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The role of C-reactive protein as a biomarker in dogs with lymphosarcoma

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Submitted in fulfilment of the requirements for the Degree of Master of Science (MSc)

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List of Abbreviations

Clinical abbreviations:

$\beta_{2\text{M}}$ - Beta-2 microglobulin

$\text{AGP}$ - Alpha-1 acid glycoprotein

$\text{APP}$ - Acute phase protein

$\text{APR}$ - Acute phase response

$\text{CBC}$ - Complete blood count

$\text{CD}$ - Cluster of differentiation e.g. CD79a stains for B lymphocytes and CD3 stains for T lymphocytes

$\text{CNS}$ - Central nervous system

$\text{Cp}$ - Ceruloplasmin

$\text{CR}$ - Complete remission

$\text{CRP}$ - C-reactive protein

$\text{CT}$ - Computed tomography

$\text{DFI}$ - Disease-free interval

$\text{DIC}$ - Disseminated intravascular coagulation

$\text{DNA}$ - Deoxyribonucleic acid

$\text{EBV}$ - Epstein-Barr virus

$\text{ELISA}$ - Enzyme-linked immunosorbent assay
EOD - Every other day

FNAB - Fine needle aspirate biopsy

GI - Gastrointestinal

HIV - Human immunodeficiency virus

HL - Hodgkin’s lymphoma

HLA - Human leukocyte antigen

HMGB-1 - High mobility group box 1 protein

Hp - Haptoglobin

HTLV-1 - Human T-cell leukaemia virus - type 1

ICC - Immunocytochemistry

IHC - Immunohistochemistry

IL - Interleukin e.g. IL-1 and IL-6

IPI - International Prognostic Index

LAK - Lymphokine-activated killer cell

LDH - Lactate dehydrogenase

LogCRP - Log-transformed C-reactive protein

LSA - Lymphosarcoma / Lymphoma

MCP1 - Monocyte chemotactic protein 1

MI - Mitotic index
MMP - Matrix metalloproteinase
MRD - Minimum residual disease
NHL - Non-Hodgkin’s lymphoma
NK cell - Natural killer cell
OST - Overall survival time
PCR - Polymerase chain reaction
PD - Progressive disease
PET - Positron emission tomography
PR - Partial remission
Pred - Prednisolone (glucocorticoid)
REAL - Revised European-American Classification of Lymphoma
RECIST - Response evaluation criteria in solid tumours
SAA - Serum amyloid A
SD - Stable disease
SID - Once daily
SMLN - Submandibular lymph node
TFR - Time to first relapse
TK - Thymidine kinase
TNF-α - Tumour necrosis factor-alpha
**VEGF** - Vascular endothelial growth factor

**WHO** - World Health Organization

**Statistical abbreviations:**

**AD** - Anderson-Darling test of normality

**AUROC** - Area under the receiver operator curve

**Chi-sq** - Chi-square test

**CI** - Confidence interval (usually stated as the 95% CI)

**d.f.** - Degrees of freedom

**GBW** - Gehan-Breslow-Wilcoxon test

**IQR** - Interquartile range

**KM** - Kaplan-Meier survival curve

**KW** - Kruskal-Wallis test

**LR** - Log rank test

**MW** - Wann-Whitey U test

**n** - Sample number

**SD** - Standard deviations

**SR** - Spearman rank test

**WSR** - Wilcoxon signed-rank test
Abbreviations assigned to groups for analysis:

CD - Concurrent disease

GM - Generalised multicentric disease

HA - Hypoalbuminaemic

HC - Hypercalcaemic

ICD - Inflammatory concurrent disease

NCD - No concurrent disease

NGM - Non-generalised multicentric disease

NHA - Not hypoalbuminaemic

NHC - Not hypercalcaemic

NICD - Non-inflammatory concurrent disease

No-Tx - No previous treatment

NPS - No previous treatment with steroids

PD - Point of relapse/progressive disease

PD-1 - One week prior to relapse

PD-2 - Two weeks prior to relapse

Pre-Tx - Time point prior to chemotherapy treatment (time of diagnosis)
Summary

C-reactive protein (CRP) is a highly sensitive but non-specific acute phase protein that is produced in the liver in response to injury, inflammation and disease. CRP has been by shown by several authors to be elevated in dogs with lymphoma (LSA) compared to healthy control dogs, however the findings of their small studies have suggested that CRP is too non-specific to be used in a clinical setting to monitor dogs with LSA that are receiving treatment with chemotherapy. Studies in the human literature have shown that CRP concentration is significantly affected by extent of disease and by the presence of clinical signs in patients with both non-Hodgkin’s and Hodgkin’s lymphoma, and it has been suggested that an elevated concentration of CRP at time of diagnosis carries a poorer prognosis. No veterinary studies to date have been large enough to investigate the effect of patient clinical parameters on CRP concentration, or to determine whether CRP concentration carries any prognostic significance.

The aim of this study was to determine whether serum CRP was a clinically relevant biomarker in dogs with LSA using a large population. Specific aims included confirming if differences existed between the CRP concentration of healthy dogs and dogs with LSA, and determining if any patient variables had a relationship with CRP concentration. The effect on CRP concentration in dogs receiving chemotherapy treatment was investigated, with an aim to determine whether CRP could be used for categorisation of remission status. Finally, the role of CRP concentration as a prognostic indicator was investigated with respect to disease relapse and survival.

This study included 59 control dogs and 73 dogs with LSA. Spare serum was prospectively harvested and stored at -70°C prior to batch analysis on an immunoturbidimetric assay (Pentra 400, Horiba ABX). Serial samples were obtained at time of diagnosis, throughout treatment with chemotherapy, at routine re-check appointments and at time of recrudescence of disease. Dogs with LSA were fully staged at time of diagnosis to determine the extent of disease and all dogs were assigned a WHO stage and substage. Of the dogs that received treatment with chemotherapy, a remission status (i.e.
complete/partial remission, stable disease or progressive disease) was assigned at each visit based on the subjective response to treatment from the palpation of peripheral lymph nodes. Patient clinical data was retrospectively obtained from both paper and electronic records. CRP was investigated as both a continuous and categorical variable, and categorisation was based on the degree of elevation of concentration. Significance was set at a p-value of ≤0.05. CRP concentration was deemed normal if ≤10 mg/L.

