecular techniques, immunohistochemistry, sample processing and equipment sourcing in general, and to Wendi Roe and Stu Hunter for keeping me somewhat sane throughout the process.

Finally, and at the risk of sounding like a bad Academy award acceptance speech, I would like to thank my family, most of whom thought I was insane for embarking on this madness but have supported me nonetheless. I promise this is the last time. Probably. Particular thanks to my parents, Keith and Joy, for their endless encouragement, support, and (I suspect, often feigned) interest in the lymphoproliferative diseases of the cat and the conundrum of the double negative T-cell. Last, but definitely not least, thank you to Thomas, my ginger kiss, and to Bella, the best dog in the world. Your patience and love is unrivalled. And yes, Thomas, I think your mum has finally finished her “P Haitch D”.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAFCO</td>
<td>American Association of Feed Control Officials</td>
</tr>
<tr>
<td>AI</td>
<td>apoptotic index, a measure of the level of apoptosis present within the relevant tissue field(s) using the number of apoptotic cells as the basis for calculation</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>ALPS</td>
<td>autoimmune lymphoproliferative syndrome, a human disease of defective lymphocyte apoptosis resulting in lymphoproliferation including lymphadenopathy and splenomegaly and variable manifestations of autoimmunity</td>
</tr>
<tr>
<td>ALPS-CASP10</td>
<td>People fulfilling the diagnostic criteria for autoimmune lymphoproliferative syndrome (ALPS) with either germline or somatic mutations in the caspase 10 (CASP10) gene</td>
</tr>
<tr>
<td>ALPS-FAS</td>
<td>People fulfilling the diagnostic criteria for autoimmune lymphoproliferative syndrome (ALPS) with either heterozygous or homozygous germline mutations in the Fas gene</td>
</tr>
<tr>
<td>ALPS-FASLG</td>
<td>People fulfilling the diagnostic criteria for autoimmune lymphoproliferative syndrome (ALPS) with germline mutations in the Fas ligand gene</td>
</tr>
<tr>
<td>ALPS related disease</td>
<td>Diseases in people with similar clinical signs and pathology to autoimmune lymphoproliferative syndrome (ALPS) but not meeting the diagnostic criteria for ALPS. ALPS-related disorders include caspase-8 deficiency state, RAS-associated autoimmune leukoproliferative disease, Dianzani autoimmune lymphoproliferative syndrome and X-linked lymphoproliferative syndrome.</td>
</tr>
<tr>
<td>ALPS-sFAS</td>
<td>People fulfilling the diagnostic criteria for autoimmune lymphoproliferative syndrome (ALPS) with somatic mutations in the Fas gene</td>
</tr>
<tr>
<td>ALPS-U</td>
<td>People fulfilling the diagnostic criteria for autoimmune lymphoproliferative syndrome (ALPS) without a determined genetic defect</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>apoptotic rate, a measure of the level of apoptosis present within the relevant tissue field(s) using apoptotic area as the basis for calculation</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia-telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>the ataxia telangiectasia mutated gene</td>
</tr>
<tr>
<td>BSH</td>
<td>British shorthair breed of cat</td>
</tr>
<tr>
<td>CASP10</td>
<td>the caspase 10 gene</td>
</tr>
<tr>
<td>CBC</td>
<td>complete blood count</td>
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</table>
CD

cluster of differentiation

CD3

cluster of differentiation antigen 3, a protein complex associated with the T-cell receptor (TCR) complex on mature T-cells and composed of 4 distinct chains (γ, δ and two ε chains)

CD4

cluster of differentiation antigen 4, a glycoprotein predominantly expressed by and on the surface of helper T-cells

CD8

cluster of differentiation antigen 4, a transmembrane glycoprotein predominantly expressed by and on the surface of cytotoxic T-cells usually composed of α and β chains

CMA

chromosomal microarray analysis

Coombs test

blood test used to detect the presence of antibodies to erythrocytes and providing evidence of immune-mediated erythrocyte destruction and immune-mediated haemolytic anaemia

ConA

Concanavalia ensiformis type IV-S

CRT

capillary refill time

CTLA-4
cytotoxic T-lymphocyte antigen 4

CVID

combined variable immunodeficiency

Cy5
cyanine 5, a fluorescent dye used in flow cytometry

DAB

3,3-diaminobenzidine, a chromagen used in immunohistochemistry

DMEM

Dulbecco's Modified Eagle Medium

DN
double negative, lymphocyte development stages expressing neither CD4 nor CD8 cell surface proteins, and including the thymic DN1, DN2, DN3 and DN4 stages of T-cell development

DNA
deoxyribonucleic acid

DNT cell

double negative T-cell ) a CD3+ T-cell expressing neither CD4 nor CD8 cell surface markers

DP
double positive, lymphocyte development stages expressing both CD4 and CD8 cell surface proteins

DSH
domestic shorthair breed of cat

dUTP
deoxyuridine triphosphate

EBV

Epstein-Barr virus (alternatively termed human herpesvirus-4 (HHV-4))

EDTA

ethylenediaminetetraacetic acid

ELISA

enzyme-linked immunosorbent assay

F

inbreeding co-efficient, calculated as a percentage of the chances that two alleles in offspring will be identical by descent

FACS

fluorescence activated cell sorting

FADD

Fas-associated death domain protein

Fas

The Fas protein
**Fas** the Fas gene (also termed CD95, Apo1, Apt and TNFRSF6 (tumour necrosis factor receptor superfamily member 6)

**FasL** the Fas ligand protein

**Fasl** the Fas ligand gene

**FeLV** feline leukaemia virus

**FFPE** formalin-fixed paraffin-embedded

**FITC** fluorescein isothiocyanate

**FIV** feline immunodeficiency virus

**FOCMA** feline oncornavirus cell membrane antigen

**FSC** forward scatter

**Gld** the generalised lymphoproliferative disease gene, one of the genes mutated in the mice with lymphoproliferative disease resembling autoimmune lymphoproliferative syndrome (ALPS) in people

**H&E** haematoxylin and eosin

**HEPES** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

**HHV-4** human herpesvirus-4, alternatively termed Epstein Barr virus (EBV)

**HHV-8** human herpesvirus-8, alternatively termed Kaposi’s sarcoma-associated virus

**HIV** human immunodeficiency virus, the causative agent of acquired immunodeficiency syndrome (AIDS) in people

**HNPCC** hereditary non-polyposis colorectal cancer

**hpf** high power field

**HRP** horseradish peroxidase

**HSC** haematopoietic stem cell

**HTLV-1** human T-cell leukaemia virus-1 type 1

**IGH** immunoglobulin heavy chain locus, a gene present in B-cells that undergoes rearrangement early in B-cell development and used to assess B-cell clonality in lymphoid proliferations

**IL** interleukin, one of a group of cytokines involved in a wide range of signalling processes

**IFA** immunofluorescence antibody

**LGL** large granular lymphocyte, a subset of lymphocytes characterised by intracytoplasmic azurophilic granules, and which in humans may be of natural killer (NK)-cell (CD3–) or mature T-cell (CD3+) lineage

**LPD** lymphoproliferative disease (any disease, either neoplastic and non-neoplastic, in which abnormally excessive numbers of lymphocytes are produced)

**Lpr** the lymphoproliferation gene, one of the genes that mutated in the mice with lymphoproliferative disease resembling autoimmune lymphoproliferative syndrome (ALPS) in people
lymphoma  a clonal proliferation of lymphocytes (lymphoid neoplasia)
lymphoblast  large lymphocyte
MAA  morphometric area analysis, used to calculate the apoptotic rate (AR) within tissue fields, determined by calculating the area of the relevant field(s) showing evidence of apoptosis (positive staining)
MALT  mucosa-associated lymphoid tissue, aggregations of lymphoid tissue at various mucosal sites throughout the body, particularly the gastrointestinal tract
MCC  morphometric cell count, used to calculate the apoptotic index (AI) within tissue fields, determined by counting the number of cells within the relevant field(s) showing evidence of apoptosis (morphology and positive staining)
MHC  major histocompatibility complex
MLH1  MutL homolog 1 protein, encoded by the MLH1 gene
MLH1  the MLH1 gene, one of the mismatch repair (MMR) genes, defects in which are associated with hereditary non-polyposis colorectal cancer (HNPCC) in people
MMR  mismatch repair, a highly conserved system within prokaryotes and eukaryotes that recognises and repairs errors in base insertion and deletion during DNA replication and recombination and repairs DNA damage
MMR  the mismatch repair genes, particularly including MLH1, MSH2 and MSH6
mRNA  messenger ribonucleic acid
MSH2  MutS protein homolog 2 protein, encoded by the MSH2 gene
MSH2  the MSH2 gene, one of the mismatch repair (MMR) genes, defects in which are associated with hereditary non-polyposis colorectal cancer (HNPCC) in people
MSH6  mutS homolog 6 protein, encoded for by the MSH6 gene
MSH6  the MSH6 gene, one of the mismatch repair (MMR) genes, defects in which are associated with hereditary non-polyposis colorectal cancer (HNPCC) in people
NBS  Nijmegen breakage syndrome
NK cells  natural killer cells, a type of cytotoxic lymphocyte distinct from B-cells and T-cells, which in people usually express surface markers CD16 and CD56
NK T-cells  natural killer T-cells, a heterogeneous subset of T-cells with properties of both T-cells and natural killer (NK) cells
NMD  nonsense-mediated decay (of RNA)
NRAS  the neuroblastoma RAS gene
nRBCs  nucleated (immature) erythrocytes, typically metarubricytes
NZVP  New Zealand Veterinary Pathology
xx
PALS  periaarteriolar lymphoid sheath, a cuff of lymphocytes (mainly T-cells) surrounding small splenic arterioles and part of the splenic white pulp
PBMCs  peripheral blood mononuclear cells (includes lymphocytes and monocytes)
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PCV  packed cell volume
PE  phycoerythrin
PEL  primary effusion lymphoma, an uncommon B-cell lymphoma typically presenting as an effusion without a tumour mass or nodal involvement, and most commonly associated with human herpes virus 8 (HHV-8) and human immunodeficiency virus (HIV) infection in people
PTGC  progressive transformation of germinal centres
Q1  British shorthair-Manx cross queen, dam of LPD-affected Litters 1 and 2
Q2  British shorthair queen, dam of LPD-affected Litter 0 and granddaughter of T1
qPCR  real-time polymerase chain reaction
REAL  Revised European/American Lymphoma classification
RIPA  radio-immunoprecipitation assay
RNA  ribonucleic acid
RT  room temperature
RT-PCR  reverse transcriptase polymerase chain reaction
SCID  severe combined immunodeficiency
SLE  systemic lupus erythematosus
SSC  side scatter
T1  British shorthair tom; sire of LPD-affected Litters 1 and 2, and both grand-sire and great-grand sire of LPD-affected Litter 0
T2  Oriental tom; sire of unaffected Litter 3
T3  Unknown tom; sire of Litter 4
T4  Unknown tom; sire of Litter 5
T5  British shorthair tom; sire of LPD-affected Litter 0 and son of T1
T6  British shorthair tom; common ancestor off all LPD-affected litters
TBS  tris-buffered saline
TCR  T-cell receptor
TCRαβ  a T-cell receptor composed of α and β protein chains
TCRγδ  a T-cell receptor composed of γ and δ protein chains
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>TCRG</td>
<td>the T-cell receptor γ locus, a gene present in T-cells that undergoes rearrangement early in T-cell development, and used to assess T-cell clonality in lymphoid cell proliferations</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labelling, a method for used to detect the DNA fragmentation typically present when cells undergo apoptosis by labeling the terminal ends of nucleic acids</td>
</tr>
<tr>
<td>WAS</td>
<td>Wiskott-Aldrich Syndrome</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XLP</td>
<td>X-linked lymphoproliferative disease</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>chi-square</td>
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1.1 INTRODUCTION AND PROBLEM STATEMENT

In 2009, three British shorthair (BSH) kittens from a litter of five presented with lymphoproliferative disease (LPD) characterised by massive enlargement of multiple lymph nodes. As these kittens were siblings, this suggested a possible inherited predisposition to the disease. While aspects of the disease presentation in affected kittens suggested a diagnosis of lymphoma, other features were inconsistent with lymphoid neoplasia. In particular, the consistently young age of affected kittens, the pattern of disease affecting multiple littermates, and the presence of such marked generalised lymphadenopathy, were atypical for feline lymphoma. This unusual constellation of clinical and pathological features present in affected BSH kittens has not been previously reported in cats.
Preliminary investigations indicated that the disease in affected BSH kittens differed to previously described LPDs associated with lymphadenopathy in cats. However, it appeared to have several similarities to the human disease autoimmune lymphoproliferative syndrome (ALPS). Autoimmune lymphoproliferative syndrome is a rare inherited disorder that causes persistent lymphoproliferation together with variable manifestations of autoimmunity and predisposition to lymphoma in people (Sneller et al. 1992; Sneller et al. 1997; Straus et al. 1999; Rao and Straus 2006). The majority of human ALPS patients have inherited Fas gene mutations causing defective lymphocyte apoptosis, although for a proportion of ALPS patients the cause of disease is unknown (Fisher et al. 1995; Rieux-Laucat et al. 1999; Jackson et al. 1999a; Rao and Straus 2006).

It is often difficult to identify familial disease predispositions in companion animal species as related animals are frequently raised in geographically distant locations with limited communication between the animals’ owners. In addition, breeders are often reluctant to acknowledge possible genetic diseases in their animals. Opportunities to investigate such diseases are therefore rare. The identification of an apparently novel disease in sibling kittens with similar features to a disease in people therefore warranted further investigation. A more complete definition of the clinico-pathological features of the disease in affected kittens was necessary to confirm or exclude similarities to previously documented feline diseases. A definition of the disease in BSH kittens would also provide a rational basis for further investigations and allow the identification of future and historical cases in other kittens. Determining the pathogenesis of disease in the kittens could allow informed decisions to be made regarding treatment and the prevention of future cases. In particular, identification of an inherited basis for the disease could ultimately allow selective breeding measures to be undertaken to eliminate likely carriers of any genetic defect from the BSH breeding population and reduce future cases.

Further investigations of the basis for disease development in the kittens were also desirable to identify whether affected kittens could be used as an animal model for ALPS in people. While mouse models for ALPS exist, these models have several limitations and differences to ALPS in people, including differences in the mode of disease inheritance (Cohen and Eisenberg 1991; Nagata and Suda 1995). If studies in affected kittens
revealed similarities between the feline disease and ALPS in people, this may suggest that these kittens could be a valuable animal model.

1.2 RESEARCH AIM

The aim of this project is to describe and investigate this unusual LPD in BSH kittens, particularly the likely basis for disease development, and identify similarities with comparable diseases in people.

1.3 OVERVIEW OF THESIS STRUCTURE

In order to achieve the project aim, a range of investigative techniques were used to better define and describe the disease in affected BSH kittens. Chapter 2 reviews the background information and literature relevant to LPDs in cats and people. The clinical presentation, pathology, diagnosis, and other features of similar LPDs in cats and people are reviewed. Particular attention is given to ALPS in people and the equivalent diseases in mice, lpr and gld diseases. To investigate the hypothesis that LPD in the initial litter of BSH kittens was inherited, breeding trials and pedigree investigations were performed, and the results of these are contained in Chapter 3. In Chapter 4, the clinical features and pathology of the LPD in affected BSH kittens are described and the implications of the findings discussed in detail. The results of further investigations to better characterise the LPD in affected kittens are discussed in Chapters 5 and 6. Chapter 5 reports studies of the immunophenotype and clonality of the lymphoid proliferation in affected kittens. Chapter 6 contains the immunochemical and molecular techniques used to investigate evidence of defective lymphocyte apoptosis and Fas-gene abnormalities in LPD-affected kittens, changes typically present in the majority of people with ALPS. Chapter 7 describes the Western blot and immunohistochemical techniques used to investigate whether the LPD in BSH kittens may be due to the presence of inherited defects in the mismatch repair (MMR) genes, sometimes present in lymphoma in people. Those techniques are also applied more broadly to investigate whether small intestinal lymphomas in young cats generally may be due to the presence of inherited
MMR defects. In Chapter 8, overall conclusions that can be drawn about the nature of LPD in BSH affected kittens are hypothesised and the limitations of the research and directions for future studies identified. The Appendix contains a suggested protocol for sample collection from any future cases of similar feline LPD.

1.4 TERMINOLOGY

There is considerable inconsistency in the use of the term LPD in both human and veterinary medicine. While the many forms of lymphoma are consistently included within the definition, diseases such as those involving histiocytic or non-neoplastic lymphocyte proliferations are only variably included within the ambit of the term. Throughout this thesis, the term LPD is defined to refer to any disease in which abnormally excessive numbers of lymphocytes are produced. This definition differs to the narrow interpretation of LPD often used in the veterinary literature as limited to neoplastic diseases, primarily lymphoma (Valli 2007b), and reflects the broader construction of the term more typically applied in the human medical literature to also include atypical and non-neoplastic lymphoid proliferations (Brown and Skarin 2004; Warnke et al. 2007). The term LPD as used here therefore includes a wide spectrum of diseases from neoplastic monoclonal lymphoid proliferations through to non-neoplastic disorders involving polyclonal lymphoid proliferations (Greiner et al. 2000). In this thesis, the term lymphadenopathy indicates enlargement of a lymph node or nodes regardless of cause.

1.5 REFERENCES


2.1 INTRODUCTION

Preliminary findings in affected British shorthair (BSH) kittens indicated an unusual lymphoproliferative disease (LPD), primarily affecting the lymph nodes, with a likely familial predisposition. Although most commonly due to lymphoid neoplasia, non-neoplastic LPDs causing widespread lymphadenopathy are also reported, and both neoplastic and non-neoplastic diseases were considered possible. Accordingly, following a brief review of lymphocyte development (section 2.2), the second part of this chapter (section 2.3) reviews neoplastic LPDs of people and cats to identify similarities between previously described diseases and the disease observed in BSH kittens. Differentiating between neoplastic and non-neoplastic LPDs can be difficult, and general principles and methods of lymphoma diagnosis are discussed in this section. Given the breadth of literature concerning human lymphoma, the discussion here concentrates on lymphomas with a familial predisposition similar to disease in the BSH kittens. In the third part of the chapter (section 2.4), potentially relevant non-neoplastic causes of lymphadenopathy and LPD in people and cats are reviewed. Subsequent investigations
of affected kittens revealed several similarities with the human inherited LPD autoimmune lymphoproliferative syndrome (ALPS), and features of ALPS are reviewed in detail in the final part of the chapter (section 2.5).

2.2 LYMPHOCYTE DEVELOPMENT

Lymphocytes play a key role in the execution of the immune response to antigens. Mature lymphocytes comprise B-cells, T cells and natural killer (NK) cells. B-cells are central to the humoral immune response, primarily through the production of antigen-neutralising antibodies. T-cells regulate the immune response and play a fundamental role in cell-mediated immunity, principally by recognising antigens (especially viral antigens) on the surface of other cells and eliminating those cells. Natural killer cells also have a role in the cytotoxic destruction of abnormal (particularly neoplastic) cells, but do so in the absence of either antibody or major histocompatibility complex (MHC) -bound antigen (Abbas et al. 2012; Owen et al. 2013a). Lymphocytes differentiate from pluripotent haematopoietic stem cells (HSCs) primarily located within the bone marrow to form mature B-cells, T-cells and NK cells, and lymphocyte development involves a complex series of events controlled by multiple different genes (Paul 2008; Abbas et al. 2012). Different “cluster of differentiation” (CD) proteins are expressed by lymphocytes of particular lineages at specific developmental stages and are used to identify cells by immunophenotyping techniques (discussed in section 2.3.1.2 below). A schematic representation of lymphocyte development is presented in Figure 2.1.

In mammals, B-cells initially develop within the bone marrow (or fetal liver) from HSCs through pro B-cell, pre-B-cell, early B-cell and ultimately mature B-cell stages, although the exact number of stages and terminology used to describe them varies (Hardy and Hayakawa 2001; Owen et al. 2013c). During the later stages of B-cell development, B-cells with the potential to react to self-antigens are identified and eliminated (Monroe and Dorshkind 2007; Abbas et al. 2012). Mature B-cells leave the bone marrow and migrate to secondary lymphoid organs such as the lymph nodes and spleen, where they may become activated by exposure to appropriate antigen (with the assistance of helper T-cells) and differentiate into antibody-producing plasma cells, become memory B-cells,
or undergo programmed cell death following a lack of antigenic stimulation (Abbas et al. 2012; Owen et al. 2013a).

B-cells have an antigen-specific cell surface B-cell receptor (BCR), and binding of the BCR to the appropriate antigen activates the cell. The BCR comprises a membrane-bound immunoglobulin molecule complexed to the transmembrane protein CD79, which itself comprises the CD79a and CD79b chains. CD79a is often regarded as a pan-B-cell marker as it is expressed at most stages of B-cell development, from pro-B-cell through to mature B-cell and most plasma cell stages; many other proteins (e.g. CD21) tend to be expressed at a more limited number of stages (Minegishi et al. 1999; Warnke and Isaacson 2001).

The vast majority of T-cells develop in the thymus following the migration of pro-thymocytes from the bone marrow. Within the thymus, T-cell precursors undergo progressive differentiation through several double negative (DN) stages (expressing neither CD4 nor CD8 cell surface proteins), to double positive (DP) cells (expressing both CD4 and CD8), and finally to mature single positive T-cells expressing either CD4 (primarily helper T-cells) or CD8 (primarily cytotoxic T-cells); natural killer T-cells (NK T-cells) show variable expression of CD4 and CD8 (Godfrey et al. 2004; Zuniga-Pflucker 2004; Koch and Radtke 2011; Owen et al. 2013b). The DN stages DN1, DN2, DN3 and DN4 can be differentiated by their variable expression of various surface markers, particularly CD25 and CD44 (Zuniga-Pflucker 2004; D’Acquisto and Crompton 2011; Koch and Radtke 2011). The γδ T-cell lineage (discussed further in the following paragraph) diverges at the DN2/DN3 cell stage (Xiong and Raulet 2007; D’Acquisto and Crompton 2011; Juno et al. 2012). During the later stages of thymic T-cell development, T-cells with potential reactivity to self-antigens are identified and eliminated (D’Acquisto and Crompton 2011). Following thymic maturation, T-cells leave the thymus and migrate to peripheral lymphoid organs such as the lymph nodes and spleen (Abbas et al. 2012).

Development of the cell surface antigen-specific T-cell receptor (TCR) occurs during thymic T-cell development. The TCR recognises antigens bound to MHC molecules on other cells, a critical step in T-cell activation and the successful elimination of antigens. The TCR is composed of two protein chains: either an α and a β chain (TCRαβ), or a γ and a δ chain (TCRγδ). In people, the vast majority (reportedly up to 95%) of T-cells express
TCRαβ, with γδ T-cells constituting only a small minority (Kabelitz 1992; Haas et al. 1993; Germain 2002). The TCR (either TCRαβ or TCR γδ) associates with the 4 chains of the CD3 protein: CD3γ, CD3δ, and two CD3ε chains, as well as CD3ζ, to form the fully assembled TCR complex (Kuhns and Badgandi 2012). Expression of one or more of these CD3 chains within the TCR complex is frequently used in the immunochemical identification of T-cells: the fully assembled CD3 complex is present on the later stages of T-cell development, including some DP and single positive thymocytes, γδ T-cells, cytotoxic T-cells, helper T-cells and NK T-cells (Warnke and Isaacson 2001; Brodeur et al. 2009).

TCRαβ T-cells are further sub-classified into helper or cytotoxic T-cell populations based on their expression of the CD4 and CD8 surface glycoproteins. Helper T-cells (CD4+) regulate the immune response to antigens and modulate the activity of B-cells and other T-cells. Regulatory T-cells are specific types of CD4+ T-cell that downregulate immune responses, particularly to self-antigens. Cytotoxic T-cells (CD8+) are particularly involved in the destruction of virally infected cells and neoplastic cells (Owen et al. 2013a). The role of NK T-cells (variable CD4 and CD8 expression) is poorly understood but involves both cytotoxic and helper T-cell functions (Juno et al. 2012). The role of TCRγδ cells is also incompletely understood, but their response to antigens appears to involve aspects of both innate and acquired immunity (Xiong and Raulet 2007).

NK cells also differentiate from the common lymphoid progenitor cell (and possibly also the DN1 stage) prior to commitment to either the B-cell or T-cell lineage to form a third lymphocyte category (D'Acquisto and Crompton 2011; Koch and Radtke 2011). In people, the majority of NK cells express CD56 and CD16, with variable expression of CD8. Natural killer cells often show large granular lymphocyte (LGL) cytological features such as the presence of azurophilic cytoplasmic granules (Lanier et al. 1986; Morice 2007), although LGL morphology is also reported in other T-cell subpopulations (Greer et al. 2001). While NK cells do not express the fully formed CD3-TCR complex (and are typically described as CD3-), they may express individual subunits of the TCR complex, including the epsilon (ε) chain of CD3 (Morice 2007).
Figure 2.1: Lymphocyte development and expression of selected CD markers. A simplified schematic representation of lymphocyte development from pluripotent haematopoietic stem cells in the bone marrow through to mature B-cell and T-cell forms which populate the secondary lymphoid organs. B-cell development occurs predominantly within the bone marrow; T-cell development occurs predominantly within the thymus. Selected CD surface markers expressed by the majority of the particular cell type and of relevance to the studies in this thesis are indicated. Markers shown are for humans; there is slight species variation.

DN = double negative (expressing neither CD4 nor CD8), DP = double positive (expressing both CD4 and CD8), HSC = haematopoietic stem cell, NK = natural killer.
CHAPTER 2

2.3 NEOPLASTIC LYMPHOPROLIFERATIVE DISEASES

Lymphoma results from the malignant transformation of a single lymphocyte and the subsequent clonal proliferation of that cell (Stricker and Kumar 2010). Depending on the type of lymphocyte that undergoes transformation, lymphoma can be sub-classified as B-cell, T-cell, or occasionally NK cell. The term “lymphoma” typically refers to a discrete tissue mass of neoplastic lymphocytes; “lymphoid leukaemia” is used to describe neoplastic cell proliferations predominantly located within the blood or bone marrow (Valli 2007b; Kumar et al. 2010).

2.3.1 GENERAL PRINCIPLES OF LYMPHOMA DIAGNOSIS

Lymphomas encompass an extensive and diverse group of neoplastic diseases. In both humans and animals, lymphoma has a diverse clinical presentation depending on the particular anatomic site or sites affected, the extent of organ involvement, and the characteristics of the particular lymphoid cell proliferation (Jacobs et al. 2002; Valli 2007b; Kumar et al. 2010). Given this diversity, generalisations about the typical clinical presentation and gross pathology of lymphoma are difficult. While lymphomas that primarily involve the lymph nodes often present as diffusely enlarged non-painful nodes which bulge on cut section, and are white or cream coloured with effacement of the normal cortico-medullary demarcation, this is not always the case (Jacobs et al. 2002; Kumar et al. 2010; Fry and McGavin 2012). The definitive diagnosis of lymphoma is therefore based on a combination of supportive microscopic pathology, the presence of a single immunophenotype within the proliferating lymphocytes, and the demonstration of clonality within the proliferating lymphoid cells (Stricker and Kumar 2010; Moore et al. 2012).

2.3.1.1 MICROSCOPIC PATHOLOGY

Lymphomas involving lymph nodes must be distinguished from other potential causes of lymphadenopathy, particularly lymphoid hyperplasia (Jacobs et al. 2002). Microscopic pathology is an important initial step in diagnosis, and both cytology and histology are used.
In the cytological evaluation of aspirates or impression smears from enlarged lymph nodes, the presence of a monomorphic population of morphologically atypical lymphoid cells with an absence or paucity of other cell types is considered supportive of a preliminary diagnosis of lymphoma (Messick 2008; Raskin 2010; Fry and McGavin 2012). In general, if more than half the cells present within cytological samples from the affected node comprise medium or large lymphocytes (lymphocytes more than 1.5x the diameter of a normal erythrocyte), a diagnosis of lymphoma is likely (Messick 2008; Raskin 2010). Other cytological features suggestive of lymphoma include many lymphocytes with large nuclei and nucleoli, anisocytosis and anisokaryosis, lymphocytes with intensely basophilic cytoplasm, the presence of many cells in mitosis, and abundant background cytoplasmic fragments (previously termed lymphoglandular bodies) (Messick 2008; Raskin 2010). It is not always possible to make a definitive diagnosis of lymphoma cytologically, especially when nodes are sampled early in the course of neoplastic disease or if concurrent inflammatory disease is present.

Principles of lymphoma diagnosis by histology are similar to those of cytology regarding the morphological features of the proliferating cells, but also allow for assessment of the architecture of affected nodes (Jacobs et al. 2002). Normal nodal architecture seen on histology includes the outer capsule surrounding the peripheral superficial cortex containing defined lymphoid follicles and germinal centres, deeper paracortex, and distinct inner medullary cords and sinuses (Figure 2.2). Lymphocytes of the germinal centres are encircled by a denser mantle zone of lymphocytes. Interfollicular regions of the superficial cortex and the paracortex contain mainly T-cells, while the follicles, germinal centres and mantle zones predominantly contain B-cells. The medulla primarily contains macrophages and plasma cells (Willard-Mack 2006; Fry and McGavin 2012).

Nodes affected by lymphoma show variable disruption of the normal architecture (Fry and McGavin 2012). In broad terms, two main histologic patterns are seen in lymph nodes affected by lymphoma: diffuse and follicular. In the diffuse pattern, the proliferating lymphocytes initially distort and ultimately efface normal nodal architecture. The diffuse expansion of the node with loss of normal architecture is a histological characteristic strongly suggestive of a diagnosis of lymphoma (Jacobs et al. 2002). The diffuse pattern is more commonly observed in animals, possibly due to the fact that diagnosis is often made relatively late in the clinical course of disease (Vezzali
et al. 2010). In the follicular pattern of lymphoma, multiple aggregates of proliferating lymphocytes are typically present within affected lymph nodes and diagnosis is often more challenging, as remnants of normal architecture are still present and the appearance of the node may be similar to that seen in nodes reacting to antigen exposure. The follicular form of lymphoma is common in people but rarely recognised in animals (Bienzle 2003; Vezzali et al. 2010).

2.3.1.2 IMMUNOPHENOTYPE

Lymphomas are predominantly of either B-cell or T-cell origin; tumours of NK cell origin are recognised in people and animals, but appear rare and detailed descriptions are limited (Valli et al. 2002; Kumar et al. 2010). In both humans and animals, immunophenotyping is used to identify the particular type of cell proliferating within a lymphoma or suspected lymphoma based mainly on the combination of CD antigens expressed by those cells, and results are often useful in differentiating between neoplastic and non-neoplastic lymphadenopathy. The presence of a single immunophenotype within an enlarged lymph node is suggestive of a diagnosis of lymphoma, as it indicates the cells present have a single lineage and is therefore more
consistent with neoplasia. In contrast, in nodes reacting to antigenic stimulation, a mixture of cellular immunophenotypes is typically seen (Renshaw 2007; Moore 2008).

Immunophenotyping techniques apply a labelled CD antibody to the cells in question: if the cells are of a type that expresses the relevant CD antigen, the labelled CD antibody will bind to that CD antigen. The labelled cells may then be identified using various means, including the application of chromagen substrates such as 3,3’-diaminobenzidine (DAB) in immunohistochemical methods, or fluorescent emission at particular wavelengths in flow cytometry. Techniques may be applied to various sample types to identify the lineage(s) of cells present in the sample, including cytological samples, frozen tissue sections, formalin-fixed paraffin-embedded (FFPE) histological specimens, blood samples and other cell suspensions (Carey et al. 2007; Renshaw 2007; Craig and Foon 2008; Oliver and Jamur 2008). Immunocytochemistry involves the application of immunophenotyping methods to individual cells, while immunohistochemistry involves the use of tissue sections, either snap frozen or FFPE material. Flow cytometric methods may be used to immunophenotype cells in suspension, including blood and other fluids (Vernau and Moore 1999; Raskin 2010).

Few CD antigens are expressed by a single lineage of cells, so the application of multiple antibodies specific for particular CD antigens to the relevant samples is desirable to allow definitive identification of particular types of leukocyte (Moore 2008). However, while a large number of CD and other antigens have been identified and are routinely used in lymphoma diagnosis in people (Zola et al. 2005; Zola et al. 2007), many of these antibodies have not been characterised or do not cross-react with feline cells. Consequently, only a limited number of antibodies are available for immunophenotyping lymphocytes in cats (Table 2.1). The practical use of these antibodies in feline samples is further restricted by their limited commercial availability. In addition, many of the CD antibodies useful in confirming neoplastic cell lineage and identifying lymphocyte subtypes do not react in FFPE tissue, limiting their use in historical cases where fresh tissue is unavailable. Due to cost considerations and the limited practical impact of a definitive diagnosis in ultimate outcome for the animal, any immunophenotyping of suspected lymphomas in clinical veterinary practice is usually restricted to the application of CD79a (B-cells) and CD3 (T-cells). However, while
immunophenotyping tends to be limited in clinical feline medicine, it can be extremely useful in the research environment.

Table 2.1: Selected CD antibodies previously used in the identification of feline lymphocyte subpopulations by various immunophenotyping techniques (immunohistochemistry, immunocytochemistry and flow cytometry) (Lanier et al. 1992; Affolter and Moore 2006; Raskin 2010; Vermeulen et al. 2012). Non-feline antibodies listed have been previously demonstrated to cross-react with feline cells. Other than CD3ε and CD79a, commercial availability of assays for these antigens is largely limited to research laboratories in the United States and Europe.

<table>
<thead>
<tr>
<th>Antigen/Antibody</th>
<th>Clone</th>
<th>Species</th>
<th>Reactive in FFPE</th>
<th>Main cell reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>Fe1.5F4</td>
<td>Feline</td>
<td>No</td>
<td>B-cell subpopulation, monocytes, dendritic cells</td>
</tr>
<tr>
<td>CD1c</td>
<td>Fe5.5C1</td>
<td>Feline</td>
<td>No</td>
<td>B-cell subpopulation, monocytes, dendritic cells</td>
</tr>
<tr>
<td>CD3ε</td>
<td>CD3-12</td>
<td>Human</td>
<td>Yes</td>
<td>T-cells (including NK T-cells)</td>
</tr>
<tr>
<td>CD4</td>
<td>Fe1.7B11</td>
<td>Feline</td>
<td>No</td>
<td>T-cell subpopulation (helper)</td>
</tr>
<tr>
<td>CD5</td>
<td>Fe1.1B11</td>
<td>Feline</td>
<td>No</td>
<td>T-cells (and B-cell subpopulation)</td>
</tr>
<tr>
<td>CD8a</td>
<td>Fe1.10E9</td>
<td>Feline</td>
<td>No</td>
<td>T-cell subpopulation (cytotoxic)</td>
</tr>
<tr>
<td>CD8β</td>
<td>Fe5.4D2</td>
<td>Feline</td>
<td>No</td>
<td>T-cell subpopulation (cytotoxic)</td>
</tr>
<tr>
<td>CD11b</td>
<td>Ca16.3E10</td>
<td>Canine</td>
<td>No</td>
<td>Granulocytes, histiocytes, monocytes</td>
</tr>
<tr>
<td>CD11d</td>
<td>Ca11.3D3</td>
<td>Canine</td>
<td>No</td>
<td>T-cell subpopulation, macrophages</td>
</tr>
<tr>
<td>CD18</td>
<td>Fe3.9F2</td>
<td>Feline</td>
<td>Yes</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>CD21</td>
<td>Ca2.1</td>
<td>Canine</td>
<td>No</td>
<td>Mature B-cells</td>
</tr>
<tr>
<td>CD56</td>
<td>S2K1</td>
<td>Feline</td>
<td>No</td>
<td>NK cells, NK T-cells</td>
</tr>
<tr>
<td>CD45R</td>
<td>B220</td>
<td>Mouse</td>
<td>Yes</td>
<td>B-cells, T-cell subpopulation</td>
</tr>
<tr>
<td>CD79a</td>
<td>HM57</td>
<td>Human</td>
<td>Yes</td>
<td>B-cells</td>
</tr>
</tbody>
</table>

FFPE = formalin-fixed paraffin-embedded tissue (histological samples suitable for immunohistochemistry), NK cells = natural killer cells, NK T-cells = natural killer T-cells.

2.3.1.3 CLONALITY

As neoplasia results from the clonal expansion of a single transformed cell, clonality is a fundamental characteristic of neoplasia (Werner et al. 2005). While clinical, morphological and immunophenotypic features may all suggest a diagnosis of lymphoma, demonstration of a clonal population of lymphocytes is generally regarded as the most reliable basis on which to differentiate lymphoid neoplasia from non-neoplastic lymphocyte proliferations and clonality studies are a valuable adjunctive test in lymphoma diagnosis (Griesser et al. 1989; Weiss and Spagnolo 1993; Vernau and Moore 1999). The clonality of a cell population is assessed by determining if differences in the cells’ gene rearrangements are absent (monoclonal and likely neoplastic) or present (polyclonal and likely non-neoplastic). When T-cell receptor genes and immunoglobulin genes rearrange during normal T-cell and B-cell development...
respectively, the resulting length and sequence of the genes produced varies between cells. A polyclonal population will contain multiple different lymphocytes and therefore T-cell receptor or immunoglobulin genes of different sequences and sizes. In a monoclonal population due to neoplastic transformation and proliferation of a single lymphocyte, the T-cell or immunoglobulin genes present are typically of a single sequence and size (Moore 2008; Avery 2012).

In human samples, the detection of lymphocyte antigen receptor gene rearrangements using polymerase chain reaction (PCR) methods is used to assess clonality of lymphoid proliferations (Medeiros and Carr 1999; Kumar et al. 2005; Werner et al. 2005). Clonality assays use primers designed to amplify the T-cell receptor γ locus (TCRG) to assess T-cell clonality, or the immunoglobulin heavy chain locus (IGH) to assess B-cell clonality. The primers used are directed at the conserved regions of the relevant T-cell or immunoglobulin genes that flank the hypervariable regions of the genes, and so enable amplification of those variable regions. Following immunophenotyping, DNA is extracted from the lymphocyte population and PCR using the relevant primers is performed. The products of amplification are then separated electrophoretically. Polyclonal proliferations typically appear as a broad smear in the gel or broad (Gaussian) peak on capillary electrophoresis corresponding to the multiple gene rearrangements in the different lymphoid cells within the cell population. The presence of a smear or broad peak is therefore consistent with a non-neoplastic lymphoproliferation. Monoclonal proliferations typically show one or two strong sharply defined bands or spikes of the same size, as most cells present are clones of the initial single transformed neoplastic cell and thus have the same gene rearrangement. The presence of one or two defined and repeatable bands or spikes is therefore evidence of lymphoma (Moore 2008; Avery 2012). The interpretation of oligoclonal results (samples exhibiting 3 to 5 reproducible bands or peaks) and pseudoclonal results (samples exhibiting one or two non-identical bands or peaks) is more equivocal.

Although not currently available in New Zealand, PCR techniques for the amplification of TCRG or IGH loci to assess clonality have been validated for use in the lymphoma diagnosis in cats (Moore et al. 2005; Werner et al. 2005). Clonality assays are generally indicated when microscopic features and immunophenotyping results for a suspected lymphoma are inconclusive. T-cell assays in cats reportedly detect clonality in
approximately 90% of feline T-cell lymphomas, if both monoclonal and oligoclonal results are interpreted as clonal (Moore et al. 2005; Moore et al. 2012). By comparison, B-cell clonality assays in cats are less sensitive, with clonality reportedly detected in approximately 50%-68% of B-cell lymphomas (Werner et al. 2005; Moore et al. 2012).

2.3.2 LYMPHOMA IN PEOPLE

Lymphoma in people represents a hugely diverse clinical and biological disease entity. The most recent classification of lymphoma in people lists over 80 different types of lymphoma, broadly grouped into 5 categories (Swerdlow et al. 2008). A discussion of the multiple different types of lymphomas in people is therefore beyond the scope of this review. Instead, selected aspects of the human literature of particular relevance to the presentation of LPD in the kittens are discussed: first, the lymphoma classification systems used in people are briefly summarised, and then inherited and other diseases that predispose people to lymphoma are reviewed.

2.3.2.1 CLASSIFICATION SYSTEMS

The categorisation of lymphomas in people is controversial, and multiple classification systems have been described. Early lymphoma classification systems were based largely upon tumour and cell morphology (Gall and Mallory 1942; Rappaport 1966), while more recent classification schemes incorporate additional factors including tumour grade, cell lineage and patient survival. The Kiel classification (Gerard-Marchant et al. 1974; Lennert et al. 1975) divided lymphomas into low and high grade based on the rate of tumour progression, and as either B-cell or T-cell based on cell morphology. The publication of the National Cancer Institute Working Formulation in 1982 (Anonymous 1982) divided lymphomas into low, intermediate and high grade neoplasms based on their rate of progression, but given the limited availability of immunohistochemistry at that time, did not attempt to classify lymphomas as B-cell or T-cell neoplasms.

From the 1980s, the increasing availability of antibodies against lymphocyte CD antigens enabled more reliable determination of tumour immunophenotype, and immunophenotype began to be incorporated into subsequent lymphoma classifications. The Kiel classification was updated (Stansfeld et al. 1988) to incorporate immunophenotype and divided lymphomas into B-cell and T-cell subtypes, with each
category further subdivided into low, intermediate or high grade tumours. The Revised European/American Lymphoma (REAL) classification (Harris et al. 1994) used immunophenotypic, morphologic and genotypic features of the neoplasm to compile a list of specifically identified lymphomas. The World Health Organization (WHO) classification of tumours of haematopoietic and lymphoid tissues, published in 2001 and updated in 2008 (Jaffe et al. 2001; Swerdlow et al. 2008), is currently the most commonly used lymphoma classification in people. Based on the REAL classification, the WHO system separates lymphomas into B-cell, T-cell and NK cell lineages and considers phenotypic, molecular and cytogenetic characteristics of the tumour to differentiate between mature and immature cell types. The distinction between Hodgkin lymphoma (lymphoma arising in a single node or chain of nodes and spreading first to contiguous nodes) and non-Hodgkin lymphoma (all other lymphomas) emphasised by older categorisations is much less important under current classifications, although is often still used in the literature.

2.3.2.2 FAMILIAL LYMPHOMA

Familial clusters of lymphoma in people are unusual. Reported cases usually occur with rare inherited conditions such as genomic instability syndromes, immune deficiency syndromes or autoimmune diseases. An apparent familial predisposition also may be observed if family members share environmental exposure to viral agents or other carcinogens (Linet and Pottern 1992; Mueller and Pizzo 1995; Segel and Lichtman 2004; Siddiqui et al. 2004). However, regardless of cause, the simultaneous development of lymphoma by multiple family members is extremely uncommon.

Genomic instability syndromes

Genomic instability syndromes associated with a familial predisposition to lymphoma include ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS), Bloom syndrome, Li-Fraumeni syndrome and hereditary non-polyposis colorectal cancer (HNPCC) (Siddiqui et al. 2004).

Ataxia-telangiectasia, NBS and Bloom syndrome are rare autosomal recessive disorders of chromosomal instability (Taylor 2001). They are characterised by multiple and varied clinical signs affecting various organs, including progressive neurodegeneration and telangiectasia in AT (Meyn 1999), growth retardation and microencephaly in NBS.
Clinical signs common to all three syndromes include immunodeficiency and an increased predisposition to B-cell and T-cell lymphoid neoplasia, typically in childhood (Bloom 1954; Taalman et al. 1983; Peterson et al. 1992; Seidemann et al. 2000; Michallet et al. 2003). All three syndromes are caused by germline genetic mutations. Ataxia-telangiectasia is due to mutations in the ATM gene (Gatti et al. 1988; Savitsky et al. 1995), NBS is due to mutations in the NBS1 or NBN gene (Matsuura et al. 1997), while Bloom syndrome is due to mutation in the BLM (RECQL3) gene (Ellis et al. 1999). A loss of the protein coded for by these genes causes chromosomal instability, resulting in a high level of spontaneous chromosome abnormalities. Diagnosis involves detection of increased cellular sensitivity to ionising radiation in AT and NBS, or detection of increased frequency of sister chromatid exchanges in Bloom syndrome. Additional diagnostic tests look at defective expression and function of the relevant proteins in blood or tissue. Genetic screening using PCR to detect mutations is also used in diagnosis.

Li-Fraumeni syndrome is a rare autosomal dominant disease characterised by a familial predisposition to the early development of a wide range of neoplasms, predominantly soft tissue and bone sarcomas (Li and Fraumeni 1969). Li-Fraumeni syndrome is primarily attributed to the presence of germline mutations in the TP53 tumour suppressor gene, which encodes the p53 protein (Malkin et al. 1990). Cases of B-cell lymphoma and lymphocytic leukaemia are occasionally reported in patients meeting Li-Fraumeni syndrome criteria (Fraumeni et al. 1969; Kleihues et al. 1997; Pepper et al. 2003; Schiffman et al. 2008). However, whether TP53 mutations predispose to familial lymphoma and lymphocytic leukaemia has not been definitively established, as these mutations are absent from the majority of cases of familial lymphoma in Li-Fraumeni patients (Weintraub et al. 1996).

Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) is an autosomal dominant inherited disease that results in a predisposition to colorectal neoplasia at a young age (Mecklin and Jarvinen 1991), as well as neoplasia at multiple other sites (Lynch and Krush 1971; Niessen et al. 2006). The syndrome is caused by germline mutations in one or more of the DNA mismatch repair (MMR) genes. Although seven genes have been associated with HNPCC (MLH1, MSH2, MLH3, MSH6, PMS1, PMS2 and
EXO1), mutations in MLH1, MSH2 and MSH6 only are currently considered to cause HNPCC (Lagerstedt Robinson et al. 2007). While lymphomas are generally not recognised to form part of the spectrum of HNPCC neoplasia in people, MMR gene knockout mice frequently develop lymphoma at a young age (de Wind et al. 1995; Reitmair et al. 1995; Edelmann et al. 1997; Lowsky et al. 1997; Kawate et al. 1998; Prolla et al. 1998; de Wind et al. 1999; Yao et al. 1999; Wei et al. 2002; Chen et al. 2005). Biallelic recessive mutations in the MSH2 gene are occasionally reported in people presenting with early childhood T-cell lymphoma and lymphocytic leukaemia (Bougeard et al. 2003; Scott et al. 2007), while other studies suggest that approximately one-quarter of sporadic lymphoid tumours in people exhibit signs consistent with underlying somatic defect in the MMR genes (Indraccolo et al. 1999).

**Immune deficiency syndromes**

Immune deficiency syndromes associated with familial lymphoma include X-linked lymphoproliferative disease (XLP), Wiskott-Aldrich syndrome (WAS), common variable immunodeficiency (CVID) and severe combined immunodeficiency (SCID). Both XLP and WAS are caused by mutations in the X chromosome and so are seen almost exclusively in males (Siddiqui et al. 2004). As discussed in Chapter 3, the disease in the kittens is unlikely to be X-linked and XLP and WAS are not reviewed further.

Common variable immunodeficiency encompasses a large group of primary immunodeficiency diseases with diverse clinical presentations including persistent infections and autoimmune diseases (Cunningham-Rundles and Bodian 1999; Cunningham-Rundles 2012). A diagnosis of CVID is usually made in adults between the ages of 20 and 40 years, although clinical signs often begin in childhood (Cunningham-Rundles and Bodian 1999; Cunningham-Rundles 2012). Although most cases of CVID occur sporadically, up to 25% are due to inherited genetic mutations, typically with autosomal-dominant inheritance (Schroeder et al. 2004). Multiple genetic defects have been implicated in CVID, including mutations in ICOS (inducible T-cell co-stimulator) (Grimbacher et al. 2003), tumour necrosis factor receptor superfamily members TNFRSF13B (or TACI) (Salzer et al. 2005) and TNFRSF13C (or BAFFR) (Warnatz et al. 2005) and CD19 93 (van Zelm et al. 2006) genes. In very broad terms, B-cells fail to differentiate in CVID patients, resulting in hypogammaglobulinaemia and immunosuppression (Cunningham-Rundles and Bodian 1999). The overall risk of cancer
is also increased, with lymphoma most commonly reported (Kinlen et al. 1985; Cunningham-Rundles et al. 1991; Mellemkjaer et al. 2002). Lymphomas are typically B-cell in origin and involve sites other than the lymph nodes (Cunningham-Rundles and Bodian 1999; Cunningham-Rundles 2002; Resnick et al. 2012).

Severe combined immunodeficiency is another primary immune deficiency resulting from a heterogeneous group of conditions affecting the immune system. The clinical signs of SCID are typically more severe than those seen with CVID, as SCID involves defects in T-cell, B-cell and occasionally NK cell differentiation and function, resulting in severe and often fatal infections within the immediate post-natal period (Fischer 1992). The thymus is usually atrophic in SCID patients, and peripheral lymphoid tissue is absent or markedly decreased. Multiple genetic defects have been implicated in SCID. The most common genetic mutation responsible for SCID involves an X-linked mutation of the interleukin (IL) receptors (Fischer 1992), but other less common autosomal recessive forms involve mutations in JAK3 (Janus-associated kinase 3) (Macchi et al. 1995), ADA (adenosine deaminase) (Hirschhorn et al. 1979) or RAG-1 and RAG-2 (recombination-activating gene) (Villa et al. 1999). Children with SCID have an increased risk of lymphoma development, the majority of which occur in the first year of life. Lymphomas are most commonly of B-cell origin (Garcia et al. 1987; Mueller and Pizzo 1995).

**Autoimmune diseases**

Autoimmune diseases associated with familial lymphoma include hyper-IgM syndrome and ALPS. Hyper-IgM syndrome is a disease with an X-linked mode of inheritance (Winkelstein et al. 2003) and so appears unlikely to be relevant to the cause of the disease in kittens and is not reviewed further. Autoimmune lymphoproliferative syndrome is a recently recognised disease involving genetic defects in lymphocyte apoptosis that cause widespread but non-neoplastic LPD, variable autoimmune manifestations, and an increased development of malignancies, most commonly B-cell lymphomas (Sneller et al. 1997; Infante et al. 1998; Jackson and Puck 1999b; Straus et al. 2001; Poppema et al. 2004). As there are many similarities between ALPS and the disease the in kittens, ALPS is discussed in detail in section 2.5 of this chapter.
Shared environmental exposures

In addition to inherited diseases associated with lymphoma development in people, an apparent familial predisposition to lymphoma may result from the common exposure of multiple family members to environmental lymphomagens (Segel and Lichtman 2004). A range of chemicals and infectious agents, predominantly viruses, have been associated with an increased risk of lymphoma in people. However, in such cases the development of lymphoma typically occurs many years after exposure to, or infection with, the relevant agent, and only a small minority of exposed people develop lymphoid neoplasia.

A number of chemicals have been associated with lymphoma in people (Merhi et al. 2007). Pesticides containing organophosphates and organochlorines are most commonly implicated (Cantor et al. 1992; McDuffie et al. 2001; Waddell et al. 2001), although exposure to various herbicides, fungicides and even wood dust has also been suggested to be associated with an increased risk of lymphoma in some studies (Hoar et al. 1986; Cantor et al. 1992; McDuffie et al. 2001). Lymphomas are generally described as non-Hodgkin lymphoma, with B-cell lymphomas more commonly reported (van Balen et al. 2006). However, the literature is contradictory, with other studies providing more limited support for an increased risk of lymphoma in association with pesticide and herbicide exposure (Pearce and McLean 2005; Cocco et al. 2013).