Results suggest that serum CRP concentration is significantly higher at time of diagnosis in dogs with LSA compared to the control dogs, however not all dogs with LSA exhibit an elevated CRP concentration. CRP concentration returns to within normal limits for the majority of dogs following 4 weeks of treatment with chemotherapy. Of the patient variables investigated, WHO stage and substage, and pre-treatment albumin concentration were shown to be significant following univariate analysis. Significant differences between the median CRP concentration of the different remission statuses exists, however the overlap of ranges of CRP concentration in each group suggests that it could not be used to categorise remission status. The median CRP concentration at time of diagnosis is significantly higher than at time of relapse of disease; however CRP concentration does become elevated in most dogs at time of relapse. CRP concentration was not shown to be significantly elevated in the weeks leading up to relapse, indicating that this biomarker is not useful for predicting early relapse prior to recrudescence of disease becoming clinically apparent. Finally, results of the survival analysis revealed that pre-treatment CRP concentration is not a significant variable, however immunophenotype and WHO substage are.

This is the largest study to date investigating CRP concentration in dogs with LSA and is the first study to suggest a relationship between CRP concentration at time of diagnosis and pre-treatment albumin, WHO stage and substage. Findings from this study have confirmed those of previous authors; for example CRP concentration becomes elevated in dogs with LSA both at time of diagnosis and at time of relapse of disease, and that immunophenotype and WHO substage carry prognostic significance. Although CRP concentration did not show any prognostic significance, there was a trend for those dogs with a moderate and marked elevation in CRP concentration (>30 mg/L) to have a reduced overall survival time.
Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature........................................................................................................................................

Printed name..................................................................................................................................
1 Literature Review

1.1 Canine lymphosarcoma

1.1.1 Prevalence of canine lymphosarcoma

Cancer is a major cause of death in dogs. Prevalence increases with age (Edwards et al., 2003), and one third of all tumours occur in patients greater than 9 years of age (Dobson et al., 2002). Lymphosarcoma (LSA, malignant lymphoma) is the most common neoplastic condition of the haemolymphatic system in this species, and accounts for 83% of all haematopoietic malignancies (Dobson, 2004, Vail et al., 2001). Reported annual incidence rates range from 24-79 in 100,000 dogs (Dorn et al., 1967, Edwards et al., 2003, Teske et al., 1994, Dobson et al., 2002). A study by Dobson et al (2002) looking at a UK population of insured dogs showed an age-standardised annual incidence rate of 107 in 100,000 dogs, however the actual incidence rate may be even higher when the non-insured population is considered.

1.1.2 Aetiology

LSA is a round cell tumour that can develop in any lymphoreticular tissue and is characterized by a clonal expansion of lymphoid cells.

Figure 1 Cytology from a canine lymph node infiltrated with a centroblastic lymphosarcoma
LSA occurs spontaneously in the dog and although the aetiology is largely unknown, it is likely to be multi-factorial.

1.1.2.1 Sex and breed

It is generally accepted that there is no sex predisposition for canine LSA, however an increased prevalence is reported amongst males in some studies (Dobson and Gorman, 1993, Merlo et al., 2008, Ponce et al., 2003a). A genetic predilection has been suggested with a number of breeds reported to be at higher risk including boxers, bulldogs, Scottish terriers, Basset hounds and bullmastiffs (Edwards et al., 2003, Vail and MacEwen, 2000, Onions, 1984). A familial incidence is reported in bullmastiffs and predisposition by heritable mutation of the tumour suppressor gene p53 has been identified (Veldhoen et al., 1998, Nasir and Argyle, 1999, Onions, 1984).

1.1.2.2 Environmental factors

Several environmental causes have been suggested including living in industrial areas, exposure to magnetic fields, and chemicals such as pesticides, paints and solvents (Hayes et al., 1991, Gavazza et al., 2001, Reif et al., 1995). However, studies suffer from selection bias and recall bias due to their retrospective case-control design; hence no definitive environmental causes can thus far be listed.

1.1.2.3 Viruses

Several viral agents have been implicated in the pathogenesis and progression of different forms of human LSA including the Epstein-Barr virus (EBV), human T-cell lymphoma virus type-1 (HTLV-1) and human immunodeficiency virus (HIV) (Bower et al., 2008, Shuh and Beilke, 2005, Gandhi et al., 2004). Recent veterinary literature suggests a possible gamma herpes viral pathogenesis for canine LSA (Milman et al., 2011, Huang et al., 2012), however, the significance of viral particles with properties similar to retroviruses in short-term cultures of canine LSA tissue is unclear (Tomley et al., 1983). Investigation into a viral aetiology of canine lymphoma is ongoing and as molecular biology techniques improve so may the likelihood of isolating a causative agent.
1.1.3 Patient presentation

Dogs with LSA commonly present with an asymptomatic peripheral lymphadenopathy, however clinical signs may also include anorexia, lethargy, weight loss, polyuria, polydipsia and dyspnoea (Dobson and Gorman, 1993, Gavazza et al., 2009). The type and severity of clinical signs are often related to the anatomic site of the LSA and whether there are associated paraneoplastic syndromes such as hypercalcaemia (Dobson and Gorman, 1993, Weller et al., 1982a).

1.1.4 Obtaining a diagnosis

Round cell tumours exfoliate cells readily and as such LSA is often diagnosed by cytological examination of samples obtained by fine needle aspirate biopsy (FNAB). In dogs with equivocal cytological results or where histopathological diagnosis would be preferred, excision of a lymph node or surgical biopsy of the affected organ is performed. In recent years, flow cytometry and polymerase chain reaction (PCR) assay techniques have been described to provide a more sensitive and specific diagnosis (Gibson et al., 2004, Lana et al., 2006, Reggeti and Bienzle, 2011).

1.1.5 Characterisation of LSA

LSA has been characterized and classified in many different ways including its anatomical location, morphological type, tumour grade and phenotype.

1.1.5.1 Anatomical classification

LSA is most commonly classified on the basis of anatomic location. The multicentric form occurs most frequently and is reported in more than 80% of dogs with the disease (Weller et al., 1982b, Gavazza et al., 2009). Other anatomic classifications include alimentary or gastrointestinal (GI), cranial mediastinal and cutaneous (Cotchin, 1984, Rallis et al., 1992). Extranodal forms are less commonly observed and some sites include the central nervous system (CNS), eye, nose and kidney (Rallis et al., 1992, Dobson and Gorman, 1993).
1.1.5.2 Morphological and grade classification

Canine LSA is described as a spontaneous model for human non-Hodgkins lymphoma (NHL) (Teske, 1994). To allow this model to work the pathological classification of lymphoma in the dog has often been performed using human NHL classification systems. The Working Formulation was adopted in the 1980’s and grouped LSA into 4 grades based on cell morphology: low, intermediate, high, and miscellaneous (National Cancer Institute, 1982, Carter et al., 1986). This system is now largely obsolete, having been replaced by the updated Kiel classification (Stansfeld et al., 1988, Lennert and Feller, 1991) and the Revised European-American Classification of Lymphoma (REAL) system (Harris et al., 1994). Both of these systems changed LSA classification to low or high grade and introduced the concept of classification based on molecular and genetic characteristics. The most recent system has been proposed by the World Health Organisation (World Health Organization, 2008), where the REAL system has been revised and now groups LSA based on molecular, phenotypic and cytogenic characteristics. In veterinary medicine, the updated Kiel classification is currently considered the most useful for canine patients (Fournel-Fleury et al., 1997, Ponce et al., 2010)

These classification systems allow LSA to be given a “grade” according to its aggressiveness and features of malignancy. Tumour grading when performed using the updated Kiel classification considers the size of cell and the mitotic index (MI). Low grade LSAs have small sized cells with a low or medium MI and high grade LSAs have medium to large sized cells and a high MI (Ponce et al., 2010). Most canine LSA are high grade large B-cell lymphomas of an immunoblastic or centroblastic polymorphic subtype (Dobson and Gorman, 1993, Greenlee et al., 1990, Ponce et al., 2010).

High grade lymphomas progress more rapidly, but due to their high mitotic rate are highly responsive to treatment with chemotherapy and radiation therapy, and may rarely be curable (Vail et al., 2001, Chun, 2009). Low grade LSA is less common and incidence ranges from 11-26% of cases depending on the classification system used (Greenlee et al., 1990, Fournel-Fleury et al., 1997).
Low grade lymphomas usually progress much more slowly and are less responsive to drug therapy (Gear, 2009a, Carter et al., 1986).

1.1.5.3 Immunophenotypic classification

Leukocytes have surface antigen markers which are referred to by a cluster of differentiation (CD) number, with CD79a (B-cell) and CD3 (T-cell) commonly used in veterinary medicine for immunophenotyping canine LSA (Fontaine et al., 2009, Ponce et al., 2003b).

LSA is an oversimplified term used to describe a myriad of different pathological presentations of lymphoid neoplasia in the dog. This differs greatly from human medicine where LSA is classified very precisely both pathologically and immunophenotypically using a wide panel of markers. In human medicine the treatment given and the prognosis for the patient varies depending on their exact diagnosis. These markers are currently not available commercially for dogs but have become the subject of much veterinary research in recent times and may improve our understanding of this complex disease in the future. Currently, immunophenotyping can be performed using specific monoclonal antibodies to determine the antigens on a cell surface and several techniques have been described. Microscopic detection by immunohistochemistry (IHC) and immunocytochemistry (ICC) are the most widely available techniques and allow for concurrent assessment of cell morphology and tissue architecture. With these techniques interpretation is subjective and there is often limited quantification of antigen expression (Culmsee et al., 2001). Flow cytometry can be used to assess large numbers of cells by objective and defined criteria to yield robust results. This technique is being increasingly used in veterinary medicine as a wider range of monoclonal antibodies become more readily available allowing more subtle classification (Gibson et al., 2004, Reggeti and Bienzle, 2011). Whatever the technique used, canine LSA is most commonly classified as being B-cell or T-cell, however other immunophenotypic classifications are less commonly assigned including mixed B/T cell and null cell (natural killer (NK) cell) (Ponce et al., 2010).
The most common immunophenotype for canine LSA is B-cell (Dobson et al., 2001, Ponce et al., 2010), however many low grade and all primary cutaneous LSAs are T-cell (Fontaine et al., 2009, Fournel-Fleury et al., 1997). When comparing high grade multicentric LSA, immunophenotyping has been shown to have prognostic importance and historically B-cell LSA has carried a better prognosis with improved survival times when compared with T-cell LSA (Greenlee et al., 1990, Ponce et al., 2004, Dobson et al., 2001, Marconato, 2011).

1.1.6 Clinical staging

Following a diagnosis of LSA the patient is “staged” to determine the anatomical extent of the disease according to the World Health Organisation (WHO) system (Owen, 1980), which is outlined in Table 1, as this may influence treatment choice and can have prognostic significance. Staging usually includes a thorough clinical examination, complete blood count (CBC), serum biochemistry, urinalysis, thoracic radiography, abdominal ultrasonography and ultrasound-guided FNAB of organs and internal lymph nodes if abnormalities are detected. Bone marrow cytology or histopathology is indicated particularly when haematologic abnormalities are seen (Flory et al., 2007). Computed tomography (CT) can be combined with functional positron emission tomography (PET) using radiolabelled tracers such as $^{18}$F-fluorodeoxyglucose (FDG) to allow the visualization of various metabolic processes within cancer cells. PET-CT forms an important part of the standard diagnostic and staging protocol in human patients with LSA and has been shown to have prognostic significance (Hutchings and
Barrington, 2009). Its use has been investigated in dogs with LSA and initial studies show promise of improved detection of disease and more complete staging of disease (Lawrence et al., 2010). Flory et al (2007) has highlighted a need for a standardized staging regime as stage migration can occur as more sensitive diagnostic procedures are introduced.