Solvent exposure, particularly involving benzene, has also been suggested as a risk factor for lymphoma development in people. Again, results are conflicting, with some studies finding benzene exposure increases risk of B-cell lymphoma (Smith et al. 2007; Wang et al. 2009; Cocco et al. 2010), but others finding no increased risk (Wong and Raabe 2000; Alexander and Wagner 2010).

In addition to environmental exposure to a number of chemicals, several viruses are known to cause lymphoma in people, particularly Epstein-Barr virus (EBV), human herpesvirus-8 (HHV-8), and human T-cell leukaemia virus-1 type 1 (HTLV-1).

Infection with EBV is associated with the development of a heterogeneous group of lymphomas in people (Hsu and Glaser 2000). Epstein-Barr virus is a γ-herpesvirus (human herpesvirus-4 or HHV-4) predominantly spread through saliva and blood (Wolf
et al. 1993). It is one of the most common viral infections in people and it is estimated that more than 90% of the world’s population are infected; however, the vast majority do not develop disease (de-The et al. 1975; Cohen 2000). Lymphomas associated with EBV infection in people include Burkitt lymphoma (a highly aggressive B-cell non-Hodgkin lymphoma), Hodgkin lymphoma and post-transplant lymphoproliferative disorder (PTLD) (Hsu and Glaser 2000; Khanna and Burrows 2000; Parkin 2011). Post-transplant lymphoproliferative disorder is a life-threatening disease that may develop following haematopoietic stem cell or organ transplantation and encompasses a wide group of LPDs ranging from non-neoplastic polyclonal hyperplasia through to non-Hodgkin lymphoma (Gottschalk et al. 2005).

Infection with the retrovirus human T-cell leukaemia virus-1 (human T-cell lymphotropic virus type 1 or HTLV-1) is associated with adult T-cell leukaemia and lymphoma in people (Davey and Hutchison 1991; Parkin 2006; Lairmore et al. 2012). Infection rates vary widely between different geographical areas throughout the world, and infection is endemic within parts of Japan (Nicot 2005). The virus is primarily spread by sexual and blood contact (Tajima et al. 1987; Take et al. 1993) although transmission from mother to child in breast milk and across the placenta is also reported (Takahashi et al. 1991; Fujino and Nagata 2000; Nicot 2005). The majority of people infected by HTLV-1 remain asymptomatic carriers with only a small proportion developing lymphoid malignancies, usually many years after infection (Nicot 2005). Both T-cell leukaemia and lymphoma are reported in association with HTLV-1, with lymphomas typically characterised by the involvement of multiple lymph nodes (Nicot 2005).

Human herpes virus 8 (HHV-8) or Kaposi’s sarcoma-associated virus infections are also associated with an increased risk of lymphoma development, most commonly primary effusion lymphoma (PEL). Primary effusion lymphoma is a rare type of B-cell lymphoma that typically presents as a pleural, peritoneal or pericardial neoplastic effusion without a solid tumour mass or evidence of nodal involvement (Nador et al. 1996). Most cases of PEL occur in people co-infected with human immunodeficiency virus (HIV), and the vast majority of tumours are also positive for EBV (Chen et al. 2007). Transmission by saliva appears the most common route of HHV-8 infection, and while sexual, blood and transplant-related transmission are also reported, the precise mechanisms by which transmission occurs are often uncertain (Martin 2003; Pica and Volpi 2007). Human
herpesvirus-8 infection is also associated with the development of multicentric Castleman's disease, a non-neoplastic LPD which may itself develop into lymphoma (Dupin et al. 2000; Oksenhendler et al. 2002). Castleman's disease is discussed further with the non-neoplastic LPDs of people in section 2.4.1.2 below.

Other infectious agents also associated with an increased incidence of lymphoma in people include hepatitis C virus, human immunodeficiency virus (HIV) and the bacterium Helicobacter pylori. While more commonly associated with cirrhosis and subsequent hepatocellular carcinoma, Hepatitis C virus infection is also linked with the development of a range of LPDs, particularly B-cell lymphomas (Dal Maso and Franceschi 2006; Zignego et al. 2012). Infection with HIV is associated with an increased incidence of many disease states including various types of lymphoma, predominantly of B-cell origin (Kaplan 2012). Helicobacter pylori infection is commonly associated with chronic gastritis and the subsequent development of B-cell lymphomas of the gastric mucosa-associated lymphoid tissue (MALT) (Du and Atherton 2006; Parkin 2006; Kim et al. 2011).

2.3.3 LYMPHOMA IN CATS

Lymphoma is a common neoplasm in cats (Dorn et al. 1967; Hardy 1981; Jacobs et al. 2002) and may occur sporadically, in association with a familial predisposition, or in association with retroviral infection (Louwerens et al. 2005). The viruses most commonly associated with lymphoma development are feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV).

Various attempts have been made to apply the different classification systems used in people to lymphomas of animals, including cats. However, differences in lymphoma morphology and behaviour between people and animals (Bienzle 2003) have somewhat restricted the veterinary application and use of the classification schemes used in people. In 2002, the WHO proposed a new classification system for lymphomas of domestic animals based upon the human REAL system but also incorporating aspects of the other classification systems (Valli et al. 2002). While often used in veterinary research (Day et al. 2004; Waly et al. 2005; Pohlman et al. 2009; Vezzali et al. 2010), the complexity of the WHO system and practical difficulties in its application have meant that in routine clinical practice, diagnosis of lymphoid neoplasms seldom attempts to classify tumours further than the anatomic site, and sometimes, the immunophenotype.
A detailed review of classification systems as they have been applied to feline lymphoma has only limited relevance to the current study and is beyond the scope of this review. However, the importance of neoplastic cell lineage or immunophenotype and morphology in classification is a consistent feature of the lymphoma classification systems currently employed in cats.

Feline lymphoma is often classified according to the anatomic site of primary neoplastic involvement, although in practice the tumour is seldom confined to a single location (Jacobs et al. 2002). Various anatomic classification systems have been used by different investigators. Common categories used include alimentary (or abdominal), mediastinal (or thymic), multicentric, cutaneous and miscellaneous (or atypical) (Valli et al. 2000; Jacobs et al. 2002; Valli 2007b). However, precise definition of the various categories is frequently lacking, and interpretation of the categories by different investigators is often inconsistent, making comparisons between the results of different studies challenging. In particular, there is wide variability regarding interpretation of the category of multicentric lymphoma. For example, some authors (Gruffydd-Jones et al. 1979; Court et al. 1997; Gabor et al. 1998; Louwerens et al. 2005) class tumours as abdominal (lymphoma involving the intestines and abdominal organs including spleen, liver and kidneys), mediastinal (including cranial mediastinal lymph nodes or the thymus), nodal (where the lymph nodes are the primary site of involvement), atypical (central nervous system and nasopharyngeal lymphoma) or mixed (where the lymphoma can be defined by multiple categories). Other authors categorise lymphomas as alimentary (involving the gastrointestinal tract and regional lymph nodes), multicentric (involving multiple lymph nodes or other organs such as spleen and liver), mediastinal, cutaneous and miscellaneous (affecting other organs such as the eye, kidney or central nervous system) (Jackson et al. 1996; Jacobs et al. 2002; Bienzle 2003). Still others add separate categories for oral, nasal, ocular cutaneous, renal or other tumours and appear to class tumours as multicentric if they affect multiple anatomic sites regardless of whether they involve the lymph nodes (Valli et al. 2000; Wang et al. 2001).

2.3.3.1 **Sporadic Lymphoma**

Sporadically occurring lymphomas are tumours not associated with any known cause. It should be emphasised that the reliability of data concerning sporadic feline lymphomas is often limited by the fact that in many studies, the FeLV and FIV status of the cats for
which tumours are included is not known, and accordingly an incomplete distinction is made between sporadic and virus-associated lymphomas (Gabor et al. 1998; Gabor et al. 1999; Louwerens et al. 2005; Vezzali et al. 2010). The classification of a lymphoma as “sporadic” rather than virus-associated is therefore often assumed and cannot always be definitively confirmed. Data reported may therefore include a number of retrovirus-associated lymphomas as well as truly sporadic disease.

All anatomic forms of sporadic lymphoma occur in cats, with the abdominal (or alimentary) form reportedly the most common type (Gabor et al. 1998; Vail et al. 1998; Louwerens et al. 2005; Vezzali et al. 2010; Chino et al. 2013). In contrast to dogs, primary nodal forms of sporadic lymphoma appear relatively uncommon in cats, although the inconsistent interpretation of the multicentric category of lymphomas limits firm conclusions. While lymphoma involving the lymph nodes usually causes lymphadenopathy, node enlargement in such cases is typically confined to one or several nodes; widespread involvement of multiple nodes is a rare clinical presentation of feline lymphoma (Nielson 1969; Moore et al. 1986; Gabor et al. 1998; Jacobs et al. 2002; Louwerens et al. 2005). When lymphoma affects the lymph nodes of cats, the nodes of the head and neck are most commonly affected, particularly the mandibular or cervical nodes (Gabor et al. 1998). Clinical features and pathology are sometimes similar to Hodgkin lymphoma in people, which typically involves a single axial group of nodes with subsequent spread to contiguous nodes (Day et al. 1999; Walton and Hendrick 2001; Steinberg and Keating 2008; Kumar et al. 2010).

Cats with all forms of sporadic lymphoma are typically older than 10 years of age, and the disease is not reported in young kittens. North American studies have reported a median age of 11 years for cats with sporadic lymphoma, with age ranges of 7 months to 20 years. (Vail et al. 1998; Louwerens et al. 2005). A recent Japanese study reported a median age of 12 years for cats with lymphoma, with an age range of 3 to 17 years (Chino et al. 2013), although several cases of retrovirus-positive lymphoma were included in the Japanese data. An older Australian study reported that cats with lymphoma have a median age of 10 years and an age range of 5 months to 17 years (Gabor et al. 1998). However, the FeLV or FIV status of cats included in the Australian study was unknown, and it is therefore possible that a proportion of included cats were affected by retroviral-associated rather than sporadic lymphoma. In addition, stringent
diagnostic criteria such as clonality assessment were not applied in most studies, raising the possibility that some cats diagnosed with lymphoma in fact had non-neoplastic LPD.

There is some variation in the ages of cats with different forms of disease, most notably the mediastinal form of lymphoma, which tends to affect younger cats. Recent studies report a median age of 2 years (age range 1 to 19 years) for the mediastinal form of the disease (Louwerens et al. 2005). In contrast, cats with the multicentric or nodal form of sporadic lymphoma are reported to have median ages of 10 years (age range 2 to 15 years) (Louwerens et al. 2005) and more than 8 years of age (age range 5 to 16 years) (Gabor et al. 1998) depending on the number of affected nodes and involvement of extranodal sites. Cats with nodal lymphoma resembling Hodgkin lymphoma in people are also older, with a median age of 11 years (age range 1-18 years) (Day et al. 1999; Walton and Hendrick 2001). Similarly, cats with the abdominal (or alimentary) form of lymphoma are typically older, with a reported median or mean age of more than 10 years (Gabor et al. 1998; Vail 2007; Pohlman et al. 2009). Published age data for cats in New Zealand affected by sporadic lymphoma is limited.

As in human lymphoma, the morphology and immunohistochemistry of sporadic feline lymphomas are diverse (Gabor et al. 1999; Valli et al. 2000; Valli 2007b; Vezzali et al. 2010). Diffuse effacement appears more common than follicular morphology, possibly due to the relatively late stage at which diagnosis is made in cats compared to people (Jackson et al. 1996; Valli 2007b; Vezzali et al. 2010). The predominant immunophenotype of lymphomas occurring at particular anatomic sites in cats is variable, and the literature is frequently contradictory. Most studies report that B-cell lymphomas are more common than T-cell or NK cell lymphomas in the alimentary tract (Vail et al. 1998; Patterson-Kane et al. 2004; Pohlman et al. 2009), but other studies have found a predominance of T-cell lymphomas at this site (Zwahlen et al. 1998; Moore et al. 2012; Chino et al. 2013). Mediastinal lymphomas are predominantly T-cell (Holmberg et al. 1976; Rojko et al. 1989; Jackson et al. 1996; Jacobs et al. 2002; Chino et al. 2013), but in most studies a proportion of the tumours included occurred in FeLV-positive cats and were likely associated with the presence of FeLV infection rather than truly sporadic. Interpretation of data concerning the immunophenotype of nodal lymphomas in cats is complicated by the variable inclusion of tumours involving the lymph nodes, liver, spleen and other organs within the multicentric category.
Multicentric tumours are reportedly more commonly of T-cell origin (Vezzali et al. 2010), although other studies report no clear predominance of immunophenotype (Wang et al. 2001; Chino et al. 2013). For tumours involving only the lymph nodes, B-cell lymphomas appear more common (Gabor et al. 1999). Several reports have also identified cases of nodal lymphoma in cats with histological features consistent with Hodgkin lymphoma in people (Day et al. 1999; Walton and Hendrick 2001; Steinberg and Keating 2008). These features include a pleomorphic cell population including both T-cell and B-cell lymphocytes and other leukocytes with a minority of large and often binucleate or multinucleate cells (interpreted as Reed-Sternberg variant cells) scattered throughout the tumour. While Reed-Sternberg cells are thought to arise from transformed B-cells in people, they frequently fail to react with either B-cell or T-cell antibodies in feline cases (Walton and Hendrick 2001; Steinberg and Keating 2008).

2.3.3.2 Familial Lymphoma

Familial predispositions to lymphoma have not been well-documented in cats. However, multiple studies suggest an increased incidence of mediastinal lymphoma with an early age at onset in Oriental breeds of cat (Dorn et al. 1967; Gruffydd-Jones et al. 1979; Hardy 1981; Hardy 1993; Court et al. 1997; Gabor et al. 1998; Lorimer 1999; Teske et al. 2002; Louwerens et al. 2005; Rissetto et al. 2011), suggesting a possible inherited predisposition to the development of this form of lymphoma. Cases are most commonly reported in Siamese cats (Gruffydd-Jones et al. 1979; Court et al. 1997), but other Oriental breeds including Oriental shorthair, Colourpoint shorthair and Javanese breeds of cat also appear to be affected (Louwerens et al. 2005). An autosomal recessive mode of inheritance has been suggested (Louwerens et al. 2005), but is speculative only, and the precise mechanism for disease development in these cats is currently unknown. The majority of cases appear to occur in cats 2 years of age or younger (Court et al. 1997; Gabor et al. 1998; Louwerens et al. 2005), with dyspnoea and regurgitation the most common presenting signs (Gruffydd-Jones et al. 1979; Court et al. 1997). Where immunophenotyping is performed, tumours are more commonly of T-cell origin (Gabor et al. 1999).

Other than this, familial predispositions to lymphoma have not been described in cats. In particular, familial predispositions to nodal lymphoma or lymphoma in BSH breeds of cat have not been reported.
2.3.3.3  **Feline Leukaemia Virus-Associated Lymphoma**

Feline leukaemia virus is an exogenous γ-retrovirus that can cause a wide range of clinical signs in infected cats including lymphoma (Cotter *et al*. 1975; Francis *et al*. 1979; Hardy *et al*. 1981; Shelton *et al*. 1990; Lutz *et al*. 2009). Since its initial identification in the 1960s (Jarrett *et al*. 1964), FeLV infection has been reported in cats worldwide, although infection rates vary between different geographical regions (Gabor *et al*. 2001a; Valli 2007b; Lutz *et al*. 2009; Chhetri *et al*. 2013). Prior to the institution of control measures in the 1980s, FeLV infection was suggested to be the main cause of lymphoma in cats, and was it was estimated that FeLV was associated with up to 70% of cases of feline lymphoma in Europe and North America (Cotter *et al*. 1975; Francis *et al*. 1979; Shelton *et al*. 1990; Hardy 1993). Recently there has been a significant decrease in the occurrence of FeLV infection in cats in North America and Europe, and the number of lymphomas attributed to FeLV infection has also decreased (Louwerens *et al*. 2005; Lutz *et al*. 2009; Stutzer *et al*. 2011). Interestingly, Australian studies have only ever suggested a limited role for FeLV infection in lymphoma development in Australian cats (Gabor *et al*. 2001a). While studies in the early 1980s indicated a prevalence of FeLV infection of up to 6.5% in cats in New Zealand (Jones and Lee 1981; Jones *et al*. 1983), FeLV infection is currently regarded as uncommon in New Zealand cats and the prevalence of FeLV in cats in New Zealand was recently estimated to be approximately 1% (Galloway 2003).

The outcome of FeLV infection is variable, and depends largely on whether the cat’s immune response successfully eliminates the virus (Hoover and Mullins 1991; Hartmann 2012). Abortive infections are terminated by an effective immune response and the cat never becomes viraemic or develop signs of disease (Major *et al*. 2010). Regressive infections also follow an effective immune response, but result in a transient viraemia and integration of a copy of the viral genome (provirus) into the cat’s cells (Torres *et al*. 2005; Cattori *et al*. 2006). This is referred to as “latent” infection: these cats are non-viraemic, test negative for the presence of FeLV antigen in the blood on routine testing, and seldom develop FeLV-associated disease. However, reactivation of regressive infections can occur, for example, following immunosuppression (Rojko *et al*. 1982). Finally, progressive infections follow an ineffective immune response to the virus, resulting in persistent viraemia and the subsequent development of various FeLV-
CHAPTER 2

associated diseases, including lymphoma (Hardy 1981). The cat’s age is a significant
factor in the outcome of infection; older cats are more likely to mount a successful
immune response and eliminate the virus, while young kittens are highly susceptible
(Hoover et al. 1976; Grant et al. 1980; Shelton et al. 1990; Hoover and Mullins 1991;
Hartmann 2012).

Feline leukaemia virus is shed in saliva, nasal secretions, faeces, urine and milk (Hardy et
al. 1973; Hardy et al. 1976; Pacitti et al. 1986). Transmission between cats occurs mostly
through friendly contacts, including mutual grooming or the sharing of food or water
bowls. (Jarrett et al. 1973; Hardy et al. 1976; Lutz et al. 2009). In pregnant queens, FeLV
viraemia usually leads to embryonic death, stillbirth, or the birth of viraemic kittens that
die rapidly (Jacobs et al. 2002). In latently infected queens, FeLV is not usually
transmitted to the fetuses, but kittens can become viraemic after birth due to
transmission of the virus in the queen’s milk (Pacitti et al. 1986).

A variety of methods are available for diagnosing FeLV infection in cats. The tests most
commonly used in clinical practice are based on the direct detection of the FeLV p27
antigen by enzyme-linked immunosorbent assay (ELISA) or immunochromatography
(Lutz et al. 2009). The reported sensitivity of these tests in detecting FeLV antigen is
typically at least 92%, and specificity is more than 97% (Hartmann et al. 2001; Hartmann
et al. 2007; Pinches et al. 2007; Diagnostik Megacor 2012; Idexx Laboratories 2013). The
immunofluorescent assay (IFA) tests for the presence of FeLV structural antigens within
the cytoplasm of leukocytes within the peripheral blood or bone marrow. It is most
commonly used to confirm a prior positive FeLV result obtained on ELISA or
immunochromatographic in-clinic tests, and performed correctly on a bone marrow
sample, has a reported sensitivity of detection of 98% (Hardy 1991; Hardy and
Zuckerman 1991a; Hardy and Zuckerman 1991b). The IFA cannot be performed in-clinic,
and at present, is not available in New Zealand. Virus isolation involves the isolation and
propagation of FeLV in cell cultures, and has been traditionally regarded as the gold
standard in FeLV diagnosis (Jarrett et al. 1982; Jarrett and Ganiere 1996). However, it is
complex and time-consuming to perform, detects only replication-capable virus, may
result in false negative results in incorrectly stored samples, and is no longer used
routinely (Hartmann et al. 2007; Lutz et al. 2009). Polymerase chain reaction, primarily
using real-time PCR, has also been used to detect the presence of FeLV and proviral DNA
and RNA in a range of samples, including blood, saliva, faeces, and FFPE tissue (Jackson et al. 1993; Wang et al. 2001; Tandon et al. 2005; Torres et al. 2005; Cattori et al. 2006; Gomes-Keller et al. 2006; Gomes-Keller et al. 2009; Stutzer et al. 2011).

Following progressive FeLV infection and the development of persistent viraemia, affected cats typically remain asymptomatic for a lengthy period, and clinical signs associated with infection often take months or even years to develop (Hardy et al. 1976; Francis et al. 1979; Ladiges et al. 1981; Hartmann 2012). The most common clinical signs associated with FeLV infection are immunosuppression and consequent susceptibility to a range of opportunistic infections, immune-mediated disease, anaemia and lymphoma (Cotter et al. 1975; Hardy et al. 1976; Ogilvie et al. 1988; Hartmann 2012). Peripheral hyperplastic lymphadenopathy is also described in a small number of young cats and may be associated with FeLV infection; this condition is described further in section 2.4.2.2 below. Anaemia in FeLV-infected cats is typically non-regenerative, primarily due to bone marrow suppression (Cotter 1979; Quigley et al. 2000), but haemolytic regenerative anaemia associated with *Mycoplasma haemofelis* infection or immune-mediated destruction of erythrocytes are also occasionally reported (Shelton et al. 1995). The clinical outcome of progressive FeLV infection is determined by a combination of viral and host factors, particularly the viral subgroups involved in particular infections (Jarrett 1992).

Infection with FeLV is associated with a range of neoplasms in cats, but lymphoma is most commonly reported (Hartmann 2012). Cats infected with FeLV have been reported to be up to 60 times more likely to develop lymphoma than uninfected cats (Reinacher 1989; Shelton et al. 1990). The exact mechanism by which FeLV induces lymphoma development is still uncertain, and is beyond the scope of this review. However, an important mechanism by which the virus causes lymphoma is by the insertion of its genome into the cat’s genome near a cellular oncogene (most commonly myc), resulting in activation and over-expression of that gene and uncontrolled cell proliferation (Tsatsanis et al. 1994; Hartmann 2012).

Cats developing lymphoma in association with FeLV infection tend to be significantly younger than cats with sporadic lymphoma, and are typically less than 4 years of age at the time of diagnosis, with a significant proportion less than 2 years of age (Hardy 1981;
Shelton et al. 1990; Gabor et al. 2001a; Stutzer et al. 2011). Although lymphoma has been reported in kittens as young as 9 weeks of age following experimental FeLV infection at birth, lymphoma in kittens under 6 months of age following natural infection appears rare (Hoover et al. 1973).

Lymphoma in FeLV-infected cats is reported at various anatomic sites but multicentric and mediastinal tumours are reportedly more common than other anatomic forms (Hardy 1981; Shelton et al. 1990; Jackson et al. 1996; Gabor et al. 2001a; Weiss et al. 2010). Lymphomas in FeLV-infected cats are more likely to have a T-cell than B-cell immunophenotype, although a proportion do not react with either T-cell or B-cell antibodies (Jackson et al. 1996; Gabor et al. 2001a; Weiss et al. 2010; Stutzer et al. 2011).

The significance of latent FeLV infection in the development of lymphoma is controversial. Feline leukaemia virus proviral DNA has been detected in lymphomas of FeLV antigen-negative cats using PCR, causing speculation that FeLV may still be associated with the development of these tumours regardless of the cat’s apparent FeLV status (Jackson et al. 1993; Gabor et al. 2001a; Weiss et al. 2010). However, the existence of any causative association is dubious, and the results of more detailed studies conclude latent FeLV infection is unlikely to play a significant role in lymphoma development (Herring et al. 2001; Stutzer et al. 2011).

2.3.3.4 FELINE IMMUNODEFICIENCY VIRUS-ASSOCIATED LYMPHOMA

Feline immunodeficiency virus is a retrovirus of the genus Lentivirus that is closely related to HIV (Miller et al. 2000). Infection with FIV is associated with a range of clinical signs including lymphadenopathy (discussed in further detail in section 2.4.2.1), and less commonly, lymphoma (Shelton et al. 1990; Callanan et al. 1992; del Fierro et al. 1995; Callanan et al. 1996). Since its identification in 1986 (Pedersen et al. 1987), FIV infection has been reported in cats worldwide, although infection rates vary between different geographical regions (Gabor et al. 2001b; Gleich et al. 2009; Hosie et al. 2009). Five genetically distinct subtypes of FIV (A, B, C, D and E) have been identified and clustering of particular subtypes is seen in different geographical locations (Hosie et al. 2009). Overseas studies have estimated the prevalence of FIV infection to be between 1% and 14% in healthy cats, but up to 44% in cats that were clinically unwell (Ishida et al. 1989;
Yamamoto et al. 1989; Levy et al. 2006; Goldkamp et al. 2008; Gleich et al. 2009; Little et al. 2009). Recent data on FIV prevalence in cats in New Zealand are lacking, but older studies have reported prevalences of 6.8% in healthy cats and 20.9%-27.3% in clinically unwell cats (Swinney et al. 1989; Jones et al. 1995).

The virus is present in saliva, blood and milk, and is primarily transmitted through bites from infected cats during activities such as fighting or mating (Yamamoto et al. 1989; Matteucci et al. 1993; Allison and Hoover 2003). Viral transmission from infected queens to their kittens is documented but appears rare in natural infections (Medeiros Sde et al. 2012). Transmission to kittens may occur in utero or through the milk, but is more likely if the queen is in the acute stages of FIV infection during gestation and the perinatal period (Wasmoen et al. 1992; Sellon et al. 1994; O’Neil et al. 1995; O’Neil et al. 1996; Weaver et al. 2005). Intrauterine FIV infection is reported to cause variable outcomes for kittens, including arrested fetal development, abortion and stillbirth, low birth weight, and the birth of infected but asymptomatic kittens (Rogers and Hoover 1998; Weaver et al. 2005).

Several methods are available for diagnosing FIV infection in cats. The tests most commonly used in clinical practice are based on the indirect detection of antibodies recognising FIV structural proteins (usually p24 core protein or gp40 transmembrane protein) by ELISA or immunochromatography using whole blood or serum (Hosie et al. 2009). Applied correctly, the reported sensitivity of these tests in the detection of FIV antibody is at least 92%, and specificity is more than 98% (Hartmann et al. 2007; Pinches et al. 2007; Diagnostik Megacor 2012; Idexx Laboratories 2013). Western blot analysis is regarded as the gold standard for the indirect identification of FIV and is used to confirm inconclusive or unexpected results obtained from in-clinic tests (Egberink et al. 1991), but is not available in New Zealand. False negative results using indirect methods may be seen early in the course of infection prior to seroconversion, in the terminal stages of FIV-associated disease due to immunodeficiency, or when the presence of high concentrations of FIV in the blood result in antibody sequestration within immune complexes (Swango 1991; Hosie et al. 2009). False-positive results using indirect methods may also be seen in kittens born to FIV-infected queens due to the presence of maternal antibody (Barr 1996; Hosie et al. 2009). In addition, the presence of
vaccination-derived antibody in cats vaccinated against FIV is another potential cause of false-positive results (Andersen and Tyrrell 2004).

Methods for the direct identification of FIV are also available for research purposes, but have significant limitations. Virus isolation is reliable, but complex and time-consuming to perform and is not used routinely (Hosie et al. 2009). Polymerase chain reaction methods for the detection of proviral DNA are available, but results appear unreliable and performance is often inferior to in-clinic methods (Bienzle et al. 2004; Crawford and Levy 2007; Hosie et al. 2009).

Following FIV infection, cats progress through several stages (Hartmann 2011; Hartmann 2012). The acute phase of infection occurs in the first few weeks or months after exposure. After viral replication and the development of progressive viraemia, affected cats typically develop transient and mild clinical signs including fever, anorexia, lethargy and generalised lymphadenopathy (Pedersen et al. 1987; Callanan et al. 1992; del Fierro et al. 1995; Obert and Hoover 2000). A neutropenia and lymphopenia may be present (Pedersen et al. 1987; Callanan et al. 1992). While the majority of these signs are typically of short duration, lymphadenopathy may persist for several months (Callanan et al. 1992). Following the initial phase of infection, clinical signs typically resolve and infected cats become asymptomatic. The asymptomatic phase of FIV is of variable duration but often lasts for many years, with some cats reportedly remaining asymptomatic for the remainder of their lives (Ishida et al. 1992; Addie et al. 2000). The terminal phase, during which severe clinical signs develop, is characterised by a gradual depletion of lymphocytes, ultimately resulting in signs of immunosuppression (Pedersen et al. 1989; Bendinelli et al. 1995). Many of the clinical effects of infection during the terminal phase are therefore secondary to immunosuppression, and include chronic inflammatory disease, particularly gingivitis and stomatitis, various secondary and opportunistic infections, and an increased incidence of neoplasms, particularly lymphoma (Tenorio et al. 1991; Callanan et al. 1996; Hosie et al. 2009; Hartmann 2011; Hartmann 2012).

Cats infected with FIV are reportedly 5 times more likely to develop lymphoma than uninfected cats (Hartmann 2011; Hartmann 2012). Due to its lengthy asymptomatic phase, cats developing lymphoma in association with FIV infection are usually older
adult cats, and are typically more than 6 years of age; FIV-associated lymphomas have not been reported in kittens (Callanan et al. 1996; Hosie et al. 2009). Lymphomas associated with FIV infection may affect a wide range of anatomic sites, but are typically extranodal. Involvement of alimentary, renal, hepatic, cardiac, nasal and central nervous system tissues have been reported; lymphoma involving multiple lymph nodes appears rare (Poli et al. 1994; Callanan et al. 1996; Gabor et al. 2001b). The majority of FIV-associated lymphomas are high grade, of diffuse morphology and predominantly of B-cell immunophenotype (Poli et al. 1994; Terry et al. 1995; Callanan et al. 1996; Gabor et al. 2001b).

2.4 NON-NEOPLASTIC LYMPHOPROLIFERATIVE DISEASES

2.4.1 DISEASES IN PEOPLE

There are many non-neoplastic causes of lymphadenopathy in people that can clinically mimic lymphoma, including systemic bacterial, viral and protozoal infections, autoimmune disorders and various atypical LPDs (Chau et al. 2003; Brown and Skarin 2004). However, the majority of these diseases primarily cause lymphadenitis or histiocytosis rather than lymphoproliferation (McCabe et al. 1987; Leung and Robson 2004; Asano 2012). Non-neoplastic LPDs causing lymphadenopathy as a primary presenting sign are relatively uncommon, but include various viral infections and a range of rare diseases such as Castleman’s disease, Kikuchi disease, and autoimmune disorders such as ALPS. Causes of non-neoplastic LPDs potentially relevant to the LPD in BSH kittens are reviewed in the following sections of this chapter. Conditions such as post-transplant LPDs and drug-associated lymphadenopathies may also cause LPD and lymphadenopathy in people, but given the specific context in which these conditions arise, are considered unlikely to be relevant to the present studies and are not reviewed further.

2.4.1.1 VIRAL INFECTIONS

In addition to their association with lymphoma (described in section 2.3.2.2 above) various viral infections, particularly HIV and EBV, may also cause generalised lymphadenopathy and LPD in people.
Persistent generalised lymphadenopathy is commonly reported in HIV infection. The initial histological features of affected nodes are florid follicular hyperplasia progressing to a mix of follicular hyperplasia and follicular involution, to predominantly follicular involution, and finally lymphoid depletion (Chadburn et al. 2013). The initial florid hyperplastic stage is characterised by the presence of large irregular follicles composed of macrophages and a mixed lymphoid cell population with many mitoses; increased CD8+ cytotoxic T-cells are present within the expanded germinal centres. Interfollicular areas contain a similar mixed cell population with B-cells showing a high proliferation rate. Medullary sinuses often contain many neutrophils (Garcia et al. 1986; Pileri et al. 1986; Turner et al. 1987; Chadburn et al. 2013). As the nodes progress towards involution, the germinal centres become depleted of B-cells and are often hyalinised. Finally, germinal centres are lost and scant lymphocytes remain (Turner et al. 1987; Chadburn et al. 2013). As discussed in section 2.4.1.2 below, HIV infection is also associated with the multicentric form of Castleman’s disease, which may itself also cause LPD and lymphadenopathy (Mylonas et al. 2008).

In addition to lymphoma, the acute stage of EBV infection is also commonly associated with a transient non-neoplastic lymphadenopathy primarily affecting the cervical lymph nodes, a condition often termed infectious mononucleosis (Vetsika and Callan 2004; Kutok and Wang 2006). Additional clinical signs including a sore throat, pyrexia and splenomegaly are also often reported, with adolescents and young adults more commonly affected. The lymphadenopathy associated with acute EBV infection typically resolves over 3-6 weeks (Vetsika and Callan 2004). Histology of affected lymph nodes may show distortion of normal nodal architecture by a marked paracortical B-cell proliferation that may superficially resemble lymphoma (Childs et al. 1987; Louissaint et al. 2012).

2.4.1.2 Castleman’s Disease

Castleman’s disease (also termed giant or angiofollicular lymph node hyperplasia, and angiomatous lymphoid hamartoma) is an uncommon non-neoplastic LPD in people. It typically affects older adults and has a highly variable presentation which may include single or widespread lymphadenopathy (Greiner et al. 2000; Brown and Skarin 2004). Historically, the disease has been classified into unicentric and multicentric forms. The
more common unicentric disease typically involves a single lymph node or site, often within the thorax, and is often asymptomatic. The multicentric form is a more serious systemic disease that can affect multiple organs and is commonly associated with more severe clinical signs, including peripheral lymphadenopathy, hepatosplenomegaly, fever and weight loss (Peterson and Frizzera 1993; Brown and Skarin 2004). The clinical course of Castleman’s disease varies from rapid progression and death to a more chronic and insidious clinical course (Weisenburger et al. 1985; Cronin and Warnke 2009; Talat and Schulte 2011). The aetiology is unknown, although a significant proportion of cases of multicentric disease occur in people with HHV-8 infection in association with HIV infection or other immunosuppressive conditions (Mylona et al. 2008; Stebbing et al. 2008). In addition to non-neoplastic LPD, the multicentric form of Castleman’s disease is also associated with an increased risk of predominantly B-cell lymphoma, particularly in HHV-8 and HIV-infected patients (Weisenburger et al. 1985; Larroche et al. 2002; Talat and Schulte 2011).

More recent classifications focus more on the histological features and pathogenesis of the different forms of Castleman’s disease and distinguish between hyaline-vascular forms, plasma cell-dominant forms, HHV-8 infection-associated forms, and other multicentric forms of disease (Cronin and Warnke 2009; Bonekamp et al. 2011). Histological features of the hyaline-vascular form of Castleman’s disease include lymphoid follicles with expanded mantle zones containing many B-cells and the proliferation of interfollicular capillaries showing perivascular hyalinisation (Kojima et al. 2008; Cronin and Warnke 2009; Bonekamp et al. 2011). The plasma cell form of disease is less distinctive histologically, but large numbers of plasma cells within paracortical regions and mantle zones is a common feature. There is variable germinal centre hyperplasia and mantle zone expansion with relatively preserved lymph node architecture (Keller et al. 1972; Enomoto et al. 2007). Unicentric disease typically involves the hyaline-vascular form of disease, while multicentric disease is more commonly of the plasma cell variant (Talat and Schulte 2011).

### 2.4.1.3 Kikuchi-Fujimoto Disease

Kikuchi-Fujimoto disease is a rare disease that typically affects young adults (Lin et al. 2003; Bosch et al. 2004). Clinically, the disease is characterised by a transient cervical lymphadenopathy, pyrexia and leukopenia; generalised lymphadenopathy is
occasionally reported (Lin et al. 2003). While typical histological findings are of a histiocytic necrotising lymphadenitis, in some cases, large numbers of T-cells are also present within affected nodes and can resemble lymphoma (Kuo 1995; Bosch et al. 2004). The aetiology is unknown, although viral infections and autoimmune causes, particularly systemic lupus erythematosis (SLE), have been speculated to play a role in disease development (Brown and Skarin 2004).

2.4.1.4 PROGRESSIVE TRANSFORMATION OF GERMINAL CENTRES

Progressive transformation of germinal centres (PTGC) is a persistent lymphadenopathy of unknown aetiology that occurs more commonly in young adults (Osborne et al. 1992; Hicks and Flaitz 2002). Typically, PTGC presents as an asymptomatic lymphadenopathy involving the lymph nodes of the head and neck (Hicks and Flaitz 2002; Kojima et al. 2003). Histologically, affected lymph nodes show follicular hyperplasia and the loss of definition between the lymphocytes of the germinal centre and those of the mantle zone as the mantle zone B-cells gradually expand and efface the germinal centre, often extending into adjacent sinusoids (Hansmann et al. 1990; Nguyen et al. 1999; Hicks and Flaitz 2002; Verma et al. 2002). Progressive transformation of germinal centres is also associated with the development of certain types of lymphoma, particularly Hodgkin lymphoma (Hansmann et al. 1990; Nguyen et al. 1999; Hicks and Flaitz 2002).

Progressive transformation of germinal centres is also part of the spectrum of lymph node changes present in IgG4-related disease, referred to in section 2.4.1.6 below (Grimm et al. 2012).

2.4.1.5 COMMON VARIABLE IMMUNODEFICIENCY

In addition to the increased risk of lymphoma and other characteristic clinical signs including persistent infections (see section 2.3.2.2 above), people with CVID often also develop a marked lymphadenopathy typically involving the mediastinal, abdominal or cervical nodes (Ardeniz et al. 2010; Cunningham-Rundles 2012). On histology, affected nodes most commonly show reactive lymphoid hyperplasia, but other changes including chronic granulomatous inflammation and atypical lymphoid hyperplasia with preservation of nodal architecture and marked expansion by both B-cells and T-cells, are also reported (Sander et al. 1992).
2.4.1.6 AUTOIMMUNE DISEASES

A lymphadenopathy due to LPD may occur as part of the clinical spectrum associated with various systemic autoimmune diseases, including SLE, rheumatoid arthritis and Sjögren’s syndrome (Brown and Skarin 2004). However, these disorders typically present with an extensive and varied range of clinical signs and while lymphadenopathy is often present, it is seldom a primary presenting clinical sign. Lymph node histology is variable in such cases, but reactive follicular hyperplasia with interfollicular plasmacytosis is a common finding in both SLE and rheumatoid arthritis (Kojima et al. 1990; Kojima et al. 2006; Kojima et al. 2007). Lymph nodes in SLE may also show varying degrees of coagulative necrosis with hematoxylin bodies (extracellular nuclear material bound by immune complexes) (Segal et al. 1993; Kojima et al. 2007). Lymph nodes from people with Sjögren’s syndrome commonly show reactive follicular or atypical lymphoid hyperplasia (McCurley et al. 1990).

IgG4-related disease is a recently recognised entity that most likely has an autoimmune basis. The disease encompasses a group of previously disparate disorders characterised by mass-forming lesions in a range of extranodal organs, increased serum IgG4, and many IgG4-positive plasma cells within affected tissues (Hamano et al. 2001; Kamisawa and Okamoto 2008; Masaki et al. 2009). Regional or generalised lymphadenopathy is frequently present in IgG4-related disease, with the mediastinal and intra-abdominal nodes most commonly involved (Hamano et al. 2006; Sato et al. 2009; Sato et al. 2010). Affected nodes show a spectrum of reactive histological changes, including follicular hyperplasia, proliferation of interfollicular lymphocytes, PTGC, varying degrees of fibrosis and inflammation, and occasionally similar morphology to the plasma cell variant of Castleman’s disease discussed above in section 2.4.1.2. However, all forms of IgG4 related disease are characterised by the presence of increased numbers of IgG4-producing plasma cells within affected nodes (Cheuk et al. 2008; Cheuk and Chan 2012; Grimm et al. 2012). While the disease is typically progressive, it is often successfully managed with immunosuppressive treatment, however, there is limited information concerning longer-term outcomes (Guma and Firestein 2012).

A marked generalised lymphadenopathy due to LPD is typically one of the initial presenting signs in people with ALPS. Due to its multiple similarities with the LPD in BSH kittens, ALPS is discussed separately and in greater detail in section 2.5 below.
2.4.2 DISEASES IN CATS

As with people, lymphadenopathy in cats can result from antigenic stimulus due to acute inflammation. Such changes tend to be of short duration and confined to nodes within one area of the body. The non-neoplastic causes of feline lymphadenopathy due to LPD include FIV-associated lymphadenopathy and three poorly defined diseases in cats: the so-called “distinctive peripheral lymph node hyperplasia” (DPLNH) described by Moore et al. (1986), the “generalised lymphadenopathy resembling lymphoma” (GLRL) described by Mooney et al (1987), and the “plexiform vascularisation of lymph nodes” described by Lucke et al (1987).

2.4.2.1 FELINE IMMUNODEFICIENCY VIRUS-ASSOCIATED LYMPHADENOPATHY

As mentioned in section 2.3.3.4, cats often present with a generalised lymphadenopathy during the acute phase of FIV infection (Pedersen et al. 1987; Callanan et al. 1992; del Fierro et al. 1995; Obert and Hoover 2000). In affected cats, lymphadenopathy typically develops within the first few weeks following FIV infection, and may persist for weeks or months (Yamamoto et al. 1988; Callanan et al. 1992). There is uniform enlargement of multiple peripheral lymph nodes, with the nodes of the hindlimbs often enlarged more than those of the forelimbs and head (del Fierro et al. 1995). Enlargement of the visceral nodes is also described (Callanan et al. 1992), but is usually less obvious than that of the peripheral nodes (del Fierro et al. 1995). The degree of typical nodal enlargement present is not well described, but appears to be mild, as gross differences between the nodes of FIV-infected cats and controls are not always detectable (Rideout et al. 1992; del Fierro et al. 1995). Cortico-medullary architecture of affected nodes is retained, both on gross and histological examination (Callanan et al. 1992). On histology, affected nodes typically show irregular and often prominent cortical follicular and paracortical hyperplasia with thinning of the mantle zone and large numbers of medullary plasma cells (Callanan et al. 1992; Rideout et al. 1992; Parodi et al. 1994). Germinal centres are expanded and increased in number with many large lymphocytes admixed with macrophages and fewer plasma cells (Callanan et al. 1992; del Fierro et al. 1995). Erythrophagocytosis is also described (Brown et al. 1991). There is often expansion of the splenic white pulp and MALT of the intestinal tract (Callanan et al. 1992). Lymphoid follicles or aggregates are also commonly present within other tissues, including the
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salivary glands, thymus, bladder, lung, bone marrow, kidneys, sclera and choroid of the eye (Callanan et al. 1992).

2.4.2.2 DISTINCTIVE PERIPHERAL LYMPH NODE HYPERPLASIA OF YOUNG CATS

A generalised peripheral lymphadenopathy with a possible association to FeLV infection has also been described in a small number of young domestic shorthair (DSH) cats, and has been termed distinctive peripheral lymph node hyperplasia (DPLNH) (Moore et al. 1986). Cats affected by DPLNH are less than 2 years of age (5 months to 2 years) and present with variable clinical signs including fever, lethargy, anorexia, hepatosplenomegaly, and lymphadenopathy. Anaemia is typically present; other haematologic parameters are more variable. In most cats, the lymphadenopathy resolved over the following weeks or months regardless of treatment. One cat subsequently developed intrathoracic lymphoma 2 years after presentation for lymphadenopathy and was euthanased.

The majority of cats affected by DPLNH show moderate enlargement of all peripheral lymph nodes; the lymphadenopathy is less commonly confined to the mandibular or popliteal nodes. Enlargement of visceral lymph nodes is not reported, but the possibility that visceral nodes were enlarged was not investigated in the study. Affected lymph nodes are typically two to three times normal size, firm, yellow-tan to white with a smooth capsular surface and marked distortion of nodal architecture. Histologically, cortico-medullary architecture is distorted or effaced with variable loss of follicular structures and sinuses and expansion of the paracortical regions with increased numbers of macrophages, lymphocytes and plasma cells and increased prominence of post-capillary venules.

Of the 14 cats included in the original study describing DPLNH, antibodies against FeLV were present in 6 out of 9 cats tested (by either ELISA or IFA methods). However, as FeLV vaccination status was not known in almost half of the 14 cats, it is possible that at least some of the cats testing positive for the presence of FeLV antibody may have been previously vaccinated for FeLV and so tested positive due to vaccination rather than true FeLV infection.
Subsequent investigations of cats described in the original report of DPLNH identified small pleomorphic intracellular coccobacilli in the mandibular and popliteal lymph nodes of several cats affected by DPLNH (Kirkpatrick et al. 1989). The bacteria were not visible on routine haematoxylin and eosin stained sections, stained positive with silver stains, but stained negative with a range of other stains including Gram’s method and acid-fast stains. The identity and significance of these bacteria in both DPLNH and feline lymphadenopathy more generally is unknown.

**2.4.2.3 Generalised Lymphadenopathy Resembling Lymphoma**

A transient generalised peripheral lymphadenopathy of unknown aetiology has also been described in a small number of adult cats and has been termed generalised lymphadenopathy resembling lymphoma (GLRL) (Mooney et al. 1987). Cats affected by GLRL are aged between 1 and 4 years and Maine Coon cats appear over-represented. Of the six cats included in the initial report of the disease, three were Maine Coon while the remainder were DSH cats. Four of the six cats in the study had concurrent clinical signs of upper respiratory or urinary tract infection at presentation. Although no cat tested positive for the presence of FeLV antigen, two cats had a history of chronic FeLV exposure and tested positive for antibodies to feline oncornavirus cell membrane antigen (FOCMA), indicative of prior regressive FeLV infection. Five of the six cats had a moderate leukocytosis, while other haematologic parameters were more variable.

Cats affected by GLRL typically show mild to moderate enlargement of multiple peripheral lymph nodes. Involvement of other organs or visceral nodes is not described. Histology of affected nodes is variable, but includes several features consistent with lymphoma, including effacement of normal nodal architecture by large numbers of lymphocytes, lymphoid cellular infiltration within the subcapsular and perinodal tissues, the presence of large follicular structures without germinal centres or mantle zones and the presence of many lymphocytes in mitosis. However, features less suggestive of lymphoma are also present in some nodes, including increased reactive follicular structures with germinal centres, lack of capsular invasion, low mitotic activity and a mixed cell population that includes plasma cells, macrophages and other leukocytes. Often neoplastic and non-neoplastic features are present within the same node. While the initial histological diagnosis was lymphoma, in the majority of cats the lymphadenopathy resolved in the months following presentation. Although the
aetiology of GLRL has not been determined, it is speculated to be related to immune stimulation and the cat’s response to viral infection.

As with DPLNH, subsequent investigations of the lymph nodes from the cats described in the original report of GLRL also identified small pleomorphic intracellular coccobacilli in the mandibular lymph nodes of several cats affected by GLRL (Kirkpatrick et al. 1989). Again, the identity and significance of these bacteria is unknown.

2.4.2.4 PLEXIFORM VASCULARISATION OF LYMPH NODES

A peripheral lymphadenopathy involving a single cervical, retropharyngeal or inguinal lymph node characterised by marked capillary proliferation and lymphoid follicular atrophy is also reported in a small number of cats and has been termed plexiform vascularisation of lymph nodes (Lucke et al. 1987; Welsh et al. 1999). Affected cats are adult domestic shorthair or longhair breeds aged between 3 and 14 years. Lymphadenopathy is not associated with other clinical signs and does not typically recur following surgical excision of the affected node. While the cause is unknown, an ischaemic pathogenesis similar to the vascular transformation of lymph node sinuses occasionally reported in people has been postulated (Haferkamp et al. 1971; Steinmann et al. 1982).

2.5 AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

Autoimmune lymphoproliferative syndrome, previously also termed Canale-Smith syndrome (Canale and Smith 1967), is a recently recognised human disease involving genetic defects in Fas-mediated lymphocyte apoptosis (Fisher et al. 1995). Patients typically present in infancy or childhood with a non-neoplastic but often dramatic lymphadenopathy and splenomegaly, together with variable manifestations of autoimmunity (Sneller et al. 1997; Straus et al. 1999). Lymph nodes and other affected organs are expanded by markedly increased numbers of an unusual population of T-cells that express CD3, but neither CD4 nor CD8 surface antigens, commonly referred to as “double negative T-cells” (DNT cells) (Lim et al. 1998). In contrast to the other diseases
reviewed in this chapter, ALPS has multiple similarities with the presentation of disease in BSH kittens in the present studies, and is discussed in greater detail below.

2.5.1 MOLECULAR AND GENETIC BASIS FOR DISEASE

2.5.1.1 LYMPHOCYTE APOPTOSIS

The molecular basis for the development of lymphocyte proliferation and autoimmunity in ALPS is typically due to defects in the Fas-mediated apoptotic pathway. Apoptosis is a specific form of programmed cell death which occurs through an ordered pathway of self-degradation and terminates with the phagocytosis and removal of cellular remnants (Kerr et al. 1972; Evan et al. 1995). Apoptotic processes are highly conserved across species and have a regulatory role in normal tissue development and maintenance, as well as in the removal of self-reactive cells and cells damaged beyond repair (Kerr et al. 1972). Apoptosis can be initiated by events both internal and external to the target cell but ultimately involve the activation of caspase enzymes through death-receptor-ligand interactions at the cell surface (the extrinsic pathway) or through enzyme induction through mitochondria (the intrinsic pathway) (Danial and Korsmeyer 2004). The death receptors involved in the extrinsic pathway are members of the tumour necrosis factor (TNF) receptor family and contain a cytoplasmic death domain critical to the delivery of apoptotic signals (Tartaglia et al. 1993). One of the most important and extensively studied death receptors is the Fas receptor, also known as CD95, Apo1, Apt and TNFRSF6 (tumour necrosis factor receptor superfamily member 6) (Siegel et al. 2000). The binding of Fas to Fas ligand (FasL) or agonistic antibodies triggers trimerisation and recruitment to the Fas death domain of the corresponding region of the cytoplasmic adaptor protein, Fas-associated death domain protein (FADD), followed by caspase activation and subsequent cell death (Golstein et al. 1991; Itoh et al. 1991; Kischkel et al. 1995).

In the normal animal, apoptosis plays a particularly important role in T-cell development and regulation, and in ensuring appropriate immune responses by mature peripheral T-cells. In general, T-cells able to mount an appropriate immune response to antigens bound to MHC complex molecules will undergo positive selection within the thymus and survive to ensure the development of a range of T-cells capable of effectively responding to foreign antigens. The immune system is therefore able to respond to a range of
foreign antigens by maintaining small numbers of a broad range of antigen-specific lymphocytes. On subsequent antigen recognition, T-cells that recognise that antigen undergo rapid and marked proliferation; once the antigen has been eliminated, those lymphocytes undergo apoptosis and are removed. Conversely, strongly self-reactive T-cells are eliminated by negative selection within the thymus before they fully mature to prevent potentially damaging autoimmune reactions and ensure the development of immunologic tolerance to ubiquitous self-antigens. The vast majority of developing T-cells are eliminated either before emerging from the thymus and bone marrow or maturing in the peripheral lymphoid organs (Owen et al. 2013b). Fas-mediated apoptosis of activated lymphocytes is particularly important in maintaining immune homeostasis by eliminating self-reactive T-cells and limiting the accumulation of activated and aging lymphocytes (Lenardo 1996; Lenardo et al. 1999; Worth et al. 2006; Yolcu et al. 2008).