Table 1 World Health Organisation (WHO) staging system in dogs with lymphoma (Owens, 1981)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Single lymph-node involvement</td>
</tr>
<tr>
<td>II</td>
<td>Regional lymph nodes (on one side of the diaphragm)</td>
</tr>
<tr>
<td>III</td>
<td>Generalised lymph node involvement (on both sides of the diaphragm)</td>
</tr>
<tr>
<td>IV</td>
<td>Liver and/or spleen involvement, ± stages I-III</td>
</tr>
<tr>
<td>V</td>
<td>Haematologic or bone marrow involvement, ± stages I-IV</td>
</tr>
<tr>
<td>Substage a</td>
<td>No clinical symptoms</td>
</tr>
<tr>
<td>Substage b</td>
<td>Clinical symptoms present</td>
</tr>
</tbody>
</table>

Patients with a lower stage and without clinical signs generally carry a better prognosis than those with a higher stage and which are showing clinical signs of disease (Keller et al., 1993, Vail and MacEwen, 2000, Carter et al., 1986, Baskin et al., 2000), however there is disparity in the literature and several studies have shown no significant association between WHO stage and prognosis (Keller et al., 1993, Flory et al., 2007).

1.1.7 Treatment and prognosis

Regardless of WHO stage at time of diagnosis, without treatment most dogs with LSA will die of their disease within 4-6 weeks (Vail et al., 2001), and systemic treatment in the form of prednisolone or cytotoxic drugs is required to prolong survival (Dobson et al., 2001, Hahn et al., 1992, Chun, 2009). Patients with stage I or single site extranodal lymphoma may benefit from a local treatment such as surgical excision of the tumour or radiation therapy at the local site. For those patients with multicentric LSA, chemotherapy is the most common form of treatment and many protocols have been suggested. Treatment with prednisolone alone increases patient median survival time to only 1-2 months.
Multi-drug chemotherapy protocols produce higher response rates and longer survival times than single agent protocols (with the exception of doxorubicin) (Simon et al., 2008, Hahn et al., 1992, Chun, 2009). Conventional chemotherapy protocols include the low dose COP (cyclophosphamide, vincristine, prednisolone) protocol and the CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) protocol (Appendix 1). Complete response is achieved in 60-90% of dogs on these protocols, with a median survival time of 6-12 months (Dobson et al., 2001, Kaiser et al., 2007, Vail et al., 2001, Chun, 2009).

Half-body radiation therapy has also been used in combination with chemotherapy and early results suggest that this may produce significantly longer disease-free intervals and median survival times (Williams et al., 2004, Gustafson et al., 2004, Lurie et al., 2009). Radiation therapy is not commonly performed due to the limited number of linear accelerators in the UK and the high cost associated with this treatment modality.

### 1.1.8 Monitoring and categorising response

Monitoring the response to treatment is important in patients with LSA. To improve survival time, a change of chemotherapy protocol may be indicated in refractory cases or where there is evidence of tumour recrudescence. For those patients with a peripheral lymphadenopathy, response to treatment is usually assessed by the attending veterinary surgeon palpating the lymph nodes and taking caliper measurements. Despite the objective measurement of lymph nodes, determining remission status is subjective. Variation in the recorded remission status of human patients with both Hodgkin’s (HL) and NHL, and canine patients with LSA has been shown depending on the monitoring methods used (Carde et al., 2002, Williams et al., 2005).

#### 1.1.8.1 RECIST guidelines

The response evaluation criteria in solid tumours (RECIST) guidelines v1.1 (Eisenhauer et al., 2009) were developed to standardize the assessment of tumour burden and the monitoring of response to treatment. These guidelines were initially developed for solid tumours but have been shown to be applicable
in the monitoring of LSA in people and dogs (Assouline et al., 2007, Vail et al., 2010). Tumour response is recorded according to the following categories:

- **Complete response (CR):** No detectable disease.
- **Partial response (PR):** Target lesions reduced by >30% but <100%.
- **Stable disease (SD):** <30% decrease and <20% increase in target lesions.
- **Progressive disease (PD):** Target lesions increased by >20%.

The guidelines state that a complete response should not be assigned until 4 weeks of treatment has been given (Eisenhauer et al., 2009).

**1.1.8.2 Minimum residual disease**

Minimum residual disease (MRD) is the term used to indicate there are cells remaining within the lymph nodes that are resistant to therapy despite no clinical detection of gross disease. As these cells continue to multiply, tumour recrudescence or relapse will ultimately occur. Repeat cytology or flow cytometry on samples from clinically normal lymph nodes can reveal evidence of MRD in patients with LSA which may facilitate treatment modification (Williams et al., 2005); however these techniques are not sensitive. Recent advances in PCR based detection methods have allowed the quantification of MRD in canine LSA and may have prognostic significance (Thilakaratne et al., 2010, Yamazaki et al., 2008).

For those patients with other anatomical forms of LSA, diagnostic imaging including ultrasonography and radiography is often used to monitor response to treatment, however these techniques may not be sensitive enough to show MRD when compared to advanced imaging techniques such as PET-CT (Zinzani et al., 2006, Spaepen et al., 2001). Early results show that PET-CT may be sensitive in detecting MRD disease in dogs with LSA and in detecting early response and early recrudescence of disease (Lawrence et al., 2009, LeBlanc et al., 2009). However the financial implications to fee-paying clients, combined with the limited
availability of scanners and radio-isotopes, has stopped this technique from being widely adopted in veterinary medicine.

1.2 The acute phase response

The body has many homeostatic mechanisms in place to maintain an optimal internal environment. When subjected to tissue injury or immunological stress, the body responds with a rapid, complex and non-specific reaction known as the acute phase response (APR). The APR is part of the innate defense system of many different species including dogs and humans and occurs when disturbances in homeostasis give rise to systemic and metabolic changes (Eckersall and Conner, 1988, Gabay and Kushner, 1999, Heinrich et al., 1990, Kushner and Mackiewicz, 1993, Murata et al., 2004, Paltrinieri, 2007, Tecles et al., 2005). Causes include infection, tissue injury from surgery and trauma, malignant neoplasia and immunological disorders (Ebersole and Cappelli, 2000, Gabay and Kushner, 1999, Hogarth et al., 1997, Lin et al., 2000, Nakamura et al., 2008, Pepys, 1981, Tecles et al., 2005).

1.2.1 Local and distant effects

During the APR a local reaction occurs at the site of injury which includes blood vessel dilatation and leakage, platelet aggregation and clot formation and activation of granulocytes and mononuclear cells within the inflamed tissue (Kushner, 1982). These produce cytokines and inflammatory mediators which diffuse into the systemic circulation (Castell et al., 1990, Heinrich et al., 1990, Yamashita et al., 1994).

1.2.1.1 Cytokines in the APR

The most important cytokines appear to include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Castell et al., 1989, Murata et al., 2004, Paltrinieri, 2007, Petersen et al., 2004, Yamashita et al., 1994). The cytokines mediate a systemic reaction which is characterised by fever, leukocytosis, increased synthesis of hormones (including insulin, cortisol and catecholamines), alterations in serum cations (decreased zinc and iron
concentrations), increased erythrocyte sedimentation rate, activation of the clotting cascade, metabolic changes, and the modulation of protein synthesis by hepatocytes. This hepatic modulation gives rise to a number of proteins collectively termed the acute phase proteins (APPs) (Eckersall, 1995, Gabay and Kushner, 1999, Kushner and Feldmann, 1978, Kushner, 1982). Although APP production is mainly hepatocyte-derived and driven by the action of the inflammatory cytokines on hepatocytes (Castell et al., 1990, Castell et al., 1989, Hurlimann et al., 1966, Kushner and Mackiewicz, 1993), extrahepatic production has also been described in tissues including kidney, intestine, lung, heart, spleen, testis, bone marrow and lymphocytes (Kalmovarin et al., 1991, Lecchi et al., 2009, Ramadori et al., 1985, Skovgaard et al., 2009).

There are at least 15 different cytokines known to exist and these are composed of very small molecules with a very short half-life (Gruys et al., 2005). There are similarities between human and dog cytokines (Soller et al., 2007). The relationship between the different cytokines is complex, and studies have shown that there is an overlapping pathway of APP induction (Heinrich et al., 1990, Lin et al., 2000). Cytokines can network to combine with other cytokines to promote or augment the production of APPs or they can directly regulate the production of other cytokines and APPs (Gabay and Kushner, 1999, Ganapathi et al., 1991).

### 1.2.1.2 IL-6, IL-1 and TNF-α

IL-6 is the major regulator of APP production, having been shown to promote hepatic synthesis of all positive APPs, but in particular C-reactive protein (CRP) (Castell et al., 1990, Mackiewicz et al., 1991, Yamashita et al., 1994). IL-1 has a moderate effect on the synthesis of some APPs and inhibits the production of others; it also induces IL-6 (Ebersole and Cappelli, 2000, Gabay and Kushner, 1999). TNF-α is the major stimulator of IL-1 and induces catabolism of the muscles allowing increased flow of amino acids to the liver (Gabay and Kushner, 1999, Paltrinieri, 2007).

### 1.2.2 The acute phase proteins

APPs have been defined empirically by Kushner and Mackiewicz (1993) as proteins whose plasma concentrations change by more than 25% following an
inflammatory response. Kushner (1982) previously defined the positive APPs as those whose serum concentrations increase and includes CRP, serum amyloid A (SAA), haptoglobin (Hp), α-1 acid aminoglycoprotein (AGP) and ceruloplasmin (Cp) (Eckersall and Conner, 1988, Caspi et al., 1987). The term ‘negative’ APPs describes those whose serum concentrations decrease during the APR. This occurs as the body preferentially uses any available amino acids to build the positive APPs. Examples include albumin and transferrin (Kushner, 1982, Tecles et al., 2009, Yamashita et al., 1994). Kushner (1982) further divided the positive APPs into 3 classes based on the magnitude of their response: Class I includes the proteins whose concentration have a 2-fold increase (Cp); Class II includes those whose concentration increases approximately 2- to 4-fold (AGP, Hp); and Class III includes those whose concentration increase several hundred fold (CRP, SAA).

In both people and dogs the “major” positive APPs are CRP and SAA, and the “major” negative APP is albumin (Cerón et al., 2005, Kushner, 1982, Murata et al., 2004, Paltrinieri, 2007). There are species differences which occur however, and CRP for example is a major APP in dogs and humans but only a moderate APP in cats and horses (Cerón et al., 2005, Murata et al., 2004).

1.2.3 C-reactive protein

CRP was first described by Tillet and Francis (1930) and was so named because it exhibited a high binding affinity to the C-polysaccharide of Streptococcus pneumoniae. It has been shown in both man and dogs to rise in concentration as quickly as 4 hours following an inflammatory stimulus, to achieve its maximum concentration within 24-72 hours, and to have a doubling time of 8 hours. Its concentration rapidly declines within 24-36 hours once the inflammatory stimulus is removed (Caspi et al., 1987, Kushner and Mackiewicz, 1993, Pepys, 1981).

1.2.4 The role of the APPs

The biological function of the individual APPs is still not fully understood. They are known to protect against infection, help repair damaged tissue, and regulate the immune and inflammatory response (Cerón et al., 2005). Several APPs also
have the ability to initiate and sustain inflammation as well as having an anti-
inflammatory effect (Gabay and Kushner, 1999, Murata et al., 2004). There is
growing evidence to suggest that APPs can be produced by neoplastic tissue and
may contribute to tumour development and progression (Chan et al., 2007,
Kovacevic et al., 2008, Malle et al., 2009).

### 1.3 The role of APPs as general biomarkers of disease

A biomarker has been defined by the National Cancer Institute as “a biological
molecule found in blood, other body fluids, or tissues that is a sign of a normal
or abnormal process or of a condition or disease”. Although measuring APPs is
highly non-specific due to the numerous conditions that can result in tissue
injury or inflammation, its measurement is highly sensitive. Due to this high
sensitivity, APPs have become extensively used as biomarkers in human medicine
over the last few decades. They are a marker of infection and inflammation and
have been shown to have prognostic importance in many diseases (Herishanu et
al., 2007, Hogarth et al., 1997, Kompoti et al., 2008, Ebersole and Cappelli,
2000). Several APPs including CRP have been shown to be elevated in human
patients with a variety of malignancies and they have therefore gained
popularity in the diagnosis and monitoring of different cancers (Beer et al.,
2008, Chan et al., 2007, Falconer et al., 1995, Yoshida et al., 2008). Pre-
operative CRP has been significantly associated with the pathological stage of
disease and of disease progression in patients with solid malignant tumours
(Yudoh et al., 1996, Crozier et al., 2007, Shimada et al., 2003, Koike et al.,
2008).