2.5.1.2 DEFECTS IN FAS-MEDIATED APOPTOSIS

Although ALPS and ALPS-related diseases can be caused by a range of underlying genetic defects, functional impairment of Fas-mediated apoptosis is present in most cases. This typically results in a failure to eliminate self-reactive, chronically activated and senescent lymphocytes. Those lymphocytes then accumulate within the lymph nodes and other organs (Worth et al. 2006).

Fas is a 35kDa transmembrane protein that is expressed by a wide variety of cells, including those in the thymus, liver, heart, kidney and lymph nodes (Itoh et al. 1991; Watanabe-Fukunaga et al. 1992; Nagata 1997). Fas protein is encoded by the Fas gene. In people, the Fas gene has been mapped to chromosome 10 (Inazawa et al. 1992; Lichter et al. 1992). The gene comprises 9 exons and 8 introns encoding 325 amino acids (Nagata and Golstein 1995). Exon 1 contains the 5’ untranslated region and initial part of the signal sequence. Exons 2, 3, 4 and 5 encode the extracellular region, and exon 6 encodes the transmembrane region. The intracellular region comprises exons 7, 8 and 9: the membrane proximal portion of the cytoplasmic region is encoded by exons 7 and 8, while exon 9 encodes the death domain and the 3’ untranslated region (Behrmann et al. 1994; Cheng et al. 1995).
Approximately three-quarters of human ALPS patients have heterozygous germline mutations in the \textit{Fas} gene (ALPS-FAS, previously ALPS type 1a), most of which involve substitutions, insertions of deletions of one or more nucleotides into the coding exons or splice sites of the gene (Rao and Straus 2006; Anonymous 2012a). Rare patients with homozygous germline \textit{Fas} mutations (ALPS-FAS, previously ALPS type 0) are also reported and typically display a severe clinical phenotype (Rieux-Laucat \textit{et al.} 1995; van der Burg \textit{et al.} 2000; Oliveira \textit{et al.} 2010). There are also a small number of people with phenotypic features of ALPS associated with somatic \textit{Fas} mutations (ALPS sFAS, previously ALPS type 1m) (Holzelova \textit{et al.} 2004; Puck and Straus 2004; Oliveira \textit{et al.} 2010).

Mutations are reported at more than 70 different locations throughout the \textit{Fas} gene in ALPS patients, but the majority (approximately 70\%) affect the death domain of the intracellular region and most commonly involve exon 9 (Fisher \textit{et al.} 1995; Rieux-Laucat \textit{et al.} 1999; Jackson \textit{et al.} 1999a; Rao and Straus 2006). The distribution of mutations affecting the \textit{Fas} genes in ALPS patients (ALPS-FAS) is set out in Figure 2.3. In people, mutations affecting the death domain of \textit{Fas} appear associated with a higher disease penetrance and overt clinical signs of disease (Infante \textit{et al.} 1998; Jackson \textit{et al.} 1999a). As the \textit{Fas} gene mutations that are present in people with ALPS are so diverse, the consequences of those mutations also vary, but typically result in the production of truncated \textit{Fas} protein or modification of the amino acid sequence, which in turn causes defective \textit{Fas}-mediated apoptosis due to inactivation of the death domain and other regions critical to successful \textit{Fas}-mediated apoptosis (Fisher \textit{et al.} 1995; Rieux-Laucat \textit{et al.} 1999; Vaishnaw \textit{et al.} 1999). In other cases normal \textit{Fas} protein is produced and detectable but at a reduced level insufficient for full physiological function (Hsu \textit{et al.} 2012).

In addition to \textit{Fas}, mutations in several other genes involved in \textit{Fas}-mediated apoptosis including \textit{Fas ligand (Fasl)}, caspase 10 (\textit{CASP10}), caspase 8 (\textit{CASP8}) and neuroblastoma \textit{RAS (NRAS)} genes are also less commonly implicated in ALPS and ALPS-related disorders (Rao and Straus 2006; Oliveira \textit{et al.} 2010). Germline mutations in the \textit{Fasl} gene (ALPS-FASLG, previously ALPS type Ib) and \textit{CASP10} gene (ALPS-CASP10, previously ALPS type Ila) are occasionally reported in people with ALPS, while rare germline mutations in the caspase 8 (\textit{CASP8}) gene (Chun \textit{et al.} 2002) and somatic mutations in the \textit{NRAS} gene
(Oliveira et al. 2007) have been reported to cause clinical signs similar to those seen in ALPS (ALPS related disease) (Oliveira et al. 2010). Reported mutations in FasL include nucleotide deletions (Wu et al. 1996) and substitutions (Del-Rey et al. 2006; Bi et al. 2007) resulting in abnormalities in FasL function and consequently ineffective Fas-mediated apoptosis. Reported mutations in CASP10 include nucleotide substitutions which decrease caspase activity and impair effective apoptosis (Wang et al. 1999). Further, in a significant proportion of ALPS patients and people with similar clinical signs but not meeting the diagnostic criteria for ALPS (approximately one-third), a causative genetic defect has not been identified (ALPS-U) (Rao and Straus 2006; Oliveira et al. 2010).

![Diagram of human Fas gene showing the location and nature of mutations known to cause ALPS-FAS](https://example.com/fas_diagram.png)

**Figure 2.3:** Diagram of human Fas gene showing the location and nature of mutations known to cause ALPS-FAS (courtesy: National Institute of Allergy and Infectious Diseases (Anonymous 2012a)).

2.5.1.3 **DOUBLE NEGATIVE T-CELLS**

As discussed in section 2.5.2 below, an increased number of circulating DNT cells is a defining feature of ALPS (Sneller et al. 1997; Bleesing et al. 2001a; Worth et al. 2006).
Double negative T-cells are a subpopulation of T-cells which express the surface marker CD3, but which do not express either CD4 or CD8. They are typically present in very low numbers in the peripheral blood and organs of healthy people (Fuss et al. 1997; Rieux-Laucat and Magerus-Chatinet 2010).

In people, T-cells develop in the thymus from pro-thymocytes along a well-characterised pathway of differentiation, and T-cell development includes several intermediate DN (CD4-/CD8-) cell stages prior to maturity (see section 2.2 and Figure 2.1 above). The origin and function of the small number of DNT cells normally found within peripheral blood and other organs in healthy people is controversial. (D’Acquisto and Crompton 2011). Some authors propose a thymic origin for these DNT cells, followed by migration to and proliferation at the periphery upon encountering antigen (Mixter et al. 1999; Priatel et al. 2001; Hayes et al. 2005). Other authors propose that DNT cells found within the periphery are generated there, citing studies demonstrating the presence of DNT cells in thymectomised mice (Kadena et al. 1997; Ford et al. 2006). The origin and significance of the greatly expanded population of DNT cells present in ALPS patients is also incompletely understood (Steinberg et al. 1980; Mehal and Crispe 1998; Marlies et al. 2007; Bristeau-Leprince et al. 2008; Lev et al. 2012). It has been proposed that these cells derive from previously activated and post-thymic mature T-cells that subsequently lose CD4 or CD8 receptor expression, and represent an arrested or altered stage of T-cell differentiation which then become chronically activated in a particular *in vivo* environment (Bleesing et al. 2002; Worth et al. 2006; Puck et al. 2007; Bristeau-Leprince et al. 2008; Rieux-Laucat and Magerus-Chatinet 2010). However, other aspects of the DNT cell profile in ALPS patients, including the expression of CD45RA and lack of CD25 expression, suggest a naïve (non-activated) T-cell phenotype and are less consistent with this theory (Bleesing et al. 2002).

### 2.5.1.4 Penetrance of the Genotype

Although the majority of ALPS patients are heterozygous for a *Fas* gene mutation with an autosomal dominant pattern of inheritance, there is incomplete penetrance of the genotype and highly variable expression of overt clinical signs (Fisher et al. 1995; Infante et al. 1998; Rieux-Laucat et al. 1999; Jackson et al. 1999a; Rao and Straus 2006). Even ALPS patients from a single family and with the same genetic mutation may show marked differences in clinical phenotype, and while some patients will show obvious
clinical signs of disease, in others the disease may be clinically silent and is only diagnosed following the investigation of another family member for unexplained lymphoproliferation (Worth et al. 2006). Mutations affecting the intracellular portion and death domain of Fas show the highest clinical penetration (up to 90%), while mutations affecting the extracellular domain have a lower penetrance of clinical signs (approximately 30%) (Rieux-Laucat et al. 1999; Vaishnaw et al. 1999; Jackson et al. 1999a).

Penetrance of the other genotypes associated with ALPS such as ALPS-FASLG and ALPS-CASP10 is not well-documented, most likely because of the small number of people affected by these other defects.

2.5.2 CLINICAL FEATURES

2.5.2.1 LYMPHOPROLIFERATION

Autoimmune lymphoproliferative syndrome is typically characterised by the onset in infancy or early childhood of a chronic and often marked non-neoplastic lymphoproliferation. Enlargement of multiple lymph nodes and the spleen are typically the primary presenting clinical signs in ALPS. Hepatomegaly is more variably present (Sneller et al. 2003; Rieux-Laucat and Magerus-Chatinet 2010) and thymic enlargement is only occasionally reported (Sneller et al. 1992; Le Deist et al. 1996; Sneller et al. 1997; Lim et al. 1998; Avila et al. 1999). The degree of lymph node enlargement ranges from mild to massive; the spleen is typically markedly enlarged. (Canale and Smith 1967; Sneller et al. 1992; Sneller et al. 1997; Straus et al. 1999). The median age at presentation is 24 months, but cases have been reported from birth onwards (Sneller et al. 1997; Avila et al. 1999; Straus et al. 1999; Rao and Straus 2006; Worth et al. 2006; Puck et al. 2007). While lymphadenopathy may persist for many years, it is often more marked in infancy and frequently regresses during adolescence and early adulthood (Infante et al. 1998; Rieux-Laucat et al. 1999).

2.5.2.2 AUTOIMMUNITY

Autoimmune manifestations are less consistent and are reportedly present in less than 70% of ALPS patients (Infante et al. 1998; Rieux-Laucat et al. 2003; Sneller et al. 2003;
Worth et al. 2006). Autoimmunity is due to a failure to eliminate autoreactive lymphocytes due to defective apoptosis (Bleesing 2003). Peripheral cytopenias are the most common autoimmune disorders seen, and include immune-mediated anaemia, neutropenia and thrombocytopenia (Straus et al. 1999; Teachey et al. 2010). Anaemia usually results from immune-mediated haemolysis of erythrocytes, and a positive Coombs’ test is often present (Infante et al. 1998; Straus et al. 1999; Teachey et al. 2010). However, the anaemia is highly variable, with some patients demonstrating transient haemolytic anaemia, while in other cases, anaemia is present but the cause is undetermined (Alvarado et al. 2004). In other cases, circulating autoantibodies to erythrocytes are identified, but are not associated with a concurrent haemolytic anaemia (Infante et al. 1998). Other autoimmune manifestations seen less frequently include glomerulonephritis, uveitis, primary biliary cirrhosis, and urticaria (Pensati et al. 1997; Sneller et al. 1997; Avila et al. 1999; Rieux-Laucat et al. 1999; Lim et al. 2005; Rao and Straus 2006). Cytopenias and other manifestations of autoimmunity are frequently absent at the time of initial presentation when patients typically show marked lymphoproliferation, but tend to develop and become more severe as patients age (Straus et al. 1999; Worth et al. 2006).

2.5.2.3 LYMPHOMA DEVELOPMENT

People with ALPS have an increased risk of developing a range of malignancies, particularly lymphoma. Lymphomas subsequently develop in an estimated 10-20% of ALPS patients following presentation, and people with germline mutations of the intracellular domain of Fas (the majority of ALPS cases) are at particular risk of lymphoma development (Straus et al. 2001; Poppema et al. 2004; Worth et al. 2006). The median age at presentation for lymphoma development in ALPS patients is reportedly 17 years (range 2-50 years) (Rao and Straus 2006). Lymphomas are almost exclusively of B-cell origin, but show a range of histological morphologies, including both Hodgkin and non-Hodgkin forms of disease. The relative risk of developing Hodgkin lymphoma is reportedly 51 times greater than controls, and 14 times greater for non-Hodgkin lymphomas (Straus et al. 2001).

2.5.3 LABORATORY FINDINGS

Common laboratory findings in peripheral blood samples from ALPS patients include the presence of circulating autoantibodies, elevations in various biomarkers, and the
presence of an expanded population of peripheral DNT cells. The demonstration of
defective apoptosis in ALPS patients’ peripheral lymphocytes using specialised in vitro
assays is also a hallmark of disease (Straus et al. 1999; Bleesing et al. 2000), although is
no longer considered an absolute criterion for diagnosis (see section 2.5.5.below).

Defective lymphocyte apoptosis is assayed in vitro by exposing the patient’s peripheral
blood mononuclear cells (PBMCs) to a T-cell activation stimulus, typically
phytohaemagglutinin (PHA). Activated T-cells are then cultured in interleukin-2 (IL-2),
and after up to three weeks, are cross-linked to induce (Fas-mediated) apoptosis using
anti-Fas monoclonal antibodies, recombinant Fas ligand, or by TCR restimulation.
Induction of apoptosis may be quantified by various methods, including assays that use
terminial deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL)
or annexin V to label apoptotic cells which are then detected and quantified using flow
cytometry (Sneller et al. 1997; Muppidi et al. 2004).

Autoantibodies are commonly present and are often detected in the absence of any
overt clinical manifestations of autoimmunity such as cytopenia (Kwon et al. 2003).
Their presence is likely due to the disease-related failure of elimination of a sub-
population of lymphocytes reactive to self-antigens due to defective apoptosis (Bleesing
2003). The most common autoantibodies found are those directed against erythrocytes
and detected by various tests including a positive Coombs’ (direct antiglobulin) test.
However, antibodies to neutrophils and platelets are also often present, and often
antibodies to more than one blood cell type or other component are present (Carter et
antibodies, anti-nuclear antibodies and rheumatoid factors are also variably present in
ALPS patients (Bleesing 2003; Kwon et al. 2003; Sneller et al. 2003).

Consistent elevations in various blood biomarkers including interleukin 10 (IL-10),
interleukin 18 (IL-18), soluble Fas ligand, vitamin B12, as well as a polyclonal
hypergammaglobulinaemia, are also commonly present in ALPS (Fuss et al. 1997;
Lopatin et al. 2001; Magerus-Chatinet et al. 2009; Caminha et al. 2010; Bowen et al.
2012). Interleukin-10 is increased due to alterations in helper T-cell responses in ALPS
patients and the likely constitutional expression of IL-10 by the expanded population of
DNT cells (Fuss et al. 1997; Lopatin et al. 2001; Magerus-Chatinet et al. 2009; Caminha et
Vitamin B12 is likely increased due to increased lymphocyte expression of haptocorrin (one of the plasma proteins to which Vitamin B12 normally binds), although the mechanism for the elevation in haptocorrin is not known (Caminha et al. 2010; Bowen et al. 2012). The amount of soluble Fas ligand present is presumably elevated due to the presence of ongoing stimuli for FasL production following the failure of Fas-mediated apoptosis. Gamma-globulins are thought to be elevated due to the concurrent dysregulation of humoral immunity and aberrant B-cell responses often present in ALPS (Sneller et al. 1997). The mechanism for the increase in IL-18 is not known.

As mentioned above, the expansion of an unusual population of circulating DNT cells is a defining feature of ALPS, and is the one of the most consistent haematological differences between ALPS patients and controls (Sneller et al. 1997; Bleesing et al. 2001a; Worth et al. 2006). In healthy controls, very low numbers of DNT cells are usually present, typically constituting less than 1% of circulating lymphocytes (Fuss et al. 1997). The increased numbers of DNT cells in people with ALPS comprise a homogenous population of CD3+ T-cells that express the α/β TCR but do not express either CD4 or CD8. These DNT cells therefore express a CD3+ TCR αβ+ CD4- CD8- immunophenotype when assayed by flow cytometric analysis of a peripheral blood sample (Sneller et al. 1997; Bleesing et al. 2001a). In addition, the population of DNT cells in ALPS patients typically expresses CD45RA, CD57, CD27, CD28, perforin and HLA-DR but not CD45RO and CD56, although these additional surface markers are not routinely assayed in a diagnostic setting (Bleesing et al. 2001b; Bleesing et al. 2002; Oliveira et al. 2010).

### 2.5.4 Pathologic Features

Histology of affected lymph nodes in ALPS patients typically shows a marked expansion of the interfollicular or paracortical regions by a population of lymphocytes at various stages of immunoblastic transformation, admixed with variable numbers of plasma cells (Sneller et al. 1992; Sneller et al. 1997; Lim et al. 1998; Alvarado et al. 2004). While there is often retention of cortico-medullary architecture with follicular hyperplasia, in other cases the interfollicular lymphocyte proliferation effaces nodal architecture and may be sufficiently florid to suggest a diagnosis of lymphoma (Canale and Smith 1967; Sneller et al. 1997; Lim et al. 1998; Weintraub et al. 1998; Strobel et al. 1999; Bleesing et al. 2000). The interfollicular proliferation of lymphocytes comprise a population of polyclonal DNT
cells (CD3+ TCRαβ+ CD4- CD8- by immunochemical methods) similar to those elevated in circulation. These DNT cells have a high proliferation rate, evidenced by the presence of many cells in mitosis and a high proliferation index by Ki67 immunopositivity, but show relatively few cells undergoing apoptosis identified by cellular morphology or other in situ apoptosis assays such as TUNEL (Lim et al. 1998).

Splenic enlargement in ALPS patients is predominantly due to the expansion of the red and sometimes white pulp by a similar T-cell population to that present within the lymph nodes, again often admixed with plasma cells (Le Deist et al. 1996; Lim et al. 1998). Hepatic enlargement in ALPS is more variable (Canale and Smith 1967; Sneller et al. 1992; Le Deist et al. 1996; Infante et al. 1998) and histological changes are generally limited to extramedullary haematopoiesis, erythrophagocytosis and a mild lymphocytic infiltrate within sinusoids and portal areas (Le Deist et al. 1996; Lim et al. 1998).

2.5.5 DIAGNOSIS

The definitive diagnostic criteria for ALPS have been recently revised and the current criteria are set out in Table 2.2. A definitive diagnosis is of ALPS is made if both required criteria and at least one primary accessory criterion are present. A probable diagnosis of ALPS is made if both required criteria and at least one secondary accessory criterion are present.

The first absolute requirement is the presence of a non-neoplastic and non-infectious lymphadenopathy, splenomegaly, or both, that has persisted for at least 6 months. The lymphadenopathy must be generalised or affect at least two nodal chains. While often also present, hepatomegaly is not a diagnostic criterion (Oliveira et al. 2010). The second requirement is the presence of elevated DNT cells in circulation, which must be immunochemically identified as CD3+ TCRαβ+ CD4- CD8- cells. Increased DNT cells are present if they comprise 1.5% or greater of all circulating lymphocytes or 2.5% or greater of all circulating CD3+ T-cells. Elevations of DNT cells greater than 3% of all lymphocytes or 5% of T-cells are rarely seen in conditions other than ALPS and are essentially considered pathognomonic for ALPS diagnosis (Teachey et al. 2005; Magerus-Chatinet et al. 2009; Caminha et al. 2010).
Table 2.2: Current diagnostic criteria for ALPS (Oliveira et al. 2010)

<table>
<thead>
<tr>
<th>Required criteria</th>
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<tbody>
<tr>
<td>1. Chronic (&gt; 6 months) non-malignant, non-infectious lymphadenopathy or splenomegaly or both</td>
</tr>
<tr>
<td>2. Increased peripheral CD3+ TCRαβ+ CD4- CD8- DNT cells (&gt;1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes) in the setting of normal or elevated lymphocyte counts</td>
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<table>
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<tr>
<th>Accessory criteria Primary</th>
<th>1. Defective lymphocyte apoptosis (in 2 separate assays)</th>
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<tr>
<td></td>
<td>2. Somatic or germline pathogenic mutation in Fas, FasL or CASP10 genes</td>
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<table>
<thead>
<tr>
<th>Accessory criteria Secondary</th>
<th>1. Elevated plasma levels of soluble FAS ligand protein (&gt;200pg/mL) OR Elevated plasma interleukin-10 levels (&gt;20pg/mL) OR Elevated vitamin B12 levels (&gt;1500ng/mL) OR Elevated plasma interleukin-18 levels (&gt;500pg/mL)</th>
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<td></td>
<td>2. Typical immunohistological findings (discussed in section 2.5.4)</td>
</tr>
<tr>
<td></td>
<td>3. Autoimmune cytopenias (haemolytic anaemia, thrombocytopenia, or neutropenia) AND Elevated immunoglobulin G levels (polyclonal hypergammaglobulinaemia)</td>
</tr>
<tr>
<td></td>
<td>4. Family history of a non-malignant/non-infectious lymphoproliferation with or without autoimmunity</td>
</tr>
</tbody>
</table>

DNT cells = double negative T-cells

Primary accessory criteria include demonstration of abnormal lymphocyte apoptosis on 2 separate assays (Straus et al. 1999; Bleesing et al. 2000). Fas-mediated apoptosis is activated in cultured T-cells using cross-linked agonistic Fas antibodies, recombinant Fas ligand, or TCR restimulation; ALPS patients typically show significantly reduced apoptosis compared to controls (Sneller et al. 1997; Muppidi et al. 2004). However, demonstrating defects in lymphocyte apoptosis is no longer considered an absolute requirement for ALPS diagnosis as patients with ALPS-sFAS (fulfilling the diagnostic criteria for ALPS with somatic mutations in the Fas gene) and ALPS-FASLG typically show normal Fas-mediated apoptosis when assayed by these methods (Holzelova et al. 2004; Del-Rey et al. 2006; Bi et al. 2007).

An alternative primary accessory criterion is the demonstration of germline or somatic mutations in the Fas, FasL or CASP10 genes by DNA amplification and sequencing methods. Patients with CASP8 and NRAS mutations are currently classified separately as ALPS-related disorders due to differences in clinical presentation to other ALPS patients. Patients with CASP8 mutations typically present with recurrent bacterial and viral infections due to defects in T-cell, B-cell and NK cell activation (Chun et al. 2002; Su et al. 2005). Patients with NRAS mutations also show differences in clinical phenotype to the
typical ALPS presentation, and do not consistently have elevated DNT cells in circulation (Oliveira et al. 2007; Oliveira et al. 2010).

Secondary accessory criteria are present with greater variability and include elevations in a range of serum or plasma biomarkers (interleukin 10, interleukin-8, soluble Fas ligand and vitamin B12) (Magerus-Chatinet et al. 2009; Caminha et al. 2010; Seif et al. 2010), the presence of autoimmune cytopenias and hypergammaglobulinemia (Seif et al. 2010), and the presence of other family members with a history of non-malignant lymphadenopathy or splenomegaly (Oliveira et al. 2010). The presence of characteristic microscopic pathology is also a secondary accessory criterion for ALPS diagnosis, and typical findings are discussed in section 2.5.4 above.

2.5.6 TREATMENT AND PROGNOSIS
The lymphadenopathy seen in ALPS is seldom life-threatening, does not usually require specific treatment, and may resolve as the patient ages (Infante et al. 1998; Rieux-Laucat et al. 1999; Teachey et al. 2010). Immunosuppressant therapies including corticosteroids, cyclosporine, azathioprine and mycophenolate mofetil have been used but do not consistently reduce the lymphoproliferation. They are generally only recommended if the enlargement of lymph nodes or spleen is causing significant anatomic obstruction (Worth et al. 2006).

In contrast, the autoimmune cytopenias associated with the disease may be severe and sometimes fatal, and the majority of therapeutic interventions in ALPS patients are directed at managing these manifestations. Previously, splenectomy was often performed to manage chronic and severe cytopenias in ALPS patients by reducing lysis of various haematological cell types (Rao and Straus 2006; Teachey et al. 2010). However, while splenectomy produced resolution of many of the autoimmune manifestations of the disease in some cases, clinical improvement of cytopenias was variable, and it was also associated with increased development of sometimes fatal opportunistic infection or sepsis in splenectomised patients despite antibiotic prophylaxis and vaccination (Bleesing et al. 2000; Bleesing et al. 2002). Consequently, medical management of refractory or life-threatening autoimmune cytopenias is now preferred, with splenectomy a treatment of last resort (Teachey et al. 2010). A course of prednisone is the initial treatment of choice, followed by mycophenolate mofetil and/or
sirolimus, and finally chemotherapeutics such as vincristine, methotrexate and rituximab for non-responsive cases (Rao et al. 2005; Rao et al. 2009; Teachey et al. 2009; Teachey et al. 2010).

The prognosis for most ALPS patients primarily depends on the development, severity and successful management of any autoimmune cytopenias and also on the subsequent development of lymphoma (Worth et al. 2006). While on-going monitoring and surveillance for the development of these signs is recommended, for well-managed patients the overall prognosis is currently considered relatively good (Rao and Straus 2006).

2.5.7 ANIMAL MODELS – LPR AND GLD DISEASE

In mice, mutations in the *lpr* and *gld* genes induce a syndrome similar to ALPS in people and affected mice are used as models for the human disease (Sneller et al. 1992). The clinical manifestations of the disease in mice vary widely between different murine strains but often include additional features not typically present in people with ALPS, such as glomerulonephritis and vasculitis (Izui et al. 1984; Cohen and Eisenberg 1991). However, the development of autoimmune disease characterised by autoantibody formation, the development of a marked generalised lymphadenopathy due to accumulation of large numbers of non-malignant DNT cells at a young age, and the presence of defective apoptosis are consistent features of both *lpr* and *gld* disease in mice (Cohen and Eisenberg 1991; Watanabe-Fukunaga et al. 1992; Nagata and Golstein 1995).

There is limited description of the histological changes present within lymph nodes and other organs of *lpr* and *gld* mice, but effacement and loss of normal lymph node architecture by an atypical population of DNT cells in mice has been described (Roths et al. 1984; Kimura et al. 1990). Variable but less extensive infiltration of other organs including spleen and liver by DNT cells also may be present (Kimura et al. 1990; Cohen and Eisenberg 1991; Weintraub et al. 1998). Thymic enlargement is not described in mice with *gld* or *lpr* disease (Roths et al. 1984).

In contrast to ALPS, the murine diseases show an autosomal recessive mode of inheritance (Cohen and Eisenberg 1991). Mice homozygous for the *lpr* mutation have
defects in the \textit{Fas} gene (Watanabe-Fukunaga et al. 1992; Nagata 1994). In contrast to people, only two \textit{lpr} mutations are reported, \textit{lpr} and \textit{lpr}^{ca}, that produce a disease phenotype in affected mice similar to ALPS (Cohen and Eisenberg 1991). In \textit{lpr} mice, the insertion of 183 nucleotides into intron 2 of the \textit{Fas} gene causes premature termination and aberrant splicing of the \textit{Fas} transcript resulting in defective and markedly reduced \textit{Fas} expression and function (Watanabe-Fukunaga et al. 1992; Adachi et al. 1993). In \textit{lpr}^{ca} mice, a point mutation of the \textit{Fas} gene involving a single substitution of arginine for thiamine at nucleotide 786 results in the replacement of isoleucine with asparagine in the cytoplasmic region of the gene (Watanabe-Fukunaga et al. 1992). This mutation in \textit{lpr}^{ca} mice results in the expression of normal amounts of dysfunctional \textit{Fas} which are unable to initiate effective apoptosis (Nagata 1994). There is also variability in clinical expression of the disease in mice that is primarily associated with differences in the genetic background of the mouse (Cohen and Eisenberg 1991). Mice homozygous for the \textit{gld} mutation have defects in the \textit{FasL} gene (Takahashi et al. 1994; Nagata and Suda 1995). In \textit{gld} mice, a point mutation (the replacement of thiamine to cytosine) near the C-terminus of the coding region for \textit{FasL} causes phenylalanine to be replaced by leucine, which renders \textit{FasL} unable bind to \textit{Fas}, consequently preventing the induction of \textit{Fas}-mediated apoptosis in affected cells (Takahashi et al. 1994; Nagata and Suda 1995).

Although \textit{lpr} and \textit{gld} diseases in mice are useful models for the study of ALPS in people, there are differences between the species that limit direct comparisons of aspects of the disease. In particular, the mode of inheritance and typical spectrum of clinical signs differ between people with ALPS and mice with \textit{lpr} or \textit{gld} mutations. In addition, there are differences in the proteins involved in the apoptotic pathways between the two species (Puck et al. 2007). For example, the absence of a functional \textit{CASP10} gene in mice restricts investigations of the role of the different caspases in \textit{Fas}-mediated apoptosis (Reed et al. 2003; Janicke et al. 2006; Eckhart et al. 2008). The development of other animal models with greater similarity to ALPS in people could therefore be a useful adjunct to the existing murine models of disease.
2.6 REFERENCES


CHAPTER 2


CHAPTER 3

DISEASE INHERITANCE

3.1 INTRODUCTION

Most lymphoproliferative diseases (LPDs) in humans and animals are neoplastic and are considered to be sporadic. However, environmental and inherited factors have also been reported to predispose to LPD (Shelton et al. 1990; Linet and Potten 1992; Segel and Lichtman 2004; Siddiqui et al. 2004), with hereditary forms best described in humans (Segel and Lichtman 2004) and mice (Cohen and Eisenberg 1991; Tivol et al. 1995). While an inherited basis for LPD has been suspected in other animal species, including cats, examples are rare and supporting evidence is lacking (Louwerens et al. 2005).

Evidence to support an inherited basis for disease includes observations that the disease occurs more frequently in multiple related animals, or more frequently in some breeds within a species. The presence of a high degree of inbreeding within the pedigrees of affected animals may also provide support for particular modes of inheritance (Nicholas
However, the potential contributions to disease development of other non-genetic factors, for example viral infection, must also be considered, and ideally excluded (Nicholas 2010a).

In 2009, an unusual multicentric LPD was identified in three 5 to 7-week-old sibling kittens from a single litter of British shorthair (BSH) kittens. The development of this disease in multiple related animals therefore suggested a possible inherited basis. Non-genetic causes of LPD, such as infection with feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV) were considered unlikely as both parents of the initial affected litter and two of the three affected kittens showed no evidence of infection with either virus. Further, LPD due to natural infection with FeLV or FIV has not been previously reported in kittens less than 6 months of age (Grant et al. 1980; Callanan et al. 1996). While there are anecdotal reports of LPD also occurring in BSH kittens in Australia (pers. comms. Mary Hefford, Iris Baker and Daniel Wheeler) and in young domestic shorthair (DSH) cats in the United States of America (pers. comm. Niels C. Pedersen), these cases are not reported in the literature and the cause of any such LPD in these animals is unknown.

The aim of this chapter was to confirm or exclude the hypothesis that LPD in the initial litter of BSH-cross kittens was inherited, through breeding trials and analysis of pedigree and other records. First, the initial mating was repeated to determine if the results of the initial litter were repeatable. Outcross, backcross and sibling matings were then undertaken to help determine the likely mode of inheritance. Finally, further information from retrospective surveys of available pathology and pedigree records was used to identify further cases of LPD and to reconstruct a more extensive pedigree of LPD-affected animals. The aims of the breeding trials and the further surveys were to verify that this form of LPD in young kittens was inherited, to determine the mode of inheritance, and, if possible, to generate animals for further investigation of the disease phenotype.
CHAPTER 3

3.2 MATERIALS AND METHODS

3.2.1 BREEDING TRIALS

Breeding trials were based upon the results of the initial mating of a BSH-Manx cross queen (Q1) and a BSH tom (T1). This mating produced five kittens, three of which developed a rapidly progressive multicentric LPD at 5 to 7 weeks of age (Litter 1). Both male \( n = 1 \) and female \( n = 2 \) kittens were affected by the disease. The dam of Litter 1 (Q1) had not been previously mated, and it was initially hypothesised that she was more likely to be the source of any defective gene; the tom (T1) had reportedly sired multiple litters (>30) without LPD in any of his progeny. Prior to mating, whole blood from Q1 was tested using an in-clinic rapid ELISA test for the presence of feline leukaemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies (AGEN FeLV/FIV Simplify™, AGEN Biomedical Ltd, Australia). T1’s owner reported that his blood had been previously tested for evidence of FeLV antigen and FIV antibody (Fastest® FeLV-FIV, Vetlab Supplies Limited, England). Blood from two of the LPD-affected kittens (K1 and K2) from Litter 1 were also tested for evidence of FeLV antigen and FIV antibody (AGEN FeLV/FIV Simplify™) after the development of lymphadenopathy.

All matings described in this chapter were natural matings and details of animals used are presented in Table 3.1. In all cases, the intended dams were observed for behavioural signs of oestrus such as calling, lordosis, rolling and ultimately, acceptance of copulation (neck grip, tail deflection and penile intromission). Following observation of signs of oestrus, queens were confined with the intended sire until mating was observed and the queen confirmed pregnant by trans-abdominal ultrasound examination or abdominal palpation.

Prior to mating, queens and toms were blood-tested for the presence of FeLV antigen and FIV antibody using in-clinic rapid ELISA testing (Fastest® FeLV-FIV, (Litter2) or Snap® Combo FeLV Ag/FIV Ab Test Kit (IDEXX Laboratories, United States (Litters 3-7))). With the exception of Litter 2, queens were confined indoors in Palmerston North throughout gestation. Apart from the final two weeks of gestation, during which they were fed a commercial wet diet suitable for feline growth, queens were fed a commercial wet diet suitable for adult feline maintenance throughout gestation. No treatments were
administered during gestation. Kittens resulting from matings were confined indoors with their dam in isolation from other cats from birth until 12 weeks of age. The kittens’ diet from birth to 12 weeks consisted of a combination of the queen’s milk and a commercial wet diet meeting the requirements for feline growth. All diets fed met the relevant nutritional guidelines appropriate for either feline growth or feline maintenance established by the Association of American Feed Control Officials (AAFCO). No vaccinations were administered to the kittens during their first 12 weeks of life.

Approval was obtained from the Massey University Animal Ethics Committee for all procedures outlined here (protocol numbers 09/49, 10/36, 10/120, 12/04).

3.2.1.1 Repeat of Initial Mating – Litter 2

The mating of Q1 and T1 was repeated in September 2009 to determine if animals affected by LPD were consistently produced from this mating. Following confirmation of pregnancy at approximately 3 weeks gestation, Q1 was transported from Christchurch to Massey University in Palmerston North where she was housed indoors and in isolation from other animals for the remainder of gestation.

3.2.1.2 Outcross Mating - Litter 3

In this experiment, Q1 was mated to an unrelated Oriental tom (T2) in July 2010. Based on the results of Litters 1 and 2 and the information available at that time, Q1 was hypothesised to be the most likely source of any genetic defect. If kittens produced by this outcross mating were also affected by LPD, this would support a modified and likely autosomal dominant mode of inheritance carried by Q1.

3.2.1.3 Sibling Matings – Litters 4, 5 and 7

Following Litter 3, the following matings of surviving sibling littermates of kittens affected by LPD were planned:

- K5 (Litter 1) x K8 (Litter 2);
- K6 (Litter 2) x K7 (Litter 2);
- K6 (Litter 2) x K9 (Litter 2);
- K4 (Litter 1) x K8 (Litter 2); and
- K9 (Litter 2) x K11 (Litter 2).
If a sibling mating produced kittens affected by LPD, this would provide support for an autosomal recessive mode of inheritance.

3.2.1.4 BACKCROSS MATING – LITTER 6
To achieve a backcross, it was planned that Q1 be successively mated to both of her surviving male progeny from affected Litters 1 and 2 (K6 and K8).

3.2.1.5 DATA AND SAMPLE COLLECTION
Kittens born as a result of matings were monitored for signs of LPD from birth to 12 weeks of age. A full physical examination was performed on all kittens weekly from birth to 12 weeks of age. Blood was collected by jugular or cephalic venipuncture at 2, 4, 6, 8, 10 and 12 weeks of age for a complete blood count (CBC). For the blood collection performed at 8 weeks of age, kittens were sedated with Zoletil (Zoletil-50 5mg/kg (tiletamine hypochloride and zolazepam hypochloride, Virbac, Australia,) to enable a larger volume of blood to be drawn. Blood collected at 8 weeks was also tested for evidence of FeLV and FIV infection (Snap® Combo FeLV Ag/FIV Ab Test Kit) and used for the various other assays detailed in Chapters 5 and 6. In kittens that developed signs of LPD, blood was also collected for serum biochemical analysis. Fine needle aspirates of enlarged lymph nodes were also taken for cytological analysis following the first appearance of clinical signs of LPD. Bone marrow aspirates from LPD-affected animals were further tested at a commercial laboratory (UC Davis Veterinary Medicine Clinical Laboratory Service, California, United States) for evidence of the presence of intracellular p27 FeLV structural antigen by immunofluorescence antibody (IFA) assays as previously described (Hardy and Zuckerman 1991b).

3.2.2 IDENTIFICATION OF FURTHER HISTORICAL CASES
A retrospective review of available New Zealand diagnostic laboratory (New Zealand Veterinary Pathology (since 2004) and Gribbles Veterinary NZ (since 2006)) and Massey necropsy records (since 1990) was undertaken to identify further similar cases of LPD to those seen in Litter 1. The search criteria used comprised a kitten less than 12 weeks of age with a diagnosis of multicentric LPD or lymphoma.
CHAPTER 3

3.2.3 PEDIGREE RECONSTRUCTIONS

Further information provided by feline breeders and published pedigrees for LPD-affected animals and their ancestors was used to reconstruct a pedigree of LPD-affected animals (Cottonwood Cattery 2007; Harris 2008; Russian Somali Abyssinian and British Cat Club 2008; Ueda 2011; Boreal Cattery 2012; Philip 2012; Power 2012; Heddle 2013; Mayne 2013). As it was provided by breeders, this information could not be verified. Pedigree constructions were performed using GenoPro genealogy software (2011, United States, GenoPro Inc.).

3.2.4 DATA ANALYSIS

3.2.4.1 GOODNESS OF FIT TESTS

The chi-square ($\chi^2$) test can be used as a goodness of fit test to determine whether the frequencies of observed categorical variables in sample data comply with the frequencies hypothesised. A Chi-square test can also be used to examine the significance of the association between two variables, such as the identity of a litters’ sire with the occurrence of LPD in kittens of that sire. However, when expected values are low, an exact test may be more accurate than $\chi^2$. Both $\chi^2$ and exact binomial test calculations were performed for potential mode of inheritance hypotheses for the data, while both $\chi^2$ and Fisher’s exact test calculations were performed for tests of association of the identity of the sire in producing LPD-affected kittens. All calculations were performed using Minitab 15 statistical software (2007, Minitab Inc.) with $p > 0.05$ regarded as statistically significant.

Calculations of the number of animals expected to be affected by a disease under a particular mode of inheritance hypothesis should also allow for the potential ascertainment bias introduced by the methods of sampling only through the identification of affected offspring. In the present investigations, much of the data concerning LPD-affected kittens and cats was collected simply by identifying LPD-affected offspring. In this situation, the strict application of simple $\chi^2$ or exact tests may not be appropriate. Accordingly, the modified goodness of fit approach described by Nicholas (1987) which accounts for this potential ascertainment bias was also applied to the data when investigating potential mode of inheritance hypotheses.
3.2.4.2 Inbreeding Co-efficient

Inbreeding coefficients (F) for animals from Litters 0-7 were calculated based on the available pedigree information according to Wright’s equation (Wright 1922) using Brian Kinghorn’s Pedigree Viewer software (version 6.5b, 2011).

3.3 Results

The results of the various breeding trials, retrospective surveys, and further pedigree information are summarised in the pedigree chart in Figure 3.1 and the table of animals in Table 3.1.

3.3.1 Breeding Trials

All queens and toms used in matings were negative for the presence of FeLV antigen and FIV antibody on blood-testing prior to mating. All kittens tested were also negative for the presence of FeLV antigen and FIV antibody on blood-testing at 8 weeks of age. Additional IFA testing of LPD-affected animals did not reveal evidence of intracellular FeLV antigen within the bone marrow of these animals. These results were interpreted as demonstrating no evidence of FeLV or FIV infection in all cats and kittens tested.

3.3.1.1 Repeat of Initial Mating – Litter 2

The repeat mating of T1 x Q1 produced 6 kittens (Litter 2), which were born without intervention in December 2009. One male kitten (K10) from this litter developed rapidly progressive multicentric LPD at 6 weeks of age and was euthanased at 8 weeks of age. The LPD observed in K10 had similar clinical and pathological findings to those observed in affected animals from Litter 1. A second male animal (K11) from Litter 2 developed a milder form of multicentric LPD with mild enlargement of multiple lymph nodes and severe hepatic disease (biliary fibrosis) at 12 months of age and was euthanased. The LPD observed in K11 had several differences to the clinical and pathological findings observed in affected animals from Litter 1 (discussed in detail in Chapter 4). No other animals from this litter (currently 3.5 years of age) have developed LPD.
CHAPTER 3

Two female animals from Litter 2 were euthanased at 2.5 years (K7 and K9) due to persistent obesity and behavioural issues.

3.3.1.2 OUTCROSS MATING - LITTER 3

The mating of the queen (Q1) to an unrelated Oriental breed tom (T2) produced a litter of 7 kittens born in September 2010 (Litter 3). No kittens from this litter (currently 2.5 years of age) have developed LPD.

One female kitten (K18) from Litter 3 was born with palatoschisis (cleft palate), had difficulty suckling and showed markedly reduced post-natal weight gain compared to littermates. This kitten subsequently developed aspiration pneumonia (diagnosed clinically and confirmed at post mortem) and was euthanased at 3 days of age. A second male kitten (K14) from Litter 3 was killed at 1 year of age in a road traffic accident following re-homing.

3.3.1.3 SIBLING MATINGS – LITTERS 4, 5 AND 7

Only one of the planned sibling matings was successful. After 10 months of attempted matings, the mating of K6 (Litter 2) x K9 (Litter 2) produced a single female kitten (K36), born in April 2012. This kitten (currently 12 months of age) has not developed LPD.

Sibling matings were undertaken between K5 and K8 on multiple occasions over 6 months but were not successful. Behavioural signs of oestrus and copulation were observed but K5 failed to become pregnant to K8. On two separate occasions, K5 was instead successfully mated by unknown toms (T3 and T4) after the queen escaped confinement. The first of these unintended matings produced 5 kittens (Litter 4, born September 2010), 4 of which (K19, K20, K21 and K22) were of low birthweight (<80g) and died in the immediate perinatal period. The sole surviving kitten (K23) from Litter 4 did not develop LPD before being killed in a road traffic accident at 18 months following re-homing. The second mating produced 3 kittens (Litter 5, born February 2011), one of which (K24) was of low birthweight (<80g) and died in the immediate perinatal period. The two surviving kittens (K25 and K26, currently 2 years of age) have not developed LPD.
Sibling matings were also undertaken between K6 and K7 on multiple occasions over 10 months but were not successful. Behavioural signs of oestrus but not copulation were observed, and K7 failed to become pregnant.

It was also not possible to perform the proposed sibling matings between K4 (Litter 1) and K8 (Litter 2), and K9 (Litter 2) x and K11 (Litter 2). Prior to the initiation of mating, K4 was euthanased at 15 months of age for progressive urinary and faecal incontinence that was non-responsive to treatment. Similarly, K11 was euthanased prior to initiation of mating after he developed severe progressive liver disease and mild LPD at 12 months of age.

3.3.1.4 BACKCROSS MATING – LITTER 6

The mating of the Q1 to one of her surviving male progeny from Litter 2 (K8) produced a litter of 5 kittens born in March 2011 (Litter 6). No kittens from this litter (currently 2 years of age) have developed LPD.

One female kitten from Litter 6 (K29) was euthanased at 2 weeks of age with mild spina bifida and persistent clinical evidence of neurological disease. A post mortem exam revealed marked hydrocephalus. One male kitten (K31) from Litter 6 was killed at 12 months of age in a road traffic accident following re-homing.

3.3.2 IDENTIFICATION OF FURTHER HISTORICAL CASES

A retrospective review of available records identified three additional cases of LPD in kittens with features similar to those seen in LPD-affected animals from Litters 1 and 2. The three additional animals identified were BSH kittens from a single litter (Litter 0) of 4 kittens born approximately 16 months before Litter 1. According to the breeder of Litter 0, the fourth kitten from this litter (K35) reportedly developed feline infectious peritonitis at approximately 11 months of age and was euthanased. The sire and dam of this Litter 0 (Q2 and T5) were different animals to the parents of Litters 1 and 2, and were also reportedly unaffected by LPD. The parents of all three affected litters (Q2 and T5 (Litter 0) and Q1 and T1 (Litters 1 and 2)) were all from the same geographical area of New Zealand (Christchurch/Rangiora region).
3.3.3. PEDIGREE RECONSTRUCTIONS

The use of further information from breeders and pedigree analyses allowed the construction of a pedigree of LPD-affected animals. This information is summarised in the pedigree chart in Figure 3.1. While incomplete and unverifiable, this further information indicates the presence of multiple consanguineous matings (F>1.56%, second cousin or closer) (Bittles and Black 2010) present throughout the pedigree and indicated by a double line. In particular, T1 is involved as both the maternal great grand-sire and paternal grand-sire, and another queen (Q3) involved as both the maternal great grand-dam and paternal grand-dam of the LPD-affected Litter 0. In addition, the sire and dam of affected Litters 1 and 2 (T1 and Q1) share at least one common ancestor (T6). Finally, and contrary to the initial information provided by his breeder, the original tom (T1) is involved in the pedigree of all LPD-affected animals, either directly, as the sire of Litters 1 and 2, or indirectly, as both the maternal great grand-sire and the paternal grandsire of Litter 0.

3.3.4 DATA ANALYSIS

Litters 0, 1 and 2 all produced kittens affected by LPD, while Litters 3 to 7 did not. Litters 0, 1 and 2 produced a total of 15 kittens, 7 of which (3 male and 4 female) developed LPD at 5 to 7 weeks of age, giving an observed incidence of 46.6%. One further animal (K11, Litter 2) appeared to develop a milder form of LPD with concurrent liver disease at 12 months of age (discussed in detail in Chapter 4). For the purposes of statistical analysis, K11 has not been included as a LPD-affected animal as his clinical presentation and pathology were significantly different to the other 7 affected animals. The parents of the three affected litters were T1 and Q1 (Litters 1 and 2), and Q2 and T5 (Litter 0). Q1 also produced two further litters with two toms other than T1 (T2 and K8, Litters 3 and 6) with 0/7 and 0/5 animals affected by LPD respectively, compared with 4/11 (36.4%) affected kittens in her two litters sired by T1. All LPD affected animals produced by Q1 were sired by T1.