#### 1.3.1 Acute phase proteins and cancer

Serum APPs have been widely investigated in both human and veterinary
medicine. CRP, SAA and AGP are often elevated with neoplasia (Biran et al.,
1986, Falconer et al., 1994, Chan et al., 2007, Raynes and Cooper, 1983,
Fournier et al., 2000, Duché et al., 2000, Fujita et al., 1999), however CRP
appears to be the most useful systemic biomarker for cancer in humans. It is
elevated in a wide variety of malignancies including NHL, HL, hepatocellular,
colorectal, urinary bladder, pancreatic, breast, prostatic and gastric cancer and
is a predictor of recurrence of disease and of overall poor survival (Wieland et al., 2003, Beer et al., 2008, Yoshida et al., 2008, Crozier et al., 2007, Legouffe et al., 1998, Ito et al., 2006, Hashimoto et al., 2005). Similarly, CRP, AGP and SAA can become elevated in many canine malignancies including LSA, acute lymphoblastic leukaemia, and a variety of sarcomas and carcinomas (Nakamura et al., 2008, Tecles et al., 2005, Planellas et al., 2009). Of these canine APPs, CRP has been most widely investigated due to its widespread clinical application in human medicine.

1.3.2 Current suggested biomarkers for human and canine LSA

Biomarker analysis is widely used in the diagnosis and prognostication of human cancers including NHL (Kim, 2006, Khalifa et al., 2008). Using proteomic analysis, canine specific biomarkers have been identified that are capable of differentiating LSA patients from non-LSA patients, though currently it has not been determined whether these markers are suitable to monitor the response to treatment of previously diagnosed patients (Ratcliffe et al., 2009, Mian et al., 2006).

1.3.2.1 Cytokines as biomarkers

Cytokines are altered in humans and dogs with breast, lung, gastric, renal and bone tumours, lymphoma and leukaemia, with high serum concentrations being associated with advanced stages of disease (Khalifa et al., 2008, Itoh et al., 2009, Tsimberidou et al., 2008, Preti et al., 1997, Seymour et al., 1995). IL-6 is the most studied plasma cytokine and has been shown to promote the antitumour activity of macrophages to produce lymphokine-activated killer (LAK) calls and stimulate the production of CRP which binds to tumour cells and causes tumour cell lysis. IL-6 also prevents the apoptosis of neutrophils which aid the killing of tumour cells (Wang and Sun, 2009). The use of IL-6 as a biomarker in dogs may be limited as the measurement of plasma cytokines is difficult due to their short half-lives (Gabay and Kushner, 1999). Recent investigation of TNF-α in dogs with LSA has shown that the current assays commercially available for detection of human TNF-α may be of limited use in this species (Hofer et al., 2011).
1.3.2.2 Tissue biomarkers

Survivin is a member of the inhibitor of apoptosis protein family and has a role in tumour cell proliferation. Survivin expression is a negative prognostic indicator in both human and canine patients with lymphoma (Schlette et al., 2004, Adida et al., 2000, Rebhun et al., 2008). A current limitation of the use of survivin in veterinary patients is that biopsied tissue is required and there is limited availability of the test.

1.3.2.3 Serum enzyme biomarkers

Serum enzyme biomarkers such as lactate dehydrogenase (LDH) and thymidine kinase (TK) have prognostic significance in human and canine patients with LSA and may be capable of predicting recurrence of disease (Dumontet et al., 1999, Ferraris et al., 1979, Hallek et al., 1992, von Euler et al., 2004, Nakamura et al., 1997). LDH is higher in canine patients with LSA when compared to normal or diseased dogs; however there is disparity in the literature as to whether there is a relationship between LDH levels and clinical stage or survival time (Marconato et al., 2009, Greenlee et al., 1990, Zanatta et al., 2003, von Euler et al., 2006, Nakamura et al., 1997, Marconato et al., 2010). Serum TK is elevated in canine patients with LSA and appears to have prognostic significance (von Euler et al., 2004, von Euler and Eriksson, 2011). In a study by von Euler et al (2004) serum TK correlates with WHO stage and substage, and may be an early marker of relapse. Serum TK is potentially a very useful tumour biomarker in veterinary LSA patients, and recent development of an ELISA assay may allow full exploitation of its use in the near future (von Euler et al., 2006).

1.3.3 The current role of CRP in human and canine LSA

In human medicine, serum CRP concentration provides prognostic information for patients with NHL and HL. It is a significant marker for disease remission and relapse, and can be used as a predictor of overall survival (Herishanu et al., 2007, Legouffe et al., 1998, McMillan et al., 2001, Wieland et al., 2003). Serum CRP concentration is similarly significantly elevated in dogs with LSA. This elevation decreases to within the normal reference range when the patient
achieves complete remission during treatment with chemotherapy (Mischke et al., 2007, Merlo et al., 2007, Nielsen et al., 2007). CRP becomes elevated at time of relapse though there is disparity in the literature over whether this elevation achieves statistical significance (Merlo et al., 2007, Nielsen et al., 2007). The elevated concentration of serum CRP at time of relapse may be related to tumour burden (Merlo et al., 2007). If this is so, CRP would not be useful as an indicator of early relapse as serum concentration would only become elevated once tumour burden was significant and relapse was already clinically apparent.

1.4 CRP assay

The current assay for canine serum CRP is relatively rapid, cheap and readily available for general veterinary practitioners due to the development of immunoturbidimetric methodology (Eckersall et al., 1991, Kjelgaard-Hansen et al., 2003a), making it an ideal biomarker for dogs with LSA. Lipaemic, haemolytic or hyperbilirubinaemic serum may not be suitable for use in some assays including the enzyme-linked immunosorbent assays (ELISA) due to significant changes in the values of CRP obtained (Martinez-Subiela and Ceron, 2005), however immunoturbidimetric assays can be much less affected and the change in CRP is often within an acceptable 10% limit (Tecles et al., 2007). CRP has been shown to be a stable biomarker for analysis which can be successfully stored at -70°C for long periods and can be subjected to multiple freeze-thaw cycles without a decline in concentration (Hartweg et al., 2007, Aziz et al., 2003). It is recommended that multiple samples be run at the same time to reduce interassay variability (Aziz et al., 2003). Substantial variation in CRP concentration between dogs has been noted, as has individual dog variation in multiple measurements (Carney et al., 2011, Kjelgaard-Hansen et al., 2003b). It has been suggested that a population-based reference range may be unsuitable for interpreting serum CRP concentrations in dogs due to the individual dog variation, and that comparison between serial results may be more appropriate (Kjelgaard-Hansen et al., 2003b, Carney et al., 2011).
1.5 Study Aims