Based on these data, either simple autosomal recessive or modified autosomal dominant (variable expression or incomplete penetrance) were considered as possible modes of inheritance.
Figure 3.1: Extended pedigree of LPD-affected kittens and cats

Animal’s pedigree or study names are located immediately below or within the animal’s symbol. Where deceased, the animal’s age at death is immediately above the animal’s symbol.
Table 3.1: Kittens and cats included in mode of inheritance studies

DoB = date of birth, F = female, M = male, U = unknown, BSH = British Short Hair, DSH = Domestic Short Hair, L = long (full) tail, S = stumpy (partial) tail, R = rumpy (no tail), LH = long-haired, † = deceased, K = LPD affected

<table>
<thead>
<tr>
<th>Animal</th>
<th>Litter</th>
<th>Parents</th>
<th>DoB</th>
<th>Age at death</th>
<th>Breed</th>
<th>Sex</th>
<th>Colour</th>
<th>Tail</th>
<th>LPD (age developed)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>1</td>
<td>Q1 x T1</td>
<td>22/12/08</td>
<td>7 weeks</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Tortoiseshell</td>
<td>R</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>1</td>
<td>Q1 x T1</td>
<td>22/12/06</td>
<td>9 weeks</td>
<td>BSH</td>
<td>F</td>
<td>Blue</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>U</td>
<td></td>
<td>11/04/07</td>
<td></td>
<td>Oriental</td>
<td>M</td>
<td>Grey + white</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>U</td>
<td>Presumed DSH</td>
<td></td>
<td></td>
<td>Grey + white</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>U</td>
<td>Presumed DSH</td>
<td></td>
<td></td>
<td>Grey + white</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Blue</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>U</td>
<td>BSH</td>
<td></td>
<td></td>
<td></td>
<td>F</td>
<td>Grey Tabby</td>
<td>S</td>
<td>Yes (6 weeks)</td>
<td></td>
</tr>
<tr>
<td>K1†</td>
<td>1</td>
<td>Q1 x T1</td>
<td>27/01/09</td>
<td>7 weeks</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Grey Tabby</td>
<td>S</td>
<td>Yes (6 weeks)</td>
<td>Urinary + faecal incontinence, megacolon - euthanased</td>
</tr>
<tr>
<td>K2†</td>
<td>1</td>
<td>Q1 x T1</td>
<td>27/01/09</td>
<td>9 weeks</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Grey Tabby</td>
<td>S</td>
<td>Yes (7 weeks)</td>
<td></td>
</tr>
<tr>
<td>K3†</td>
<td>1</td>
<td>Q1 x T1</td>
<td>27/01/09</td>
<td>8 weeks</td>
<td>BSH x Manx</td>
<td>M</td>
<td>Grey Tabby</td>
<td>S</td>
<td>Yes (7 weeks)</td>
<td></td>
</tr>
<tr>
<td>K4†</td>
<td>1</td>
<td>Q1 x T1</td>
<td>27/01/09</td>
<td>1 year</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Grey Tabby</td>
<td>S</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K5</td>
<td>1</td>
<td>Q1 x T1</td>
<td>27/01/09</td>
<td></td>
<td>BSH x Manx</td>
<td>F</td>
<td>Grey Tabby</td>
<td>S</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K6</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td>2.5 years</td>
<td>BSH x Manx</td>
<td>M</td>
<td>Red Tabby</td>
<td>R</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K7†</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td>2.5 years</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Chocolate</td>
<td>L</td>
<td>No</td>
<td>Obesiy + behavioural issues – euthanased</td>
</tr>
<tr>
<td>K8</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td></td>
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<td>M</td>
<td>Black</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K9†</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td>2.5 years</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Lilac</td>
<td>R</td>
<td>No</td>
<td>Obesity + behavioural issues – euthanased</td>
</tr>
<tr>
<td>K10†</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td>8 weeks</td>
<td>BSH x Manx</td>
<td>M</td>
<td>Cream</td>
<td>R</td>
<td>Yes (6 weeks)</td>
<td></td>
</tr>
<tr>
<td>K11†</td>
<td>2</td>
<td>Q1 x T1</td>
<td>01/12/09</td>
<td>12 months</td>
<td>BSH x Manx</td>
<td>M</td>
<td>Cream</td>
<td>L</td>
<td>Yes (12 months)</td>
<td>+ marked biliary fibrosis</td>
</tr>
<tr>
<td>K12</td>
<td>3</td>
<td>Q1 x T2</td>
<td>04/09/10</td>
<td></td>
<td>BSH/Manx x Oriental</td>
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<td>Tortoiseshell</td>
<td>(LH)</td>
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<td>K13</td>
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<td>Q1 x T2</td>
<td>04/09/10</td>
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<td>BSH/Manx x Oriental</td>
<td>M</td>
<td>Lilac (LH)</td>
<td>L</td>
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<td>04/09/10</td>
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<td>BSH/Manx x Oriental</td>
<td>M</td>
<td>Grey + white</td>
<td>(LH)</td>
<td>L</td>
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<td>3</td>
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<td>04/09/10</td>
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<td>BSH/Manx x Oriental</td>
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<td>(LH)</td>
<td>L</td>
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<td>04/09/10</td>
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<td>04/09/10</td>
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<td>04/09/10</td>
<td>3 days</td>
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<td>Grey + white</td>
<td>L</td>
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<td>Litter</td>
<td>Parents</td>
<td>DoB</td>
<td>Age at death</td>
<td>Breed</td>
<td>Sex</td>
<td>Colour</td>
<td>Tail</td>
<td>LPD (age developed)</td>
<td>Other</td>
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<td>4</td>
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<td>16/09/10</td>
<td>Perinatal</td>
<td>BSH/Manx x DSH</td>
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<td>16/09/10</td>
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<td>BSH/Manx x DSH</td>
<td>F</td>
<td>Black</td>
<td>L</td>
<td>No</td>
<td>Low birth weight + dystocia – died</td>
</tr>
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<td>K5 x T3</td>
<td>16/09/10</td>
<td>Perinatal</td>
<td>BSH/Manx x DSH</td>
<td>M</td>
<td>Red tabby</td>
<td>R</td>
<td>No</td>
<td>Low birth weight – died</td>
</tr>
<tr>
<td>K22†</td>
<td>4</td>
<td>K5 x T3</td>
<td>16/09/10</td>
<td>Perinatal</td>
<td>BSH/Manx x DSH</td>
<td>F</td>
<td>Grey tabby</td>
<td>L</td>
<td>No</td>
<td>Low birth weight + dystocia- died</td>
</tr>
<tr>
<td>K23†</td>
<td>4</td>
<td>K5 x T3</td>
<td>16/09/10</td>
<td>18 months</td>
<td>BSH/Manx x DSH</td>
<td>M</td>
<td>Red tabby</td>
<td>S</td>
<td>No</td>
<td>Road traffic accident – found dead</td>
</tr>
<tr>
<td>K24†</td>
<td>5</td>
<td>K5 x T4</td>
<td>19/02/11</td>
<td>3 days</td>
<td>BSH/Manx x DSH</td>
<td>F</td>
<td>Chocolate</td>
<td>S</td>
<td>No</td>
<td>Low birth weight – died</td>
</tr>
<tr>
<td>K25</td>
<td>5</td>
<td>K5 x T4</td>
<td>19/02/11</td>
<td></td>
<td>BSH/Manx x DSH</td>
<td>M</td>
<td>Red tabby</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K26</td>
<td>5</td>
<td>K5 x T4</td>
<td>19/02/11</td>
<td></td>
<td>BSH/Manx x DSH</td>
<td>F</td>
<td>Grey tabby</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K27</td>
<td>6</td>
<td>Q1 x K8</td>
<td>12/03/11</td>
<td></td>
<td>BSH x Manx</td>
<td>M</td>
<td>Black</td>
<td>S</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K28</td>
<td>6</td>
<td>Q1 x K8</td>
<td>12/03/11</td>
<td></td>
<td>BSH x Manx</td>
<td>M</td>
<td>Red tabby</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K29†</td>
<td>6</td>
<td>Q1 x K8</td>
<td>12/03/11</td>
<td>2 weeks</td>
<td>BSH x Manx</td>
<td>F</td>
<td>Tortoiseshell</td>
<td>R</td>
<td>No</td>
<td>Spina bifida + hydrocephalus – euthanased</td>
</tr>
<tr>
<td>K30</td>
<td>6</td>
<td>Q1 x K8</td>
<td>12/03/11</td>
<td></td>
<td>BSH x Manx</td>
<td>M</td>
<td>Red tabby</td>
<td>R</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>K31†</td>
<td>Q1 x K8</td>
<td>12/03/11</td>
<td>12 months</td>
<td></td>
<td>BSH x Manx</td>
<td>M</td>
<td>Grey tabby</td>
<td>R</td>
<td>No</td>
<td>Road traffic accident – found dead</td>
</tr>
<tr>
<td>K32†</td>
<td>0</td>
<td>Q2xT5</td>
<td>U/10/07</td>
<td>8 weeks</td>
<td>BSH</td>
<td>M</td>
<td>U</td>
<td>Y</td>
<td>Yes (5 weeks)</td>
<td></td>
</tr>
<tr>
<td>K33†</td>
<td>0</td>
<td>Q2xT5</td>
<td>U/10/07</td>
<td>8 weeks</td>
<td>BSH</td>
<td>F</td>
<td>U</td>
<td>Y</td>
<td>Yes (6 weeks)</td>
<td></td>
</tr>
<tr>
<td>K34†</td>
<td>0</td>
<td>Q2xT5</td>
<td>U/10/07</td>
<td>10 weeks</td>
<td>BSH</td>
<td>F</td>
<td>U</td>
<td>Y</td>
<td>Yes (6 weeks)</td>
<td></td>
</tr>
<tr>
<td>K35†</td>
<td>0</td>
<td>Q2xT5</td>
<td>U/10/07</td>
<td>11 months</td>
<td>BSH</td>
<td>U</td>
<td>U</td>
<td>Y</td>
<td>No</td>
<td>Suspected feline infectious peritonitis – euthanased</td>
</tr>
<tr>
<td>K36</td>
<td>7</td>
<td>K6 x K9</td>
<td>27/04/12</td>
<td></td>
<td>BSH x Manx</td>
<td>F</td>
<td>Grey tabby</td>
<td>S</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
For the hypothesis of a simple autosomal recessive mode of inheritance with all parents of Litters 0, 1 and 2 being heterozygotes (LPD carriers), the null hypothesis (H₀) is that the expected incidence of LPD-affected offspring over Litters 0, 1 and 2 (25%) is not significantly different to the incidence of LPD actually observed (46.6%). Chi-square (p=0.053) gives some support for an autosomal recessive mode of inheritance, but Fisher’s exact test (p= 0.039) indicates that the data do not support an autosomal recessive mode of inheritance for LPD. However, if the ascertainment bias is accounted for using the modified goodness of fit approach proposed by Nicholas (1987), the observed incidence of LPD decreases to 42.9% (range 19.4% to 56.1%). As this range includes 25% (the proportion of expected animals with LPD if the mode of inheritance was autosomal recessive), this indicates that the data are potentially consistent with an autosomal recessive mode of inheritance.

The alternate hypothesis is of a modified autosomal dominant mode of inheritance with either variable clinical expression of the LPD phenotype or incomplete penetrance of the LPD genotype. Under this hypothesis, either of the parents of Litters 1, 2 and 0 would be a heterozygote not displaying the LPD phenotype, and the null hypothesis (H₀) is that the expected incidence of animals affected by LPD over Litters 0, 1 and 2 is <50% and is not significantly different to the incidence of LPD actually observed (46.6%). As the expected variability in the expression or incompleteness in disease penetrance is an unknown value, the calculations needed to apply goodness of fit tests are difficult to perform. For example, if an autosomal dominant mode of inheritance with 90% expression or penetrance of LPD in the heterozygous genotype is assumed, both Chi-square (p=0.897) and exact tests (p=0.201) provide strong support for the hypothesis. If an autosomal dominant mode of inheritance with 70% expression or penetrance of LPD with the heterozygous genotype is assumed, both Chi-square (p=0.343) and exact tests (p=0.132) also support the hypothesis. However, if an autosomal dominant mode of inheritance with 50% expression or penetrance of LPD with the heterozygous genotype is assumed, Chi-square (p=0.053) and exact tests (p=0.039) give identical goodness of fit test values to those for a simple autosomal recessive mode of inheritance, with Chi-square providing only weak support and the exact test not supporting the hypothesis.

If the ascertainment bias is accounted for using the modified goodness of fit approach of Nicholas (1987), the observed incidence of LPD decreases to 42.9% (range 19.4% to 56.1%). As this range includes the proportions of expected animals with LPD if the mode
of inheritance is autosomal dominant with 90% expression (45%), 70% expression (35%) and 50% expression (25%), this indicates that the data here are potentially consistent with any of these modes of inheritance.

In addition to the LPD-affected Litters 1 and 2 with T1 as sire, Q1 also produced two further litters with different sires (Litter 3 with T2, and Litter 6 with K8). None of the 12 kittens produced from these matings with sires other than T1 developed LPD. These results indicate that the identity of the sire (T1) is likely to be associated with the presence of LPD in Q1’s offspring (Chi-square p= 0.022; Fisher’s exact test p=0.037). Similar tests of association of the identity of the queen with the presence of LPD in offspring cannot be performed due to unavailable information about LPD in T1’s offspring from matings with other queens.

Inbreeding coefficients (F) calculated for animals from Litters 0 to 7 according to Wright’s equation (Wright 1922) are set out in Table 3.2 below.

### Table 3.2: Inbreeding coefficients (F) for Litters 0-7 based on available pedigree information

<table>
<thead>
<tr>
<th>Animals</th>
<th>Litter</th>
<th>LPD Affected?</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1-K5</td>
<td>1</td>
<td>Yes (3/5)</td>
<td>0.037</td>
</tr>
<tr>
<td>K6-K11</td>
<td>2</td>
<td>Yes (1/6)</td>
<td>0.037</td>
</tr>
<tr>
<td>K12-18</td>
<td>3</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>K19-23</td>
<td>4</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>K24-K26</td>
<td>5</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>K27-K31</td>
<td>6</td>
<td>No</td>
<td>25.0</td>
</tr>
<tr>
<td>K32-K35</td>
<td>0</td>
<td>Yes (3/4)</td>
<td>13.464</td>
</tr>
<tr>
<td>K36</td>
<td>7</td>
<td>No</td>
<td>25.0</td>
</tr>
</tbody>
</table>

### 3.4 DISCUSSION

In the absence of obvious non-genetic causes of LPD, the results of the various matings and further retrospective survey and pedigree information together support an inherited basis for LPD in BSH kittens, but do not yet allow definitive identification of the mode of inheritance. The results provide support for both simple autosomal recessive and modified autosomal dominant (with variable expression or incomplete penetrance).
modes of inheritance. Further data are needed to confirm the likely mode of inheritance of LPD in BSH kittens.

In the majority of people with autoimmune lymphoproliferative syndrome (ALPS), the disease is caused by Fas gene mutations and is inherited in an autosomal dominant manner, but with a high degree of variability in expression of the disease phenotype (Infante et al. 1998; Jackson et al. 1999; Bleesing et al. 2001a). The disease in people is occasionally caused by mutations in the Fas ligand (FasL) genes, which also appear to be inherited in an autosomal dominant manner in most cases (Wu et al. 1996; Bi et al. 2007). Although ALPS in people has an autosomal dominant mode of inheritance in most cases, the degree of clinical expression of the ALPS genotype reportedly ranges from approximately 30% through to 90% in different families (Le Deist et al. 1996; Sneller et al. 1997; Infante et al. 1998; Jackson et al. 1999; Rieux-Laucat et al. 1999; Vaishnaw et al. 1999). A significant proportion of people with the ALPS genotype therefore do not show overt signs of disease such as lymphoproliferation or autoimmunity. There is evidence for an autosomal recessive mode of inheritance in a small minority of cases of ALPS in people. For example, ALPS patients with homozygous mutations in either Fas (van der Burg et al. 2000) or FasL (Del-Rey et al. 2006) are occasionally reported, suggesting an autosomal recessive mode of inheritance; these patients generally show a severe disease phenotype. Rare cases of ALPS due to underlying caspase 10 (CASP10) gene defects are also reported, with evidence for both autosomal recessive and autosomal dominant modes of inheritance (Wang et al. 1999). In approximately one-third of ALPS patients both the underlying genetic defect and mode of inheritance are unknown (Oliveira et al. 2010).

In contrast to ALPS in people, LPD in mice due to either lpr or gld gene mutations is inherited in an autosomal recessive manner (Cohen and Eisenberg 1991). Mice homozygous for the lpr mutation have defects in the Fas gene (Watanabe-Fukunaga et al. 1992), while mice homozygous for the gld mutation have defects in the FasL gene (Takahashi et al. 1994). Variability in clinical expression of the disease in mice appears to be associated with differences in the genetic background of the mouse (Cohen and Eisenberg 1991).
The results of the present study support an autosomal recessive mode of inheritance for LPD in cats. If so, this would be similar to the mode of inheritance seen in mouse models of ALPS (*lpr* and *gld* mice) and in rare cases of the disease in humans. While the results of the basic goodness of fit tests provide only limited support for this mode of inheritance, the data show a better fit with an autosomal recessive mode of inheritance if the ascertainment bias likely to be present here is addressed by a modified test. Ascertainment bias is the sampling bias which often occurs in the initial investigations of a new disease where cases are identified simply by identifying the presence of disease in offspring and subsequently calculating the proportion of disease-affected offspring. This method of sampling is commonly used in the initial investigations of a new disease, and was the method predominantly used to identify LPD-affected animals in the present study. While pragmatic, this method potentially overestimates the observed proportion of animals affected by disease, as litters with multiple affected progeny are more likely to be identified than those with only a single affected offspring, and the results of heterozygous carrier to heterozygous carrier matings that, by chance, do not produce offspring affected by disease are excluded from the results. This tends to result in a higher apparent incidence of disease than that actually present, so that even where a disease is inherited by an autosomal recessive mode, the apparent incidence of disease reported in initial investigations is often greater than the expected 25% (Nicholas 1987; Nicholas 2010a). In such cases, application of simple Chi-square or exact tests will not account for this potential ascertainment bias, and a modified approach may be a more appropriate measure of the true goodness of fit of the data to a particular mode of inheritance hypothesis. The modified goodness of fit approach described by Nicholas (1987) attempts to account for this potential ascertainment bias, and this approach was also applied to the data when investigating potential mode of inheritance hypotheses.

It is likely that a degree of ascertainment bias is present in the data here, where cases of LPD were identified by the presence of LPD in at least one member of a litter and were highly reliant on notification of cases by the kittens’ owners to veterinarians and to veterinary diagnostic laboratories by the kitten’s veterinarian. At least one of the putative carrier animals here (T1) has reportedly been mated multiple times (>30) to many different queens. It is therefore possible that a proportion of T1’s litters which were unaffected by LPD in fact involved carrier to carrier matings, and the results should have been included in the study analysis. The inclusion of any such unaffected litters...
would have the effect of decreasing the apparent high incidence of LPD reported here (46.6%), bringing it closer to the 25% expected with an autosomal recessive mode of inheritance.

An autosomal recessive mode of inheritance for LPD is also supported by several features of the pedigree more generally. The fact that the occurrence of LPD appears to skip generations in the pedigree, that both parents of all LPD-affected animals appeared clinically unaffected by the disease, and that approximately equal numbers of male \(n = 3\) and female \(n = 4\) animals are affected are features more often seen with autosomal recessive diseases than diseases with other modes of inheritance (Pasternak 2005; Nicholas 2010a). The presence of inbreeding or consanguinity in the pedigrees of several affected kittens and of at least one common ancestor in the pedigrees of all affected animals also further supports an autosomal recessive mode of inheritance.

Inbreeding occurs when a mating involves parents which are more closely related than any two animals drawn from the population at random (Klug et al. 2012). Over time, inbreeding is likely to increase the frequency of homozygous genotypes, which in turn increases the likelihood that recessive genes will be observed or “unmasked” in progeny, which may be seen clinically as disease (Nicholas 2010b; Leroy 2011; Klug et al. 2012).

While assessment of the amount of inbreeding associated with increased risk of inherited disease is complex and dependant on multiple factors, matings between animals as or more closely related as second cousins (\(F > 1.56\%\)) are often regarded as “consanguineous” (Bittles and Black 2010), while other authors regard \(F\) values greater than 10% as indicative of a significant level of inbreeding (Calboli et al. 2008; Mucha et al. 2011; Billing et al. 2012). Information provided by pedigree reconstructions and retrospective surveys of records here indicated the presence of multiple consanguineous matings within the pedigree of LPD affected animals (see Figure 3.1) and high \(F\) values in one of the three affected litters of kittens >10% (Litter 0, \(F = 13.464\%\)). While \(F\) values for LPD-affected Litters 1 and 2 were less than 1.56% (\(F = 0.037\%\)), they are higher than the zero value expected for matings of unrelated or distantly related animals. Further, the \(F\)-values calculated in this study likely underestimate the true values of some animals and the level of inbreeding present in the pedigree, as in many cases complete pedigree information was not available (Leroy 2011). Information from the cat breeding industry suggests that inbreeding (e.g. parent-offspring, sibling, or half-sibling matings) is a relatively common practice in pure-bred...
cats (pers. comm. Daniel Wheeler, Debbie Chesterfield, Iris Baker (Robinson 1991; Gunn-Moore et al. 2008). It is therefore possible that a higher level of consanguinity than that reported is actually present in the pedigree here, and specifically within LPD-affected Litters 1 and 2. If so, this would provide further support for an autosomal recessive mode of inheritance of LPD in affected BSH kittens.

Had any of the proposed sibling matings of unaffected littermates of LPD-affected kittens produced LPD-affected kittens, this would have provided further support for an autosomal recessive mode of inheritance. However, of the proposed sibling matings, only one was successful (Litter 7). As only one kitten (K36) was produced by this mating, animal numbers are too low to draw inferences regarding the mode of inheritance.

The results of the present study also provide support for a modified autosomal dominant mode of inheritance, such as autosomal dominant inheritance with variable clinical expression or penetrance of the genotype. If so, this would be similar to the mode of inheritance seen in human cases of ALPS. The results of both the standard goodness of fit tests and the modified approach to account for ascertainment bias support this mode of inheritance. In diseases with variability in clinical expression or incomplete penetrance of the genotype, initial assessments of pedigree information may lead to the erroneous conclusions about the mode of inheritance. An autosomal recessive mode of inheritance is often initially suspected in these cases, as the disease appears to skip generations and the parents of affected patients do not always show the disease phenotype themselves (Pasternak 2005). As there may be wide variability in the degree of clinical expression or genotype penetrance seen with this mode of inheritance, the proportions of LPD-affected animals expected as a result of any mating involving a carrier animal are difficult to predict. However, assuming a penetrance of 70% (the approximate average proportion of ALPS genotype which show overt clinical signs of lymphoproliferation and/or autoimmunity over multiple studies) (Fisher et al. 1995; Le Deist et al. 1996; Sneller et al. 1997; Infante et al. 1998; Jackson et al. 1999; Rieux-Laucat et al. 1999; Vaishnaw et al. 1999; Poppema et al. 2004), the expected proportion of LPD-affected animals from all affected litters if one parent of each litter had a heterozygous dominant genotype (Litters 0, 1 and 2) would be 35%, which is similar to the LPD incidence of 46.6% actually observed. With higher penetrance of clinical expression or genotype (e.g. 90%), support for this mode is even stronger.
Further support for an autosomal dominant mode of inheritance with variable clinical expression may be provided by the fact that the parents of affected litters are unaffected by overt clinical signs of disease, a finding not uncommon in pedigrees of human ALPS patients (Infante et al. 1998). However, with modified autosomal dominant inheritance it would be expected that at least some of the many litters (reportedly >30) sired by T1 with queens other than Q1 and Q2 would have contained LPD-affected kittens. While the reported absence of any such kittens argues against this mode of inheritance, the reliability of breeder-provided information regarding disease in progeny may be questionable.

The results of the present study do not support simple autosomal dominant, X-linked recessive or X-linked dominant modes of inheritance. The absence of LPD in any parents of affected litters indicate that a simple autosomal or X-linked dominant mode of inheritance is very unlikely (Pasternak 2005; Nicholas 2010a). In addition, the presence of LPD in several females \( n = 4 \) as well as males \( n = 3 \) indicate that an X-linked recessive mode of inheritance is also unlikely. If the disease were inherited in an X-linked recessive manner, it would be expected that matings between 2 clinically normal animals (normal male and carrier female, for example the parents of Litters 0, 1 and 2) would produce only male kittens affected by LPD; female offspring could be carriers but would not be clinically affected by disease. This was not the case, where kittens of both sexes resulting from these matings were affected by LPD. In the unlikely event that both parents were carrying the defective (X-linked) gene, both male and female offspring could be affected by disease, but the sires of all affected litters would also be expected to show clinical signs of LPD, which they did not (Pasternak 2005; Nicholas 2010a). More complex modes of inheritance, such as multifactorial inheritance are possible, but are beyond the scope of the present study (Lane 2009).

It was initially hypothesised that Q1 was the likely source of any genetic defect, based on the fact that Q1 had not been mated prior to Litter 1 and that T1 had reportedly been mated numerous times with no reported LPD in his progeny. However, following the subsequent identification of Litter 0, it appeared that the assumptions upon which this hypothesis were based were incorrect, and that LPD had occurred previously in the indirect progeny of T1. In addition, the pattern of LPD in Q1’s litters suggest that the identity of T1 as sire was likely associated with the occurrence of LPD in offspring. It was
therefore hoped to undertake further matings of T1 as a putative carrier animal (for example, to his daughters to test the hypothesis of an autosomal recessive mode of inheritance, and to an unrelated queen to test the hypothesis of a modified autosomal dominant mode of inheritance). The ability to use a putative male carrier animal as the sire of multiple litters in a single season would have dramatically increased the number of kittens produced and therefore the amount of data generated. Unfortunately, T1’s breeder would not allow him to be used for further matings after Litter 2. T1’s role in the LPD therefore remains speculative.

Several matings of unaffected sibling littermates of LPD-affected animals were also planned to test the hypothesis of an autosomal recessive mode of inheritance. Unfortunately, only one of these proposed sibling matings was successful (Litter 7). Difficulties in finding suitable long-term housing for the sibling littermates of affected kittens meant it was necessary to permanently re-home several kittens and cats into private households during the study, including K4, K5 and K8. The planned mating of K4 and K11 could not be completed as both animals were euthanased prior to the onset of sexual maturity. K4 developed signs of progressive urinary and faecal incontinence at 12 months of age and her owner elected euthanasia, while K11 developed mild LPD and severe and progressive idiopathic liver disease at 12 months of age which required euthanasia. Repeated attempts to mate K5 and K8 were unsuccessful after K5 twice escaped confinement over periods of oestrus resulting in the unintended but successful matings by unknown toms (T4 and T5). K6 and K7 were housed together for 8 months with K7 showing signs of regular behavioural oestrus over this period but K7 failed to become pregnant to K6. Attempts to mate K6 and K9 were finally successful after 8 months, but produced only a single kitten (K36, Litter 7). While it is unknown why the mating of K6 and K7 was not successful, it is possible that housing the cats indoors under restricted light conditions for parts of the mating period may have adversely affected K7’s reproductive performance (Tsutsui et al. 2004; Faya et al. 2011). It is unknown why Litter 7 produced only a single kitten. However, the mating of two short tailed sibling animals (K6 and K9) may have been a factor here, as it would be expected that 25% of kittens resulting from this mating would carry the lethal short-tailed homozygous (Manx) genotype and would likely be spontaneously aborted (Robinson 1991).
It is possible that there have been additional BSH litters where one or more kittens were affected by LPD but which have not been reported. In this context, the anecdotal reports of a LPD in BSH cats in Australia are interesting and potentially supportive of an inherited basis. The existence of further unreported cases may alter the observed incidence of disease and provide more or less support for a particular mode of inheritance. There are several possible reasons why cases of LPD may have occurred but not been reported. The disease affects kittens at an age (less than 8 weeks) when they are generally still with their breeder. A breeder may have many incentives not to report or further investigate disease in affected kittens. The loss of a single kitten in a litter is not necessarily unexpected and may not be investigated further. The cost of a diagnostic workup and necropsy by a veterinarian can be high and may be prohibitive. For some breeders, the potential negative consequences on their reputation and future financial return from stud animals that flow from identification of a suspected genetic disease in the stud’s progeny may also provide an incentive not to report such cases. Furthermore, unless a full clinical examination of affected kittens was performed, the presence of enlarged lymph nodes may not have been detected, particularly by less experienced breeders. Such animals may present only with non-specific signs of reduced weight gain and lethargy or simply be found dead (as was the case with one animal in Litter 1). Finally, even if affected animals are examined by a veterinarian, unless further biopsy of affected lymph nodes or necropsy examination was performed, these cases would not have been identified by the retrospective studies undertaken here. It is therefore possible that other cases of LPD have occurred but were not identified and included in the studies here.

There was no evidence that LPD-affected animals were infected with either FeLV or FIV, both of which are known causes of LPD in cats. No animal tested showed evidence of the presence of either virus and they are therefore considered unlikely to have contributed to the development of LPD (discussed further in Chapter 4). The possibility that a novel infectious agent caused the development of LPD cannot be completely excluded, but is considered unlikely for several reasons. None of the parents or littermates of the affected litters showed any reported signs of similar illness at any stage. In addition, while LPD-affected Litters 1 and 2 had the same parents, both the gestation period and the kittens’ first 12 weeks of life occurred under different environmental conditions, with the gestation and rearing of Litter 2 in isolation from all
other animals. Furthermore, it is likely that any infectious agent would have also affected kittens of breeds other than the BSH; to date, this has not been reported.

It is acknowledged that the different form of disease observed in K11, the adult cat affected by LPD (mild lymphadenopathy, biliary fibrosis, a much later age at presentation, discussed in detail in Chapter 3), may represent a variation of the clinical expression of the LPD phenotype seen in affected kittens. If so, K11 should have been included as an affected animal in the statistical analyses, which was not done. However, if this were the case, it would have been expected that other animals from affected litters (Litters 0, 1 and 2) would have also developed similar variations in clinical signs to those seen in K11. To date they have not. K11 was therefore excluded from consideration as an affected animal for the purposes of the present study, however, this approach may require amendment depending on the occurrence and clinical presentation of any future LPD cases.

The results of the studies reported in this chapter therefore suggest an inherited LPD with either autosomal recessive inheritance or autosomal dominant inheritance with incomplete penetrance or variable expression. However, the conclusions that can be drawn from the data here are limited by the relatively low numbers of animals affected by LPD, the inability to perform several of the proposed matings (sibling matings and further matings involving T1), and the fact that the complete pedigree data was unavailable. It is unknown if any genetic defect in affected kittens arose recently de novo (for example, in T1) or has been transmitted through the BSH pedigree for a longer period of time. If a similar form of LPD is also confirmed in BSH kittens in Australia, this would provide further support for an inherited basis for the disease and may help confirm the likely mode of inheritance. It is hoped that the identification of further cases of LPD will further clarify and characterise the syndrome, and definitively identify its likely mode of inheritance.

3.5 SUMMARY

The results of planned matings, retrospective surveys and further pedigree information support an inherited basis for LPD in BSH kittens, but do not yet allow definitive
identification of the mode of inheritance. The results do not support a simple autosomal dominant, X-linked recessive or X-linked dominant mode of inheritance. The results provide support for either a simple autosomal recessive or modified autosomal dominant (with variable clinical expression or incomplete penetrance) mode of inheritance.

3.6 REFERENCES


CLINICAL PRESENTATION AND PATHOLOGY

4.1 INTRODUCTION

A definitive diagnosis of the lymphoproliferative disease (LPD) that developed in British shorthair (BSH) kittens was not possible from the preliminary clinical findings and pathology. While multicentric lymphoma was considered most likely, histological features of the disease in some kittens were more consistent with a reactive lymphadenopathy. Furthermore, the presence of features suggesting an inherited basis for disease including the young age of kittens at presentation and the development of disease in multiple littermates and related animals (discussed in Chapter 3), as well as the massive enlargement of all lymph nodes throughout the body, would be considered unusual for feline lymphoma. A more complete definition of LPD in affected BSH kittens and cats was therefore required to enable a definitive diagnosis of the disease, to provide a rational basis for further investigations, and to help identify any future cases.
The aim of the investigations described in this chapter was to define the clinical and pathological features of the LPD in BSH kittens and cats. The main clinical features, haematological abnormalities, gross pathology and histopathology present in LPD-affected BSH kittens and cats are described and interpreted, and the implications of the results considered. The LPD in BSH kittens and cats is also compared to other LPDs in humans and cats that share common features. In particular, the feline LPD in BSH kittens and cats is compared to autoimmune lymphoproliferative syndrome (ALPS), a rare inherited disease which causes marked lymphadenopathy and autoimmune manifestations in people (Sneller et al. 1992; Sneller et al. 1997; Rao and Straus 2006). The results of the studies in this chapter form a basis for the additional studies of the disease described in subsequent chapters.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS USED

Kittens from Litters 0-7 (as described in Chapter 3, Figure 3.1 and Table 3.1) were used for the studies described in this chapter. Kittens from Litters 0 and 1 remained with their breeders during the period of LPD development and received only sporadic monitoring and sample collection by referring veterinarians. In contrast, kittens from Litters 2-7 were regularly monitored for signs of LPD and repeated samples collected. Unaffected littermates of LPD-affected animals, healthy kittens from litters which did not develop LPD, healthy kittens and cats from the Centre for Feline Nutrition at Massey University, and kittens and cats unaffected by LPD that were presented to the Massey University necropsy service for post mortem, were all used as controls.

4.2.2 CLINICAL FEATURES

All kittens from Litters 2-7 were regularly monitored for clinical signs of LPD. A full physical examination was performed on these kittens weekly from birth to 12 weeks of age and the results recorded. Data collected included bodyweight, demeanour, heart rate, respiration rate, mucous membrane colour, capillary refill time (CRT), hydration status, presence and degree of abdominal distension, and presence and degree of
peripheral lymph node enlargement. Abdominal distension was subjectively assessed as mild, moderate, marked or very marked. Lymph node enlargement was assessed by palpation of the peripheral lymph nodes (mandibular, cervical, prescapular, axillary, inguinal and popliteal) and any lymphadenopathy present classified as mild, moderate, marked or very marked. Kittens and cats that developed LPD were euthanased by intravenous pentobarbitone overdose once they developed depression or anorexia.

Kittens from Litters 0 and 1 remained with their breeders. As the breeders were unaware that this disease was about to develop, initial monitoring of these kittens prior to disease development was likely minimal. Five of the 6 LPD-affected kittens from these litters were examined by their referring veterinarians at initial presentation for LPD (5 to 7 weeks of age) and a basic clinical examination performed. With the exception of K3, which died without euthanasia, kittens that developed LPD were euthanased by intravenous pentobarbitone overdose following veterinary consultation.

4.2.3 HAEMATOLOGY AND BIOCHEMISTRY

Blood was collected by jugular or cephalic venipuncture at 2, 4, 6, 8, 10 and 12 weeks of age for a complete blood count (CBC) from all kittens in Litters 2-7. For the blood collection performed at 8 weeks of age, kittens were sedated with tiletamine hypochloride and zolazepam hypochloride (Zoletil-50, 5mg/kg, Virbac, Australia) to enable a larger volume of blood to be drawn. This larger blood volume allowed the lymphocyte immunophenotyping and apoptosis studies in Chapters 5 and 6 to be performed. For Litter 2, which contained kittens which developed LPD, blood was also collected for serum biochemical analysis at 8 weeks of age. For kittens over 6 weeks of age in Litters 2-7, a CBC was performed at a commercial laboratory (New Zealand Veterinary Pathology Ltd., Palmerston North (NZVP)) using an ADVIA 120 haematology analyser (Siemens Healthcare Diagnostics Inc., United States). Total blood protein was measured using a Roche Hitachi 911 chemistry analyser (Roche Diagnostics, Indianapolis, IN, United States) at NZVP.

Due to their low circulating blood volume, only a small sample of blood (less than 1mL) was able to be collected from kittens less than 6 weeks of age. In these kittens, a CBC was performed by centrifugation and analysis of a blood sample in duplicate micro-
haematocrit tubes and analysis of blood smears. The packed cell volume (PCV) was estimated by calculating the erythrocyte percentage of centrifuged blood in the micro-haematocrit tube, and the plasma protein concentration was estimated by refractometry of the plasma component of the centrifuged blood (g/L). In these kittens, manual evaluations of the leukocyte and platelet count and erythrocyte morphology were performed using duplicate blood smears. Leukocyte counts were performed by counting the total number of leukocytes present in 10-20 high power fields (400x magnification) and obtaining a mean leukocyte count per field, then multiplying that count by 2x to give an estimated total leukocyte count x 10^9/L. To optimise the manual leukocyte count method for use in kittens, automated leukocyte counts from 10 blood samples from control cats (processed using automated counting methods) were compared with the manual count for that blood sample calculated by multiplying the mean leukocyte count per 400x field by different factors (1.5x, 2x, 2.5x). A multiplication factor of 2x was selected as it gave a reasonable correlation (within 10%) when compared with automated leukocyte counts from that sample. Differential leukocyte counts and platelet counts were performed by standard methods as previously described (Latimer and Prasse 2003; Topper and Welles 2003). If significant abnormalities within the blood smear were identified or if kittens developed clinical signs of illness, a larger blood volume was re-drawn and re-analysed by automated methods as previously described (CBC and serum biochemistry).

Blood was also collected by jugular or cephalic venipuncture from three of the 6 LPD-affected kittens from Litters 0 and 1 following the first sign of LPD. A full CBC was performed by automated analysis using an ADVIA 120 haematology analyser on one LPD-affected kitten (K32); PCVs from the two other affected animals (K1 and K2) were performed in-clinic.

If structures consistent with of *Mycoplasma haemofelis* infection were identified in peripheral blood smears from any animal, whole blood from that animal was tested for the presence of *M. haemofelis* DNA by polymerase chain reaction (PCR) methods (Jenkins et al. 2013)).
4.2.4 FeLV and FIV Testing

As described in Chapter 3, blood collected at 8 weeks of age from kittens from Litters 2-7 was tested for the presence of feline leukaemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibody using a Snap® Combo FeLV Ag/FIV Ab Test Kit (IDEXX Laboratories, United States). In kittens that developed signs of LPD, bone marrow aspirates were also collected from the proximal femur using an 18g needle and 10mL syringe at the time of euthanasia and tested for the presence of FeLV antigen by immunofluorescence antibody (IFA) assay (performed at UC Davis, California, United States). Blood collected by jugular or cephalic venipuncture from three of the 6 LPD-affected kittens from Litters 0 (K32) and 1 (K1 and K2) was also tested for the presence of FeLV antigen and FIV antibody using either a Snap® Combo FeLV Ag/FIV Ab Test Kit or a AGEN FeLV/FIV Simplify™ Test (AGEN Biomedical Ltd, Australia).

4.2.5 Pathology

4.2.5.1 Cytology

Fine needle aspirates of enlarged lymph nodes using a 22 g needle and 5mL syringe were taken for cytological analysis following the first clinical evidence of disease in kittens from Litters 2-7 that developed LPD. Fine needle aspirates of enlarged lymph nodes were also taken for cytological analysis following the first appearance of clinical signs of LPD from 4 of the 6 LPD-affected animals from Litters 0 and 1 (K32, K33, K34 and K1).

4.2.5.2 Gross Pathology

Following euthanasia, a post mortem examination was performed on 5 of the 8 LPD-affected kittens and cats (K1, K2, K10, K11 and K32) and gross findings recorded. Two LPD-affected animals were from Litters 2-7, the remainder were from Litters 0 and 1.

4.2.5.3 Histology

Tissue samples including multiple lymph nodes, liver and spleen, were collected from 5 of the 8 LPD-affected kittens and cats (K1, K2, K10, K11 and K32) at post mortem, fixed in 10% neutral buffered formalin for 24-48 hours, processed and embedded into paraffin.
blocks. Routine haematoxylin and eosin stains were performed on 3μ sections of tissue cut from the formalin-fixed, paraffin-embedded (FFPE) blocks.

4.2.6 DATA ANALYSIS

Differences between groups were investigated by 2-sample t tests using Minitab 15 statistical software (2007, Minitab Inc.). Samples available from LPD-affected animals are summarised in Table 4.1 below.

4.3 RESULTS

4.3.1 ANIMALS USED

In total, 8 related BSH and BSH-cross kittens and cats developed LPD. Seven kittens developed LPD before 8 weeks of age: three from Litter 1 (K1, K2 and K3), one from Litter 2 (K10) and three from Litter 0 (K32, K33, K34). One adult cat from Litter 2 (K11) also developed a less marked form of LPD and evidence of liver disease at 12 months of age. Samples available from the LPD-affected animals for which results are presented in this chapter are summarised in Table 4.1 below.

Table 4.1: Sample availability for LPD-affected kittens and cats
F= female, M = male, LPD = lymphoproliferative disease, FeLV = feline leukaemia virus, FIV = feline immunodeficiency virus, †† = died without euthanasia

<table>
<thead>
<tr>
<th>Animal</th>
<th>Litter</th>
<th>Sex</th>
<th>Age at presentation</th>
<th>Age at euthanasia or death</th>
<th>Blood</th>
<th>FeLV + FIV tested (age tested)</th>
<th>Gross pathology</th>
<th>Microscopic pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>1</td>
<td>F</td>
<td>6 weeks</td>
<td>7 weeks</td>
<td>Y</td>
<td>Y (6 weeks)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>K2</td>
<td>1</td>
<td>F</td>
<td>7 weeks</td>
<td>9 weeks</td>
<td>Y</td>
<td>Y (7 weeks)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>K3</td>
<td>1</td>
<td>M</td>
<td>7 weeks</td>
<td>8 weeks††</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>K10</td>
<td>2</td>
<td>M</td>
<td>6 weeks</td>
<td>8 weeks</td>
<td>Y</td>
<td>Y (8 weeks)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>K11</td>
<td>2</td>
<td>M</td>
<td>12 months</td>
<td>12 months</td>
<td>Y</td>
<td>Y (8 weeks, 12 months)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>K32</td>
<td>0</td>
<td>M</td>
<td>5 weeks</td>
<td>8 weeks</td>
<td>Y</td>
<td>Y (6 weeks)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>K33</td>
<td>0</td>
<td>F</td>
<td>6 weeks</td>
<td>8 weeks</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>K34</td>
<td>0</td>
<td>F</td>
<td>6 weeks</td>
<td>10 weeks</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>
4.3.2 Clinical Features

Of the 8 kittens and cats with LPD, 6 kittens and 1 adult cat were examined by a veterinarian and the clinical features of disease recorded.

All affected kittens and cats appeared clinically normal from birth through to the development of LPD between 5 and 7 weeks of age (6 kittens) or as an adult cat at 12 months of age (1 cat). Affected kittens initially presented with mild non-painful bilateral enlargement of multiple peripheral lymph nodes (mandibular, cervical, prescapular, axillary, inguinal and popliteal) which rapidly progressed over several weeks to a very marked and non-painful enlargement (approximately 10-20 times larger than those of unaffected littermates). However, even when very marked, the presence of peripheral lymph node enlargement was not readily detectable on visual external examination of affected animals alone, and could be reliably detected in the live animal only when palpation of the nodes was performed (Figure 4.1A). The adult cat affected by LPD (K11) developed mild (approximately 1.5 times larger than those of control cats) non-painful enlargement of multiple peripheral lymph nodes at 12 months of age over a period of approximately 4 weeks.

Over a period of several weeks from initial presentation, kittens with LPD tended to show progressive and ultimately moderate to marked abdominal distension. In some kittens, mild to moderate enlargement of both spleen and liver was suspected on abdominal palpation. Where marked, abdominal distension was detectable on visual examination alone but was often subtle (Figures 4.1A and C) compared with normal littermates (Figures 4.1B and D). The adult cat that developed LPD (K11) did not show detectable abdominal distension at any stage.
Figure 4.1: Litter 2 kittens at 6 weeks of age, LPD-affected and unaffected.  
A. K10, LPD-affected kitten: Although all peripheral lymph nodes are markedly enlarged on palpation (see Figure 4.5), this is not evident on external visual examination of the live animal.  
B. K11, LPD-unaffected kitten: No enlargement of peripheral lymph nodes is present in this kitten but there is no obvious difference in external appearance from the LPD-affected kitten in A on visual examination alone.  
C. K10, LPD-affected kitten: Mild to moderate abdominal distension can be seen from external visual examination.  
D. K6, LPD-unaffected kitten: obvious abdominal distension is not present.

Over a period of several weeks from initial presentation, LPD-affected kittens and cats tended to show changes in demeanour from bright, alert and responsive to quiet, alert and responsive, ultimately appearing depressed and lethargic. At this stage these animals were euthanased by intravenous pentobarbitone overdose. One kitten (K3) became moribund within 3 weeks of initial presentation and died without veterinary intervention. LPD-affected kittens tended to show progressive mucous membrane pallor over a period of several weeks from initial presentation. The adult cat that developed LPD (K11) did not show pallor of the mucous membranes at any stage but did develop progressive yellow discolouration (jaundice) of the mucous membranes in the 2-week period following the initial observation of lymphadenopathy at 12 months of age. As shown in Figure 4.2, the growth of the LPD-affected kitten from Litter 2 (K10) appeared similar to unaffected littermates from birth to approximately 6 weeks of age after which the kitten failed to
gain weight at the same rate as his littermates. Detailed growth rate data was not recorded for LPD-affected kittens from other litters.

![Litter 2: Bodyweight, birth to 12 weeks](image_url)

**Figure 4.2:** Weekly bodyweight measurements for Litter 2, birth to 12 weeks. An apparent reduction in the growth rate of LPD-affected kitten K10 (in red) was observed from approximately 6 weeks of age to euthanasia at 8 weeks of age when compared with unaffected littermates (in blue).

Anorexia was not obvious in any of the affected kittens. However, a progressive reduction in appetite over the 4-week period between initial presentation and euthanasia was seen in the adult cat that developed LPD at 12 months of age.

### 4.3.3 Haematology and Biochemistry

Blood samples were taken from 4 of the 7 LPD-affected kittens (K1, K2, K10, K32) and from the adult cat that developed LPD (K11) for routine haematology. Serum biochemistry results were available for 3 of these kittens (K1, K10 and K32) and from the adult cat that developed LPD (K11). A summary of relevant haematology and biochemistry results from LPD-affected animals is present in Table 4.2.
Table 4.2: Relevant haematology and biochemistry results from LPD-affected kittens and cats.

PCV = packed cell volume; nRBCs = nucleated red blood cells (metarubricytes); TPP = total plasma protein; Plt = platelets; (L) = result lower than relevant reference range; (H) = result higher than relevant reference range ND = no data; NA = not applicable; ad = adequate numbers present.

<table>
<thead>
<tr>
<th>Animal</th>
<th>PCV (%)</th>
<th>Reticulocytes (%)</th>
<th>nRBCs (/100 leukocytes)</th>
<th>TPP (g/L)</th>
<th>Leukocytes ( \times 10^9/L )</th>
<th>Lymphocytes ( \times 10^9/L )</th>
<th>Plt</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kittens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>11 (L)</td>
<td>2.5 (H)</td>
<td>11 (H)</td>
<td>69</td>
<td>34.7 (H)</td>
<td>8.3</td>
<td>ad</td>
<td></td>
</tr>
<tr>
<td>K2</td>
<td>24 (L)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12.5 (L)</td>
<td>4.9 (L)</td>
<td>ad</td>
<td></td>
</tr>
<tr>
<td>K10</td>
<td>18 (L)</td>
<td>2.3 (H)</td>
<td>31 (H)</td>
<td>59</td>
<td>20.4 (H)</td>
<td>11.2 (H)</td>
<td>ad</td>
<td></td>
</tr>
<tr>
<td>K32</td>
<td>11 (L)</td>
<td>ND</td>
<td>19 (H)</td>
<td>ND</td>
<td>13.4 (L)</td>
<td>6.0 (L)</td>
<td>ad</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16 (L)</td>
<td>2.4 (H)</td>
<td>20.3 (H)</td>
<td>64</td>
<td>20.3 (H)</td>
<td>7.6 (L)</td>
<td>ad</td>
<td></td>
</tr>
<tr>
<td>Adult cat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K11</td>
<td>30</td>
<td>ND</td>
<td>0</td>
<td>83 (H)</td>
<td>12.8</td>
<td>2.6</td>
<td>ad</td>
<td>Liver disease (see text)</td>
</tr>
<tr>
<td>Reference ranges (Rizzi et al. 2010)</td>
<td>6-8 wk kittens</td>
<td>28.5-31.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>16.1-20.0</td>
<td>8.02-11.18</td>
<td>ad</td>
</tr>
<tr>
<td>Adult cats</td>
<td>24-45</td>
<td>&lt;1%</td>
<td>0</td>
<td>54-78</td>
<td>5.5-19.5</td>
<td>1.5-7.0</td>
<td>ad</td>
<td></td>
</tr>
</tbody>
</table>

Mild to severe anaemia was present in all 4 LPD-affected kittens tested following initial disease presentation at 6-8 weeks of age (Table 4.2). Packed cell volume values were significantly different (p=0.01) in LPD affected kittens at presentation (mean 6-8 week PCV = 16%, range 11%-24%) from those of littermates unaffected by LPD (mean 6-8 week PCV = 32.7%, range 28%-44%) (see also Figure 4.3A and B). Where taken, total plasma protein measurements for LPD-affected kittens were within reference ranges (Table 4.2).

In LPD-affected kittens for which haematology data were available, morphological changes of polychromasia and anisocytosis, moderately to markedly increased numbers of nucleated red blood cells (nRBCs) and mild reticulocytosis were present (Table 4.2).

The anaemia in LPD-affected kittens therefore appeared mildly regenerative, but the level of reticulocytosis (bone marrow response) present appeared inadequate for the severity of the anaemia present. Occasional small (0.5-1µm diameter) basophilic structures that resembled *Mycoplasma haemofelis* parasites were observed on the surface of erythrocytes and in the background of the blood smear from K10, but *M. haemofelis* DNA was not identified on PCR-testing of whole blood from this cat (Jenkins et al. 2013). While *M. haemofelis* could be excluded, the identity of these structures is unknown. Structures resembling *M. haemofelis* were not seen in examined blood smears from any of the other kittens or the adult cat.
Figure 4.3: Packed cell volume % (PCV) differences between LPD-affected and unaffected kittens.
A. Litter 2, changes in PCV % during the first 12 weeks of life; the LPD-affected kitten (K10, in red) shows a decrease in PCV % compared with unaffected littermates from 4 weeks of age compared with non LPD-affected littermates (in blue). B. PCV % at 6–8 weeks of age, LPD-affected kittens (red, mean = 16% n = 4) compared with non-LPD-affected littermates (blue, n = 19, mean = 32.7%). P=0.014, X = mean; box = interquartile range, * = outlier. PCV reference range for kittens 6–8 weeks of age = 28.5–31.1% (Rizzi et al. 2010).

Full serum biochemistry was performed on blood from three LPD-affected kittens (K1, K10 and K32) shortly after the development of clinical signs of LPD and all parameters were within reference ranges.

A peripheral leukocytosis and lymphocytosis was variably present in LPD-affected kittens. Mild to moderate peripheral leukocytosis was present in two LPD-affected kittens (K1 and K10). A mild leukopenia was present in the other two affected kittens (K2 and K32, Table 4.2). Peripheral lymphocytes were mildly elevated in one of these two kittens (K10), but within the reference range for the other kitten (K1) and a mild lymphopenia was present in the other two LPD-affected kittens for which data were available (K2 and K32). In 3 of the 4 LPD-affected kittens for which data were available, low to moderate numbers of peripheral lymphocytes showed large lymphoblast-type morphology (K1, K10 and K32). Platelet numbers could not be determined in any animal due to clumping but numbers
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appeared adequate in all 4 kittens. Platelet morphology for all 4 kittens was unremarkable.

The adult cat that developed milder LPD at 12 months of age (K11) did not become anaemic at any stage and leukocyte parameters were within reference ranges. However, serum biochemistry revealed multiple abnormalities, including marked elevations in the hepatocellular leakage enzymes aspartate aminotransferase (120 IU/L, reference range = 0-66 IU/L) and alanine aminotransferase (254 IU/L, reference range = 0-100 IU/L). A moderate bilirubinemia was also present (13µm/L, reference range = 0-5 µm/L). Alkaline phosphatase was not elevated (33 IU/L, reference range = 0-85 IU/L). Urea (4.5 mmol/L, reference range 6.0-11.8 mmol/L) and creatinine (44 µmol/L, reference range = 80-178 µmol/L) were both moderately decreased. Creatine kinase was moderately elevated (920 mmol/L, reference range = 0-344). Total protein (83 g/L, reference range = 54-78 g/L) and globulins (56 g/L, reference range = 26-51 g/L) were mildly elevated. Albumin was at the low end of the reference range (0.5 g/L, reference range = 0.5-1.1 g/L). Serum biochemistry results in K11 were interpreted as most consistent with hepatocellular disease of unknown cause.

4.3.4 FeLV AND FIV TESTING

In total, 4 of the 7 LPD-affected kittens and 27 unaffected littermates were tested for evidence of FeLV or FIV infection. As reported in Chapter 3, none had evidence of FeLV antigen or FIV antibody in blood tested at 6-8 weeks of age. Blood from K11 re-tested at 12 months of age again did not demonstrate evidence of the presence of FeLV antigen or FIV antibody. Additional IFA testing of LPD-affected animals K10 and K11 did not reveal evidence of FeLV antigen within the bone marrow of these animals. These results were interpreted as demonstrating absence of FeLV and FIV infection in all cats and kittens.

4.3.5 PATHOLOGY

4.3.5.1 CYTOLOGY

Fine needle aspirates of lymph nodes from all 5 LPD affected kittens contained large numbers (60-90%) of medium-sized to very large lymphocytes up to 20µm in diameter showing many features typically associated with malignancy, including marked
anisocytosis, anisokaryosis, karyomegaly, and increased amounts of granular basophilic cytoplasm. Nucleoli were generally single but occasionally multiple and frequently large to very large and prominent. These cells were interpreted as immature lymphocytes or lymphoblasts. Many lymphoblasts were in mitosis (Figure 4.4A) and mitotic figures were often large and atypical. In lymph node aspirates from 2 of the 5 LPD-affected kittens, low numbers of uniform plasma cells and occasional macrophages were also present, in addition to increased numbers of lymphoblasts (see Figure 4.4B).

Fine needle aspirates of lymph nodes from the LPD-affected adult cat (K11) contained predominantly small (6-8µm diameter) and uniform lymphocytes with occasional plasma cells, neutrophils and rare medium sized to large lymphoblasts. Increased mitoses of the lymphocytes in cytological samples from K11 were not observed.

Bone marrow aspirates from one LPD-affected kitten (K10) were mildly hypercellular compared to controls and showed mild erythroid hyperplasia (myeloid : erythroid ratio of 1.15, reference range 1.21-2.16 (Harvey 2001)). Megakaryocyte numbers and maturation of both erythroid and myeloid lines appeared normal. Increased numbers of lymphoid cells were not present. Bone marrow aspirates from K11 were within normal limits.
4.3.5.2 **GROSS PATHOLOGY**

In the 4 LPD-affected kittens available for necropsy (K1, K2, K10 and K32), multiple peripheral and visceral lymph nodes showed marked to massive enlargement (10-20 times larger than those of control kittens, Figure 4.5A-E). Affected lymph nodes were diffusely firm and smooth, white to cream-coloured and bulged from the cut surface, with loss of the normal cortico-medullary architecture (Figure 4.5F). Affected lymph nodes included the mandibular, cervical, prescapular, axillary, inguinal, popliteal, gastrohepatic, splenic, mesenteric, ileo-cecal, sublumbar and renal nodes. There was mild diffuse enlargement of the liver (up to 1.5 times larger than those of control kittens) and moderate enlargement of the spleen (up to 2 times larger than those of control kittens) in all LPD-affected kittens (K1, K2, K10 and K32, Figure 4.5D). Both organs were slightly darker red in colour and slightly firmer and more “meaty” in texture than normal.

The thymus was present and grossly unremarkable in all LPD-affected kittens examined except K1, in which thymic tissue was not identified during necropsy examination. All LPD-affected kittens and cats were in poor body condition with a marked loss of subcutaneous and visceral adipose tissue. Other examined organs did not contain significant gross lesions.
Figure 4.5: Gross pathology - multicentric lymphadenopathy, LPD-affected kittens (K2 and K10).

A. K2: Whole body with skin removed, showing marked to massive bilateral enlargement of peripheral lymph nodes and marked abdominal distension. B. K2: Head and ventral cervical region showing massive bilateral enlargement of mandibular and cervical lymph nodes. C. K2: Inguinal and hind limb region showing marked bilateral enlargement of inguinal and popliteal lymph nodes. D. K2: Abdominal viscera showing marked to massive enlargement of multiple visceral lymph nodes and moderate diffuse enlargement of spleen and liver. E. K2: Abdominal viscera showing marked to massive enlargement of multiple visceral lymph nodes. F. K10: Incised mesenteric lymph node (measuring approximately 5cm x 3cm x 2cm) to show cream-coloured and bulging cut surface with effacement of normal cortico-medullary architecture.
CHAPTER 4

Gross pathology in the adult cat with LPD (K11) differed from that in affected kittens. There was mild enlargement of several lymph nodes including the mandibular, tracheobronchial and visceral nodes (up to 1.5 times larger than those of control cats). Affected nodes were slightly nodular and did not bulge from the cut surface. The axillary, inguinal and popliteal lymph nodes appeared grossly unremarkable. Normal cortico-medullary architecture appeared grossly intact in all examined nodes from K11. Examination of the liver and spleen of K11 revealed diffuse moderate enlargement of both organs (approximately 1.5-2 times larger than those of control cats). The liver was very firm, pale pink-tan in colour with irregular red mottling, occasional pitting on the capsular surface, and an enhanced lobular pattern (Figure 4.6). The spleen was friable, very dark red and readily oozed blood from cut surfaces.

Other examined organs from K11 did not contain significant gross lesions.

Figure 4.6: Gross pathology - viscera, LPD-affected adult cat (K11). A: Abdominal and thoracic viscera of K11 showing a mildly enlarged and pale tan liver with multifocal red mottling, markedly enlarged dark red spleen, and moderately enlarged mesenteric lymph nodes. B. Liver of K11, diaphragmatic surface, showing pale tan liver with multifocal red mottling and an enhanced lobular pattern.
4.3.5.3 Histology

Full tissue sets were available from 4 LPD-affected kittens (K1, K2, K10 and K32) and the adult cat affected by LPD (K11). In two LPD-affected kittens (K2 and K10), all examined lymph nodes were markedly expanded by extensive and densely cellular sheets of lymphocytes in a fine fibrovascular stroma that effaced normal cortico-medullary architecture and variably expanded the sub-capsular sinus compared to controls (Figures 4.7C, D and F). Lymphocytes were generally medium to large in size, with well-defined cell borders, a small amount of eosinophilic cytoplasm and a single large, eccentrically located nucleus with marginalized, coarsely stippled or vesicular chromatin (Figures 4.8A, C and D). Nucleoli were generally single and were often large and prominent. Mitotic figures were very frequent, averaging 5-10 per 400x high power field (hpf). Low numbers of larger cells with abundant pale eosinophilic vacuolated cytoplasm and single round basophilic nuclei (histiocytes) and rare smaller cells with moderate basophilic cytoplasm containing a pale Golgi region and round basophilic nuclei eccentrically located within the cell (plasma cells) were variably admixed with the lymphocyte population within the nodes.

In lymph nodes from the two other LPD-affected kittens examined (K1 and K32), cortical areas and medullary sinuses of affected lymph nodes were expanded by a similar population of lymphocytes to those present in K1 and K10 and described above, variably admixed with low to moderate numbers of histiocytes and plasma cells (Figure 4.8B). In these kittens, cortico-medullary architecture was occasionally effaced but in the majority of nodes was variably intact with scattered follicular and germinal centre remnants still present (Figures 4.7A and B). In these nodes, there was evidence of germinal centre involution with collapse of the mantle cells to form multifocal dense clusters of darkly basophilic nuclei.

Histiocytes within the medullary sinuses from LPD-affected kittens often contained intracytoplasmic erythrocytes. Evidence of apoptosis such as shrunken, basophilic cells with condensed chromatin and cytoplasm and their fragments was not prominent within the lymphoid cellular population.
Figure 4.7: Lymph node histology (low power), LPD-affected and control animals, haematoxylin and eosin. A. K32, LPD-affected kitten: There is marked expansion of the paracortical regions and medulla by a population predominantly comprising lymphoid cells. Multifocal follicular remnants are present. B. K1, LPD-affected kitten: Similar findings to those in A but with less prominent follicular remnants. C. and D. K2 and K10 respectively, LPD-affected kittens: Effacement of normal lymph node architecture by an extensive cell population predominantly comprising lymphoid cells. Lymphoid follicles and germinal centres are not evident. E. K11, LPD-affected adult cat: Normal node architecture is present with increased small follicles showing expanded mantle zones encircling germinal centres throughout the outer and deep cortex. F. Control kitten, 10 weeks, normal cortico-medullary architecture with lymphoid follicles (germinal centres and mantle region) clearly visible. All magnification 20x.
In all 4 LPD-affected kittens, the splenic white pulp was expanded by moderate to large numbers of lymphocytes with similar morphology to those present within the lymph nodes. These cells formed large multifocal to coalescing aggregates which distorted and variably effaced the normal splenic architecture with variable extension into, and replacement of, the red pulp (Figure 4.9A, C and D). Low numbers of scattered erythroid and myeloid precursor cells were also present throughout the spleen. Splenic macrophages contained variable numbers of intracytoplasmic erythrocytes (erythrophagocytosis).
Figure 4.9: Splenic histology (low, medium and high power), LPD-affected kittens and control kitten, haematoxylin and eosin. A. K10, LPD-affected kitten, low power view (magnification 40x) showing expansion of the white pulp and replacement of parts of the red pulp by an extensive lymphoid cellular infiltrate. B. Unaffected control kitten euthanased by intravenous pentobarbitone overdose, low power view (magnification 40x) showing the marked expansion of red pulp by large numbers of erythrocytes (congestion) typically present following euthanasia by this method. C. and D. K10, LPD-affected kitten, medium power view (magnification 200x) and high power view (magnification 400x) of the splenic lymphoid infiltrate in A.

Examination of the liver in all 4 LPD-affected kittens revealed moderately increased numbers of lymphocytes. These cells were present both as small multifocal aggregates within periportal regions and more diffusely within hepatic sinusoids (Figures 4.10A and B). Both small mature lymphocytes and larger cells with more lymphoblastic morphology similar to those present within the lymph nodes were present; rare plasma cells were also present within lymphoid infiltrates. Mildly increased numbers of small cells with small round densely basophilic round nuclei and a small to moderate amount of pale eosinophilic to basophilic cytoplasm (interpreted as erythroid and myeloid precursors).
were also present throughout the liver, including within periportal areas and sinusoids. Kupffer cells throughout the liver contained variable numbers of intracytoplasmic erythrocytes (erythrophagocytosis) and granular brown pigment (interpreted as haemosiderin) was present within many hepatocytes (Figure 4.10C).

Bone marrow from one LPD-affected kitten (K10) was examined histologically. Consistent with the cytology (discussed above), there was a mild increase in overall cellularity compared with normal animals, with a mild increase in erythroid precursors. Increased numbers of cells with lymphoid morphology were not observed within the bone marrow.

Sections of thymus were histologically unremarkable in 3 of the 4 LPD-affected kittens for which tissue was available. Within sections of lung from all 4 LPD-affected kittens, alveolar septae were expanded by variable numbers of lymphoid cells with similar morphology to those present within the lymph nodes. Lymphoid cells also occasionally formed small perivascular aggregates. Sections of ileum and colon from 2 of the 4 LPD-affected kittens from which tissue was available showed mild expansion of lymphoid tissue including Peyer’s patches by a population of lymphocytes with similar morphological features to those present within lymph nodes.

Findings present on histological examination of lymph nodes, liver and spleen from the adult cat with LPD (K11) differed from those present in LPD-affected kittens. Examination of sections of lymph node from this cat revealed intact cortico-medullary architecture (Figure 4.7E). The cortex was moderately expanded and in places compressed medullary structures. Lymphoid follicles and germinal centres throughout the nodes appeared mildly increased in number but not enlarged. Mantle cell cuffs were slightly expanded and generally completely encircled germinal centres. Splenic histology from this cat revealed marked expansion of the sinuses of the red pulp by large numbers of erythrocytes which widely separated areas of white pulp. These changes were similar to those seen in the spleens of control cats euthanased by intravenous pentobarbitone overdose (Figure 4.9B) and were interpreted as consistent with and likely secondary to euthanasia by this method (Blue and Weiss 1981; Valli 2007a; Fry and McGavin 2012). Increased numbers of lymphoid cells were not observed.
Figure 4.10: Hepatic histology (low, medium and high power), LPD-affected animals, haematoxylin and eosin. A and B: K2, LPD-affected kitten, low power view (magnification 40x) and medium power view (magnification 200x) of multifocal portal lymphoid aggregates and increased cellularity within hepatic sinusoids. C. K10, LPD-affected kitten, high power view (magnification 1000x) showing erythrophagocytosis by Kupffer cells, and increased lymphocytes, erythroid precursors and myeloid precursors within sinusoids. D. K11, LPD-affected adult cat, low power view (magnification 40x) showing distortion of hepatic architecture and hepatocyte replacement by extensive and bridging areas of portal fibrosis admixed with increased numbers of small bile ducts and multifocal lymphoid follicles. E. K11, LPD-affected adult cat, medium power view (magnification 200x) of portal lymphoid
Hepatic histology from the adult cat with LPD revealed marked distortion of hepatic architecture (Figure 4.10D). The capsular surface was irregular and portal areas were markedly expanded by fibrous connective tissue containing many small bile ducts and moderate numbers of inflammatory cells including neutrophils, lymphocytes, plasma cells and occasional macrophages (Figure 4.10F). These areas often bridged portal areas and effaced large regions of hepatic parenchyma. Hepatocyte numbers were markedly reduced and occasional hepatocytes appeared swollen and hypereosinophilic (interpreted as degeneration). Multifocal aggregates of small mature lymphocytes were also scattered throughout areas of fibrosis and often formed follicular structures (Figure 4.10E).

Examined sections of bone marrow, thymus, lung and intestine, from K11 were histologically unremarkable.

### 4.4 Discussion

The clinical presentation, gross findings and microscopic pathology of the LPD in BSH and BSH-cross kittens was initially considered most consistent with a diagnosis of lymphoma involving multiple lymph nodes and the spleen with variable involvement of other organs. Multiple markedly enlarged but non-painful lymph nodes that were expanded by a monomorphic population of lymphocytes which distorted and effaced normal lymph node architecture were present in all examined LPD-affected kittens. These lymphocytes showed morphological features of neoplasia including pleomorphism, anisocytosis and anisokaryosis, large and prominent nucleoli, and many mitoses. The presence of a population of lymphocytes with similar morphological features to those expanding the lymph nodes within the spleen and other organs of LPD-affected kittens suggested metastatic spread, providing further support for a diagnosis of lymphoma rather than a non-neoplastic lymphoid proliferation (Cullen et al. 2002; Valli 2007b; Meinkoth et al.
Lymphoid leukaemia was not a feature of disease in any examined kitten. While clinico-pathological findings provided support for the preliminary diagnosis of lymphoma, many other aspects of LPD in affected kittens were not typical of those previously reported in feline lymphoma. The very young age at which LPD-affected kittens developed disease was unusual for feline lymphoma (Nielsen 1969; Hardy et al. 1981; Jacobs et al. 2002; Louwerens et al. 2005). In addition to sporadically occurring lymphoma, known causes of lymphoma in cats include infection with the viruses FeLV or FIV (Cotter et al. 1975; Francis et al. 1979; Shelton et al. 1990; Poli et al. 1994). However, irrespective of cause, cats with lymphoma are typically much older than the LPD-affected kittens here, all of which were less than 8 weeks of age at initial presentation. Cats with sporadic lymphoma reportedly have a median age of 11 years, and 7 months is earliest reported age at presentation (Louwerens et al. 2005). FeLV-positive cats with lymphoma tend to be younger than those with sporadic lymphoma, with the majority less than 4 years of age at diagnosis (Stutzer et al. 2011). The earliest reported age at presentation of a kitten with non-experimental FeLV-associated lymphoma is 4 months (Stephens et al. 1983). Lymphoma in younger kittens is only reported following experimental FeLV infection of kittens at birth. Data on the age of cats with FIV-associated lymphoma are more limited, but these cats generally appear to be significantly older than the kittens in the present study (Poli et al. 1994; del Fierro et al. 1995; Hosie et al. 2009).

In addition to the unusually young age at development of disease, there were several other features that were not typical of feline lymphoma. The development of lymphoma in multiple closely related animals of a single breed of cat was unusual. While reported, familial clusters of cases of LPD or lymphoma in people are rare, and usually occur in association with inherited conditions such as genomic instability syndromes, immune deficiency syndromes or autoimmune diseases (Linet and Pottern 1992; Segel and Lichtman 2004; Siddiqui et al. 2004). Other than an increased incidence of mediastinal lymphoma in Oriental and Siamese breeds (Dorn et al. 1967; Gruffydd-Jones et al. 1979; Hardy 1981; Hardy 1993; Court et al. 1997; Gabor et al. 1998; Teske et al. 2002; Louwerens et al. 2005; Rissetto et al. 2011), inherited predispositions to lymphoma affecting multiple related animals have not been described in cats. Furthermore, the acute development of widespread enlargement of lymph nodes up to 20 times the size of
control kittens has not been previously reported in cases of feline lymphoma (Jacobs et al. 2002; Louwerens et al. 2005). Finally, the variable presence of a population of plasma cells and retention of lymphoid follicular remnants within enlarged lymph nodes from some affected kittens was not typical of histological findings in feline lymphoma (Valli et al. 2000; Jacobs et al. 2002; Valli 2007b). Although the latter findings could simply indicate an earlier stage in nodal effacement to that present in other kittens with more advanced disease, the presence of significant numbers of plasma cells would not be expected if lymph node enlargement was due to early lymphoma.

While a viral cause could explain the presence of multiple affected kittens in a litter, a viral cause for the development of LPD in BSH kittens is considered unlikely for several reasons. No LPD-affected kitten, unaffected littermate or parent showed evidence of infection with either FeLV or FIV, including during the period when overt clinical signs of LPD were present. If infection with either virus caused LPD in the kittens it would be expected that the testing undertaken would have identified infection in at least some animals. The reported sensitivity of the blood tests used to detect the presence of either FeLV antigen or FIV antibody is typically at least 92% (Pinches et al. 2007; Diagnostik Megacor 2012; Idexx Laboratories 2013). While it is possible that a single kitten may have returned a false negative test result, the chance that all 5 LPD-affected animals tested would test falsely negative is extremely low (less than 4 in 1,000,000 for either virus). The failure to identify FeLV antigen on IFA testing of bone marrow of two LPD-affected animals tested provides further support for this conclusion. Immunofluorescent antibody testing of bone marrow for FeLV antigen has a reported sensitivity of 98% (Hardy 1991; Hardy and Zuckerman 1991a; Hardy and Zuckerman 1991b), so again the likelihood that both LPD-animals tested using both the ELISA and IFA tests returned falsely negative results is very low (less than 3 in 1,000,000).

Although it has been reported that cats with “latent” FeLV infections may show no evidence of FeLV antigen on routine blood testing, the significance of latent FeLV infection in the development of lymphoma is currently uncertain (Torres et al. 2005; Hofmann-Lehmann et al. 2007). “Latent” infection refers to the situation where FeLV provirus is integrated into the cat’s cells but antigen is not released into the blood (Hoover and Mullins 1991; Stutzer et al. 2011). Previous reports have speculated that latent FeLV infection may be associated with the development of lymphoma in cats that
are not antigenaemic on routine FeLV testing (Jackson et al. 1993; Gabor et al. 2001a; Weiss et al. 2010). However, other studies conclude that latent FeLV infection likely plays a very limited role in the development of lymphoma in cats (Herring et al. 2001; Stutzer et al. 2011). In any case, it is considered unlikely that all LPD-affected kittens and cats that tested negative for the presence of FeLV antigen on blood and bone marrow testing were latently infected with FeLV, and latent FeLV infection is therefore considered unlikely to be a factor in development of LPD in kittens in the present study.

The clinical features of disease seen in LPD-affected kittens in the present study also do not support a significant role for FeLV infection in the pathogenesis of the disease. As discussed above, lymphoma associated with FeLV usually develops in significantly older cats (Ladiges et al. 1981; Stutzer et al. 2011). Infected pregnant queens can transmit FeLV to their kittens either in utero or in the post-natal period. In utero infection usually results in fetal death in utero or weak kittens which die shortly after birth (Hardy et al. 1976; Pacitti et al. 1986; Lutz et al. 2009). While FeLV infection of kittens in the immediate post-natal period may result in viraemia, lymphoma appears to be an uncommon outcome of natural perinatal infection and takes several months after infection to develop (Hoover et al. 1973; Hoover et al. 1977; Ladiges et al. 1981; Stutzer et al. 2011). Although lymphoma in kittens as young as 9 weeks has been reported following experimental FeLV infection, the diagnosis of lymphoma in these cases appeared based on histological examination of the bone marrow only; further diagnostic confirmation by clonality assays or immunophenotyping were not performed. In addition, clinical or histological involvement of the lymph nodes in these cases was rare, and where present, involved only a single node (Hoover et al. 1973). This differs to the dramatic expansion of multiple lymph nodes by lymphocytes observed in kittens in the present study.

Clinico-pathological features of disease in affected kittens in the present study also differ to those typically reported in FIV infection. Natural transmission of FIV from infected queens to their kittens in utero or in the post-natal period is rarely documented and has been associated with abortion, stillbirth, delayed fetal development or the birth of asymptomatic FIV-infected kittens (Sellon et al. 1994; O’Neil et al. 1996; Rogers and Hoover 1998; Weaver et al. 2005). Lymphoma due to FIV transmission from queen to kitten is rare and takes months or years to develop (Poli et al. 1994; del Fierro et al. 1995; Hosie et al. 2009). Additionally, lymphoma due to FIV more commonly affects sites other
than the lymph nodes (Poli et al. 1994; Callanan et al. 1996). While generalised non-neoplastic lymphadenopathy can also develop in kittens following FIV infection, kittens tend to be older (at least 4-5 months of age) than kittens in the present study (Parodi et al. 1994). In addition, histological findings within the enlarged lymph nodes of such FIV-infected kittens are those of follicular hyperplasia with retention of nodal architecture. This is in contrast to the distortion or effacement of the node seen in LPD-affected kittens in the present study.

The features of LPD in affected BSH kittens also differ to two previously reported but poorly defined diseases in cats causing enlargement of multiple lymph nodes: the so-called “distinctive peripheral lymph node hyperplasia” (DPLNH) described by Moore et al. (1986) and the “generalised lymphadenopathy resembling lymphoma” (GLRL) described by Mooney et al. (1987). While histological changes within the lymph nodes of cats with DPLNH have some similarities to those present in LPD-affected kittens in the present study, affected cats were significantly older (5 months to 2 years) than LPD-affected kittens, and were unrelated to one another. Six of 9 cats with DPLNH showed evidence of FeLV infection on routine blood testing, suggesting a possible role for this virus in disease development (Moore et al. 1986). In most cases, the lymphadenopathy in DPLNH appeared transient and involvement of other organs was not described. Cats with GLRL were also significantly older than LPD-affected kittens. While several cats affected by GLRL were of the same breed (Maine Coon), it appears affected cats were not directly related (Mooney et al. 1987). In addition, involvement of other organs was not a described feature of disease and the lymphadenopathy generally resolved without treatment over the following months. Affected cats did not show evidence of FeLV infection and the aetiology of GLRL was not determined.

The clinical presentation and pathology of LPD in affected BSH kittens in the present study has several similarities to ALPS in people, which is discussed in detail in Chapter 2. Both diseases have an early age of onset of lymphadenopathy and the appearance of other clinical signs. The median age of onset of clinical signs in ALPS patients is 24 months of age, although cases have been reported from birth (Sneller et al. 1997; Avila et al. 1999; Strauss et al. 1999; Rao and Straus 2006; Worth et al. 2006). In addition, a striking clinical feature of both diseases is the massive non-painful enlargement of the spleen and multiple lymph nodes throughout the body, with more variable enlargement of the liver.
In ALPS patients, the degree of lymph node enlargement may be sufficient to disrupt normal anatomic landmarks (Straus et al. 1999). The enlargement of multiple lymph nodes throughout the body was similarly dramatic in affected BSH kittens, with apparent involvement of all peripheral and visceral lymph nodes. In addition, moderate enlargement of the spleen and mild enlargement of the liver was also present, similar to the typical clinical findings in ALPS.

Both ALPS in people and the LPD in affected BSH kittens affect multiple family members. In ALPS, this is due to the inherited basis for disease development. Most cases of ALPS in people have an autosomal dominant mode of inheritance but with variable expression of the clinical signs (Sneller et al. 1997; Infante et al. 1998; Jackson et al. 1999a). Families with multiple siblings affected by ALPS are often reported (Le Deist et al. 1996; Infante et al. 1998; Straus et al. 1999; Vaishnaw et al. 1999). Similarly, LPD in kittens affected multiple related animals with a shared (BSH) genetic background. Based on the results of the studies in Chapter 3, the disease in BSH kittens is also likely to be inherited, although the mode of inheritance remains uncertain.

Anaemia is frequently reported in ALPS in people and was also present in LPD-affected kittens for which blood was available. Anaemia in human ALPS patients is usually due to the immune-mediated haemolysis of erythrocytes, and a positive Coombs’ test is often reported (Infante et al. 1998; Straus et al. 1999; Teachey et al. 2010). However, manifestations of autoimmunity and anaemia in ALPS patients are highly variable. Some patients demonstrate transient haemolytic anaemia while in other cases, anaemia is present but the cause is undetermined (Alvarado et al. 2004). In some cases, circulating autoantibodies are present, but are not associated with a concurrent haemolytic anaemia. In addition, while cytopenias including anaemia but also neutropenia and thrombocytopenia are often present in people with ALPS, they are frequently absent at the time of initial presentation when patients show marked lymphoproliferation, and only develop as patients age (Straus et al. 1999; Cojocaru et al. 2010). Cytopenias other than anaemia were not identified in LPD-affected kittens.

Potential similarities between the disease in kittens and ALPS in people had not been identified when samples were taken from affected kittens, and consequently, blood
samples were not Coombs’ tested for the presence of autoantibodies. Accordingly, although haemolytic anaemia could not be definitively confirmed, several findings support a possible haemolytic basis for anaemia in LPD-affected kittens. The presence of decreased PCV with erythrocyte polychromasia, anisocytosis and reticulocytosis together with erythroid hyperplasia of the bone marrow indicate the presence of a regenerative anaemia. The lack of evidence of haemorrhage and the presence of normal total plasma protein without concurrent clinical dehydration indicate a possible haemolytic cause for that anaemia. Histological evidence of erythrophagocytosis and increased haemosiderin within the liver and spleen further suggests extravascular haemolysis as a possible mechanism for the regenerative anaemia in the kittens; this finding is also reported in ALPS patients (Le Deist et al. 1996). Extravascular haemolysis may be due to multiple causes, including erythrocytic parasites, intrinsic erythrocyte defects, erythrocyte fragmentation, and immune-mediated mechanisms (Stockham and Scott 2008). While the cause of any extravascular haemolysis in the kittens could not be determined, in the absence of other obvious causes, an immune-mediated cause is possible. Additional diagnostic testing including full CBCs and Coombs’ testing of blood from any future cases of LPD in kittens would allow confirmation or exclusion of haemolytic anaemia and investigation of the presence of circulating autoantibodies to erythrocytes. Monitoring LPD-affected kittens for a longer period of time may also reveal the development of immune-mediated disease as appears common in people with ALPS. Neither neutropenia nor thrombocytopenia were observed in LPD-affected kittens for which data were available, but again, the presence of circulating autoantibodies could not be determined.

Histological findings within the lymph nodes, spleen and other organs in LPD-affected kittens have several similarities but also some differences from the typical histological findings in people with ALPS. The lymph nodes of ALPS patients are typically enlarged by the marked expansion of interfollicular areas by a population of lymphocytes at various stages of immunoblastic transformation admixed with variable numbers of plasma cells and mature lymphocytes (Sneller et al. 1992; Sneller et al. 1997; Lim et al. 1998). Lymph node histology from some LPD-affected kittens similarly showed variable numbers of plasma cells, but this was not consistently present. Proliferating lymphocytes from human cases of ALPS show a high mitotic rate with an apparent reduction in the number of lymphocytes undergoing apoptosis (Lim et al. 1998), findings which also appeared to be present in LPD-affected kittens and which are investigated further in Chapters 5 and 6.
However, lymph nodes from ALPS patients generally show retention of nodal architecture and germinal centres. While this differs to the marked distortion or effacement of lymph node architecture present in LPD-affected kittens, histological effacement of lymph node architecture in ALPS patients is also reported. The lymphoid proliferation within the lymph nodes of some ALPS patients may be so marked that an initial histological diagnosis of lymphoma is made (Canale and Smith 1967; Sneller et al. 1997; Lim et al. 1998; Weintraub et al. 1998; Strobel et al. 1999; Blesing et al. 2000). In such cases, it is only the results of further diagnostic tests including immunophenotyping, clonality assays and investigations of defective lymphocyte apoptosis that allow the diagnosis of lymphoma to be excluded. Further investigations of the LPD in BSH kittens performed to definitively confirm or exclude lymphoma and investigate additional similarities with ALPS in people are described in Chapters 5 and 6.

Splenic and hepatic histology in LPD-affected kittens also shows both similarities and differences to that typically reported in people with ALPS. Splenic enlargement is common in ALPS, predominantly due to the expansion of the red and sometimes white pulp by a similar lymphoid cell population to that present within the lymph nodes, again admixed with plasma cells (Le Deist et al. 1996; Lim et al. 1998). The spleen was also enlarged by an abnormal lymphoid cell population in affected kittens, but lymphocytes predominantly expanded the white pulp and plasma cells were not a feature of the cellular infiltrate. Hepatic enlargement in ALPS is more variable (Canale and Smith 1967; Sneller et al. 1992; Le Deist et al. 1996; Infante et al. 1998) and histological hepatic changes are generally limited to extramedullary haematopoiesis, erythrophagocytosis and a mild lymphocytic infiltrate within sinusoids and portal areas (Le Deist et al. 1996; Lim et al. 1998). Extramedullary haematopoiesis, erythrophagocytosis and a lymphocytic infiltrate within sinusoids and portal areas were also present on hepatic histology of LPD-affected kittens.

In human ALPS patients, lymphadenopathy is generally chronic and not usually life-threatening (Sneller et al. 1997; Rao et al. 2005). This appears different to the course of disease in the kittens, whereby all were dead by 10 weeks of age. However, the majority of LPD-affected kittens were euthanased due to the poor prognosis of the presumptive diagnosis of lymphoma. It is therefore not known whether the disease in kittens would
have had a more chronic course and the lymphadenopathy ultimately diminished had the kittens lived longer.

While thymic enlargement detectable on ultrasound examination is reported in some ALPS patients (Avila et al. 1999), thymic abnormalities are generally not described in human ALPS patients (Sneller et al. 1992; Le Deist et al. 1996; Lim et al. 1998). Likewise, the thymuses from all LPD-affected kittens examined did not appear grossly enlarged compared with control kittens of similar age, and thymic histology from these kittens was also unremarkable. However, thymuses of affected kittens were not weighed and it is possible that subtle changes in thymic size were overlooked.

The disease in BSH kittens also has several similarities to that in mice with lpr or gld gene mutations. These murine diseases are similar to ALPS and are used as a model for the disease in people (Sneller et al. 1992). The clinical manifestations of the disease in mice vary widely between different murine strains and include features not typically present in people with ALPS, such as glomerulonephritis and vasculitis (Izui et al. 1984; Cohen and Eisenberg 1991). However, the development of autoimmune disease and a marked generalised lymphadenopathy at a young age due to accumulation of a subpopulation of lymphocytes which express CD3 but neither CD4 or CD8 surface markers (“double negative T cells” or DNT cells) are consistent features of both lpr and gld disease in mice (Cohen and Eisenberg 1991; Watanabe-Fukunaga et al. 1992). There is limited description of the histological changes present within lymph nodes and other organs of lpr and gld mice, but effacement and loss of normal lymph node architecture by an atypical population of lymphoid cells in mice has been described (Roths et al. 1984; Kimura et al. 1990). Variable but less extensive infiltration of other organs including spleen and liver by lymphocytes has also been described in affected mice (Kimura et al. 1990; Cohen and Eisenberg 1991; Weintraub et al. 1998). These findings appear similar to the histological changes present within the lymph nodes, spleen and liver of LPD-affected kittens. As was the case in affected kittens, thymic enlargement is not described in mice with gld or lpr disease (Roths et al. 1984).

Due to the reliance on referring veterinarians and the owners of the affected kittens to collect and record data from the initial cases, some clinical and pathologic data was unavailable in the present study. The only LPD-affected animals fully assessed were K10
and K11 from Litter 2; no other kittens from any of the litters bred as part of the study (Litters 3-7) developed LPD. In particular, the absence of full CBC information and serum biochemistry for several LPD-affected animals, and the failure to perform a Coombs’ test in any affected animal is unfortunate, as these tests may have allowed determination of the pathogenesis of disease.

It is of note that while affected kittens’ lymph nodes were markedly enlarged on palpation, lymphadenopathy was not obvious on external visual examination alone. It is therefore possible that similar cases of this disease in kittens have been previously overlooked by owners and breeders if affected animals did not receive a veterinary or post mortem examination. This may have been more likely if only a single kitten in a litter was affected.

The clinical presentation and pathological findings in samples from the adult cat affected by LPD differed significantly from those present in affected kittens. Lymph nodes were only mildly enlarged (up to 1.5x normal size) compared to the massive enlargement (10-20 times normal size) consistently observed seen in affected kittens. Histological findings present in examined lymph nodes and spleen were also different in samples from K11 compared to those from affected kittens. Cortico-medullary architecture was intact within all K11’s examined lymph nodes, and lymphoid follicles and germinal centres were easily discernible. Germinal centres throughout the expanded cortex appeared small in size but mildly increased in number with slight expansion of the mantle cell cuffs to completely encircle germinal centres (see Figure 4.7E). These changes were interpreted as consistent with follicular hyperplasia and are commonly seen in animals mounting an appropriate immune (humoral) response to the presence of antigen (Valli 2007a).

Histologically, the nodal changes in K11 appear inconsistent with lymphoma and were distinct from the florid lymphocyte proliferations present in lymph nodes from LPD-affected kittens. The reduction in the size of germinal centres in K11’s lymph nodes likely indicates early involution. Splenic changes in K11 also differed to those present in LPD-affected kittens, as well as to those typically reported in people with ALPS. While K11’s spleen was diffusely enlarged, this was due to the presence of marked vascular congestion, most likely as a consequence of barbiturate euthanasia. The abnormal population of lymphocytes present within the spleens of LPD-affected kittens and people

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with ALPS (Le Deist et al. 1996; Lim et al. 1998) was not present in splenic samples from K11.

The clinical presentation, serum biochemistry and pathology from K11 all indicated the presence of severe chronic liver disease which was not observed in any of the affected kittens. Histologically, the presence of extensive portal and bridging fibrosis, a mixed inflammatory portal infiltrate, marked bile duct proliferation and hepatocyte loss suggested chronic cholangiohepatitis (Prasse et al. 1982; Day 1995). While the cause of the chronic cholangiohepatitis in K11 is unknown, histological lesions resembled those reported in primary biliary cirrhosis in humans (Talwalkar and Lindor 2003). The pathogenesis of primary biliary cirrhosis in people is poorly understood, but genetic susceptibility and autoimmune mechanisms appear to be factors in the development of disease (Talwalkar and Lindor 2003). Of note, primary biliary cirrhosis is one of the clinical manifestations of autoimmunity associated with ALPS (Pensati et al. 1997; Avila et al. 1999; Rao and Straus 2006) and is also reported in certain strains of lpr mutant mice (Tsuneyama et al. 2001; Ohba et al. 2002). It is possible that the disease in K11 may also have had an autoimmune component. Chronic histological lesions likely to contribute to the development of cholestasis were present in hepatic samples from K11, including biliary fibrosis and bile duct hyperplasia. Although no elevation in serum alkaline phosphatase was seen in samples from K11, due to the relatively short half-life of alkaline phosphatase in cats, elevations are not consistently present in such cases. An elevation in serum bilirubin, which was present in samples from K11, is often a more sensitive indicator of cholestatic damage in cats.

In conclusion, clinical and pathological features of this LPD in multiple related BSH and BSH-cross kittens are highly unusual and do not appear to be analogous to any disease previously reported in cats. While microscopic pathology is most suggestive of lymphoma, several clinico-pathological features of disease are unusual for lymphoid neoplasia, and further investigation is warranted to confirm or exclude this diagnosis. There is no evidence supporting a viral cause for disease in affected kittens. The disease in kittens appears to have several similarities to ALPS in people and disease due to gld or lpr mutations in mice. Features of disease in the adult cat affected by LPD differ to those in the kittens, and it is uncertain whether the disease in the sole adult cat affected by LPD
here represents a different clinical form of the disease seen in kittens or is an unrelated disease entity.

4.5 SUMMARY

The clinical, haematological and pathological features of LPD present in BSH and BSH-cross kittens are highly unusual and have not been previously reported in cats or other domestic animals. Affected kittens consistently presented between 5 and 7 weeks of age with massive lymphadenopathy involving all lymph nodes. Histological and cytological findings support a diagnosis of multicentric lymphoma involving multiple lymph nodes with variable involvement of other organs, particularly the spleen. A regenerative and likely haemolytic anaemia was also consistently present in affected kittens and may have an immune-mediated aetiology. While pathological features strongly suggested a diagnosis of lymphoma, the young age of animals at presentation, the pattern of disease affecting multiple related animals, and the massive enlargement of all lymph nodes throughout the body, were all features not typically present in lymphoma and possibly suggest a different disease process. As the disease affected multiple related kittens with a shared genetic background, an inherited basis for disease is considered most likely (see Chapter 3). An underlying viral cause for disease is considered unlikely, as all affected animals and unaffected littermates repeatedly tested negative for evidence of either FeLV or FIV infection and the clinical and pathological features of disease here are different to those previously reported with these viral infections. The disease in kittens also appears to have several similarities to ALPS in people and disease due to gld or lpr mutations in mice.

4.6 REFERENCES


CHAPTER 5

FURTHER CHARACTERISATIONS OF DISEASE

5.1 INTRODUCTION

In Chapter 4, it was shown that the gross and microscopic features of lymphoproliferative disease (LPD) in British shorthair (BSH) kittens strongly supported a diagnosis of lymphoma. However, as discussed previously, the kittens’ young age at presentation, the fact that multiple related kittens were affected, the degree of lymph node enlargement and the consistent involvement of multiple lymph nodes were all unusual for feline lymphoma. As these features had not previously been reported in cats or other domestic animals, further investigation was warranted. Accordingly, additional studies were needed to better characterise the nature of disease in BSH kittens and cats and to definitively confirm the initial diagnosis of lymphoma. Characterising the disease in greater depth would enable meaningful comparisons to be made with similar diseases previously described in humans and other animals, and potentially allow identification of the basis of the disease.
The aim of the studies described in this chapter was to further describe and characterise the nature of LPD in BSH kittens and cats, to confirm or exclude the diagnosis of lymphoma, and to provide a basis for further investigations of the underlying pathogenesis. As the results of studies reported in Chapter 3 suggested that the disease had an inherited basis, the presence of an inherited chromosomal abnormality was initially considered and preliminary investigations of chromosomal abnormalities in affected kittens are described. Next, investigations to classify the phenotype of the proliferating lymphoid cells in LPD-affected BSH kittens and cats in both blood and lymphoid tissue using immunochemical methods are described. Finally, to confirm or exclude the initial diagnosis of lymphoma, studies assessing the clonality of the lymphoid cellular proliferation using polymerase chain reaction (PCR) methods are described.

5.2 MATERIALS AND METHODS

5.2.1 INVESTIGATIONS OF CHROMOSOMAL ABNORMALITIES

Eight BSH kittens and cats with LPD had been previously identified. These included 7 kittens (K1, K2, K3, K10, K32, K33 and K34) and one adult cat (K11) (see Chapters 3 and 4). Of these 8 animals, fresh blood or other cells suitable for cell culture and subsequent karyotyping were available from one kitten (K10). Suitable samples were also available from this affected kitten’s dam (Q1). Blood was also collected from other LPD-affected kittens but was not processed as required for karyotyping studies and could not be used.

Preliminary investigations of chromosomal abnormalities were therefore performed on K10 and Q1, with further karyotyping analyses of future cases of LPD–affected kittens planned if the initial studies revealed abnormalities, or if subsequent litters of kittens also developed LPD. For the LPD-affected kitten (K10), karyotypes of both peripheral lymphocytes and karyotypes of dermal fibroblasts were analysed to determine whether there were karyotypic differences between the affected kitten’s lymphocytes, which appeared to be proliferating abnormally, and their somatic cells (the fibroblasts), which did not. For the dam, karyotype analysis was undertaken on peripheral lymphocytes only. The standard karyotype of the domestic cat (*Felis catus*) as previously reported (Wurster-
Hill and Gray 1973; Nie et al. 2002) was used as a normal baseline karyotype for comparison.

For karyotype analysis of peripheral blood lymphocytes, a minimum volume of 2mL blood was drawn from the jugular vein into lithium heparinised collection tubes. Aliquots of 0.3mL heparinised whole blood were inoculated with 0.6mL of PB-MAX Karyotyping Medium (Invitrogen/Life Technologies, United States), mixed and incubated at 37°C in a 5% carbon dioxide (CO₂) atmosphere for 65 hours. For analysis of the somatic cells, fibroblast cultures were prepared using a protocol adapted from those previously described in the human literature (Rittie and Fisher 2005). Briefly, several small (approximately 5mm diameter), full thickness skin biopsies were collected aseptically from K10 at the time of euthanasia. The subcutaneous tissue and epidermis were removed and the remaining dermis macerated in a Petri dish using two scalpels and incubated overnight in 0.3% trypsin in 100mL phosphate buffered saline (PBS) at 4°C. Small pieces (1-2mm) of dermis were placed in 24-well plates and allowed to dry for 30 minutes before the addition of 1.5mL of complete media (Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen Corporation, New Zealand), 100IU/mL penicillin, 100µg/mL streptomycin (Invitrogen Corporation, New Zealand), 2.5µg/mL amphotericin B (Invitrogen Corporation, United Kingdom), 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Invitrogen), 10% fetal calf serum (FCS, Invitrogen, New Zealand). Plates were incubated at 37°C in a 5% CO₂ atmosphere until cells were confluent. When approximately 70% confluent, fibroblasts were sub-cultured into 85cm² culture flasks with 20mL of complete media and re-incubated at 37°C in a 5% CO₂ atmosphere.

Cells were harvested when approximately 70% confluent. Prior to harvesting the cells, 5µl colcemid solution (10µg/mL) was added to each culture, mixed, and the cells re-incubated at 37°C for 20 minutes. Cells then were centrifuged for 9 minutes at 1400 rpm, the supernatant removed, and the pellet re-suspended in 10mL of warmed hypotonic 0.4% potassium chloride and incubated at room temperature for 10 minutes. After re-suspending the cells, 2mL of freshly prepared ice-cold fixative (3:1 methanol:acetic acid) was added and mixed. The re-suspended pellet was then re-centrifuged for 9 minutes at 1400 rpm. The supernatant was removed and the cells resuspended in 8mL of ice-cold fixative and incubated at room temperature for 10 minutes. The centrifugation,
resuspension and incubation steps were repeated until the solution was colourless. After the final centrifugation, the cells were resuspended in 0.5mL fixative. With a transfer pipette, the cell suspension was mixed gently and 1-3 drops of cell suspension placed onto cleaned glass slides, a drop of clean fixative applied to the slide, air dried and the mitotic index assessed. The slides were heated overnight in a 65°C oven, removed and immersed in 2x saline sodium citrate buffer at 40°C for 60 minutes. Slides were rinsed in distilled water before staining in 3% Giemsa solution in phosphate buffer (pH 6.5) for 6-8 minutes, rinsed in phosphate buffer, then distilled water, and finally air-dried. For each sample, metaphases of at least 400 cells were analysed microscopically at 1000x magnification, captured and analysed using Metafer4 Auto Capt Ikaros software (MetaSystems, Germany) and a Zeiss microscope.

5.2.2 INVESTIGATIONS OF LYMPHOID CELLULAR PHENOTYPE

5.2.2.1 IMMUNOHISTOCHEMISTRY

Of the 8 LPD-affected kittens and cats previously identified, formalin-fixed, paraffin-embedded (FFPE) tissue from affected lymph nodes, spleen and liver was available from 4 kittens (K1, K2, K10, and K32) and one adult cat (K11). Formalin-fixed, paraffin-embedded lymph node, liver and splenic tissue from three unrelated cats unaffected by LPD were used as control tissues. Formalin-fixed, paraffin-embedded sections of three lymphomas diagnosed by histology from three unrelated cats were also used to compare proliferation indices.

The CD3 and CD79a immunohistochemical reactions were performed using a Ventana Benchmark Ultra autostainer (Ventana Medical Systems, United States). Sections of lymph node, liver and spleen from LPD-affected kittens and cats were cut at a thickness of 5μm, mounted on charged microscope slides, deparaffinised in xylene, rehydrated in graded ethanol and rinsed in distilled water. Antigen retrieval was achieved by incubating sections in CC2 antigen retrieval solution (Ventana) for 44 minutes at 91°C (for CD3) and in CC1 antigen retrieval solution (Ventana) for 20 minutes at 100°C (for CD79a). Slides were incubated with anti-human CD3ε (LN10 mouse monoclonal, Novocastra, Leica Biosystems, Germany) at a dilution of 1:200 at room temperature for 32 minutes, or with anti-human CD79a (HM57 mouse monoclonal, Dako, United States) at a dilution of 1:200
at room temperature for 16 minutes. Both antibodies have been previously shown to cross react with feline tissue (Darbes et al. 1997; Pohlman et al. 2009; Moore et al. 2012). The UltraView DAB (3,3’-diaminobenzidine) detection kit (Ventana) was used to visualise the immunoreaction and block endogenous peroxidase activity as per the manufacturer’s instructions. Following immunostaining, sections were rinsed in tap water, counterstained with Gill’s haematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped.

Lymphocytes within the germinal centres (CD79a) or paracortex (CD3) of lymph nodes of normal cats were used as positive controls. Cells were interpreted as T-cells if they exhibited cellular membrane immunostaining with CD3 and did not exhibit cytoplasmic immunostaining with CD79a. Cells were interpreted as B-cells if they exhibited cytoplasmic immunostaining with CD79a and did not exhibit cell membrane immunostaining with CD3. The primary antibody was omitted in negative controls.

The Ki-67 immunohistochemical reactions were performed as described above for CD79a except that antigen retrieval was achieved by incubating sections in CC1 antigen retrieval solution (Ventana) for 36 minutes at 100°C and slides were incubated with anti-human Ki-67 (MIB-1 mouse monoclonal, Dako) at a dilution of 1:150 for 36 minutes. Sections of lymph node from four LPD-affected BSH kittens, from three unrelated cats diagnosed with sporadic lymphoma on routine histology, and from three normal cats unaffected by LPD were used for the Ki-67 immunohistochemical reactions. Lymphocytes within the germinal centres of lymph nodes of normal cats were used as positive controls. The primary antibody was omitted in negative controls.

The presence of Ki-67 nuclear immunostaining within lymphocytes was used to assess the proliferation indices as previously described (Webster et al. 2007; Broyde et al. 2009). For all samples assessed, the number of lymphocytes with visible Ki-67 nuclear immunostaining and the total number of lymphocytes across 10 randomly selected 1000x fields within the lymph node were assessed by photographing using NIS Elements software (Nikon Instruments Inc., United States) and a Nikon microscope and then counted using Image J software (Schneider et al. 2012). A mean proliferation index percentage for each sample was obtained by dividing the total number of Ki-67 immunopositive lymphocytes by the total number of all lymphocytes across all 10
examined fields for that sample. The proliferation index in lymph node samples from LPD-affected BSH kittens was compared to that in samples from the adult cat affected by LPD (K11), samples from unrelated cats with lymphoma, and samples from cats unaffected by LPD. Differences between groups were investigated by 2-sample t-tests and one-way analysis of variance (ANOVA) using Minitab 15 statistical software (2007, Minitab Inc.).

5.2.2.2 IMMUNOCYTOCHEMISTRY

Of the 8 LPD-affected kittens and cats previously identified, fresh cells from the lymph nodes of LPD-affected kittens and cats suitable for immunocytochemistry were available from two animals (K10 and K11). Air-dried aspirate smears from lymph nodes from cats unaffected by LPD were used as control samples.

Direct smears were made from multiple (<20) fine needle aspirates or impression smears from affected lymph nodes either prior to or immediately after euthanasia and sent to the University of California, Davis (United States) for immunocytochemical immunophenotyping. Direct smears were fixed in acetone for 3 minutes. Endogenous peroxidase activity was quenched for 10 minutes with a solution containing 0.3% hydrogen peroxide and 0.1% sodium azide in PBS followed by washing for three minutes three times in PBS. Slides were blocked for 20 minutes with 10% heat-inactivated normal equine serum in PBS. Slides were then incubated with either anti-feline CD3ε (CD3-12 Serotec, United Kingdom), anti-feline CD4 (Fe1.7B11, Leukocyte Antigen Biology Laboratory (LABL), Peter F. Moore, UC Davis, United States), anti-feline CD8α (Fe1.10E9, LABL, United States), anti-feline CD8β (Fe5.4D2, LABL, United States), or anti-feline MHCII (42.3, LABL, United States) as tissue culture fluid supernatants diluted 1:10 for optimal reactivity as established previously (Affolter and Moore 2006). Slides were incubated with the primary antibodies for one hour and then washed three times with PBS. Slides were incubated for 30 minutes with biotinylated horse anti-mouse IgG (Vector Laboratories, United States) according to the manufacturer’s instructions and then washed again three times with PBS. Sections were stained by the biotin-streptavidase horseradish-peroxidase method according to the manufacturer’s instructions (Zymed, United States) before washing in PBS and development of the slides using aminoethylcarbazole as a chromagen. Finally, slides were counterstained with Gill’s hematoxylin, dried, coverslipped with Faramount (Dako, United States) and examined microscopically (Vernau and Moore 1999;
Affolter and Moore 2006). Cells were interpreted as CD3 positive if they exhibited cellular membrane and cytoplasmic immunostaining with CD3 antibodies, CD4-positive if they exhibited cellular membrane immunostaining with CD4 antibodies, CD8α-positive if they exhibited cellular membrane immunostaining with CD8α, CD8β-positive if they exhibited cellular membrane immunostaining with CD8β, and MHCII positive if they exhibited cellular membrane immunostaining with MHCII antibodies.

5.2.2.3 FLOW CYTOMETRY

Of the 8 LPD-affected kittens and cats previously identified, fresh or frozen whole peripheral blood samples suitable for determination of lymphocyte subpopulations by flow cytometry were available from only one animal (K11, the adult cat). However, as further cases of LPD in future litters of BSH kittens were anticipated at the time these studies were undertaken, and because elevations in peripheral double negative T-cell (DNT cell) numbers are sometimes observed in the clinically normal relatives of ALPS patients, it was decided to proceed with the development and optimisation of a flow cytometric assay to identify DNT cells in peripheral feline blood. Commercial flow cytometric testing capable of identifying DNT cells in feline blood is not currently available in New Zealand. Double negative T-cells are cells which are immunopositive for T cells markers such as CD3 or CD5, but immunonegative for both CD4 and CD8.

As feline reference ranges for peripheral blood DNT cells have not been established, two to three mL of blood was collected from 22 kittens and cats by jugular venipuncture and processed within 12 hours of collection. Animals used comprised 4 clinically healthy unrelated control cats, 6 clinically healthy littermates (Litters 1 and 2) of LPD-affected kittens, 11 clinically healthy kittens and cats from unaffected litters (Litters 3, 4 and 6) and the dam of two of the three LPD-affected litters (Q1). Blood was collected into lithium heparinised blood tubes from all animals; where possible blood was also collected into EDTA tubes as well. A complete blood count (CBC) was performed on blood samples at a commercial veterinary laboratory (New Zealand Veterinary Pathology, Palmerston North) using an ADVIA 120 Haematology Analyser (Siemens Healthcare Diagnostic Inc., United States). Where sufficient blood could not be collected for an automated CBC, the CBC was estimated manually from duplicate blood smears as previously described in Chapter 4.
Blood samples were diluted in an equal volume of PBS, layered over Histopaque®-1077 (Sigma-Aldrich, New Zealand) at a 2:1 ratio in 15mL conical tubes and centrifuged at room temperature for 20 minutes at 410 g (2000 rpm). Peripheral blood mononuclear cells (PBMCs) were collected at the interface and washed twice with 10mL DMEM containing 5% FCS, then counted and adjusted to $1 \times 10^6$ viable cells/mL. As it was not possible to analyse blood from all animals immediately after acquisition, samples from 18 animals were frozen as previously described (Bull et al. 2002; Roelke et al. 2006; Alam et al. 2012). Briefly, the cell pellet was re-suspended in 1mL Recovery™ Cell Culture Freezing Medium (Life Technologies, New Zealand) and placed in a Nunc cryovial that was wrapped in cotton wool and frozen at -80°C overnight before placing in liquid nitrogen and stored until needed. For analysis, frozen cells were thawed rapidly, washed in 9 mL DMEM containing 10% fetal calf serum, pelleted by centrifuging for 5 minutes at 1500rpm, resuspended in 1 mL medium, counted using a haemacytometer, and adjusted to $1 \times 10^6$ viable cells/mL. To identify potential differences between fresh and frozen blood samples, samples from five animals were divided into aliquots and one aliquot frozen as described above and the other analysed fresh.

To investigate percentages of DNT cells present within examined cats’ peripheral blood, three-colour flow cytometric analysis was performed using antibodies against CD5 (T-cell marker), CD21 (B-cell marker), CD4 and CD8 antibodies, with DNT cells expected to express CD5 but not CD21, CD4 or CD8. Anti-canine CD21 antibody was used as a B-cell marker due to the absence of commercially available anti-feline antibodies against CD21 and the previously reported cross-reactivity of the anti-canine antibody with feline cells (Brodersen et al. 1998). CD5 was used as a pan T-cell marker due to the absence of an available cell surface CD3 antibody cross-reactive in feline cells suitable for flow cytometry.