The aims of this retrospective study were to use a large population size and a large number of serial samples per case to:

1) Investigate whether a relationship exists between serum CRP concentration and the different patient variables,

2) Investigate whether serum CRP can be used as a clinical monitoring tool of remission status for dogs with LSA, and

3) Assess if serum CRP has any prognostic significance i.e. determine whether a relationship exists between pre-treatment CRP and time to relapse and overall survival time.
2 Materials and Methods

2.1 Study design

This prospective study was performed with full ethical approval from the Department of Ethics and Welfare, University of Glasgow. Owners of dogs included in the study signed a consent form allowing excess biological materials to be retained by the University. A retrospective search of the records was performed to determine clinical data for the dogs included in the study. All clinical data were stored in Access 2003 (Microsoft Inc, USA).

2.1.1 Study population

Untreated dogs with LSA that presented to the University of Glasgow’s Small Animal Hospital between 2004 and 2010 were eligible for recruitment onto the study. A diagnosis of LSA was confirmed by cytology or histopathology and immunophenotyping was performed by ICC or IHC using antibodies against CD3 and CD79a. Tumour grade was assigned using the Updated Kiel classification (Lennert and Feller, 1991) and the WHO classification (World Health Organization, 2008).

Clinical staging was performed on all dogs and a WHO stage and substage assigned (Owen, 1980). The minimum clinical database for all dogs included CBC, biochemistry analysis, abdominal ultrasonography and thoracic radiography. Computed tomography was performed when clinically appropriate. Splenic and hepatic cytology and bone marrow evaluation was performed to complete staging when deemed clinically important and when client finances allowed.

2.1.2 Exclusion criteria

Dogs were excluded from the study if they had concurrent infectious disease, if they had previously been treated for their LSA with surgery or chemotherapy, or if they did not have a serum sample obtained prior to treatment.
2.1.3 Initial induction treatment protocols

Dogs were treated with standard multidrug chemotherapy protocols for LSA. The decision for protocol selection was guided by the degree of client compliance and by the client financial situation. Those dogs with a high grade LSA were treated with one of the following protocols:

- **CHOP/modified CHOP**: cyclophosphamide, doxorubicin, vincristine and prednisolone +/- l-asparaginase.

- **Low dose (LD) COP**: cyclophosphamide, vincristine and prednisolone.

- **ALP**: asparaginase, lomustine and prednisolone.

Dogs with low grade LSA were treated with chlorambucil and prednisolone. For those clients that did not want to treat their pet with chemotherapy, the option of steroids was made available, where dogs were treated with anti-inflammatory doses of prednisolone (1-2 mg/Kg once or twice daily). The owners were given the option of declining any form of treatment for their pet following diagnosis.

All chemotherapy protocols included an induction and maintenance part; Appendix 1 describes the protocols in detail.

2.1.4 Relapse or ‘rescue’ protocols

At relapse, dogs were re-staged and reinduced with a rescue chemotherapy protocol. The rescue protocols included:

- **CHOP/modified CHOP**

- **LD COP**

- **ALP**

- **DMAC**: dexamethasone, melphalan, actinomycin-D and cytosine arabinoside.
• **LMP**: chlorambucil, methotrexate and prednisolone.

L-asparaginase (400 iu/Kg or 10,000 iu/m$^2$) was included as an initial induction agent in several dogs. Appendix 1 describes the rescue protocols in detail.

### 2.1.5 Clinical assessment

For those patients with peripheral lymphadenopathy, orthogonal measurements of all affected lymph nodes were obtained using calipers. Dimensions of the internal medial iliac, mesenteric and mediastinal lymph nodes were measured by metric rule using ultrasonographic equipment. Full staging was repeated at the time of tumour relapse or 1, 3, 6 and 12 months following the end of a chemotherapy protocol.

Response to treatment was assessed at each visit and remission status was determined according to the RECIST guidelines v1.1 (Eisenhauer et al., 2009). CR was not assigned until 28 days post treatment. For analysis purposes dogs SD i.e. minimal change from the previous measurements, were classed as the remission status that preceded it to reflect most accurately the level of disease present.

### 2.2 Determining the CRP concentration

Serum samples were obtained when there was excess whole blood collected at the time of initial staging, prior to any dose of chemotherapy and at time of restage and relapse. 1-2mL of whole blood was collected by jugular venipuncture into serum tubes. Blood was allowed to clot for approximately 2 hours at room temperature then centrifuged at 9,000 revolutions per minute for 3 minutes. A minimum of 200µL serum was harvested and frozen at -70°C for batch analysis at a later date. It was noted if the samples were grossly haemolytic, lipaemic or hyperbilirubinaemic. CRP concentration was measured using more than one assay type as the collection period spanned several years; during which improvements and modifications were made to the assay. Where spare serum was available, as many samples as possible were run again on the most recent assay, the Pentra 400 (Horiba ABX, UK). This was done by batch
analysis to reduce inter-assay variability. CRP concentrations were excluded if they were obtained when the patient was experiencing chemotherapy toxicities or concurrent infectious disease.

**Assay 1) 2004-2005, Solid-phase sandwich immunoassay (Canine CRP ELISA, Tridelta Development Ltd, Ireland), (Eckersall et al., 1989):**

This assay was previously validated to have a low within-run imprecision with intra-assay coefficients of variance (n=8) of 1.0% and 2.8% at mean control values of 18 mg/L and 74 mg/L respectively. This assay also has an acceptable between-run imprecision with inter-assay coefficients of variance (n=11) of 11.1% and 12.6% at mean control values of 19 mg/L and 75 mg/L of CRP respectively.

**Assay 2) 2005-2010, Immunoturbidimetric assay (Cobas Mira, Roche Diagnostics, UK), (Eckersall et al., 1991):**

This assay was previously validated to have a low within-run imprecision with intra-assay coefficients of variance (n=10) of 4.5% and 4.3% at mean control values of 37 mg/L and 66 mg/L of CRP respectively. This assay also has an acceptable between-run imprecision with inter-assay coefficients of variance (n=14) of 10% and 11% at mean control values of 42 mg/L and 102 mg/L of CRP respectively.