Cells (100μl containing $1 \times 10^5$ cells/sample) were incubated with 100μl of a cocktail of primary antibodies in PBS containing mouse anti-feline CD5:biotin (0.1mg/mL IgG1, clone FE1.1B11, conjugated to biotin; AbD Serotec, ALS, Australia; 1.5μl/sample), mouse anti-canine CD21:RPE (0.1mg/mL IgG1, clone CA2.1, conjugated to R-phycoerythrin; AbD Serotec, ALS, Australia; 3μl/sample), mouse anti-feline CD4:FITC (0.1mg/mL IgG1, clone vpg34, conjugated to fluorescein isothiocyanate (FITC); AbD Serotec, ALS, Australia; 4μl/sample) and mouse anti-feline CD8:FITC (0.1mg/mL IgG1, clone vpg34, conjugated to
FITC; Santa Cruz, United States; 10μl/sample). After 30 minutes incubation at 5°C protected from light, samples were washed twice with 2 mL cold PBS by centrifuging for 5 minutes at 1500rpm, and re-incubated with 100μl PBS containing streptavidin conjugated to PE-Cy5-streptavidin (0.1mg/mL; BD Pharming, United States; 1μl/sample). Cells were again washed twice, resuspended in 2% paraformaldehyde, and data collected by flow cytometry.

Volumes of antibody used per sample were based on previous titration curves performed using cells from control cats. Samples were acquired using a BD FACSCalibur with CellQuest software. For each experiment, background levels of fluorescence were set using control tubes of cells containing individual or mixed isotype controls (5μl PE-labelled mouse IgG2a at 0.2 mg/mL, 2μl PE-Cy5-labelled mouse IgG2a at 0.2 mg/mL, or 5μl FITC-labelled mouse IgG2a at 0.5 mg/mL; BD Pharmingen), and compensation was set using control tubes of cells containing only anti-CD4, only anti-CD8, only anti-CD21, or only anti-CD5 plus streptavidin/PE-Cy5. Lymphocytes were gated by forward and side scatter characteristics and a minimum of 10 000 events acquired per sample. Cells were regarded as DNT cells if their scatter characteristics were consistent with those previously reported for feline lymphocytes on forward and back gating and were CD5+, CD21-, CD4- and CD8- on flow cytometric analysis.

Paired sample comparisons between percentages of CD5+, CD21+ and DNT cells in fresh and frozen samples were investigated using the Wilcoxon paired signed rank test. Double negative T-cell percentages in samples from control cats were compared to those in samples from the adult cat affected by LPD (K11) (“LPD-affected adult cat”), siblings and parents of LPD-affected kittens (“LPD-related cats”), and finally, kittens from litters bred as part of the present studies but unaffected by LPD (“unaffected litters”) using 2-sample t-tests and one-way ANOVA. All statistical analyses were performed Minitab 15 statistical software (2007, Minitab Inc.).

5.2.3 Clonality Studies

Of the 8 LPD-affected kittens and cats previously identified, FFPE lymph node samples suitable for clonality assays were available from 4 LPD-affected kittens (K1, K2, K10 and K32) and also from the adult cat affected by LPD (K11). Samples of FFPE lymph nodes previously harvested from cats unaffected by LPD were used as control tissues.
To assess clonality of the population of lymphoid cells within the lymph nodes of affected BSH-kittens, lymphocyte antigen receptor gene rearrangement was assessed by polymerase chain reaction (PCR) analysis of the T-cell receptor gamma (TCRG) and immunoglobulin heavy chain (IGH) loci as previously described (Moore et al. 2005; Werner et al. 2005). Although samples of affected lymph nodes from the 4 kittens with LPD had been previously confirmed as T-cell proliferations on immunohistochemistry (see sections 5.2.2 above and section 5.3.2 below), the presence of B-cell rearrangements in the cell population was also assessed to exclude the possibility of cross-lineage gene rearrangements involving both T- and B-cell loci. Additionally, the presence of clonal cross-lineage IGH rearrangements could help indicate false negative T-cell clonality results, given that the sensitivity of the assays is not 100%. Immunohistochemistry of samples of affected lymph nodes from the adult cat affected by LPD (K11) indicated the presence of a mixed population of T- and B-cells (see section 5.3.2 below) and were also assessed for rearrangements of both T- and B-cell loci.

DNA was extracted from 4 x 25μm tissue sections cut from FFPE blocks. Tissue sections were collected into 1.5mL Eppendorf tubes, deparaffinised with xylene and washed twice in 100% ethanol. DNA extraction was performed using a DNeasy Extraction kit (Qiagen, United States) as per the manufacturer’s recommendations.

Rearrangements of TCRG in the extracted DNA were assessed by PCR amplification of the CDR3 region between the variable (V) and joining (J) segments. Approximately 100ng of genomic DNA from each sample was amplified in a 50μL reaction using a consensus primer derived from the TCRG V segment (10µmol) in conjunction with a consensus primer derived from the TCRG J segment (10µmol) of feline TCRG cDNA transcripts. Details of the primers used are contained in Table 5.1. To each thin-walled PCR tube on ice containing the relevant DNA sample was added 5µL 10X reaction buffer, 1µL MgCl₂, 1µL dNTP (containing 2.5mM each of dATP, dCTP, dGTP and dTTP), 0.25µL Taq, 2µL TCRG V primer and 1µL TCRG J primer, made up to a total reaction volume of 50µL with autoclaved distilled water. Specific amplicon size was expected to occur within a target range of 80-120 base pairs.
Rearrangements of \( IGH \) were assessed by amplification of 2 loci, including the CDR3 region encompassing the junction of the V, diversity (D), and J segments. Approximately 100ng of genomic DNA from each sample was amplified in a 50\( \mu \)L reaction using consensus primers derived from the feline \( IGH \) V segment framework 2 (FR2, 10\( \mu \)mol) and framework 3 (FR3, 10\( \mu \)mol) in conjunction with consensus primers derived from \( IGH \) J segments (J2, 10 \( \mu \)mol and JD, 10\( \mu \)mol) of feline \( IGH \) cDNA transcripts. Details of the primers used are contained in Table 5.1. To each thin-walled PCR tube containing the relevant DNA sample was added 5\( \mu \)L 10X reaction buffer, 1\( \mu \)L MgCl\(_2\), 1\( \mu \)L dNTP, 0.25\( \mu \)L Taq, and either: 1\( \mu \)L FR2 primer with 1\( \mu \)L JD primer and 1\( \mu \)L J2 primer; 1\( \mu \)L FR3 primer with 1\( \mu \)L JD primer and 1\( \mu \)L J2 primer, each made up to a total reaction volume of 50\( \mu \)L with autoclaved distilled water. Specific amplicon sizes were expected to occur within a target range of 250-300 base pairs (FR2 primers) and 130-180 base pairs (FR3 primers).

**Table 5.1: List of primers used in polymerase chain reactions (PCR) to assess TCRG and IGH rearrangements for assessment of clonality.** Primers were based on those previously reported by Moore et al., (Moore et al., 2005; Werner et al. 2005; Moore et al., 2012).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Expected amplicon size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRG V</td>
<td>AAG AGC GAY GAG GGM GTG T</td>
<td>80-120</td>
</tr>
<tr>
<td>TCRG J</td>
<td>CTG AGC AGT GTG CCA GSA CC</td>
<td></td>
</tr>
<tr>
<td>FR2</td>
<td>CCA GGC TCC AGG GAA GGG</td>
<td>250-300</td>
</tr>
<tr>
<td>FR3</td>
<td>TCC AGA GAC AAC GCC AAG AAC</td>
<td>130-180</td>
</tr>
<tr>
<td>J2</td>
<td>TGA GGA CAC TGT GAC TAT GGT TCC</td>
<td>n/a</td>
</tr>
<tr>
<td>JD</td>
<td>GGA CAC CGT CAC YAK GYV TCC</td>
<td></td>
</tr>
</tbody>
</table>

A = adenine, C= cytosine, G = guanine, T= thymine, Y = thymine or cytosine, V= guanine, cytosine or adenine, S = guanine or cytosine.

Amplification conditions used a 2-step, modified touchdown protocol to increase specificity of the reactions (Hecker and Roux 1996; Moore et al. 2012). Amplification conditions were as follows: 95\(^\circ\)C for 15 minutes, 5 cycles at 94\(^\circ\)C for 30 seconds followed by 70\(^\circ\)C for 1 minute, then 5 cycles at 94\(^\circ\)C for 30 seconds followed by 68\(^\circ\)C for 1 minute, then 35 cycles at 94\(^\circ\)C for 20 seconds followed by 65\(^\circ\)C for 1 minute, followed by final elongation at 72\(^\circ\)C for 10 minutes. All PCR reactions were performed in triplicate to exclude the possibility of pseudoclonal false positive reactions.
PCR products were separated by capillary electrophoresis (HAD-GT12 system, eGene, Inc, United States) and products in the appropriate base pair size ranges evaluated for clonality using Biocalculator software analysis (Qiagen, United States). A sample was interpreted as clonal if one (monoallelic rearrangement) or two (biallelic rearrangement) sharp spikes or peaks of the appropriate and identical sizes, in triplicate samples run side by side, were present. A sample exhibiting 3 to 5 reproducible narrow spikes in triplicate analyses was interpreted as oligoclonal. A sample was interpreted as pseudoclonal if it contained one or two clonal-appearing spikes or peaks but of differing sizes when triplicate samples were analysed. Any sample interpreted as pseudoclonal was excluded from further analysis. Any sample exhibiting the presence of a broad peak (Gaussian peak) or hump covering a range of product sizes was interpreted as polyclonal. Typical results for monoclonal, polyclonal and pseudoclonal samples are shown in Figure 5.6.

5.3 RESULTS

5.3.1 INVESTIGATIONS OF CHROMOSOMAL ABNORMALITIES

Karyotyping analysis did not reveal the presence of consistent gross chromosomal abnormalities in either dermal fibroblasts or peripheral blood lymphocytes from the LPD-affected kitten (K10) or from his dam (Q1) (see Figure 5.1 below).
Figure 5.1 Karyotype of LPD-affected kitten (K10), prepared using cultured peripheral blood lymphocytes. The karyotype of the LPD-affected kitten does not reveal the presence of gross chromosomal abnormalities and is consistent with a normal male feline karyotype. Constitutional karyotypes prepared using cultured dermal fibroblasts from the same kitten were also grossly normal.

5.3.2 INVESTIGATIONS OF LYMPHOID CELLULAR PHENOTYPE

5.3.2.1 IMMUNOHISTOCHEMISTRY

In all four LPD-affected kittens for which tissue was available (K1, K2, K10 and K32), the predominant cell population expanding lymph nodes exhibited strong cellular membrane immunostaining for CD3 and no cytoplasmic immunostaining for CD79a. This pattern of immunostaining was interpreted as a marked T-cell proliferation within affected lymph nodes (see Figure 5.2). In lymph nodes from two of the four kittens (K2 and K10), the cell population exhibiting cellular membrane immunostaining for CD3 also exhibited variable nuclear immunostaining with CD79a, which was interpreted as non-specific and immunonegative. In lymph nodes from two of the four LPD-affected kittens (K2 and K10), the CD3+/CD79a-T-cell population present effaced normal lymph node architecture with very few CD3-/CD79a+ B-cells still visible within examined sections (see Figure 5.2 A and B). In the other two cases (K1 and K3), effacement of normal nodal architecture by the
CD3+/CD79a- T-cell population was less complete, with occasional small scattered aggregates of CD3-/CD79a+ B-cells present within the cortex (see Figure 5.2 C and D). These findings were consistent with findings in lymph nodes from these kittens stained with haematoxylin and eosin (H&E) and described in Chapter 4. Low numbers of cells within the sub-capsular areas and occasionally within nodal follicular remnants exhibited strong cellular membrane immunostaining for CD79a, and were interpreted as B-cells.

Sections of spleen and liver from all 4 examined LPD-affected kittens contained increased numbers of cells with similar immunochemical characteristics (CD3+/CD79a-) to those present within the kittens’ lymph nodes and described above. In the spleen, this cell population moderately expanded the splenic white pulp and variably expanded the red pulp (see Figures 5.3 A. and B.). This pattern of immunostaining was interpreted as consistent with a moderate T-cell proliferation within the splenic white pulp, and to a lesser extent, the splenic red pulp. In sections of liver from all four LPD-affected kittens, low to moderate numbers of cells within the periportal regions and sinusoids of the liver also exhibited similar CD3+/CD79a- immunostaining characteristics to those present within the lymph nodes (see Figure 5.3 C and D). This pattern of immunostaining was interpreted as consistent with a moderate T-cell proliferation within the periportal areas and sinusoids of the liver.
Figure 5.2: Lymph node immunohistochemistry, CD3 and CD79a (low power), LPD-affected kittens and cats, 3,3′diaminobenzidine/haematoxylin. A. and B., LPD-affected kitten (K10), lymph node, CD3 (A.) and CD79a (B.) immunohistochemistry: Effacement of normal lymph node architecture by a cell population predominantly comprising CD3-positive (A.) and CD79a-negative (B.) cells. C. and D., LPD-affected kitten (K32), lymph node, CD3 (C.) and CD79a (D.) immunohistochemistry: Similar findings to those in figures A. and B. but with several small scattered CD79a-positive follicular remnants visible in D. E. and F., LPD-affected adult cat (K11), lymph node, CD3 (E.) and CD79a (F.) immunohistochemistry: Normal node architecture is present with lymphocytes predominantly CD3-positive within the paracortex (E.) and predominantly CD79a-positive within the follicles, which are increased in number and show mildly expanded mantle zones. All magnification 40x.
In the adult cat affected by LPD on clinical examination and gross post mortem (K11), the pattern of CD3 and CD79a immunostaining differed from that seen in the LPD-affected kittens. Sections of lymph node from this cat revealed cells immunopositive for CD3 located predominantly within the paracortex and cells immunopositive for CD79a predominantly within the follicles, which appeared mildly increased in number with slight expansion of the mantle zones. While the lymph nodes showed diffuse mild enlargement, the distribution of cells was the same as that seen in lymph nodes from control cats (see Figure 5.2 E and F). Similarly, sections of spleen from K11 showed a similar pattern of immunostaining to that present within sections of spleen from control cats, with cells immunopositive for CD3 predominantly within the periarteriolar lymphoid sheaths (PALS) of the white pulp and scattered within the red pulp, while cells immunopositive for CD79a were predominantly located within the follicles of the white pulp and scattered within the red pulp. Examined sections of liver from K11 contained only low numbers of scattered cells immunopositive for both CD3 and CD79a located within the sinusoids and throughout areas of fibrous connective tissue. The multifocal lymphoid follicles present on routine H&E histology were predominantly CD79a immunopositive and interpreted as B-cells.

The mean proliferation indices assessed by Ki-67 immunopositivity were 53.1% (standard deviation 11.8%, range 39.8%-68.2%) for lymph nodes from LPD-affected BSH kittens, 42.2% (standard deviation 6.5%, range 35.2%-47.9%) for lymphoma in lymph nodes from unrelated cats, and 27.2% (standard deviation 0.6%, range 26.7-28.0) in lymph nodes from cats unaffected by LPD. The mean proliferation index in lymph nodes from the adult cat affected by LPD (K11) was 29.9%. The mean proliferation index for LPD-affected BSH kittens was significantly higher than that in normal feline lymph nodes (p=0.022) but was not significantly higher than that in lymphoma from unrelated cats (p=0.195). Although higher than the mean proliferation index for the LPD-affected cat (K11), the mean proliferation index in lymph nodes from the LPD-affected kittens cannot be meaningfully compared to that seen in lymph nodes from adult BSH cats affected by LPD due to the presence of only a single LPD-affected adult animal in the latter group.
5.3.2.2 IMMUNOCYTOCHEMISTRY

All direct smears made from fine needle aspirates and impressions of multiple enlarged lymph nodes from the LPD-affected kitten (K10) examined contained a lymphoid cell population which predominantly exhibited immunopositivity for CD3 and MHCII and immunonegativity for CD4, CD8α and CD8β. This pattern of immunostaining was interpreted as consistent with a predominant DNT cell population within the lymph nodes of this kitten.
Cytological smears from the LPD-affected adult cat (K11) were unable to be assessed immunocytochemically due to the failure of any cells to be immunoreactive with CD3, MHCII, CD4, CD8α or CD8β, despite appropriate staining of control samples.

5.3.2.3 FLOW CYTOMETRY

There was a good correlation between results from fresh and frozen feline blood samples for DNT (CD5+/CD21-/CD4-/CD8-) cell percentages with the Wilcoxon paired signed rank test showing insignificant differences between the two types of samples (p=0.418). Differences between percentages of CD5+ and CD21+ cells from fresh and frozen blood were also insignificant on the Wilcoxon test (p=0.59 for both).

A summary of flow cytometry results from control cats, siblings and parents of LPD-affected kittens, and unaffected litters is set out in Table 5.2, and representative results are set out in Figure 5.4. The mean peripheral CD5+% in control cats (n=4) was 54.8% (range 49.9%-60.8%). This did not differ significantly from that in LPD-related cats (n=7), which was 51.6% (range 47.52%-57.9%) (p=0.304) but did differ from that in unaffected litters (n=11), which was 47.6% (range 40.0%-59.0%) (p= 0.036). The mean peripheral CD21+% in control cats was 17.5% (range 6.6%-26.6%), which did not significantly differ from that in LPD-related cats, which was 20.8% (range 10.7%-31.6%) (p= 0.488) but did differ from that in unaffected litters, which was 31.6% (range 23.0%-43.0%) (p=0.002). The mean peripheral DNT cell % as a percentage of T-cells in control cats was 12.9% (range 8.4%-17.1%), which did not significantly differ from that in LPD-related cats, which was 13.3% (range 10.3%-16.8%) (p= 0.799) but did differ from that in unaffected litters, which was 8.5% (range 6.6%-11.1%) (p=0.007). The percentage of peripheral DNT cells did not appear elevated in the adult cat affected by LPD (10.3%) and was in fact lower than the mean value (12.9%) of controls. Mean percentages of peripheral DNT cells as a percentage of all lymphocytes were not calculated between groups due to the presence of a significant percentage (mean = 21.0%, range 12.0%-30.3%) of unidentified cells which showed scatter characteristics consistent with feline lymphocytes on both forward and back-gating but which were both CD5- and CD21-. As these cells were consistent with neither T-cells nor B-cells on flow cytometric analysis, it was not possible to calculate the accurate DNT cell % values as a percentage of all lymphocytes accurately here.
A. All cells were collected and analysed for CD5 expression. B. and C. Those positive for CD5 ("R1") were back-gated to identify the lymphocyte population ("R2") based on their forward and side scatter characteristics. D. Cells falling within both R1 and R2 (CD5+ cells with the scatter characteristics of lymphocytes) were further assessed for CD4+ CD8 vs CD21 expression. Note the presence of CD5+ cells negative for both CD4 and CD8 (double negative T cells (DNT cells)) in the lower left quadrant of the dot plot. E. and F. In addition, all cells in the lymphocyte gate were assessed for CD4+ CD8 vs CD21 (E) and CD5 vs CD21 (F). In both cases, the presence of cells expressing both T-cell and B-cell markers (upper right quadrant of each dot plot) was negligible, as expected.

Figure 5.4: Representative (Q1) histogram and dot plots of flow cytometry analysis.
5.3.3 CLONALITY STUDIES

Clonality assays of DNA extracted from lymph nodes of all 4 LPD-affected kittens and from the adult cat affected by LPD (K11) for rearrangements of TCRG all revealed broad bands or Gaussian peaks covering a range of product sizes on PCR, consistent with a polyclonal or non-clonal proliferation of T-cells (see Figure 5.5 below). Clonality assessment of all samples for rearrangements of IGH also revealed similar broad bands or Gaussian peaks covering a range of product sizes on PCR, inconsistent with a monoclonal proliferation of T-cells with cross-lineage gene rearrangement involving B-cell loci.

Neither pseudoclonality nor oligoclonality were observed in any sample on PCR testing for either TCRG or IGH rearrangements. Typical TCRG clonality results obtained from monoclonal, polyclonal and pseudoclonal samples are shown in Figure 5.6 for comparison purposes.
Figure 5.5: TCRG clonality results for lymph node samples using capillary electrophoresis. Electrophoretic profiles of the PCR products of TCRG amplification using extracted DNA from lymph nodes and performed in triplicate. A. LPD-affected kitten (K32). B. LPD-affected adult cat (K11). C. Healthy control kitten unaffected by LPD. Samples from all animals show a polyclonal pattern with a broad peak (Gaussian peak) covering a range of product sizes between approximately 80 and 120 base pairs.

Figure 5.6: Typical TCRG clonality results for monoclonal, polyclonal and pseudoclonal samples using capillary electrophoresis. Typical electrophoretic profiles of the different PCR products of TCRG amplification using extracted DNA and performed in duplicate. A. Monoclonal (monoallelic) pattern with a single sharp peak. B. Monoclonal (bialelic) pattern with two sharp peaks. C. Polyclonal pattern with a broad peak (Gaussian peak) covering a range of product sizes. D. Pseudoclonal pattern with multiple sharp peaks of non-identical sizes in duplicate samples.
5.4 DISCUSSION

Immunophenotypic studies and molecular clonality assays are invaluable adjunctive tests in the diagnosis of lymphoma (Moore et al. 2012). In the present studies, further immunophenotypic investigations of the abnormal cell population expanding lymphoid organs of LPD-affected BSH kittens identified the presence of a rapidly proliferating and predominantly T-cell population, but clonality assays confirmed these cells as polyclonal or non-clonal in origin. The identification of a non-clonal lymphoid population in LPD-affected kittens here is significant, as it most likely indicates the presence of a non-neoplastic process. The finding of a non-clonal cell population was unexpected, as it was contrary to the initial gross and microscopic findings previously described in Chapter 4 and also to the identification of a predominantly T-cell population described in this chapter, both of which had supported a diagnosis of lymphoma. In the sole LPD-affected kitten in which CD4 and CD8 cell surface markers were able to be assessed for the proliferating cells, further characterisation of the T-cell population as lacking both CD4 and CD8 surface markers indicated that the proliferating T-cells were an unusual population of “double negative T-cells” (DNT cells). Although confirmation of this finding in other LPD-affected kittens is obviously needed, this finding is potentially significant, as markedly increased numbers of non-clonal DNT cells are also responsible for the enlarged lymph nodes and other organs in human ALPS patients and in mice with gld or lpr gene mutations (Cohen and Eisenberg 1991; Sneller et al. 1992; Lim et al. 1998). This also provides further support for the possibility raised in Chapters 3 and 4 that the basis for the development of LPD in BSH kittens may be similar to that in the human and murine diseases, both of which are most commonly due to mutations in the Fas or Fas ligand (FasL) genes regulating lymphocyte apoptosis (Watanabe-Fukunaga et al. 1992; Takahashi et al. 1994; Rao and Straus 2006). If the disease in BSH kittens does have a similar basis to the human and murine diseases, this would also provide a more satisfactory explanation for some of the unusual features of disease in these kittens, particularly the young age at presentation and the fact that multiple related animals were affected.

Clonality is a fundamental characteristic of neoplasia. Neoplasia results from the clonal expansion of a single transformed cell; in theory, all cells within a neoplasm should be clones of that original transformed cell. While clinical, morphological and
immunophenotypical features may all suggest a diagnosis of lymphoma, demonstration of the presence of a clonal population of lymphocytes is regarded as the gold standard for diagnosis, and the most reliable basis on which to differentiate lymphoma from a non-neoplastic cell proliferation. (Griesser et al. 1989; Weiss and Spagnolo 1993; Vernau and Moore 1999; Werner et al. 2005). Lymphocyte antigen receptor gene rearrangement analysis using PCR methods is commonly used to assess the molecular clonality of lymphocyte proliferations in humans and has also been validated for use in cats (Moore et al. 2005; Werner et al. 2005). The reported sensitivity of the T-cell (TCRG) clonality assay in cats is excellent, detecting clonality in approximately 90% of feline T-cell lymphomas if both monoclonal and oligoclonal results are interpreted as clonal (Moore et al. 2005; Moore et al. 2012). By comparison, B-cell (IGH) clonality assays in cats are relatively insensitive, with clonality reportedly detected in approximately 50%-68% B-cell lymphomas (Werner et al. 2005; Moore et al. 2012). However, pseudoclonality is reportedly a major cause of false positive clonality detection in B-cell lymphomas in cats; in a 2005 study by Werner et al., all feline B-cell lymphomas for which monoclonality was not detected with their assay also demonstrated a pseudoclonal result for at least one primer pair (Werner et al. 2005). Specificity of lymphoma detection can therefore be improved by identifying and accounting for the presence of pseudoclonality. Cross-lineage rearrangements involving TCRG gene rearrangements in B-cell lymphoma, IGH gene rearrangements in T-cell lymphoma or rearrangements of both loci also reported (Griesser 1995; Valli et al. 2006), and are a potential reason for the failure of the assays to detect clonality in lymphoma if the clonality of only one (B- or T-) cell lineage is assayed.

In all 4 of the LPD-affected BSH kittens assessed here, clonality assays confirmed the proliferating cells from affected lymph nodes as a polyclonal or non-clonal population inconsistent with lymphoma. The results of the clonality assays were somewhat unexpected, as gross pathology, histopathology and immunophenotyping results were all strongly suggestive of lymphoma. While it is possible that the clonality results were false, this is considered unlikely for several reasons. Immunohistochemistry unequivocally revealed a predominantly T-cell (CD3+) population within and expanding lymphoid organs in all 4 LPD-affected kittens. As noted above, the T-cell clonality assays performed on samples from affected kittens have excellent (approximately 90%) sensitivity of clonality detection in feline lymphoma (Moore et al. 2005; Moore et al. 2012). Further, the T-cell clonality assays gave repeatable and strongly polyclonal results in triplicate samples from
all 4 kittens tested. Finally, the proliferating cells in the kittens were also assessed for B-cell clonality to exclude the possibility of cross lineage rearrangements, which are a further potential reason why the assays may fail to detect clonality in a particular lymphoma (Griesser 1995; Valli et al. 2006). B-cell clonality assays also demonstrated a repeatable and strongly polyclonal result in all instances, further strengthening the reliability of the results of the clonality assays and the diagnosis of a non-neoplastic process in affected kittens.

The majority of proliferating cells within the lymph nodes in ALPS patients are reportedly CD3+, CD4- and CD8- (consistent with a DNT cell phenotype), variably CD57+ but CD16- and CD56- (indicating a non-natural killer cell phenotype), CD45RO- and CD45RA+ (indicating a “naïve” T-cell phenotype), TCRαβ+ and TCRγδ- (Lim et al. 1998; Oliveira et al. 2010). In people and mice, DNT cells are a heterogeneous and poorly understood group of T-cells. Most T-cells develop in the thymus from blood-borne progenitor cells along a well-characterised pathway of differentiation. The T cell receptor (TCR), a surface molecule on T-cells which recognises major histocompatibility complex (MHC) -bound antigens, is composed of one of two different dimeric proteins: an α and β chain (αβ TCR or αβ T cells) or a γ and δ chain (γδ TCR or γδ T-cells). In humans, the vast majority (reportedly up to 95%) of T-cells are αβ T cells, with γδ T-cells constituting the minority (Kabelitz 1992; Haas et al. 1993; Germain 2002); information on T-cell subtypes in cats is limited. During αβ T-cell development in the thymus, cells pass through several intermediate DNT stages with variable CD44 and CD25 surface marker expression before giving rise to a CD4+/CD8+ (double positive T-cell or DPT cell) population, which then further differentiate to mature CD8+/CD4- cytotoxic αβ T-cells or CD8-/CD4+ helper T-cells. γδ T-cells diverge from the αβ T-cell line during the intermediate DNT stages. (D’Acquisto and Crompton 2011). There are currently several competing theories about the origin of DNT cells found within peripheral blood and other organs. Some authors propose a thymic origin for DNT cells followed by migration to and proliferation at the periphery upon encountering antigen (Mixter et al. 1999; Priatel et al. 2001; Hayes et al. 2005), while other authors propose that DNT cells found within the periphery are actually generated there, citing in support studies demonstrating the presence of DNT cells in thymectomised mice (Kadena et al. 1997; Ford et al. 2006). Mice with lpr or gld mutations and people with ALPS have also been widely used to investigate the developmental origin of DNT cells, as these cells are markedly increased in number in the peripheral blood and
lymphoid organs in these diseases. However, these studies have not definitively
determined the origin of the DNT cells and conclusions are often conflicting (Steinberg et
al. 1980; Mehal and Crispe 1998; Marlies et al. 2007; Bristeau-Leprince et al. 2008; Lev et
al. 2012). Hypotheses include that the cells derive from previously activated mature T-
cells which have subsequently lost CD4 or CD8 receptor expression, or are a minor cell
lineage selectively expanded due to defects in Fas-mediated signaling; recent studies
seem to support a CD8+ origin for the cells (Bristeau-Leprince et al. 2008). Investigations
of DNT cell populations in cats to date have been limited, however, studies suggest that
DNT cells are uncommon in normal feline lymph nodes (Endo et al. 1997; Roccabianca et
al. 2000).

Confirming the presence of a DNT cell population expanding lymph nodes and other
organs of LPD-affected BSH kittens would be significant, as it would provide further
support that the feline disease is similar to both ALPS in people and LPD due to lpr or gld
mutations in mice. The proliferating cell population within the lymph nodes and spleen in
all 4 LPD-affected kittens evaluated here was similar to that in people with ALPS and mice
with lpr or gld mutations, in that they expressed CD3 and did not express CD79a,
confirming the presence of a predominantly T-cell population. However, whether these
cells are DNT cells in all LPD-affected kittens is uncertain, as samples from only one LPD-
affected kitten were able to be evaluated for the presence of CD4 and CD8 surface
markers and the DNT cell phenotype. Fresh or fresh-frozen samples from the other LPD-
affected kittens suitable for CD4 and CD8 analysis were not collected, as potential
similarities between the feline disease and ALPS in people were not recognised until
multiple cases had been identified and analysed and these markers are not routinely
applied in cases of feline lymphadenopathy. It is therefore possible that the results
confirming a DNT cell population from one kitten would not have been repeatable in
other LPD-affected kittens. CD4 and CD8 immunochemical testing of further cases is
needed to confirm this as a finding consistently present in LPD-affected kittens and a
feature of the disease.

Even assuming the reliability of the CD4/CD8 immunocytochemistry results in the single
kitten tested, it is still possible that the cells are not DNT cells similar to those present in
people with ALPS. The DNT cells in ALPS patients are typically CD3+ TCRαβ+ CD4- CD8-.
It is not currently possible to assess feline T-cell receptor (TCR) expression by routine
immunochemical methods, and it is therefore possible that the cells in the kittens were TCRγδ T-cells, which in people also express CD3 but not CD4 or CD8. In addition, as only expression of the epsilon subunit of CD3 (CDε) was investigated immunohistochemically in affected kittens, it is also possible that the proliferating cells are in fact natural killer (NK) cells rather than T-cells. While NK cells are CD3- (as they do not express the fully assembled TCR-CD3 complex), in people they can sometimes show partial CD3 expression which may give an apparent CD3+ immunophenotype if only a single CD3 subunit (such as CD3ε) is assessed (Morice 2007). However, this does not appear to be the case in cats, in which NK cells are reportedly CD3ε- (Vermeulen et al. 2012), and the possibility that the cells in the kittens are NK cells is considered unlikely.

To further determine the likely lineage and origin of the proliferating cells within lymphoid organs in the kittens and assist in determining the underlying molecular or genetic basis for disease, it would also have been desirable that a wider panel of immunochemical markers be applied to the cells in addition to the antibodies that were used. In humans, the current recommendations for cases of suspected ALPS include evaluation of the expanded cell population within the lymph nodes for CD57, CD45RO, CD45RA, TCRαβ and TCRγδ in addition to CD3, CD4 and CD8 (Oliveira et al. 2010). It was not possible to apply these additional markers to samples from any kitten due both to the lack of appropriate samples for testing and the lack of feline specific antibodies cross-reactive in FFPE tissue. Of the 8 LPD-affected kittens and cats, FFPE tissue was available from 4 kittens and the affected adult cat, while fresh tissue was only available from two animals, one LPD-affected kitten (K10) and the adult cat affected by LPD (K11). Of the antibodies routinely applied in humans with suspected ALPS, only CD3 is commercially available and cross-reactive in FFPE feline tissue (Affolter and Moore 2006; Raskin 2010); the other markers are either only reactive in fresh or fresh-frozen feline tissue (e.g. CD4 and CD8) or have not been previously reported as cross-reactive in feline tissue (e.g. CD45RO, CD45RA, TCRαβ and TCRγδ) (Roccabianca et al. 2000; Affolter and Moore 2006; Roccabianca et al. 2006; Raskin 2010). It is therefore possible that the population of cells within LPD-affected kittens’ lymph nodes differs from those present in people with ALPS and mice with gld or lpr mutations. If the cells in affected kittens do have a different immunophenotype to those in human ALPS or murine gld/lpr diseases, then the underlying molecular or genetic basis for the feline disease is also more likely to differ.
Lymph nodes of LPD-affected kittens exhibited high lymphocyte proliferation indices as determined by Ki-67 immunopositivity when compared with lymph nodes from control cats and were similar to proliferation indices observed in sporadic feline lymphomas. Ki-67 is expressed by actively proliferating cells and the proliferation index of a tumour is a general indication of how quickly the tumour is growing (Broyde et al. 2009). Proliferation rates determined by Ki-67 immunopositivity have been previously applied to feline lymphocytes (Madewell et al. 2001; Morita et al. 2009). The high rate of Ki-67+ lymphocyte proliferation seen in lymph nodes from the LPD-affected kittens was consistent with the high mitotic rates observed in those lymph nodes on routine H&E staining (see Chapter 4) and is similar to findings present within lymph nodes of ALPS patients (Lim et al. 1998). In ALPS, the expanded DNT cell population within patients’ lymph nodes is due both to increased lymphocyte proliferation and reduced lymphocyte apoptosis (Lim et al. 1998); the results of investigations of reduced apoptosis in LPD-affected kittens are presented in Chapter 6.

In addition to increased DNT cells within lymphoid organs, people with ALPS show elevations in the percentages of peripheral (TCRαβ) DNT cells compared with healthy controls (Sneller et al. 1992; Sneller et al. 1997; Bleesing et al. 2001a; Bleesing et al. 2001b; Magerus-Chatinet et al. 2009). While mice with lpr or gld gene mutations show increased numbers of (TCRαβ) DNT cells in peripheral lymphoid tissue and other organs, elevations in DNT cell percentages in the blood of affected mice are not specifically reported (Cohen and Eisenberg 1991). To meet the current diagnostic criteria for ALPS, (TCRαβ) DNT cells in the peripheral blood must comprise at least than 1.5% of all lymphocytes or 2.5% of T-cells on flow cytometric analysis (Oliveira et al. 2010). However, actual (TCRαβ) DNT cell percentages are often higher than these minimum values (Bleesing et al. 2001a). For example, Sneller et al. report a mean DNT cell (αβ+TCR) percentage (of total lymphocytes) in examined ALPS patients of 16.0% (range 4-28%) (Sneller et al. 1997). In addition, a proportion of the clinically unaffected relatives of ALPS patients also have elevated DNT cell counts on flow cytometry analysis of peripheral blood (Sneller et al. 1997; Bleesing et al. 2001a). These individuals usually possess the ALPS genotype (and so can transmit the disease to offspring), but do not show the overt ALPS phenotype. It was not possible to investigate DNT cell elevations in LPD-affected kittens here due to the lack of suitable samples, but investigations of the clinically unaffected siblings and parents of LPD-affected kittens did not show elevations of DNT.
cells compared with controls, nor were DNT cells in the peripheral blood of the LPD-affected adult cat elevated. Peripheral DNT cells in the unaffected litters were in fact decreased compared with control animals. This may simply reflect differences such as the younger age of animals in this group (less than 12 weeks) compared with controls, all of which were adult cats. While further studies including younger control animals are needed to confirm this, the difference is considered unlikely to be significant here.

Investigations of DNT cells in feline blood are limited and reference ranges in cats are not established. Estimates of DNT values in control cats may be extrapolated from reported data in a small number of studies, but reliability of the results is questionable and findings show wide variance in estimated DNT cell percentages, from 1.8%-3.3% of T cells (Dean et al. 1991) to 7.1% of T cells (Byrne et al. 2000). Roelke et al. report significantly higher DNT percentages in large felids (21.35% and 18.17% of T cells in the puma and lion respectively) (Roelke et al. 2006), but it is likely that these values are artificially elevated as no B-cell marker was used in that study. The DNT percentage values found in all cats in the present study appear significantly higher than those reported in human controls (Bleesing et al. 2001a; Oliveira et al. 2010). This finding may be due to inherent species differences between felids and humans in DNT cell populations. Alternatively, the higher apparent DNT cell percentages in cats here may be due to a proportion of the cells identified as DNT cells in fact being CD21+ B cells. It is known that the CD5 antibody used here will also cross react with a proportion of B cells (AbD Serotec). The use of a CD21 antibody should have excluded these cells from identification as DNT cells. However, if the surface CD21 epitope of these B cells was damaged prior to flow cytometric analysis (for example, by separation of PBMCs over a density gradient rather than by whole blood lysis, by freezing the samples prior to analysis, or by a combination of the two) and the CD21 antibody was unable to attach firmly to these cells, then these cells could have been erroneously identified as DNT cells (CD5+/CD21-/CD4- and CD8-), artificially increasing the reported DNT cell percentages in the cats in the present study. Further studies investigating differences in DNT cell percentages using different anticoagulants, PBMC separation methods and storage conditions are needed and are planned.

The failure to demonstrate elevations in DNT cells in the blood of parents or siblings of LPD-affected kittens here is difficult to interpret. This finding may truly reflect the absence of any such DNT cell elevations in these animals, suggesting that the disease in
the kittens may differ from ALPS in people. Alternatively, it is possible that DNT cell elevations are present in some related animals of the LPD-affected kittens, but were not present in any of the relatively small sample (n=7) of animals tested here. Further, as it was not possible to investigate DNT cell percentages in blood from any LPD-affected kitten, it is also possible that DNT cell elevations are present in the blood of LPD-affected animals. Testing of further LPD-affected cases, their relatives and controls is therefore needed. However, LPD in mice due to lpr or gld mutations has a similar basis to ALPS in people, but elevations in DNT cells in the peripheral blood of affected mice are not described. Therefore even if further testing does not detect the presence of elevations in DNT cells in the blood of LPD-affected kittens or their relatives, the disease in BSH kittens may still have a similar basis to ALPS in people.

Karyotyping analysis did not reveal consistent chromosomal abnormalities within either the lymphocytes or dermal fibroblasts of any kitten or cat tested in the present study. While in human medicine, karyotyping is increasingly supplemented by additional diagnostic techniques such as fluorescent in situ hybridisation, quantitative fluorescence polymerase chain reaction, multiplex ligation dependent probe amplification and chromosomal microarray analysis (CMA), it is still routinely performed as part of the initial diagnostic workup of suspected genetic disorders and lymphomas in people (pers. comm. Clive Felix, Genetics Service, Capital and Coast District Health Board, New Zealand). Karyotyping studies were undertaken following routine histological evaluation of tissues from LPD-affected kittens when a diagnosis of inherited lymphoma was still suspected (see Chapters 3 and 4), but prior to the performance of clonality assays described in this chapter which indicated a non-clonal and non-neoplastic process. Karyotyping studies were therefore performed both to exclude the presence of gross constitutional chromosomal abnormalities, and also to identify any characteristic chromosomal abnormalities of the lymphocytes of LPD-affected kittens at a stage when available evidence indicated that the disease was lymphoma. Chromosomal abnormalities are frequently present in lymphoma and leukaemia in people (Pearson and Rowley 1985; Bishop 1987) and in cats (Grindem and Buoen 1989; Gulino 1992; Wu et al. 1995), and are often detectable by routine karyotypic analysis. The location and nature of any chromosomal abnormalities present can provide information as to the underlying genetic basis or mechanism for disease (Bishop 1987). For example, certain inherited diseases in people associated with an increased occurrence of lymphoma, such as ataxia-
telangiectasia (A-T), show characteristic chromosomal changes on karyotyping analysis of the lymphocytes (Oxford et al. 1975; Humphreys et al. 1989).

The failure to detect chromosomal abnormalities here may be due to the absence of those abnormalities in LPD-affected kittens. If so, this would be similar to ALPS in people, where karyotyping analysis does not appear to reveal gross chromosomal abnormalities and is not routinely performed. Alternatively, the failure to detect gross chromosomal abnormalities in LPD-affected kittens may be due to the relative insensitivity of karyotyping in detecting subtle changes such as small or balanced chromosomal translocations. Such subtle chromosomal abnormalities may therefore be present here but were not detected by routine karyotyping analysis. Further, as only one LPD-affected kitten (K10) and his dam (Q1) were tested, it is also possible, but unlikely, that detectable chromosomal abnormalities were in fact present in the other untested LPD-affected kittens.

Consistent with the conclusions of earlier chapters, the results of immunochemical testing in the adult cat affected by LPD (K11) again indicate that the disease in this animal likely differs from that in the kittens. Immunohistochemistry did not reveal an increased T-cell (CD3+/CD79a-) population within the lymph nodes or the spleen of this cat as seen in LPD-affected kittens. Rather, the immunohistochemistry results from K11 confirm the conclusion set out in Chapter 3 that the mild expansion of K11’s lymph nodes is more likely due to reactive hyperplasia than infiltration with an abnormal population of T-cells, as the pattern of immunostaining differed markedly to that seen in all affected kittens. Sections of lymph node from K11 showed retention of normal T and B cell populations and normal nodal architecture similar to that seen in samples from control cats. Additionally, the mean proliferation index in samples from K11’s lymph nodes appeared lower than that seen in LPD-affected kittens and similar to that seen in samples from control cats. Sections of spleen from K11 were also similar on immunohistochemical evaluation to those from control cats. It is unfortunate that the further immunochemical testing with CD4 and CD8 markers on lymph node samples from K11 could not be interpreted due to antigen recovery issues, as these results may have provided further support that the disease in the adult cat was different to that in the kittens (if a significant DNT cell population was not present within K11’s lymph nodes), or supported the conclusion that the disease in K11 in fact did have a similar basis to that in affected
kittens (if a significant DNT cell population was present within K11’s lymph nodes), albeit with a different clinical and microscopic presentation of disease.

5.5 SUMMARY

Contrary to the initial gross, microscopic and immunochemical findings supporting a diagnosis of lymphoma, clonality assays indicated that the abnormal cell population expanding the lymphoid organs of LPD-affected BSH kittens was not clonal in origin. This finding indicated the presence of a non-neoplastic process inconsistent with lymphoma in LPD-affected kittens. Where assessed, the proliferating T-cell population was further characterised as an unusual population of DNT cells. Markedly increased numbers of non-clonal DNT cells are also present in human ALPS patients and in mice with *gld* or *lpr* gene mutations. This raises the possibility that the basis for the development of LPD in BSH kittens may be similar to that in the human and murine diseases, most commonly due to mutations in the *Fas* or *Fas* ligand (*FasL*) genes regulating lymphocyte apoptosis, and may provide a more satisfactory explanation for some of the more unusual features of the disease in the kittens.

5.6 REFERENCES


Marlies, A., G. Udo, et al. (2007). The expanded double negative T cell populations of a patient with ALPS are not clonally related to CD4+ or to CD8+ T cells. *Autoimmunity* 40(4): 299-301.


CHAPTER 6

INVESTIGATIONS OF DEFECTIVE LYMPHOCYTE APOPTOSIS

6.1 INTRODUCTION

As discussed in Chapters 4 and 5, some features of the lymphoproliferative disease (LPD) in British shorthair (BSH) kittens with are similar to features of human autoimmune lymphoproliferative syndrome (ALPS) and lpr and gld disease in mice. These diseases in people and mice are both due to defective lymphocyte apoptosis resulting in the accumulation of large numbers of lymphocytes at multiple anatomic sites. Most cases of ALPS in people are due to the presence of underlying mutations in the Fas genes mediating apoptosis (Jackson et al. 1999; Rieux-Laucat et al. 1999; Rao and Straus 2006), and Fas gene mutations also cause lpr disease in mice (Nagata 1994). A small number of cases of ALPS in people are due to mutations in various other genes involved in apoptosis, including Fas ligand (FasL) (Wu et al. 1996; Del-Rey et al. 2006; Bi et al. 2007) and caspase
10 (CASP10) (Wang et al. 1999). *FasL* gene mutations also cause *gld* disease in mice (Takahashi et al. 1994).

If defects in lymphocyte apoptosis or mutations in relevant genes mediating apoptosis could be detected in LPD-affected BSH kittens and cats, this would provide further evidence that the basis for development of the feline disease is similar to that of ALPS in people. The results of such studies might therefore provide a direction for future studies in affected cats and kittens and allow rational treatment decisions to be made about any further LPD-affected animals. Results could also provide an indication as to whether the feline disease had potential as a model for ALPS in people.

The aim of the studies described in this chapter was to determine whether there was evidence of defective lymphocyte apoptosis in LPD-affected kittens and cats. Routine histological evaluation of sections of lymph node from LPD-affected kittens (presented in Chapter 4) revealed relatively few cells with morphology consistent with apoptosis. To confirm that few apoptotic cells were present *in situ*, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays were performed. Preliminary investigations of lymphocyte apoptosis *in vitro* using flow cytometry were also undertaken to determine whether lymphocyte apoptosis was reduced in LPD-affected animals. Finally, the *Fas* gene was amplified and sequenced in LPD-affected kittens to detect the presence of mutations that could prevent normal lymphocyte apoptosis using polymerase chain reaction (PCR) methods. The *Fas* gene has been previously sequenced in the cat (Mizuno et al. 1998) and its reported sequence was used as the basis for preliminary investigations of the presence of *Fas* gene mutations in affected animals in this chapter.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 LYMPHOCYTE APOPTOSIS IN SITU

Of the 7 kittens and one adult cat previously identified with LPD, formalin-fixed, paraffin-embedded (FFPE) tissue from LPD-affected lymph nodes was available from 4 kittens (K1, K2, K10, and K32) and the adult cat (K11). Formalin-fixed, paraffin-embedded samples of lymph node from three unrelated cats unaffected by LPD were used as normal control
CHAPTER 6

tissues. Formalin-fixed, paraffin-embedded sections of three lymphomas diagnosed by histology from three unrelated cats were also used to compare apoptosis in sporadic neoplastic LPD (lymphoma).

The DeadEnd™ Colorimetric TUNEL System kit (Promega Corporation, Madison, WI, United States) was used to detect the presence of apoptotic cells and cell fragments in FFPE tissue sections according to the manufacturer’s instructions. Following optimisation of the procedure in feline lymph node tissue sections, sections of lymph node from all animals were cut at a thickness of 5μm, mounted on charged microscope slides, deparaffinised twice in xylene, rehydrated in graded ethanol and rinsed in 0.85% sodium chloride before immersion in phosphate buffered saline (PBS) for 5 minutes. Slides were then immersed in 4% paraformaldehyde diluted in PBS for 15 minutes before being incubated with Proteinase K (20μg/mL) for 15 minutes at 37ºC and re-immersed in 4% paraformaldehyde diluted in PBS for 5 minutes. Following equilibration with the proprietary buffer for 10 minutes at room temperature (RT), sections were incubated with terminal deoxynucleotidyl transferase (TdT) reaction mix, covered with plastic coverslips, and incubated for 60 minutes at 37ºC in a humidified chamber before being immersed in 2x saline sodium citrate buffer for 15 minutes. Slides were then immersed in 0.3% hydrogen peroxide for 7 minutes before incubation with streptavidin horseradish peroxidase (HRP) diluted in PBS for 30 minutes at RT. The reaction was visualised using 3,3'-diaminobenzidine (DAB). Following immunostaining, sections were rinsed in deionised water, counterstained with Gill’s haematoxylin, dehydrated in graded alcohols, cleared in xylene, coverslipped and examined under a light microscope. Slides were washed in PBS for a minimum of 5 minutes between all steps except following TdT incubation when no wash was applied. Between the saline sodium citrate buffer and hydrogen peroxide immersion steps, slides were washed three times in PBS with added 0.1% Triton-X and 5mg/mL bovine serum albumin. Positive control slides were incubated with DNase buffer for 5 minutes at RT following the second fixation in 4% paraformaldehyde to induce DNA strand breaks. Negative control slides were incubated with reaction mix from which TdT had been omitted.

Brown colouration of cells or cell fragments was interpreted as positive staining and evidence of apoptosis (Gavrieli et al. 1992; Cuello-Carrion and Ciocca 1999; Garrity et al. 2003; Loo 2011). A minimum of either 10 randomly selected 1000x fields or 1000 cells within each sample (Labat-Moleur et al. 1998) were captured using NIS Elements.
software (Nikon Instruments Inc., United States) and a Nikon microscope and analysed using Image J software (Schneider et al. 2012). Exposure levels were locked to ensure standardised light conditions for photomicrographs. A morphometric cell count (MCC) and a morphometric area analysis (MAA) based on previously described methods (Labat-Moleur et al. 1998; Garrity et al. 2003) were performed for each sample. The MCC was performed for each sample by counting the total number of cells or cell fragments with visible positive staining and exhibiting previously reported morphological features consistent with apoptosis (described below) across all fields. The apoptotic index (AI) for samples from each animal was then calculated by dividing the MCC by the total number of cells across all fields to give the percentage of cells showing evidence of apoptosis for that sample and the mean AI across the various groups calculated. Unless obviously contained within a single phagocytic cell, each positive fragment was counted as a separate cell (Garrity et al. 2003). Morphological features interpreted as consistent with apoptosis for the purposes of the MCC were cell shrinkage, nuclear condensation, cytoplasmic bleb and apoptotic body formation, and phagocytosis of cells or cell fragments without associated inflammation (Kerr et al. 1972; Ziegler and Groscurth 2004; Wickman et al. 2012). The MAA was performed for each sample by determining the total area showing positive staining across all fields. The apoptotic rate (AR) for samples from each animal was then calculated by dividing the MAA total by the total cellular area across all fields to give the percentage of the cellular area showing evidence of apoptosis. For normal control lymph nodes, in addition to 10 randomly selected 1000x fields across the whole node, 10 randomly selected 1000x fields within the germinal centres and 10 1000x fields within the interfollicular regions of the node were also photographed, counted and assessed for both AI and AR calculations.

The AI and AR in lymph node samples from LPD-affected BSH kittens were compared to those in samples from the adult cat affected by LPD (K11), samples from unrelated cats with lymphoma, and samples from normal cats unaffected by LPD. The mean AI and AR across the entire lymph node were calculated for animals within the different groups, and in the case of normal controls, the AI and AR across germinal centres, interfollicular regions and whole node were also calculated. Differences in AI between groups were investigated by 2 sample t-tests and one-way analysis of variance (ANOVA) using Minitab 15 Statistical Software (2007, Minitab, Inc.).
6.2.2 LYMPHOCYTE APOPTOSIS IN VITRO

Of the 7 kittens and one adult cat previously identified with LPD, peripheral blood suitable for lymphocyte apoptosis assays was only available from the adult cat (K11). However, as further cases of LPD in future litters of BSH kittens were anticipated at the time these studies were undertaken, and because in vitro defects in lymphocyte apoptosis are often observed in the clinically normal relatives of ALPS patients (Fisher et al. 1995; Sneller et al. 1997; Infante et al. 1998; Rieux-Laucat et al. 1999), it was decided to proceed with the development and optimisation of lymphocyte apoptosis assays in vitro.

Blood (two to three mL) was collected from 22 kittens and cats by jugular venipuncture and processed within 12 hours of collection. Animals used comprised 4 clinically healthy unrelated control cats, 5 clinically healthy littermates (Litters 1 and 2) of LPD-affected kittens, 11 clinically healthy kittens and cats from unaffected litters (Litters 3, 4 and 6), the dam of two of the three LPD-affected litters (Q1) and the adult cat with LPD (K11).

Blood was collected into lithium heparinised blood tubes from all animals. A complete blood count (CBC) was performed on blood samples at a commercial veterinary laboratory (New Zealand Veterinary Pathology, Palmerston North) using an ADVIA 120 haematology analyser (Siemens Healthcare Diagnostic Inc., Deerfield, IL, United States). Where sufficient blood could not be collected for an automated CBC, the CBC was estimated manually from duplicate blood smears as previously described in Chapter 4.

Blood samples were diluted in an equal volume of PBS, layered over Histopaque®-1077 (Sigma-Aldrich, New Zealand) at a 2:1 ratio in 15mL conical tubes and centrifuged at room temperature for 20 minutes at 410 g (2000 rpm). Peripheral blood mononuclear cells (PBMCs) were collected at the interface and washed twice with 10mL Dulbecco’s Modified Eagle Medium (DMEM) containing 5% fetal calf serum (FCS), then counted and adjusted to 1 x 10^6 viable cells per mL. As it was not possible to analyse blood from all animals immediately after acquisition, samples from 18 animals were frozen as described in Chapter 5. Briefly, the cell pellet was re-suspended in 1mL Recovery™ Cell Culture Freezing Medium (Life Technologies, New Zealand) and placed in a Nunc cryovial that was wrapped in cotton wool and frozen -80ºC overnight before placing in liquid nitrogen and stored until needed. For analysis, frozen cells were thawed rapidly, washed in 9 mL DMEM containing 10% FCS, pelleted by centrifuging for 5 minutes at 1500rpm,
resuspended in 1 mL medium, counted using a haemocytometer, and adjusted to $1 \times 10^6$ viable cells/mL.

Into each well of 48-well plates, 200$\mu$L cells (200 000 cells) were plated in duplicate (one treated, one control) and either 100$\mu$L complete medium with 100$\mu$L camptothecin in DMSO added (treated samples) or 100$\mu$L complete medium only added (control samples) (Nagami et al. 2002; Howard 2005; Anonymous 2013). DMSO had been previously demonstrated not to induce apoptosis. Plates were incubated for 18 hours at 37$^\circ$C.

To investigate induction of apoptosis within examined cats’ peripheral blood, two-colour flow cytometric analysis was performed using the FITC (fluorescein isothiocyanate) Annexin V Apoptosis detection kit I (BD Pharmingen, United States). Volumes of annexin-FITC and propidium iodide used per sample were based on previous titration curves performed using cells from control cats. Following incubation, cells (100$\mu$l containing $1 \times 10^5$ cells/sample) were incubated with 100$\mu$l of a cocktail of binding buffer (90$\mu$l), annexin conjugated to FITC (5$\mu$l) and propidium iodide (5$\mu$l) placed into labelled FACS tubes according to the manufacturer’s instructions. Three control tubes containing either cells only, cells with annexin-FITC only, or cells with propidium iodide only, were used to calculate baseline activity. After 15 minutes incubation at room temperature protected from light, samples were washed twice with 2mL cold PBS by centrifuging for 5 minutes at 1500rpm, and re-incubated with 100$\mu$l PBS. Cells were again washed twice, resuspended in 2% paraformaldehyde, and data collected by flow cytometry.

Samples were acquired using a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, United States) with CellQuest software (BD Biosciences, United States). For each experiment, compensation was set using control tubes of cells containing only annexin-FITC or propidium iodide. Lymphocytes were gated by forward and side scatter characteristics and a minimum of 10 000 events acquired per sample. Cells were regarded as apoptotic if their scatter characteristics were consistent with those previously reported for feline lymphocytes on forward and back gating and were annexin positive and propidium iodide negative on flow cytometric analysis.

The percentage of apoptotic lymphocytes in samples from each cat induced by camptothecin treatment was calculated by subtracting the percentage of apoptotic cells
in untreated control samples from that present in treated samples. Comparisons between apoptotic cell percentages in samples from control cats, samples from the adult cat affected by LPD (K11), siblings and parents of LPD-affected kittens (“LPD-related”), and finally, kittens from litters bred as part of the present studies but unaffected by LPD (“unaffected litters”) using 2 sample t-tests and one-way ANOVA were proposed but not completed (see section 6.3).

6.2.3 Qualitative Fas-gene abnormalities

Of the 7 kittens and one adult cat previously identified with LPD, fresh-frozen (-80°C), fresh tissue preserved in RNA later® solution (Invitrogen Corp., United States) and subsequently frozen (-80°C) or FFPE tissue from LPD-affected lymph node suitable for reverse transcriptase polymerase chain reactions (RT-PCR) (Fisher et al. 1995; Mizuno et al. 2004) was available from 4 kittens (K1, K2, K10, and K32) and the adult cat (K11). Fresh-frozen (-80°C) lymph node tissue from a healthy unrelated cat without LPD was used as a normal positive control tissue.

Total RNA was extracted from fresh-frozen samples using TRI Reagent® (Sigma-Aldrich Co., St Louis, MO, United States). Briefly, for each fresh-frozen sample, approximately 100 mg of tissue was homogenised in 1 mL of TRI Reagent® using a Mini Beadbeater-16 (BioSpec Products, Inc., Bartlesville, OK, United States) for 30 to 60 seconds. The aqueous phase RNA was mixed with 0.5 mL of isopropanol and left to stand for 10 minutes before centrifugation at 12,000 g for 10 minutes at 4°C. After removal of the supernatant, the RNA pellet was washed with 1 mL 75% ethanol, vortexed for 5 seconds and centrifuged at 7,500 g 4°C for 5 minutes. The RNA pellet was air-dried for 10 minutes before resuspension in 100 μL diethylpyrocarbonate (DEPC)-treated water. Total RNA was extracted from FFPE samples using the High Pure FFPE Micro Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, tissue sections were cut at a thickness of 10 μm and deparaffinised in xylene and then graded ethanol (absolute and 70%). An overnight buffered tissue lysis step was followed by RNA isolation on a filter column through a series of wash buffer rinses, and the RNA was finally eluted into a collection tube using elution buffer. Various modifications were made to the protocol to maximise RNA recovery, including extension of the proteinase K digestion step to 16 hours and repetition of the DNase incubation and initial wash steps to remove as much genomic DNA from the sample as possible (Chung et al. 2006; Abramovitz et al. 2008;
Samples were stored at -80°C until required. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, United States) at a wavelength of 260nm.

To amplify the full-length coding region of feline Fas three primer pairs (6S/6R, 7S/7R and 8S/8R) designed to amplify three overlapping fragments of feline Fas mRNA were synthesised. These primer pairs were based on the previously reported sequence of feline Fas cDNA (Mizuno et al. 1998) and primers previously described (Mizuno et al. 2004).

Details of the primers used are set out in Table 6.1. Reverse transcriptase polymerase chain reactions (RT-PCRs) were optimised and performed to synthesise complementary DNA (cDNA) using the Superscript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen Corp., United States) according to the manufacturer’s instructions. To each 0.2mL thin-walled PCR tube on ice was added: 12.5μL 2X reaction buffer (containing 0.4 mM of each dNTP, 2.4 mM MgSO4), 1μL Superscript™ Platinum Taq, 0.2μM of each relevant forward and reverse primer and 0.2-0.8μg RNA, made up to a total reaction volume of 25μL with autoclaved distilled water. Optimal RT-PCR amplification conditions were as follows: 55°C for 30 minutes, 94°C for 2 minutes, 40 cycles at 94°C for 30 seconds, annealing at either 55°C (primers 6S/6R), 61°C (primers 7S/7R) or 57°C (primers 8S/8R) for 1 minute followed by 72°C for 1 minute with a final elongation at 72°C for 7 minutes. RNA was omitted from negative controls. The resulting PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualised under ultraviolet light using transillumination.

Amplicons to be used for sequencing were purified using the PureLink PCR purification kit (Invitrogen Corp., United States) and with the primers listed in Table 6.1, subjected to automatic dye-terminator cycle sequencing with BigDye® Terminator version 3.1 Ready Reaction Cycle Sequencing kit (Invitrogen Corp., United States), and the ABI3730 Genetic Analyzer (Applied Biosystems Inc., United States). Expected amplicon sizes for each PCR reaction are set out in Table 6.1.
### Table 6.1: List of primer pairs used in reverse transcription-polymerase chain reactions (RT-PCR) and sequencing of feline Fas.

Primers were based on those previously reported by Mizuno et al. (Mizuno et al. 2004).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Nucleotide position (in feline Fas gene)</th>
<th>Corresponding exon(s) of feline Fas gene</th>
<th>Expected amplicon size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6S</td>
<td>GGC GGG GCG CTC CGC AGC C</td>
<td>-19–1</td>
<td>1,2 and 3</td>
<td>360</td>
</tr>
<tr>
<td>6R</td>
<td>TTC TAA GCC ATG CTT TCA T</td>
<td>322–341</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7S</td>
<td>GAA GAA GCG AAG GAC TAC ACA GAC</td>
<td>255–278</td>
<td>3,4,5,6, 7 and 8</td>
<td>442</td>
</tr>
<tr>
<td>7R</td>
<td>GTT CGG CAA TGC TAC TGA TG</td>
<td>677–696</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8S</td>
<td>GAA TCT ACA TGC TCA GTT AC</td>
<td>619–631</td>
<td>7, 8 and 9</td>
<td>354</td>
</tr>
<tr>
<td>8R</td>
<td>GCA GTT TCC ATT CTC AAG</td>
<td>945–972</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A = adenine, C = cytosine, G = guanine, T = thymine.

### 6.3 RESULTS

#### 6.3.1 LYMPHOCYTE APOPTOSIS IN SITU

Results obtained using the TUNEL assay are summarised in Table 6.2 and images of lymph node sections obtained using the assay are shown in Figure 6.1.

Using the TUNEL assay and MCC, the mean AI within examined fields of the enlarged lymph nodes of LPD-affected kittens (0.6%) was significantly lower than that present in lymph nodes of cats with lymphoma (6.1%, \( p = 0.007 \)) and that present in the lymph nodes of healthy control cats (2.9%, \( p = 0.002 \)). The mean AI within lymph nodes of LPD-affected kittens was also lower than that observed in the adult cat with LPD (K11, 3.8%), but as K11 was a single animal it was not possible to determine whether this difference was significant. The mean AI within the lymph nodes of control cats differed significantly between the germinal centres (4.1%) and the interfollicular areas of the node (1.2%, \( p = 0.03 \)). The mean AI within the enlarged lymph nodes of LPD-affected kittens was significantly lower than that present in both the lymphoid follicular and germinal centre regions of lymph nodes from control cats (\( p < 0.001 \)) and that present in interfollicular regions of lymph nodes from control cats (\( p = 0.02 \)).
Using the TUNEL assay and MAA, the mean AR within enlarged lymph nodes of LPD-affected kittens (0.6%) was again significantly lower than in lymph nodes of cats with sporadic lymphoma (3.2%, $p = 0.01$) and in lymph nodes of healthy control cats (1.7%, $p = 0.04$). The mean AR within lymph nodes of LPD-affected kittens was also lower than that observed in the adult cat affected by LPD (K11, 3.2%), but as K11 was a single animal it was not possible to determine whether this difference was significant. However, the mean AR within the lymph nodes of control cats did not differ significantly between germinal centres (1.9%) and the interfollicular areas of the node (1.1%, $p = 0.18$). The mean AR within the enlarged lymph nodes of LPD-affected kittens differed significantly from that present in the germinal centres of lymph nodes of control cats ($p=0.01$) but did not differ significantly from that present in interfollicular regions ($p=0.24$).

### Table 6.2: Mean apoptotic index (AI) and apoptotic rate (AR) of lymph nodes of LPD-affected kittens and cats, normal control cats, and unrelated cats with sporadic lymphoma by TUNEL assay.

<table>
<thead>
<tr>
<th>Animal/group +/− region of node</th>
<th>n</th>
<th>Apoptotic index (AI) %</th>
<th>Apoptotic rate (AR) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole node</td>
<td>3</td>
<td>2.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Germinal centres</td>
<td></td>
<td>4.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Interfollicular</td>
<td></td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>LPD-affected kittens</td>
<td>4</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>LPD-affected adult cat</td>
<td>1</td>
<td>3.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Cats with sporadic lymphoma</td>
<td>3</td>
<td>6.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* denotes a statistically significant difference between LPD-affected kittens and other groups

Although the MCC and MAA were significantly lower in lymph nodes of LPD-affected kittens than those observed in lymph nodes from control cats or in samples from unrelated cats with sporadic lymphoma across the whole node, low numbers of cells showing evidence of apoptosis on TUNEL staining were still present in certain regions of examined lymph nodes, particularly in samples from K1 and K32. In samples from these kittens, cells showing evidence of apoptosis were more frequently located within in the remnants of germinal centres than within interfollicular and other areas of the node (Figures 6.2A and B). In samples from K2 and K10, low numbers of cells showing evidence of apoptosis on were present throughout examined nodes.
Figure 6.1: TUNEL assay (high power), showing apoptosis in control lymph nodes, LPD-affected kittens and sporadic lymphoma, 3,3′diaminobenzidine/haematoxylin. Apoptotic cells and cell fragments show brown staining and various morphologic changes consistent with apoptosis including cell shrinkage, nuclear condensation and apoptotic body formation. A. Control cat, germinal centre of lymph node showing multiple cells with evidence of apoptosis. B. Control cat, interfollicular area of lymph node showing several cells with evidence of apoptosis. C, D and E. LPD-affected kittens (K10 (C), K2 (D), and K32 (E)) showing very few cells with evidence of apoptosis: C and D contain no cells with evidence of apoptosis, while E contains only a single apoptotic cell. F. Unrelated cat with sporadic lymphoma, lymph node showing large numbers of cells with evidence of apoptosis. All 1000x magnification.
6.3.2 LYMPHOCYTE APOPTOSIS IN VITRO

Apoptosis was successfully induced by camptothecin treatment of fresh lymphocytes from control cats and analysed by flow cytometry. However, the results of flow cytometric apoptosis studies using previously frozen and thawed lymphocytes were inconsistent and not repeatable. As the majority of samples available for this study comprised previously frozen and thawed cells, including those from the sole LPD affected adult cat (K11), the results are not reported.

6.3.3 QUALITATIVE Fas-gene ABNORMALITIES

RNA was successfully extracted from fresh-frozen lymph node tissue from two LPD-affected kittens (K2 and K10) and the adult cat with LPD (K11), from an unaffected littermate (K4) and from a healthy control cat. RNA from these animals was successfully amplified by RT-PCR and the products sequenced. For all PCR reactions using three different primer pairs, the amplicons obtained from 2 LPD-affected kittens and the adult cat with LPD, the unaffected littermate and the unrelated healthy control cat were
identical in size, and were approximately 360 base pairs (primer pair 6S/6R), 440 base pairs (primer pair 7S/7R) and 350 base pairs (primer pair 8S/8R) respectively (see Figure 6.2). The size of all amplicons obtained by RT-PCR was consistent with the amplicon size expected based on the previously reported sequence of the feline Fas gene (Mizuno et al. 1998; Mizuno et al. 2004). Sequencing of PCR products from LPD-affected animals was identical to those from both the littermate unaffected by LPD and the healthy control cat, and did not contain significant differences to the sequence for the coding region of feline Fas previously reported (Mizuno et al. 1998; Mizuno et al. 2004).

Even with multiple modifications made to the extraction protocol to improve the RNA yield (described in section 6.2.3 above), repeated attempts to extract RNA from FFPE lymph node tissue from LPD affected kittens K1 and K32 did not produce sufficient RNA for RT-PCR amplification and sequencing. Due to the small amount of FFPE lymph node tissue that by then remained from these kittens and the desirability of retaining some tissue from these kittens for future studies, no further attempts to extract RNA were made and further PCR investigations of Fas gene abnormalities from samples from these kittens were not attempted.
Figure 6.3: Reverse transcription–polymerase chain reaction (RT–PCR) analyses for the coding region of feline Fas in LPD-affected and unaffected animals. A. Primer pairs 6S and 6R, expected amplicon size = 360 base pairs. B. Primer pairs 7S and 7R, expected amplicon size 442 base pairs. C. Primer pairs 6S and 6R, expected amplicon size = 354 base pairs. All products were separated on a 1.5% agarose gel.

Lanes: M = marker lane (100 base pair increments); lane 1: LPD-affected kitten (K2); lane 3 = LPD-affected kitten (K10); lane 3 = LPD-affected adult cat (K11); lane 4 = unaffected littermate (K4); lane 5 = healthy unrelated control cat unaffected by LPD (positive control); lane 6 = negative control; bp = base pairs.
6.4 DISCUSSION

The results of in situ TUNEL assays indicate significant reductions in lymphocyte apoptosis in lymph nodes from LPD-affected kittens, supporting defective lymphocyte apoptosis as a factor in the development of disease. A similar reduction in lymphocyte apoptosis is also present in people with ALPS, and in mice with lpr or gld disease (Nagata and Suda 1995; Lim et al. 1998). These results provide further support that the basis for the feline disease may be similar to that of ALPS in people and its murine equivalents. While preliminary RT-PCR investigations did not identify qualitative Fas gene abnormalities in LPD-affected kittens, this does not exclude the possibility that the disease in LPD-affected kittens shares a similar basis to the human or murine diseases. Although qualitative defects in the Fas genes are present in the majority of cases of ALPS in people and in mice with lpr disease, defects in several other genes including FasL and CASP 10 may also cause ALPS in people and gld disease in mice. Further investigations of LPD-affected kittens that include amplification and sequencing of these additional genes are needed to definitively determine the cause of LPD in affected kittens.

Apoptosis is a specific form of programmed cell death which occurs through an ordered pathway of self-degradation terminating with the phagocytosis of cellular remnants. Apoptosis is highly conserved across species and has a role in normal tissue homeostasis and well as in the removal of self-reactive cells and damaged or senescent cells (Kerr et al. 1972; Evan et al. 1995). The apoptotic process can be triggered by multiple events but ultimately involves the activation of caspase enzymes through death receptor-ligand interactions at the cell surface (the extrinsic pathway) or through enzyme induction within mitochondria (the intrinsic pathway) (Danial and Korsmeyer 2004). The death receptors involved in the extrinsic pathway contain a cytoplasmic death domain critical to the delivery of apoptotic signals (Tartaglia et al. 1993). One of the most important and extensively studied death receptors is the Fas receptor (Fas) (Siegel et al. 2000). The binding of Fas to Fas ligand (FasL), or to agonistic antibodies, triggers trimerisation and recruitment to the Fas death domain of the corresponding region of the cytoplasmic adaptor protein, Fas-associated death domain protein (FADD), followed by caspase activation and subsequent cell death (Itoh et al. 1991; Kischkel et al. 1995). Fas-mediated apoptosis of activated lymphocytes is particularly important in maintaining immune
homeostasis by eliminating self-reactive T-cells and limiting lymphocyte accumulation (Russell 1995; Lenardo 1996; Stranges et al. 2007). The Fas and FasL genes encode the Fas and FasL proteins respectively, and both genes have been previously sequenced in the cat (Mizuno et al. 1998). Apoptosis and the role of Fas are discussed in greater detail in Chapter 2.

Certain morphological changes characterise cells undergoing apoptosis in tissue sections, including cell shrinkage, nuclear condensation, cytoplasmic bleb and apoptotic body formation, and phagocytosis of cells or their fragments without associated inflammation (Kerr et al. 1972; Ziegler and Groscurth 2004; Wickman et al. 2012). As reported in Chapter 4, there appeared to be relatively few cells showing morphological changes typically associated with apoptosis in routine haematoxylin and eosin (H&E) stained histological sections of lymph node from LPD-affected kittens, similar to findings present in the lymph nodes of people with ALPS (Lim et al. 1998). However, while the characteristic morphological changes of apoptosis can be identified by examination of H&E stained tissue sections, detection by morphology alone is unreliable and tends to underestimate the level of apoptosis present (Kerr et al. 1972; Gavrieli et al. 1992; Jerome et al. 2000; Garrity et al. 2003). For this reason, the TUNEL assay was applied to identify and quantify apoptotic cells present within lymph node sections from LPD-affected kittens and controls with greater accuracy (Garrity et al. 2003; Loo 2011).

Results confirmed the preliminary impression from routine (H&E stained) histology that apoptosis was significantly reduced in lymph node sections from LPD-affected kittens compared with both lymph nodes from control cats and lymph nodes from cats affected by lymphoma. In lymph nodes from LPD-affected kittens K2 and K10, germinal centres were largely effaced by proliferating T-cells, and relatively few cells undergoing apoptosis were present in examined sections of these kittens’ expanded lymph nodes. In lymph nodes from K1 and K32, small germinal centre remnants were still present in some parts of the nodes, and higher numbers of cells showing evidence of apoptosis were present in these remnants. As lymph nodes of people with ALPS also contain few cells undergoing apoptosis in the expanded interfollicular areas but numerous apoptotic cells in the remaining germinal centres (Lim et al. 1998), histological findings in K1 and K32 appeared similar to described changes in people. As the vast majority of cells present within the lymph nodes of affected kittens had been previously demonstrated to be T-cells (see
Chapter 5), it is likely that T-cell apoptosis is particularly reduced. However, while apoptotic cells were significantly reduced within nodes of LPD-affected kittens across the whole node, variable although generally low numbers of cells showing evidence of apoptosis on TUNEL staining were still present within the nodes of all LPD-affected kittens. The presence of some apoptotic cells, presumably T-cells, in areas of the node other than the remnants of germinal centres may indicate that any apoptotic defect in affected kittens does not completely stop T-cell apoptosis; only reduces it. Similarly, the presence of many T-cells showing evidence of apoptosis within the spleen and peripheral blood of ALPS patients is also described (Le Deist et al. 1996). In these patients, it is speculated that the presence of these apoptotic cells may be due to activation of an alternative apoptotic pathway that cannot compensate fully for the impaired apoptosis due to defects in Fas-mediated apoptosis. It possible that similar processes are also occurring in the lymph nodes of LPD-affected kittens in the present study.

Lymph node enlargement in ALPS patients is due to a combination of increased lymphocyte proliferation (determined by increased Ki67 positivity and the presence of frequent mitoses) and decreased apoptosis. Similar findings to those reported in people with ALPS were also present in LPD-affected kittens, whose lymph nodes also showed a high proliferative index (see Chapter 5) and relatively few cells in apoptosis. Within normal lymph nodes, apoptosis is most prevalent within areas of increased cell turnover, primarily the germinal centres. Apoptosis in proliferating neoplastic tissue is variable compared with control tissues, but in tumours with a high proliferative index and high cell turnover, is usually significantly increased (Vakkala et al. 1999; Xie et al. 2000; Bai et al. 2005). In the absence of abnormalities in lymphocyte apoptosis it would be expected that apoptosis in the kittens’ lymphocytes would also be occurring at a higher rate than in non-proliferating lymphoid control tissues. The reduction in lymphocyte apoptosis seen with the TUNEL assay therefore suggests a possible defect in apoptosis affecting the rapidly proliferating T-cells.

The results of the TUNEL assay assessing apoptosis in tissue sections must be interpreted with caution due to the potential for false positive staining and the consequent overestimation of apoptosis. The TUNEL assay detects the presence of DNA strand breaks that occur during apoptosis by labelling breaks with deoxyuridine triphosphates (dUTPs), which are then visualised after being linked to peroxidases (Alison 1999; Cuello-Carrion...
and Ciocca 1999; Loo 2011). Excessive digestion with proteases, such as proteinase K, during the initial tissue processing stages of the assay can induce DNA strand breaks that may also react with dUTPs. In addition, necrosis also causes DNA strand breaks and necrotic cells are a potential target for non-specific labelling in the TUNEL assay, resulting in potential false positive results (Grasl-Kraupp et al. 1995; Labat-Moleur et al. 1998; Jerome et al. 2000). Such artefactual staining and staining of necrotic cells was minimised in the present study by optimising the TUNEL procedure in feline lymph node tissue, by applying the TUNEL method together with the MCC, and by excluding cells with positive staining but morphology inconsistent with apoptosis. Application of these measures reportedly increases the accurate detection of in situ apoptosis by the TUNEL assay (Labat-Moleur et al. 1998; Garrity et al. 2003). While the presence of false positive staining cannot be completely excluded in the present study, such staining is expected to be low.

In all groups or animals except LPD-affected kittens, the AR as assessed by MAA was lower than the AI assessed by MCC for the equivalent group or animal. These differences are likely due to differences in the way apoptosis was detected and measured by the two methods. As the MAA assesses positively stained areas, small positively stained apoptotic fragments will contribute relatively less to the overall assessment of apoptosis present (the AR) than they do to assessment of the MCC, where small cells or fragment showing evidence of apoptosis have a relatively larger contribution to the assessment of apoptosis (the AI). As morphological criteria are not considered under the MAA methods, this method may fail to detect apoptotic cells or fragments with weak TUNEL staining but morphology consistent with apoptosis, meaning that these cells and fragments would be excluded from the MAA. Both factors would have likely reduced the area of apoptosis assessed by MAA and reported by the AR. It is considered likely that assessment of apoptosis using TUNEL with the MCC and AI is the more accurate and consistent of the two methods, as the inclusion of morphological criteria consistent with apoptosis mean this method is more likely to detect and include poorly stained apoptotic cells excluded by the MAA and AR method as well as reduce any potential contribution of non-specific staining potentially included under the MAA.

While these results indicate that reduced apoptosis in T-cells is likely present in LPD-affected kittens, confirmation using additional methods is necessary to confirm the
results of the present study. In situ methods of apoptosis detection such as the TUNEL assay in FFPE tissue detect indirect evidence of apoptosis in a tissue at a single point in time, and are considered less accurate than in vitro methods of apoptosis detection. In people with suspected ALPS, defects in lymphocyte apoptosis are identified by applying an apoptotic stimulus, typically a monoclonal antibody that binds activated Fas, to previously activated T-cells and then comparing the level of lymphocyte apoptosis in T-cells from ALPS patients to that in T-cells from healthy controls (Fisher et al. 1995; Oliveira et al. 2010). In the present study, the limited availability of samples from LPD-affected kittens other than FFPE tissue meant that an in situ method such as the TUNEL assay was the only available method to assess apoptosis. Attempts to develop and apply alternative flow cytometric methods of apoptosis detection were unsuccessful, most likely due to the use of previously frozen lymphocyte samples. Frozen cells had a much higher rate of cell degeneration and exhibited inconsistent uptake of propidium iodide and annexin-FITC than non-frozen cells. This may have been due to changes in the cells induced during freezing and thawing processes, or to the artefactual induction of apoptosis as an unintended consequence of cryopreservation (Baust et al. 2000; Costantini et al. 2003; Owen et al. 2007; Weinberg et al. 2009). It is therefore desirable that any future investigations of apoptosis in LPD-affected animals utilise fresh samples rather than previously frozen cells. It should also be noted that the method attempted in the preliminary study would not have identified defects in Fas-mediated apoptosis even if present, as the apoptotic stimulus used, camptothecin, induces apoptosis by the intrinsic pathway rather than by the Fas-mediated extrinsic pathway (Stefanis et al. 1999; Gunawardana et al. 2006; Zeng et al. 2012). As studies to date have not yet identified the nature of any apoptotic defect in LPD-affected kittens, any future investigations of apoptotic defects in the affected kittens’ lymphocytes will use a range of different apoptotic stimuli that induce apoptosis by both the intrinsic and extrinsic pathways to increase the likelihood that the basis for any apoptotic defect is identified.

Mutations in the Fas gene are present in lpr disease in mice (Watanabe-Fukunaga et al. 1992; Nagata 1994) and are the mutations most frequently implicated in ALPS in people (Jackson et al. 1999; Rieux-Laucat et al. 1999; Rao and Straus 2006; Anonymous 2012a). Accordingly, Fas was considered as a candidate gene for disease in LPD-affected kittens and cats, and defects in Fas mRNA expression in the feline disease were investigated using RT-PCR methods similar to those previously described in people with ALPS (Fisher et
al. 1995). Approximately three-quarters of human ALPS patients have underlying germline mutations in the Fas gene (ALPS-FAS, previously ALPS type 0 or ALPS type 1a), the majority of which involve substitutions, insertions or deletions of one or more nucleotides in the coding exons or splice sites of the gene (Rao and Straus 2006; Anonymous 2012a). While mutations have been reported at many different locations throughout the Fas gene in ALPS patients, the majority of those mutations (approximately 70%) affect the intracellular or death domain of Fas, particularly exon 9 (Jackson et al. 1999; Rieux-Laucat et al. 1999; Rao and Straus 2006; Anonymous 2012a). In people, mutations affecting the death domain of Fas appear associated with a higher disease penetrance and overt clinical signs of disease (Infante et al. 1998; Jackson et al. 1999). As the Fas gene mutations that are present in people with ALPS are so diverse, the consequences of those mutations also vary, but typically include the expression of both normal and abnormal Fas mRNAs detectable by sequencing (Fisher et al. 1995; Rieux-Laucat et al. 1999). Abnormal Fas mRNAs are often prematurely truncated, causing loss or inactivation of the death domain and other regions critical to successful Fas-mediated apoptosis (Vaishnaw et al. 1999). Similar to the majority of people with ALPS, mice with lpr mutations also have abnormalities in the Fas gene, but with much less variability. Two lpr mutations are reported, lpr and lpr<sup>cg</sup>, that produce a similar disease phenotype in affected mice (Cohen and Eisenberg 1991). In lpr mice, the insertion of 183 nucleotides into intron 2 of the Fas gene causes premature termination and aberrant splicing of the Fas transcript resulting in defective and markedly reduced Fas expression and function (Watanabe-Fukunaga et al. 1992; Adachi et al. 1993). In lpr<sup>c9</sup> mice, a point mutation of the Fas gene involving a single substitution of arginine for thiamine at nucleotide 786 results in the replacement of isoleucine with asparagine in the cytoplasmic region of the gene (Watanabe-Fukunaga et al. 1992). This mutation in lpr<sup>c9</sup> mice results in the expression of normal amounts of dysfunctional Fas that is unable to initiate effective apoptosis (Nagata 1994).

In contrast to the majority of cases of ALPS in people and mice with lpr and lpr<sup>cg</sup> mutations, qualitative abnormalities in the Fas genes of LPD-affected kittens and cats were not identified by RT-PCR and sequencing in the present study. However, this finding does not exclude the possibility that the disease in LPD-affected kittens is due to defective apoptosis. Apoptosis is a complex and multi-step process involving many different genes and their proteins. Defective apoptosis in LPD-affected kittens may
therefore result from mutations in genes other than Fas. Although Fas gene mutations are most commonly associated with ALPS in people, mutations in other genes associated with apoptosis have also been identified in a small number of human patients with ALPS and ALPS-related disorders (Rao and Straus 2006; Oliveira et al. 2010), and in mice with gld disease (Takahashi et al. 1994; Nagata and Suda 1995). Germline mutations in the FasL gene (ALPS-FASLG, previously ALPS type Ib) and CASP10 gene (ALPS-CASP10, previously ALPS type Ila) are occasionally reported in people with ALPS, while rare germline mutations in the caspase 8 (CASP8) gene (Chun et al. 2002) and somatic mutations in the neuroblastoma RAS (NRAS) gene (Oliveira et al. 2007) have been reported to cause clinical signs similar to those seen in ALPS (ALPS related disease) (Oliveira et al. 2010). Reported mutations in FasL include nucleotide deletions (Wu et al. 1996) and substitutions (Del-Rey et al. 2006; Bi et al. 2007) resulting in abnormalities in FasL function and ineffective apoptosis. Reported mutations in CASP10 cause nucleotide substitutions which decrease caspase activity and impair effective apoptosis (Wang et al. 1999). Further, in a significant proportion of ALPS patients (approximately one-third), no causative genetic defect has been identified (Rao and Straus 2006; Oliveira et al. 2010). It is therefore possible that defects in genes involved in apoptosis other than Fas are present in LPD-affected kittens. Further investigations of LPD-affected kittens for the presence of defects in these additional genes are therefore needed to confirm or exclude the involvement of these and other genes in the feline disease. Recent advances in and increased availability of SNP (single nucleotide polymorphism) chip technology for feline samples (Mullikin et al. 2010; Anonymous 2012b) may mean that alternative techniques are able to be used in place of PCR to more rapidly identify genes associated with the development of LPD in BSH kittens in future studies.

Although considered less likely, it is also possible that defects in Fas expression were present in LPD-affected kittens but were not detected by methods employed in the present study. For example, the mRNA generated by some mutations, particularly truncating mutations, can cause nonsense mediated decay (NMD) of RNA. Due to deterioration of the mutated RNA, there is a consequent failure to amplify any mutated DNA by PCR techniques (Hsu et al. 2012). In a heterozygous animal with one normal allele and one mutated allele, this phenomenon may mean that RT-PCR methods only produce DNA from the normal allele (unaffected by NMD), which does not contain sequence abnormalities (Claustres 2005). In addition, it is also possible that abnormalities in
translation or post-translational modifications to the Fas protein were present that result in the production of reduced amounts of qualitatively normal Fas protein. If the reduction in the amount of functional Fas produced was significant, this could adversely affect and ultimately reduce effective apoptosis. The methods used to evaluate the presence of Fas defects in LPD-affected kittens in the present study only assessed qualitative gene defects and so would not have detected any quantitative abnormalities present in Fas expression. To investigate the presence of such quantitative defects in Fas expression in affected kittens, alternative methods of assessment such as real-time PCR (qPCR) for Fas expression would be needed.

Alternatively, it is possible that abnormalities in the Fas genes were not identified because the disease in kittens is due to causes other than defective Fas-mediated apoptosis of lymphocytes. For example, artificially generated mice with elevated expression of the RNA gene miR-17-92 in lymphocytes develop signs of disease with some similarities to those seen in affected BSH kittens, including dramatic lymphadenopathy and splenomegaly from 5 weeks of age (Xiao et al. 2008). Disease results from defects in the intrinsic apoptotic pathway, as miR-17-92 has a role in the suppression of Bim, a pro-apoptotic protein in the Bcl-2 family involved in the intrinsic apoptotic pathway and essential for the elimination of self-reactive lymphocytes (Bouillet et al. 2002; Cory and Adams 2002). Affected mice also show autoimmune manifestations and generally die before 12 months of age, however, the LPD in affected mice occurs due to the presence of increased numbers of both T-cells and B-cells, which appears to differ from the marked proliferation of T-cells only in affected BSH kittens in the immunophenotyping studies reported in Chapter 5.

Similarly, experimental mice with defects in the expression of the CTLA-4 (cytotoxic T-lymphocyte antigen 4) receptor also develop an early onset and marked LPD which has several similarities to the disease presentation in BSH kittens. CTLA-4 has a critical role in down-regulating T-cell activation and the maintenance of immunologic homeostasis (Tivol et al. 1995). CTLA-4 defective mice show massive lymphadenopathy and splenomegaly with lymphocytic infiltration and destruction of multiple organs, including the heart, pancreas and lung (Tivol et al. 1995; Waterhouse et al. 1995). The disease in mice is rapidly progressive and death usually occurs between three and four weeks of age. However, defects in lymphocyte apoptosis are not present (Waterhouse et al. 1997), and
the LPD is due to the presence of increased numbers of non-clonal CD4+ and CD8+ T-cells. These findings again appear to differ from the features of disease in affected BSH kittens seen in the immunophenotyping and apoptosis assay studies reported in Chapters 5 and 6.

Neither elevated expression of miR-17-92 nor defects in CTLA-4 are reported to occur naturally in mice or other animals and both have apparent differences from features of LPD in BSH kittens. However, they illustrate the possibility that mechanisms other than defects in Fas-mediated apoptosis may be responsible for the development of LPD in affected kittens. The mechanisms of disease development in both diseases remain as possible bases for development of the disease in kittens and are potential areas for future investigations.

The failure to extract RNA from two LPD-affected kittens (K1 and K32) was most likely due to the poor quality and limited quantity of samples available from these animals. As these kittens were necropsied prior to the initiation of the present studies, fresh frozen samples were not collected, and accordingly, only FFPE tissue was available for further testing. Although RNA recovery from FFPE tissue is possible, RNA quality is frequently poor and often provides a poor template for cDNA synthesis using RT-PCR, particularly with prolonged tissue fixation in formalin (Bresters et al. 1994; Masuda et al. 1999; Penland et al. 2007). Despite these issues, it was decided to use RNA rather than DNA (which would have been easier to extract) as RT-PCR using RNA detects a much wider range of mutations than PCR using genomic DNA, including intron and splice site mutations. RNA extraction using a commercial kit (High Pure) designed for RNA recovery from FFPE tissue was attempted but was repeatedly unsuccessful. Further modifications previously reported to improve RNA yield were also applied to the extraction procedure (extension of the proteinase K digestion step and repetition of the DNase incubation and initial washes (Chung et al. 2006; Abramovitz et al. 2008)) but without success. It was proposed to increase the starting amount of FFPE tissue by using multiple 10µm scrolls (Hardcastle 2011), but by this time, very little FFPE tissue from these two kittens remained for RNA extraction after use in multiple previous studies (histology, immunohistochemistry, clonality and TUNEL assays). As RNA had been successfully extracted from three other LPD-affected animals (K2, K10 and K11) and analysed by RT-PCR without detecting Fas abnormalities, it was decided to preserve the small amount of FFPE tissue from K1 and
K32 remaining for future studies rather than to continue with attempts to extract RNA from these animals. Although multiple different Fas gene and other mutations are documented in people with ALPS, ALPS-affected members of a single family all share the same mutation (Fisher et al. 1995; Sneller et al. 1997; Rieux-Laucat et al. 1999). It is therefore unlikely that a different mutation would have been present in these two untested kittens than the kittens already successfully tested.

The results of studies reported in this chapter therefore support the presence of reduced T-cell apoptosis in affected kittens, and provide some support that the basis for disease may be similar to ALPS people and mice with gld or lpr disease. Samples from the adult cat affected by LPD (K11) did not show evidence of reduced apoptosis, confirming the results from studies reported in previous chapters that the disease in this animal likely differs to that in the kittens. The cause of the apparent defects in apoptosis in LPD-affected kittens remains uncertain. Results do not support the presence of qualitative Fas gene mutations in LPD-affected kittens, but do not rule out quantitative abnormalities in Fas expression nor the presence of abnormalities in the multiple other genes involved in apoptosis, some of which have been implicated in ALPS in people and gld disease in mice. These results provide a basis for further investigations into mechanisms of defective apoptosis and defects in the additional genes involved in apoptosis in LPD-affected kittens and cats.

6.5 SUMMARY

The results of TUNEL assays indicate significant reductions in T-cell apoptosis in LPD-affected kittens, supporting defective apoptosis as a causative factor in the development of this disease. A similar reduction in lymphocyte apoptosis is present in people with ALPS and in mice with lpr or gld disease. Results therefore provide further support that the underlying basis for the feline disease may be similar to that in ALPS in people and its murine equivalents. While preliminary RT-PCR investigations did not identify qualitative Fas gene abnormalities in LPD-affected kittens, this does not exclude the possibility of quantitative defects in Fas expression or defects in one of the many other genes involved in apoptosis.
6.6 REFERENCES


INVESTIGATIONS OF DEFECTIVE MISMATCH REPAIR

7.1 INTRODUCTION

The highly conserved mismatch repair (MMR) system preserves genomic stability by recognising and repairing DNA replication errors and also plays a role in multiple other cellular interactions (Bellacosa 2001). Studies suggest that all cells express MMR proteins, with increased expression observed within replicating cells (Marra et al. 1996; Wei et al. 1997). Inherited MMR defects in people predispose to some types of cancer. While MMR defects are only occasionally reported in cases of human lymphoma (Scott et al. 2007; Pineda et al. 2008; Ripperger et al. 2010), MMR defects in mice are frequently implicated in the development of lymphoid neoplasms (Reitmair et al. 1995; Edelmann et al. 1997; Prolla et al. 1998; de Wind et al. 1999). To date, few studies have evaluated the role of MMR defects in neoplasia of veterinary species (Munday et al. 2008; Munday et al. 2009) and the presence of MMR dysfunction in feline tumours has not been investigated.

* The study detailed in this chapter has been published in part as: Aberdein D, Munday JS, Howe L, French, AF, Gibson IR. Widespread mismatch repair expression in feline small intestinal lymphomas. J Comp Path 2012 147(1): 24-30.
Prior to clonality testing, the original histological diagnosis of the LPD affecting British shorthair (BSH) kittens and cats was lymphoma, and defective MMR was investigated as a possible cause of disease. As an adjunct study, the possibility that MMR expression could be impaired in a proportion of small intestinal lymphomas in the wider feline population was also investigated. Lymphoma is the most common intestinal tumour of cats (Jacobs et al. 2002; Head et al. 2003; Rissetto et al. 2011), and although the majority of intestinal lymphomas develop in cats older than 8 years of age (Vail 2007), a proportion of tumours also develop in younger cats (Valli et al. 2000; Cesari et al. 2009). While feline leukaemia virus (FeLV) infection has been associated with the development of multicentric and thymic lymphoma in cats, there is little evidence supporting a causative role for FeLV in the development of feline intestinal lymphomas (Richter 2003; Vail 2007; Stutzer et al. 2010).

The aims of this study were to investigate the hypotheses first, that LPD in BSH cats and kittens affected by LPD may be due to the presence of inherited MMR defects; and second, that small intestinal lymphomas in young cats may be due to the presence of inherited MMR defects.

Following confirmation of the cross-reactivity of the human MMR antibodies with feline tissue using Western blotting, the presence of MMR defects was investigated immunohistochemically. In the first part of the study, MMR protein expression in enlarged lymph nodes from BSH animals affected by LPD was assessed. If, as hypothesised, inherited MMR defects predispose to the development of LPD in affected animals, loss of MMR expression within cells in affected lymph nodes would be expected to be observed. In the second part of the study, MMR protein expression in small intestinal lymphomas from young cats was compared to that in lymphomas from older cats. If inherited MMR defects predispose to intestinal lymphoma in young cats, loss of MMR expression would be expected to be more common in this group. MMR expression within high and low grade tumours was also compared as a preliminary investigation of the possible prognostic significance of MMR expression in cats.
7.2 MATERIALS AND METHODS

Eight BSH kittens and cats with LPD had been previously identified (see Chapters 3 and 4). Of those 8 animals, formalin-fixed, paraffin-embedded (FFPE) tissue from affected lymph nodes was available from five animals (K1, K2, K10, K11 and K32). All samples were from affected lymph nodes taken at necropsy.

Feline small intestinal lymphomas were identified by retrospective surveys of the databases of New Zealand Veterinary Pathology Limited and the Massey University necropsy service. Samples included both surgical biopsies and samples collected at necropsy. The diagnosis recorded within the relevant database was confirmed by histological examination of a 5μm haematoxylin and eosin stained section of the neoplasm, and samples confirmed as lymphoma were classified and graded as either low, intermediate or high grade according to the National Cancer Institute Working Formulation (Anonymous 1982) as applied to feline lymphomas (Valli et al. 2000). Tumours were excluded from the study if the original diagnosis of lymphoma could not be confirmed on histology or if the cat’s age at diagnosis was not recorded.

Small intestinal tissue from three previously healthy adult cats with no clinical or histological evidence of LPD or small intestinal disease was used for control tissues for antibody validation purposes. All samples were taken at necropsy.

7.2.1 VALIDATION OF HUMAN MMR ANTIBODIES IN FELINE TISSUE

Crude tissue lysates of small intestinal tissue from three healthy adult cats were prepared by finely dicing a 50mg tissue sample which was agitated in 1mL Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, United States) for 90 minutes at 4°C and then centrifuged for 20 minutes at 12 000 rpm at 4°C. The protein concentration of each sample was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, United States) by measuring absorbance at 280nm. Lysates were diluted in Laemmli buffer to a final protein concentration of 2.5mg/mL and heated at 100°C for 5 minutes. Samples were loaded (25g μg per lane) onto a SDS-PAGE 7.5% gel (Criterion Precast Tris-HCl gel, BioRad, United States) and separated by electrophoresis at 100V for 60 minutes in 1X Tris-Glycine-SDS buffer. As recommended by the manufacturers, A431
cell lysate (BD Biosciences, United States) and Jurkat cell lysate (Santa Cruz Biotechnology, United States) were used as positive controls for MLH1 (Jurkat), MSH2 (A431) and MSH6 (A431). The primary antibody was omitted in negative controls. Proteins were transferred onto nitrocellulose membrane (BioRad) by immunoblotting at 75V for 45 minutes in 1X Tris-Glycine buffer. After blocking non-specific binding by incubating in 5% skimmed milk in phosphate buffered saline (PBS) with Tween 20 with gentle agitation for 1 hour at room temperature (RT) (MLH1 and MSH6) or overnight at 4°C (MSH2), membranes were incubated with gentle agitation overnight at 4°C with either mouse anti-human MLH1 (clone G168-15, mouse monoclonal, BD Biosciences) at a dilution of 1:100 or mouse anti-human MSH6 (clone 44, mouse monoclonal, BD Biosciences) at a dilution of 1:250, or for 1 hour at room temperature with gentle agitation with rabbit anti-human MSH2 (clone H-300, rabbit polyclonal, Santa Cruz) at a dilution of 1:100. Primary antibodies were diluted in 5% skimmed milk in PBS/Tween 20. Following a 5 minute rinse with PBS-Tween 20, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT with gentle agitation. For MLH1 and MSH6, an HRP goat anti-mouse antibody (BD Biosciences) was used and for MSH2 a HRP goat anti-rabbit antibody (Santa Cruz) was used, both at a dilution of 1:1000. Secondary antibodies were diluted in 5% skimmed milk in PBS/Tween 20. Following rinsing in PBS-Tween 20 for 20 minutes, immune complexes were visualised using a chemiluminescent detection system (Immun-Star HRP Chemiluminescent kit, BioRad) and exposed to chemiluminescent film (Lumi-Film, Roche Applied Science, United States) for 10-30 seconds and the film developed.

### 7.2.2 Immunohistochemical Analysis of MMR Expression

Sections of LPD-affected lymph nodes from and small intestinal lymphomas were cut at a thickness of 5μm, mounted on charged microscope slides, deparaffinised in xylene, rehydrated in graded ethanol and rinsed in distilled water. Antigen retrieval was achieved by heating slides in a pressure cooker for 10 minutes at full pressure in citrate buffer (pH 6.0, 0.01M) with a 10 minute cool-down. Endogenous peroxidases were blocked by incubating slides in 0.3% hydrogen peroxide in Tris buffered saline (TBS, pH 7.6, 0.05M). Non-specific staining was blocked by incubating slides with equine serum (Vector Laboratories, United States) for 20 minutes. Slides were incubated for 60 minutes with anti-human MLH1 (BD Biosciences) at a dilution of 1:50, anti-human MSH2 (Santa Cruz) at a dilution of 1:100, or anti-human MSH6 (BD Biosciences) at a dilution of 1:500. Slides
were then incubated for 30 minutes with biotinylated horse-anti-mouse/rabbit secondary antibody (Vectastain Universal Elite kit, Vector Laboratories) diluted according to the manufacturers’ instructions. Slides were finally incubated for 30 minutes with a biotin-avidin complex (Vectastain Universal Elite ABC Reagent, Vector Laboratories) diluted according to the manufacturers’ instructions. To visualise the reaction, slides were incubated with 3,3-diaminobenzidine substrate (Liquid DAB Substrate Chromagen System, Dako, United States), rinsed in tap water, counterstained with Gill’s haematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped. Slides were rinsed for a minimum of 5 minutes in TBS between steps and all steps were carried out at RT. For the LPD cases, non-neoplastic stromal and vascular endothelial cells were used as positive controls. For the small intestinal lymphomas, non-neoplastic intestinal epithelial, stromal and vascular endothelial cells were used as positive controls. The primary antibody was omitted in negative controls and slides incubated with TBS instead.

The intensity of the immunohistochemical reaction within the lymphocytes or neoplastic cells was evaluated for each section as previously described (Munday et al. 2009). This was graded on a scale of 0 to 5, with 0 indicating no visible immunoreactivity and 5 indicating intense immunoreactivity. The percentage of lymphocytes with visible nuclear immunoreactivity was estimated by examining 5 different 400x fields within the neoplasm. Each tissue was assessed to see if immunoreactivity was distributed evenly within the neoplasm. For the lymphomas, all evaluations were performed without knowledge of the group of origin of the lymphoma. Differences between groups were investigated by 2-sample t tests and one-way ANOVA using Minitab 15 statistical software (2007, Minitab Inc.).
CHAPTER 7

7.3 RESULTS

Five LPD-affected lymph nodes and 40 small intestinal lymphomas were included in the study. The 40 small intestinal lymphomas were divided into two groups: group 1, which included tumours from cats that were either 8 years of age or younger at the date of diagnosis (n=10, mean age 4.5 years, range 1 to 8 years), and group 2, which included tumours from cats which were 9 years of age or older at the date of diagnosis (n=30, mean age 12.6 years, range 9 to 18 years). Of the 40 tumours examined, low grade tumours were the most common (19/40), with 6/40 of intermediate grade and 15/40 high grade. The majority of low grade tumours were of the small lymphocytic cell type (16/30), while most high grade tumours were of the immunoblastic cell type (9/30). Lymphomas from cats in group 1 were significantly more likely to be high grade tumours than lymphomas from cats in group 2 (p=0.001).

7.3.1 VALIDATION OF HUMAN MMR ANTIBODIES IN FELINE TISSUE

Western blot analysis of samples of small intestinal tissue from three healthy adult cats confirmed that the three human MMR antibodies reacted with proteins in feline tissue that were of the approximate expected molecular weight (approximately 80kDa for MLH1, 100kDa for MSH2 and 150-160kDa for MSH6). These are the respective molecular weights for each of the three MMR proteins previously reported in human and other species (Li and Modrich 1995; Palombo et al. 1995).
Figure 7.1: Western blot analysis showing cross-reactivity of human mismatch repair (MMR) antibodies with feline tissue. M: marker lane, lane 1: positive control (Jurkat or A431); lanes 2-4 feline small intestinal tissue. A. MLH1, showing bands at approximately 75kDa (Jurkat positive control). B. MSH2, showing bands at approximately 100kDa (A431 positive control). C. MSH6, showing bands at approximately 150kDa (A431 positive control).
7.3.2 IMMUNOHISTOCHEMICAL ANALYSIS OF MMR EXPRESSION

Intranuclear expression of MLH1, MSH2 and MSH6 was observed within both positive control cells and lymphocytes within sections of lymph node from all 5 BSH kittens. In samples from K11, where lymph node architecture was retained, the percentage of cells showing expression of all three MMR proteins was consistently higher within the lymphoid follicles than within the interfollicular regions of the node, although the intensity of expression was similar in all areas. In LPD-affected lymph nodes, the average intensity of MLH1 immunoreactivity within lymphocytes was 2.4, with intensity ranging between grades 1 and 4. Overall, MLH1 expression was visible in 66.0% (range 20-90%) of proliferating lymphocytes (Figures 7.2A and B). The average intensity of MSH2 immunoreactivity within the lymphocytes was 3.8, with intensity ranging between grades 3 and 4. Overall, MSH2 expression was visible in 71% (range 55-80%) of proliferating lymphocytes (Figures 7.2C and D). The average intensity of MSH6 immunoreactivity within lymphocytes was 3.6, with intensity ranging between grades 3 and 4. Overall, MSH6 expression was visible in 75% (range 55–90%) of proliferating lymphocytes (Figures 7.2E and F).
Figure 7.2: Lymph node immunohistochemistry, MLH1, MSH2, and MSH6 (low and high power), LPD-affected kitten (K2), 3,3’diaminobenzidine/haematoxylin. A. and B. The majority (more than 90%) of lymphocytes within the node show intense (grade 4) nuclear immunoreactivity to antibodies against MLH1, low power (A.) and high power (B.) views. C. and D. Approximately 80% of lymphocytes within the node show moderate to intense (grades 3-4) nuclear immunoreactivity to antibodies against MSH2, low power (C.) and high power (D.) views. E. and F. The majority (more than 90%) of lymphocytes within the node show intense (grade 4) nuclear immunoreactivity to antibodies against MSH6, low power (E.) and high power (F.) views.
Results of the immunohistochemical evaluation for the small intestinal lymphomas are summarised in Table 7.1. Intranuclear expression of MLH1 was observed within non-neoplastic positive control cells in all 40 small intestinal lymphoma samples. Non-neoplastic positive control cells and neoplastic lymphocytes within all sections demonstrated diffuse intranuclear MLH1 expression. The average intensity of immunoreactivity within the neoplastic cells was 2.7, with intensity ranging between grades 1 and 5. Overall, MLH1 expression was visible in 66.0% (range 20–100%) of neoplastic lymphocytes. There were no significant differences between groups 1 and 2 in the intensity of immunoreactivity or the percentage of neoplastic cells expressing MLH1 (p= 0.09 (intensity) and p= 0.10 (percentage of neoplastic cells)). However, both the intensity of immunoreactivity and the percentage of neoplastic cells expressing MLH1 were significantly higher in the high grade lymphomas than in the low grade tumours (average intensity of immunoreactivity: low grade tumours = 1.8; high grade tumours = 3.7; average percentage of neoplastic cells expressing MLH1: low grade tumours = 51.6%; high grade tumours = 81.3%, p<0.001 for both) (Figures 7.3 A and B).

### Table 7.1: Summary of mismatch repair (MMR) protein expression within feline small intestinal lymphomas.*

<table>
<thead>
<tr>
<th></th>
<th>Intensity of immunoreactivity</th>
<th>Cell expression within neoplasm (%)</th>
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<tbody>
<tr>
<td></td>
<td>All</td>
<td>Group 1</td>
</tr>
<tr>
<td>MLH1</td>
<td>2.7 (1.3)</td>
<td>3.3 (1.3)</td>
</tr>
<tr>
<td>MSH2</td>
<td>3.4 (1.3)</td>
<td>3.6 (1.1)</td>
</tr>
<tr>
<td>MSH6</td>
<td>3.1 (1.5)</td>
<td>3.7 (1.1)</td>
</tr>
</tbody>
</table>

* Results with different letters are significantly different

Diffuse intranuclear expression of MSH2 was also observed in non-neoplastic positive control cells and neoplastic lymphocytes within all 40 samples. The average intensity of the immunoreactivity within the neoplastic cells was 3.4, with intensity ranging between grades 1 and 5. Overall, MSH2 expression was visible in 94.3% (range 70-100%) of neoplastic lymphocytes (Figures 7.3C and D). As with MLH1, differences between groups 1 and 2 in the intensity of immunoreactivity and the percentage of neoplastic cells expressing MSH2 were not significant (p=0.545 (intensity) and p= 0.115 (percentage of neoplastic cells)). There were also no significant differences between high and low grade.
lymphomas in either the average intensity of MSH2 expression in neoplastic cells (p=0.41) or the percentage of neoplastic cells showing positive immunostaining (p=0.05).

Intranuclear expression of MSH6 was also observed within non-neoplastic positive controls in all 40 tumours. Non-neoplastic positive control cells and neoplastic lymphocytes demonstrated a similar pattern of immunoreactivity to that observed using anti-MLH1 and anti-MSH2 antibodies. The average intensity of the immunoreactivity within the neoplastic cells was 3.1 with intensity ranging between grades 1 and 5. Overall, MSH6 expression was visible in 63.0% (range 10–100%) of neoplastic lymphocytes (Figures 7.3E and F). As with both MLH1 and MSH2, differences between groups 1 and 2 in the intensity of immunoreactivity and the percentage of neoplastic cells expressing MSH6 were not significant (p=0.08 (intensity) and p=0.13 (percentage of neoplastic cells). There were no significant differences between high and low grade lymphomas in either the average intensity of MSH6 expression in neoplastic cells (p=0.05) or the percentage of neoplastic cells showing positive immunostaining (p=0.05).

The mean intensity of MSH2 immunoreactivity in neoplastic lymphocytes over all tumours was significantly higher (3.4) than MLH1 (2.7, p=0.01) but similar to MSH6 (3.1, p=0.39). Mean percentages of neoplastic lymphocytes showing positive immunostaining for MSH2 were significantly higher (94.3%) than either MLH1 (66.0%, P<0.005) or MSH6 (63.0%, P<0.005).
Figure 7.3: Small intestinal immunohistochemistry, MLH1, MSH2, and MSH6 (low power), low and high grade tumours, 3,3′diaminobenzidine/haematoxylin. A. Feline low grade small intestinal lymphoma; approximately 50% of lymphocytes within the neoplasm show moderate (grade 3) nuclear reactivity to antibodies against MLH1. B. Feline low grade small intestinal lymphoma; approximately 90% of lymphocytes within the neoplasm show intense (grade 4) nuclear reactivity to antibodies against MLH1. C. Feline small intestinal lymphoma, low grade; more than 90% of lymphocytes within the neoplasm show very intense (grade 4) nuclear reactivity to antibodies against MSH2. D. Feline small intestinal lymphoma, high grade; more than 90% of lymphocytes within the neoplasm show very intense (grade 5) nuclear reactivity to antibodies against MSH2. E. Feline small intestinal lymphoma, low grade; approximately 70% of lymphocytes within the neoplasm show intense (grade 4) nuclear reactivity to antibodies against MSH6. F. Feline small intestinal lymphoma, high grade; approximately 90% of lymphocytes within the neoplasm show intense (grade 4) nuclear reactivity to antibodies against MSH6.
7.4 DISCUSSION

In the present study, Western blot analyses of feline tissues confirmed the cross-reactivity of the human antibodies MLH1, MSH2 and MSH6 with the corresponding proteins in the cat. The ability of these antibodies to detect a band of the appropriate molecular weight on Western blot analysis supports the cross-reactivity of the human MMR antibodies with the corresponding proteins in feline tissues and suggests that these antibodies can also be applied to detect MLH1, MSH2 and MSH6 in feline tissues, including by immunohistochemistry. The MMR system is highly conserved across multiple mammalian and other species (Buermeyer et al. 1999), and the cross-reactivity of the human MMR antibodies with feline tissue in this study is not unexpected. However, to the author’s knowledge, evidence of the cross-reactivity of these human MMR antibodies in feline tissue has not been previously reported.

All three MMR proteins were detected immunohistochemically within all LPD-affected lymph nodes from BSH kittens and cats as well as within all feline small intestinal lymphomas assessed in this study. Therefore, loss of MMR expression does not appear to be significant in the development of LPD in BSH kittens and cats, nor in the development of small intestinal lymphoma in cats more generally. As MMR expression was similar in small intestinal lymphomas from young and old cats, inherited germline defects of MLH1, MSH2 or MSH6 do not appear to be a factor in the development of these tumours. This is similar to the situation in people, where loss of MMR expression in gastrointestinal lymphomas appears uncommon (Cuilliere-Dartigues et al. 2007). To the author’s knowledge, this is the first time MMR expression has been evaluated in feline tissues.

In people, hereditary non-polyposis colorectal carcinoma (HNPCC) or Lynch syndrome is due to a deleterious germline mutation in one of the genes encoding the MMR proteins: a subsequent somatic mutation or loss of the second normal allele in a particular cell inactivates the gene. This can result in impaired MMR function and neoplastic transformation of the affected cell. In addition to loss of MMR protein expression, Lynch syndrome tumours are also associated with microsatellite instability (MSI) (Buermeyer et al. 1999; Abdel-Rahman et al. 2006).
Several studies have suggested that defects in the MMR genes play a role in lymphomagenesis and the development of lymphoproliferative disease. First, mice with MMR defects frequently develop lymphomas at a young age (Reitmair et al. 1995; Edelmann et al. 1997; de Wind et al. 1999; Wei et al. 2002; Chen et al. 2005). Second, MSI is present in a proportion of human lymphomas (Bandipalliam, 2005; de Vos et al., 2005), particularly in tumours associated with immunodeficiency (Bedi et al. 1995; Duval et al. 2004). Third, biallelic mutations in MMR genes are reported in individuals presenting with childhood lymphoma and lymphocytic leukaemia (Bougeard et al. 2003; Felton et al. 2007; Scott et al. 2007; Ripperger et al. 2010), while other studies suggest that MMR mutations may play a role in the development of lymphoid neoplasia in people more generally (Indraccolo et al. 1999; Kotoula et al. 2002).

Detection of a mutation within the MMR genes is the ultimate diagnostic test for Lynch syndrome, but as mutation screening is time-consuming and expensive, immunohistochemical evaluation of tumours is used as an alternative screening method to identify cases of Lynch syndrome and MMR dysfunction in humans (Shia 2008). Immunohistochemical examination of a neoplasm from a person with Lynch syndrome reveals normal expression of the relevant MMR protein in non-neoplastic cells but no expression in neoplastic cells.

Although the majority of feline small intestinal lymphomas occur in older animals, a proportion also occurs in younger cats (Valli et al. 2000; Cesari et al. 2009). In the second part of the present study, feline small intestinal lymphomas were divided into those from cats 8 years of age or younger and those from cats 9 years of age of older. If the hypothesis that inherited MMR defects predisposed young cats to the development of lymphomas was correct, immunohistochemical loss of MMR protein expression should have been commonly observed within tumours from young cats and rarely observed within lymphomas from old cats. The failure to detect significant differences in MMR expression between the two groups of tumours suggests that inherited MMR defects are unlikely to predispose younger cats to tumour development. Similarly, the failure to detect loss of MMR expression in any of the lymph node samples from LPD-affected BSH kittens or cats suggests that inherited MMR defects are unlikely to predispose to the development of this disease either.
However, it is possible that the studies failed to detect defects of MMR protein expression for other reasons. In both the LPD-affected lymph nodes and the group 1 small intestinal lymphomas, it is possible that the proliferating or neoplastic lymphocytes contained MMR proteins that retained their immunoreactivity but were no longer functional. Although this possibility cannot be excluded, it is considered unlikely as studies in humans suggest that the majority of MMR gene mutations prevent protein expression and only a small proportion of MMR-deficient cells retain their immunoreactivity (Lanza et al. 2006). However, further analysis of tumours by alternative methods such as MSI analysis would help exclude this possibility.

Alternatively, it is possible that the proliferating or neoplastic lymphocytes contained defects of a MMR protein other than the three evaluated in this study. Although multiple genes have been associated with Lynch syndrome (including MLH1, MSH2, MLH3, MSH6, PMS1, PMS2 and EXO1), the vast majority of Lynch syndrome neoplasia is due to mutations in MLH1, MSH2 and MSH6 (Peltomaki and Vasen 2004; Woods et al. 2007). In humans, the biological significance of mutations identified in PMS2 and other genes is less certain (Lagerstedt Robinson et al. 2007), but may play a role in tumour development. Previous studies of MMR defects in animals have reported that antibodies against human PMS2 protein do not react with PMS2 protein in ovine (Munday et al. 2008) or canine (Munday et al. 2009) tissues, most likely due to a low affinity of the human antibody for the corresponding non-human protein, and PMS2 expression was not evaluated in the current study. However, studies in mice suggest that mutations in PMS2 and MLH3 have a role in lymphomagenesis in mice (Chen et al. 2005). Further studies to evaluate the possible role of defects in additional MMR genes including MLH3 and PMS2 may be needed.

The FeLV status of none of the cats with small intestinal lymphoma in the second part of the study was known, and the possibility that a proportion of tumours included in the sample were in fact associated with undiagnosed FeLV infection cannot be excluded. However, recent studies have failed to detect a significant association between FeLV and intestinal lymphoma development (Louwerens et al. 2005; Rissetto et al. 2011). As mice with MMR defects develop both T-cell and B-cell lymphomas (Reitmair et al. 1995; Edelmann et al. 1997; Peled et al. 2010), the T- and B- lymphocyte lineage of the tumours included in this part of the study was not investigated. In the case of the BSH LPD cases,
3/5 animals included in this part of the study tested were negative for FeLV, and all LPDs involved a T-cell proliferation. This suggests that LPD development in BSH kittens and cats is unlikely to be associated with FeLV infection.

In contrast to previous studies in animals (Munday et al. 2009), the lymphomas showed significantly greater intensity in immunoreactivity to the anti-MSH2 antibody than that observed using an anti-MLH1 antibody, and significantly higher percentages of neoplastic cells expressed MSH2 than expressed either MLH1 or MSH6. The higher intensity of immunoreactivity using an anti-MSH2 antibody in this part of the study was consistent within both neoplastic cells and internal positive controls, suggesting the variation in intensity between the antibodies was due to a higher affinity of the anti-MSH2 antibody to the feline protein rather than true differences in protein expression within neoplastic cells. The use of a polyclonal anti-MSH2 antibody compared to the monoclonal antibodies used against MLH1 and MSH6 in this study may explain this increased affinity. Polyclonal antibodies often produce greater non-specific staining than monoclonal antibodies, which are usually highly specific (Ramos-Vara et al. 2008).

The higher average intensity of MLH1 immunostaining in neoplastic cells and the higher average percentage of neoplastic cells expressing MLH1 in high grade lymphomas compared to low grade tumours likely reflects the higher mitotic activity of the higher grade tumours. Although studies suggest that all viable cells express MMR proteins, increased expression is observed within replicating cells (Marra et al. 1996; Wei et al. 1997) and MMR protein expression has been shown to parallel the proliferation rate as assessed by immunohistochemical expression of Ki67 (Cuilliere-Dartigues et al. 2007). A frequent feature of high grade tumours is a high mitotic rate, indicative of the tumour proliferation rate, and it would be expected that these rapidly dividing tumour cells show stronger and higher levels of MMR protein expression. It is unclear why higher levels of intensity of expression and percentages of cells expressing immunoreactivity were not also seen in the high grade tumours with MSH2 and MSH6.
7.5 SUMMARY

In conclusion, this is the first investigation of MMR protein expression in feline tissues. The study demonstrates the cross-reactivity and validates the future use of the human antibodies MLH1, MSH2 and MSH6 in feline tissue. However, loss of MMR expression was not detected in any of the LPD-affected lymph nodes from BSH animals nor from any of the feline small intestinal lymphomas included in the study. Loss of MMR expression therefore appears unlikely to be a significant factor in the development of LPD in BSH cats and kittens. Loss of MMR also appears to be rare in feline small intestinal lymphomas. The results of this study do not support either hypothesis that the development of LPD in BSH cats and kittens or the development of a proportion of small intestinal lymphomas in younger cats is predisposed to by inherited mutations of the MMR genes.

7.6 REFERENCES


STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Danielle Aberdein

Name/Title of Principal Supervisor: John Munday

Name of Published Research Output and full reference:
Widespread mismatch repair expression in feline small intestinal lymphomas
Aberdein D, Munday JS, Howe L, French AF, Gibson IR.

In which Chapter is the Published Work: 7

Please indicate either:
- The percentage of the Published Work that was contributed by the candidate:
  
  and / or

- Describe the contribution that the candidate has made to the Published Work:
  80% contribution by the candidate

______________________________  ______________________________
Danielle Aberdein  3 May 2013
Candidate’s Signature  Date

______________________________  ______________________________
John Munday  3 May 2013
Principal Supervisor’s signature  Date
CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 INTRODUCTION

The central aim of this project was to further describe and investigate a lymphoproliferative disease (LPD) in British shorthair (BSH) kittens initially identified in 2009. Particular emphasis was placed on describing the clinical and pathological features of the disease, investigating the likely basis for disease development, and exploring similarities between the disease in the kittens and previously reported diseases in cats and people. The studies described in the thesis confirm a likely non-neoplastic T-cell LPD in BSH kittens with a probable inherited basis not previously reported in cats or other domestic animals. The disease in kittens has several similarities to the rare human disease autoimmune lymphoproliferative syndrome (ALPS), an inherited disorder of T-cell regulation typically due to defects in Fas-mediated apoptosis (Sneller et al. 1992; Fisher et al. 1995; Sneller et al. 1997; Jackson and Puck 1999b). The results of preliminary studies support defects in T-cell apoptosis as a probable factor in disease development in BSH kittens but have not identified the presence of Fas-gene mutations.
8.2 FEATURES OF LYMPHOPROLIFERATIVE DISEASE IN BRITISH SHORTHAIR KITTENS

The results of the various matings, retrospective surveys and further pedigree information confirm a LPD affecting multiple related kittens with a shared genetic background and support an inherited basis for disease. Results do not support a simple autosomal dominant mode of inheritance, as clinical signs of LPD were not reported at any stage in the parents of any LPD-affected kitten. Results also do not support an X-linked mode of inheritance, as both male and female offspring were affected by disease in similar proportions. Results do provide support for either a simple autosomal recessive or modified autosomal dominant (with variable clinical expression or incomplete penetrance) mode of inheritance, with the BSH tom T1 involved in the pedigree of all affected kittens and appearing to have a significant role in disease transmission.

Based on the pattern of disease observed, autosomal recessive inheritance is considered more likely than modified autosomal dominant inheritance. Features that support an autosomal recessive mode of inheritance include that all parents of affected kittens were clinically unaffected by LPD, that the disease appears to skip generations, and that a reasonable degree of inbreeding is present within the known pedigrees of affected kittens. While the proportion of LPD-affected offspring observed (46.6%) is higher than that typically expected from carrier-to-carrier matings for a disease with autosomal recessive inheritance (25%), the likely ascertainment bias in identifying affected animals could have resulted in the higher than expected incidence of disease observed (Nicholas 1987; Nicholas 2010a). If the disease in kittens does have an autosomal recessive mode of inheritance, it would differ to the majority of cases of ALPS in people, but have a similar mode to that seen in mouse models of ALPS (lpr and gld disease) and rare cases of human ALPS.

Most human ALPS cases are inherited by a modified autosomal dominant mode of inheritance with variable clinical expression. It is also possible that this is the mode of inheritance in the feline disease, but this is regarded as less likely. The proportion of LPD-affected offspring observed (46.6%) is similar to the proportion expected with autosomal dominant inheritance with a clinical penetrance of approximately 90% (45%). However,
with a modified autosomal dominant mode of inheritance, it would be expected that at least some kittens resulting from the reported multiple (>30) other matings by the putative carrier tom cat (T1) would have also developed LPD. The apparent absence of any such LPD-affected kittens argues against this mode of inheritance, although the reliability of the breeder-supplied data concerning LPD in T1’s offspring may be questionable.

Definitively diagnosing the LPD in affected BSH kittens was more difficult than anticipated. Affected kittens consistently presented between 5 and 7 weeks of age with massive non-painful lymphadenopathy involving all lymph nodes, abdominal distension due to visceral lymphadenopathy and splenomegaly, and a regenerative and likely haemolytic anaemia with a possible immune-mediated aetiology. The disease was initially thought to be lymphoma due to the presence of a monomorphic population of rapidly proliferating lymphocytes of a single T-cell immunophenotype (CD3+/CD79a-) within the enlarged lymph nodes. Where assessed, the proliferating T-cell population was further characterised as a population of double negative T-cells (DNT cells), cells which are CD3+ (indicating a T-cell phenotype) but express neither CD4 nor CD8 surface proteins. Cells had characteristics of malignancy, and distorted or effaced normal lymph node architecture. These features were considered suggestive of lymphoma. The presence of a similar population of T-cells within the spleen and other organs suggested possible metastatic spread, providing further support for a diagnosis of lymphoma rather than that of a non-neoplastic lymphoid proliferation.

However, lymphoma was considered very unusual in such young kittens. The pattern of disease affecting multiple related animals, and the massive enlargement of all lymph nodes throughout the body, were also clinical features not typically present in feline lymphoma. To further investigate the disease in the kittens, clonality assays were performed. Clonality assays are widely considered the most reliable basis on which to differentiate lymphoma from a non-neoplastic lymphoid proliferation (Griesser et al. 1989; Weiss and Spagnolo 1993; Vernau and Moore 1999). These assays consistently identified the proliferating T-cells as a non-clonal (or polyclonal) population that was consistent with a non-neoplastic LPD. While there is a small possibility that the clonality assays failed to detect a monoclonal and therefore neoplastic T-cell proliferation in affected kittens, this is considered unlikely. The reported sensitivity of the T-cell (TRCG)
clonality assay in cats is close to 90% (Moore et al. 2005; Moore et al. 2012), and results obtained were strongly polyclonal, repeatable and performed in triplicate. In addition, assessment of the kittens’ proliferating cells for the presence of B-cell clonality helped exclude the possibility that the proliferating cells contained cross-lineage rearrangements, another possible source of false polyclonal results if only T-cell clonality is assessed (Griesser 1995; Valli et al. 2006). B-cell clonality assays also demonstrated a strongly polyclonal result and further strengthened the conclusion that the clonality results are reliable and indicative of a non-neoplastic T-cell proliferation in affected BSH kittens. Overall, results from the kittens are consistent with a non-neoplastic LPD.

The clinical presentation and pathological findings in samples from the sole adult cat affected by LPD (K11) differed significantly to those present in LPD-affected kittens. The LPD observed in this animal was much milder, did not involve the spleen, and developed significantly later than that in affected kittens. Affected lymph nodes showed follicular hyperplasia and retention of normal architecture, which differed to the marked distortion or effacement by proliferating T-cells seen in the kittens. In addition, chronic liver disease, with marked hepatic fibrosis, bile duct proliferation and mixed inflammation, were the most prominent features of disease in the adult cat, signs not present in any of the affected kittens. It is of interest that biliary cirrhosis, a condition with histological similarities to those present in K11, is one of the autoimmune manifestations occasionally reported in people with ALPS (Pensati et al. 1997; Avila et al. 1999; Rao and Straus 2006) and also in some strains of lpr mutant mice (Tsuneyama et al. 2001; Ohba et al. 2002). However, in the absence of any other similar clinical or pathological features to affected kittens, it is considered prudent that disease in K11 is regarded separately to that in the kittens at the present time. This conclusion may need to be revisited as further information or future cases are identified, particularly regarding the basis for disease development.

8.3 Basis for Disease Development

As discussed, available data support an inherited basis for the LPD in BSH kittens, most likely with an autosomal recessive or modified autosomal dominant mode of inheritance. While shared exposure to an infectious agent could potentially cause an apparent familial
predisposition to disease if multiple littermates were simultaneously infected, there is no evidence to support an infectious aetiology for the LPD in BSH kittens. Feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) have both been reported to cause LPD in cats (Hosie *et al.* 2009; Lutz *et al.* 2009; Hartmann 2011; Hartmann 2012). However, all affected BSH animals, parents and unaffected littermates repeatedly tested negative for evidence of either FeLV or FIV infection, including during the period when overt clinical signs of LPD were present. Although false negative results occur with tests for both viruses, the likelihood that multiple infected kittens tested falsely negative for evidence of either virus is extremely low. Accordingly, neither FeLV nor FIV is considered likely to have contributed to the development of LPD in BSH kittens.

In addition, the clinical and pathological features of disease seen in LPD-affected BSH kittens differ significantly to those previously reported with either FeLV or FIV infection, which typically affect significantly older cats and show different gross and microscopic pathology. Infection with FeLV may be associated with LPD, but lymphoma rather than non-neoplastic LPD is more typical and takes many months or years to develop following infection (Hoover *et al.* 1973; Hardy 1981; Stutzer *et al.* 2011). Cats infected with FIV may also develop lymphoma, but again this typically takes many months or years to develop (Callanan *et al.* 1996; Hosie *et al.* 2009). The non-neoplastic lymphadenopathy often present in FIV-infected cats is typically much milder than that seen in affected kittens, and lymph nodes show follicular hyperplasia and retention of normal nodal architecture histologically (Callanan *et al.* 1992; Rideout *et al.* 1992; Parodi *et al.* 1994; del Fierro *et al.* 1995). This differs significantly to the nodal distortion and effacement seen in nodes from affected BSH kittens.

The possibility that a novel infectious agent caused the development of LPD in the kittens cannot be excluded, but is considered unlikely for several reasons. At no stage did any parent or littermate of the affected kittens show signs of LPD. In addition, while LPD-affected Litters 1 and 2 had the same parents (T1 and Q1), the gestation period and the kittens’ first 12 weeks of life occurred under different environmental conditions, with the gestation and rearing of Litter 2 taking place in isolation from other animals. Furthermore, if a novel infectious agent is associated with the development of LPD in the kittens, it would be expected that this agent also would have affected kittens of breeds other than the BSH. To date, this has not been reported.
The molecular basis for disease development remains speculative. Results of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays indicate significant reductions in T-cell apoptosis in LPD-affected kittens, supporting defective apoptosis as a possible causative factor in the development of disease, but further confirmation of this is needed. No such reduction in apoptosis was observed in samples from the adult cat (K11), further supporting the conclusion that the disease in this animal likely differs to that in the kittens. A reduction in T-cell apoptosis is also present in people with ALPS and in mice with lpr or gld disease (Nagata and Suda 1995; Lim et al. 1998). Reverse transcriptase polymerase chain reaction (RT-PCR) investigations did not identify the presence of qualitative Fas gene abnormalities in LPD-affected kittens similar to those present in the majority of people with ALPS. However, this does not exclude the possibility of quantitative defects in Fas expression or defects in one or more of the many other genes involved in T-cell apoptosis, particularly Fasl and CASP10. Neither defects in the MMR genes nor karyotype abnormalities were identified in LPD-affected kittens.

8.4 SIMILARITIES TO REPORTED LYMPHOPROLIFERATIVE DISEASES

There was no evidence to suggest that the LPD in affected BSH kittens was due to any of the reported causes or syndromes of LPD in cats. The clinical and pathological features of disease in affected kittens were significantly different to disease due to FeLV or FIV infection, the distinctive peripheral lymph node hyperplasia of young cats (DPLNH), the generalised lymphadenopathy resembling lymphoma (GLRL), or the plexiform vascularisation of lymph nodes previously described (Moore et al. 1986; Lucke et al. 1987; Mooney et al. 1987).

There are several similarities between the disease in kittens and ALPS in people. Both diseases cause a non-malignant, generalised and often very marked lymphadenopathy due to LPD with variable involvement of the spleen. In both diseases, lymph nodes and spleen contain a marked proliferation of non-clonal T-cells (CD3+) (Sneller et al. 1997; Rao and Straus 2006; Oliveira et al. 2010). Cytopenias, including haemolytic anaemia, are often present in ALPS (Infante et al. 1998; Straus et al. 1999; Teachey et al. 2010); evidence of a haemolytic anaemia was also present in several LPD-affected kittens. Both
diseases typically affect young children or animals: the median age of presentation for ALPS patients is approximately 24 months (Avila et al. 1999; Rao and Straus 2006; Puck et al. 2007), while all affected kittens were less than 8 weeks of age at the first development of clinical LPD. ALPS in people is typically due to inherited defects in T-cell apoptosis (Infante et al. 1998; Jackson and Puck 1999b; Rao and Straus 2006; Anonymous 2012a); the feline disease also appears likely to have an inherited basis and there is evidence of a significant reduction in T-cell apoptosis in lymph nodes from affected kittens. The disease in people typically has an autosomal dominant mode of inheritance, although with variable expression of the clinical signs (Fisher et al. 1995; Infante et al. 1998; Jackson et al. 1999a). The disease in kittens is also likely to have an inherited basis, although the mode of inheritance is not certain, and both autosomal recessive and autosomal dominant with variable expression of clinical signs remain possibilities.

However, there are also differences between the two diseases. The typical histological changes seen within lymph nodes in ALPS patients are described as marked paracortical T-cell expansion with retention of nodal architecture (Lim et al. 1998). This differs to the marked nodal distortion or complete effacement seen in affected kittens. However, it is of note that in some cases of ALPS, the T-cell proliferation can be sufficiently florid to suggest a diagnosis of lymphoid neoplasia, and many of the early cases of ALPS were misdiagnosed as lymphoma (Canale and Smith 1967; Sneller et al. 1997). In addition, in the majority of ALPS patients, disease is due to the presence of Fas-gene mutations (Rao and Straus 2006; Anonymous 2012a). Investigations to date have not identified qualitative Fas-gene mutations in affected kittens, although quantitative Fas-gene mutations and mutations in the other genes mediating apoptosis such as FasL have not yet been investigated in the kittens.

There are also many relevant aspects of the disease in kittens about which information is lacking, and comparisons with ALPS regarding these aspects remain speculative. Disease in ALPS patients is typically chronic; one of the diagnostic criteria for ALPS is that the LPD is of more than 6 months duration (Oliveira et al. 2010). It is uncertain whether the disease in kittens is chronic or not, as all but one of the affected kittens were euthanased early in the clinical course of disease following a presumed diagnosis of lymphoma. Further, the presence of elevated numbers of DNT cells both in circulation and within the enlarged lymph nodes is a defining feature of ALPS (Sneller et al. 1997; Bleesing et al.)
2001a; Oliveira et al. 2010), but the presence of DNT cells was able to be assessed in the
lymph node of only one LPD-affected kitten, and in the blood of none. In addition,
cytopenias additional to anaemia or circulating autoantibodies are often present in ALPS
(Straus et al. 1999; Kwon et al. 2003; Teachey et al. 2010). While there was no evidence
of leukopenia or thrombocytopenia in any affected kitten, no kittens’ blood was assessed
for the presence of circulating autoantibodies, and it is not known whether such
antibodies were present. Of interest, significant autoimmune manifestations in ALPS
patients are often absent at initial presentation and only develop in later life (Straus et al.
1999; Worth et al. 2006); given this, the absence of clear autoimmune manifestations in
the kittens may not be surprising. Finally, people with ALPS typically show defective Fas-
mediated lymphocyte apoptosis on *in vitro* assays (Sneller et al. 1997; Oliveira et al.
2010), tests which were not able to be performed on samples from any affected kitten.

### 8.5 STUDY LIMITATIONS

A major limitation of the present studies is the low number of LPD-affected kittens
available for the various investigations of the disease. Only 8 BSH kittens and cats with
LPD were identified or generated in the course of the study, and the disease in one of
these 8 animals (K11, the adult cat) appeared to have a different clinico-pathological
presentation and likely different basis for development to the LPD in the remaining 7
kittens, further reducing animal numbers. The seasonal nature of feline breeding and the
financial constraints preventing use of dedicated and secure housing for breeding animals
were factors that limited the number of affected kittens that could be generated for the
studies. One of the most important constraints was the inability to persuade the owner of
the putative carrier BSH tom (T1) to allow him to be used in further studies after Litter 2.
The further matings of T1 as a putative carrier animal and the matings of unaffected
sibling littermates proposed, but not completed, would have further tested the mode of
inheritance hypotheses and may have allowed definitive conclusions to be drawn. In
addition, the ability to use a putative male carrier animal as the sire of multiple litters
would have dramatically increased the number of kittens produced and therefore the
amount of data potentially generated. The low number of affected animals somewhat
limits the strength of the conclusions that can be drawn from the studies, particularly
regarding the likely mode of inheritance for the disease, and while it is considered likely that T1 has a significant role in the transmission of disease, his role remains speculative.

Several other limitations of the study have been alluded to in the previous paragraphs. For many animals, the appropriate samples or relevant data were unavailable, and this is another significant restriction of the study. In particular, the absence of fresh or fresh frozen tissues, fluids or cells from the majority of LPD-affected kittens was a real limitation. The absence of these samples from most animals meant that confirmation of the type of anaemia present, the flow cytometry assays investigating blood DNT cell populations and the lymphocyte apoptosis assays, could not be performed in affected kittens. Similarly, RNA extractions used for the Fas-gene RT-PCR investigations were only successful from the two affected kittens where fresh tissue had been frozen. While it is considered unlikely that tissue from additional kittens would have revealed a different result, the fact that only two affected kittens are were included in these studies limits the strength of the conclusion that Fas-gene mutations are not present in affected kittens. Finally, the absence of fresh cells meant that apoptosis investigations were limited to less reliable in situ methods using formalin-fixed paraffin-embedded tissue samples rather than in vitro methods where live lymphocytes are subjected to an apoptotic stimulus and responses compared with controls.

Lymph node samples from only one LPD-affected kitten were able to be evaluated for the presence of CD4 and CD8 cell surface markers due to the absence of fresh samples. While results indicated that these cells were DNT cells, the significance of this finding is questionable. First, it is not known whether similar DNT cells are present in all LPD-affected kittens, as fresh samples from the other affected kittens suitable for CD4 and CD8 analysis were not collected. Potential similarities between the feline disease and ALPS in people were not recognised until multiple cases of LPD had been identified and analysed, and CD4 and CD8 markers are not routinely applied in cases of feline lymphadenopathy in New Zealand. It is therefore possible that the results confirming a DNT cell population from one kitten would not have been repeatable in other LPD-affected kittens. Confirming the presence of a DNT cell population expanding lymph nodes and other organs of LPD-affected BSH kittens would be significant, as it would provide further support that the feline disease is similar to both ALPS in people and LPD.
due to *lpr* or *gld* mutations in mice. If DNT cells were not also present in other kittens, this would seem to limit the similarities between the feline disease and ALPS.

To further confirm the likely lineage and origin of the proliferating cells within lymphoid organs in the kittens and the underlying basis for disease, it would have been desirable that a wider panel of immunochemical markers was applied in addition to the antibodies that were used. The limited antibody panel used limits conclusions concerning the lineage of the proliferating cells. Even assuming the reliability of the CD4-/CD8-immunocytochemistry results in the single kitten tested, it is still possible that the cells are not DNT cells similar to those present in people with ALPS at all. The DNT cells in ALPS patients are typically CD3+ TCRαβ+ CD4- CD8-. It is not currently possible to assess feline T-cell receptor (TCR) expression by routine immunochemical methods, and it is therefore possible that the cells in the kittens were TCRγδ T-cells, which in people also express CD3 but not CD4 or CD8 (a DNT cell phenotype). While a feline LPD involving proliferation of TCRγδ T-cells would still be novel, it would likely indicate a different basis for development to ALPS in people.

A further limitation of the study relates to the unreliability or lack of information provided by breeders, and difficulties in verifying the information that was provided. Initial information that the putative carrier tom (T1) had sired multiple litters (>30) with no reported LPD in his progeny appeared inaccurate, at least regarding indirect offspring, following the identification of the further affected Litter 0, of which T1 was both grandsire and great-grand-sire. In addition, many breeders were reluctant to provide information or assist with the study, which particularly limited completeness of the pedigree information used in the mode of inheritance studies. Anecdotal evidence that a similar disease has occurred previously in BSH kittens in Australia may have helped confirm the mode of inheritance, but could not be verified.

Finally, while investigations indicate that reduced apoptosis in T-cells is likely present in LPD-affected kittens, additional confirmatory methods are needed to confirm the results of the present study. *In situ* methods of apoptosis detection such as the TUNEL assay in FFPE tissue detect indirect evidence of apoptosis in a tissue at a single point in time, and are less accurate than *in vitro* methods of apoptosis detection where an apoptotic
stimulus is applied to previously activated T-cells, and the level of apoptosis in cells from affected animals compared to that in cells from controls.

8.6 CONCLUSIONS AND FUTURE DIRECTIONS

The series of studies reported in this thesis should be regarded as important but initial steps towards characterising this novel disease in BSH kittens. Dissemination of information about the disease is considered critical to raise awareness of the disease and ensure that future cases of LPD in kittens are identified and investigated appropriately. To increase the likelihood that all appropriate samples and data are collected from any future cases, a protocol for sample collection is set out in the Appendix to this thesis.

Two further studies investigating causative genetic defects in affected kittens using available tissue from historical cases are currently proposed. First, it is planned to investigate quantitative defects in $Fas$ using real-time PCR (qPCR) based on the primers and probes for feline $Fas$ previously described (Mizuno et al. 2003). If abnormalities in quantitative Fas expression are not identified in this study, it is then proposed to investigate qualitative $Fasl$ using conventional RT-PCR. The results of these studies may provide further information about the molecular basis for the disease in kittens and whether the disease is similar to ALPS or its murine equivalents. Recent advances in and increased availability of SNP (single nucleotide polymorphism) chip technology for feline samples (Mullikin et al. 2010; Anonymous 2012b) may mean that alternative techniques are able to be used in place of PCR to more rapidly and accurately identify genes associated with the development of LPD in BSH kittens in these or other studies.

In view of the limited data on DNT cells in normal cats or feline LPD generally, further investigations of these cells are warranted. Studies building on the flow cytometry assays described in Chapter 5 to investigate differences in DNT cell percentages using different anticoagulants, PBMC separation methods and storage conditions are planned. Descriptions of expected DNT cell proportions within normal tissues or other forms of LPD in cats are also inadequate; while it is often assumed that DNT cells represent an uncommon cell phenotype in healthy cats (Endo et al. 1997; Roccabianca et al. 2000), evidence is limited. Further investigation of the immunophenotype of T-cells normally
present within the lymphoid organs of control cats and in T-cell lymphomas and other
LPDs in cats, as well as in any future cases of LPD in BSH kittens, would be useful to
establish expected parameters for DNT cells in these organs and lesions and confirm the
significance of any increase in DNT cell numbers in LPD-affected BSH kittens.

The performance of some of the additional studies proposed relies to some extent on the
identification of additional cases while animals are still alive. In particular, confirmation of
the results of the *in situ* TUNEL assays in this thesis by *in vitro* methods in future cases of
LPD could provide very useful information about the basis for disease development. As
studies to date have not yet identified the nature of any apoptotic defect in LPD-affected
kittens, it would therefore be desirable that any future investigations of apoptotic defects
in the affected kittens’ lymphocytes use a range of different apoptotic stimuli that induce
apoptosis by both the intrinsic and extrinsic pathways to increase the likelihood that the
basis for any apoptotic defect is identified.

Despite the various limitations of the studies reported in this thesis, results confirm and
further characterise a novel non-neoplastic T-cell LPD in BSH kittens with a probable
inherited basis. The results of preliminary studies support defects in T-cell apoptosis as a
possible factor in disease development but have not identified the presence of *Fas*-gene
mutations in affected kittens. The disease has several similarities to the human disease
ALPS, an inherited disorder of T-cell regulation most commonly due to defects in *Fas*-mediated apoptosis.

### 8.7 References

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Mutations. Retrieved 15 February 2013, from


associated with inherited genetic defects that impair lymphocytic apoptosis--CT and US


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Mizuno, T., Y. Goto, et al. (2003). Quantitative analysis of Fas and Fas ligand mRNAs in a feline T-lymphoid cell line after infection with feline immunodeficiency virus and primary peripheral blood mononuclear cells obtained from cats infected with the virus. Vet Immunol Immunopathol 93(3-4): 117-123.


APPENDIX

PRE- AND POST-MORTEM SAMPLING PROTOCOL FOR KITTENS AFFECTED BY LYMPHOPROLIFERATIVE DISEASE

1. PRE-MORTEM SAMPLE COLLECTION

If possible, blood samples and samples from lymph nodes for cytology and immunocytochemistry (ICC) should be taken prior to euthanasia (see Table A.1 for a list of samples). In fractious or small kittens and cats, sedation is recommended (Zoletil-50, 5mg/kg, Virbac, Australia) to minimise stress and discomfort for the animal and to facilitate sample collection. If the lymphadenopathy is bilateral, it is recommended that pre-mortem samples are taken from lymph nodes on the left side of the body to leave the nodes on the right side undamaged for subsequent sample collections (fresh frozen samples, weight, dimensions, histology, immunohistochemistry, etc). It is important that blood and cytological preparations do not come into contact with formalin fumes at any time.

In young kittens with a small circulating blood volume, it is recognised that may not be possible to take all blood samples listed in the table below. In this
situation, blood samples should be prioritised in the order of priority indicated by bracketed numbers in Table A.1 as follows:

<table>
<thead>
<tr>
<th>Tissue/Fluid</th>
<th>Test</th>
<th>Sample</th>
<th>Preservation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td>CBC (1)</td>
<td>Minimum 1mL in EDTA tube gently mixed</td>
<td>Either process immediately or refrigerate at 4°C until processed (&lt;24 hours)</td>
</tr>
<tr>
<td></td>
<td>CBC (2)</td>
<td>Blood smears x 2 (or more)</td>
<td>Stained for routine evaluation and leukocyte differential (Wrights-type stain)</td>
</tr>
<tr>
<td></td>
<td>Coombs test (even if no anaemia present) (4)</td>
<td>Minimum 1-2mL in EDTA tube mixed gently</td>
<td>Either process immediately or refrigerate at 4°C until processed (&lt;24 hours)</td>
</tr>
<tr>
<td></td>
<td>Serum biochemistry FeLV and FIV ELISA testing (3)</td>
<td>Minimum 1mL in plain tube mixed gently</td>
<td>Either process immediately or refrigerate at 4°C until processed (&lt;24 hours)</td>
</tr>
<tr>
<td></td>
<td>Flow cytometry immunophenotyping (5)</td>
<td>Minimum 1-2mL in EDTA tube mixed gently</td>
<td>Separate over Ficoll and ideally process immediately for immunophenotyping by flow cytometry (minimally CD3, CD21, CD4 and CD8); if not possible, freeze down and hold PBMCs at -80°C following cell separation</td>
</tr>
<tr>
<td></td>
<td>Future studies (6)</td>
<td>Minimum 1mL in lithium heparin tube mixed gently</td>
<td>Freeze at -80°C</td>
</tr>
<tr>
<td></td>
<td>Future studies (6)</td>
<td>Minimum 1mL in plain tube, separated and serum removed and stored</td>
<td>Freeze at -80°C</td>
</tr>
<tr>
<td><strong>Lymph nodes</strong></td>
<td>Routine cytology</td>
<td>FNAs (or impression smears), minimum of 6x slides, from multiple nodes</td>
<td>Keep away from exposure to formalin fumes, stain with DiffQuik</td>
</tr>
<tr>
<td></td>
<td>ICC for immunophenotyping (CD4 and CD8) (2)</td>
<td>FNAs (or impression smears), minimum of 12x slides, from multiple nodes</td>
<td>Keep away from exposure to formalin fumes</td>
</tr>
</tbody>
</table>

Do not stain, send to UC Davis for immunocytochemistry (minimally CD3, CD79a and CD4 and CD8; CD18 and CD56 if possible)

Abbreviations: CBC = complete blood count, FeLV = feline leukaemia virus, FIV = feline immunodeficiency virus, ICC = immunocytochemistry, PBMC = peripheral blood mononuclear cells

2. **EUTHANASIA**

Following pre-mortem collection of samples, the animal should be euthanased by intravenous pentobarbitone overdose and a post-mortem examination performed immediately.
3. **POST-MORTEM SAMPLE COLLECTION**

**Whole body photography**
The skin should be removed and where possible, the whole body photographed to show the peripheral lymph nodes before further samples are collected.

**Time-critical samples**
The desirability of obtaining gross photographs must be balanced against the need to collect samples as soon as possible after death. In particular, **the fresh frozen tissues, the various FNAs for ICC, and all bone marrow samples (regardless of preservation method) should be collected as soon as possible after euthanasia and not more than 15 minutes after death** (see Table A.2).

- Fresh frozen samples should measure no more than 0.5cm³, taken using clean instruments, placed into cryovials with 2.5mL RINAlater (or similar preservative), before transferring to a -20°C or -80°C freezer within 24 hours of sampling. If RINAlater is not available, samples may be initially placed directly in liquid nitrogen to snap freeze before transferring to a -80°C freezer for storage. These samples can subsequently be used for PCR or SNP chip analysis.

- Fine needle aspirates of lymph nodes, spleen and bone marrow for routine cytology, ICC and FeLV antigen immunofluorescent antibody (IFA) in the case of the bone marrow should be taken using a 22 gauge needle and 5 or 10mL syringe and minimum aspiration pressure, evacuated onto glass slides, and air dried. It is critical that these samples are kept away from formalin fumes at all times.

**Photographs and records**
Ideally, photographs should be taken and the weights and measurements of selected organs recorded in the course of the post mortem examination before substantial sampling of the organs is performed.

- Images of the abdominal and thoracic viscera *in situ* should be taken first, and then samples collected.

- The thymus, spleen and liver should be identified, measured, weighed and then sampled (see below).
For lymph nodes, selected nodes should be measured, weighed and sampled (see below), including the right mandibular, axillary, inguinal, popliteal, one visceral nodes and one tracheobronchial node. One or more lymph nodes should be incised, inspected for the presence of cortico-medullary architecture and photographed.

**Post-mortem examination and formalinised samples**

Following collection of the fresh frozen tissues, FNAs for ICC, and bone marrow samples, a standard post mortem examination should be performed and a full tissue set of formalinised samples collected.

- Samples should be maximally 1cm thick and have a 1:10 ratio of sample to formalin.
- If it is not possible to collect a full tissue set, **collection priorities are multiple lymph nodes, spleen, liver, bone marrow and thymus**, and multiple sites should be sampled. Samples to be taken from organs or regions not previously aspirated to reduce confounding effects of e.g. iatrogenic haemorrhage etc.

**Following the post-mortem**

Blood samples should be processed for CBC, biochemistry and FeLV and FIV testing as soon as possible (and within 24 hours).

**Cytological smears from:**

- Lymph nodes for ICC should be should be air-dried and immediately sent to UC Davis, United States, for ICC using CD4 and CD8 (and ideally also CD18 and if available, CD56).
- Bone marrow for FeLV IFA should be air-dried and immediately sent to UC Davis, United States for testing.

Formalinised samples should be preserved for a minimum of 24 hours before being embedded in paraffin wax and:

- Cut into 3µm tissue sections for routine histological evaluation using haematoxylin and eosin (H&E) stains (all tissues).
- Cut into 5µm tissue sections on positively charged slides and left unstained for application of CD79a, CD3 and Ki67 antigens for immunohistochemical studies (at least one lymph node, ideally also spleen and any other organs showing evidence of significant LPD on routine histology).
• A minimum of 4x 10µm scrolls cut from formalin-fixed paraffin-embedded (FFPE) sections of lymph nodes for clonality assays to be performed at UC Davis, United States.

If flow cytometry is available, blood samples should be separated over Ficoll and processed immediately for immunophenotyping (minimally CD3, CD21, CD4 and CD8); if flow cytometry is not immediately available (as is likely), separated PMBCs should be frozen down using standard protocols (see Chapter 5) and held at -80°C until processed.
Table A.2: Recommended post-mortem samples to be taken from LPD-affected kittens and cats

<table>
<thead>
<tr>
<th>Tissue/Fluid</th>
<th>Test</th>
<th>Sample</th>
<th>Preservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Lymph nodes]</td>
<td>Routine cytology (if insufficient samples obtained pre-mortem)</td>
<td>Further FNAs or impression smears, from multiple nodes</td>
<td>Keep away from exposure to formalin fumes, stain with Diff Quik or similar</td>
</tr>
<tr>
<td></td>
<td>ICC for immunophenotyping (CD4 and CD8) (if insufficient samples obtained pre-mortem)</td>
<td>FNAs or impression smears, from multiple nodes, minimum of 12x slides</td>
<td>Keep away from exposure to formalin fumes Do not stain, send to UC Davis for ICC</td>
</tr>
<tr>
<td>Spleen</td>
<td>ICC for immunophenotyping (CD4 and CD8)</td>
<td>FNAs or impression smears, minimum of 12x slides</td>
<td>Keep away from exposure to formalin fumes Do not stain, send to UC Davis for ICC</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Routine cytology</td>
<td>FNAs or impression smears, minimum of 4x slides</td>
<td>Keep away from exposure to formalin fumes</td>
</tr>
<tr>
<td></td>
<td>Routine histology</td>
<td>Sample of bone marrow from proximal humerus or femur in formalin</td>
<td>Collect and keep separate to other bone marrow samples</td>
</tr>
<tr>
<td></td>
<td>IFA for FeLV antigen</td>
<td>FNAs or impression smears, minimum of 4x slides</td>
<td>Keep away from exposure to formalin fumes</td>
</tr>
<tr>
<td>Fresh frozen tissues</td>
<td>Future genetic studies including DNA, RNA and possible SNP chip analysis</td>
<td>0.5cm³ samples of lymph node (minimum 5x, multiple nodes, spleen, liver, thymus, kidney, heart) in cryovials in 2mL RNAlater (or liquid nitrogen if RNAlater not available)</td>
<td>Freeze at -80°C</td>
</tr>
<tr>
<td>Additional formalinised tissues</td>
<td>Routine histology</td>
<td>Full tissue set (priorities are multiple lymph nodes, spleen, thymus, liver, bone marrow)</td>
<td>10% buffered formalin then paraffin-embedded blocks (FFPE tissue)</td>
</tr>
<tr>
<td></td>
<td>Immunophenotyping using IHC (CD3, CD79a, Ki67)</td>
<td>Lymph nodes (and spleen if possible)</td>
<td>Unstained tissue sections on glass slides made from FFPE tissues</td>
</tr>
<tr>
<td></td>
<td>Clonality assays</td>
<td>10µm scrolls (at least 4x) cut from FFPE lymph node sections and placed in eppendorf tubes for clonality assays at UC Davis</td>
<td>Scrolls made from FFPE tissue</td>
</tr>
<tr>
<td>Record of gross findings</td>
<td>Records and comparison for description of disease</td>
<td>Ideally record organ dimensions and weights—thymus, mandibular, retropharyngeal, axillary, inguinal, popliteal, mesenteric lymph nodes, spleen, liver</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ideally images including whole body, whole body skin removed, abdominal viscera in situ, thoracic viscera in situ, lymph nodes whole, lymph nodes incised, thymus, liver and spleen</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Abbreviations:
CBC = complete blood count, FeLV = feline leukaemia virus, FFPE = formalin-fixed paraffin-embedded, ICC = immunocytochemistry, IHC = immunohistochemistry, n/a = not applicable, SNP = single nucleotide polymorphism