**Assay 3) 2010-present, Latex-enhanced immunoturbidimetric assay (Pentra 400, Horiba ABX, UK):**

This assay was validated to have a low within-run imprecision with intra-assay coefficients of variance (n=12) of 0.5% and 1.2% at mean control values of 4 mg/L and 30 mg/L of CRP respectively. This assay also has an acceptable between-run imprecision with inter-assay coefficients of variance (n=12) of 4.8% and 8.5% at mean control values of 4 mg/L and 30 mg/L of CRP respectively.

A serum CRP concentration ≤10 mg/L was deemed normal based on previous findings by Eckersall *et al* (1989).
2.3 Control population

The control population comprised 59 dogs of various breeds which were used as blood donors. The dogs were aged between 2-8 years and all weighed >25Kg. They were deemed ‘healthy’ based on a 2 week history of no illness or injury, negative findings on clinical examination, normal CBC and no significant renal or hepatic changes on biochemistry analysis. Serum was harvested and frozen at -70°C for batch analysis on the Pentra 400 analyser (Horiba ABX, UK).

5 dogs in the healthy control population had slightly elevated serum CRP concentrations (>10 mg/L but <17 mg/L) and 1 dog was an outlier with a CRP concentration of 30.5 mg/L. The history for these dogs was reviewed to ensure there was no underlying clinical cause for the elevated CRP. No cause was determined for any of the dogs and they were therefore left within the control group.

2.4 Statistical methods

Data are presented as mean ± standard deviation (SD) where normally distributed, as median [interquartile range (IQR)] where not normally distributed, or as a percentage of the group from which they were derived for categorical variables. Normality was tested with the Anderson-Darling (AD) test, where p ≥0.05 indicated normal distribution. Where the distribution of CRP was not normal, data were log transformed in an attempt to improve the assumption of Gaussian distribution and variance homogeneity. Parametric testing was performed on data that were normally distributed and non-parametric testing was performed on data that were not normally distributed.

All statistical analyses were performed using Minitab v13 (Minitab Inc., USA), GraphPad Prism v5.04 (GraphPad Software Inc., USA) and Stata 10.1 (StataCorp., USA) statistical software. Statistical significance was set at a p-value ≤ 0.05.
2.4.1 Descriptive analysis

Descriptive statistics were generated for continuous data which included the number in each group (n), range, mean (95% confidence interval (CI)), SD, median (95% CI), and the IQR. Graphical representation of the data included bar charts and box-and-whisker plots (boxplots) where the box represents the IQR, the line within the box represents the median, and the whiskers represent the range. Stars beyond the whiskers represent outlier values.

2.4.2 Comparison of assays

The paired CRP concentrations from all patients were pooled and were assigned into 3 groups depending on the assays by which the values were obtained:

- ELISA v Mira
- ELISA v Pentra
- Mira v Pentra

Time-series plots were created for each patient that had >5 sets of paired data (Appendix 2). Differences in variance were investigated using Levene’s test. Correlation between the assays was determined using Spearman rank (SR) correlation. The Bland-Altman test was performed to test the agreement between the different assays. Graphs were produced which plotted the difference between the 2 CRP measurements from each assay on the y-axis against the average of the 2 CRP measurements on the x-axis.

2.4.3 Categorising CRP concentrations

The distribution of CRP concentration was evaluated for both the control group and the study cohort, and 4 bands were selected based on the degree of CRP elevation. The bands were categorised using the following CRP ranges:

- **Normal:** CRP = ≤10 mg/L,
• **Mild elevation:** CRP = \( >10 \leq 30 \) mg/L,

• **Moderate elevation:** CRP = \( >30 \leq 80 \) mg/L,

• **Marked elevation:** CRP = \( >80 \) mg/L.

Due to the small numbers of dogs in each categorical grouping, the Normal and Mild (N+M) groups and the Moderate and Marked (M+M) groups were combined at times to facilitate statistical analysis.

### 2.4.4 Investigating the differences between groups

Proportional differences were investigated using the Fisher’s exact test when \( n<5 \) in one or more groups, and using the Chi-square test when \( n\geq5 \) in all groups.

Comparison of the medians in 3 or more groups was performed using the Kruskal-Wallis (KW) test. Comparison of the medians of 2 groups was performed using the Mann-Whitney U (MW) test.

Comparison of paired or matched data was performed using the paired t-test for normally distributed data and the Wilcoxon-signed rank (WSR) test for data that were not normally distributed.

### 2.4.5 Investigating the relationship between groups

Correlation between CRP and the different patient variables was performed using Pearson’s correlation for normally distributed data and the SR test on data that were not normally distributed.

The relationship between CRP as a continuous outcome and the different patient variables was investigated using a general linear regression model. CRP data was log-transformed to improve the assumption of normal distribution. Categorical variables included site (generalised multicentric vs. not generalised multicentric), WHO stage (II-III vs. IV-V), WHO substage (‘a’ vs. ‘b’), immunophenotype (B-cell vs. T-cell) and continuous variables included albumin concentration. Univariate analysis was performed initially, and all significant
variables were placed into the final multivariate model. Fitted-line plots were produced for each variable investigated in the univariate model and the transformation of residuals was performed to improve the model.

Logistic regression was also performed to investigate CRP as a binary outcome (≤30 mg/L vs. >30 mg/L) with various predictor variables. Categorical predictor variables included site, WHO stage and sustage, immunophenotype (as described above) and albumin (hypoalbuminaemic vs. not hypoalbuminaemic). Significant predictor variables from univariate analysis were placed in the final model for multivariate analysis.

2.4.6 Determining CRP concentration cut-off values

Area under the receiver operator characteristic (AUROC) curve analysis was performed to generate a range of cut-off values. The best cut-off value was deemed the one with the best balance between sensitivity and specificity, where sensitivity is the proportion of true positives which are correctly identified and specificity is the proportion of true negatives which are correctly identified.

2.4.7 Survival analysis

Survival differences were investigated using a logistic regression model with different binary outcomes (survival time ≤6 months vs. >6 months, dead vs. alive, death due to LSA vs. death due to other reasons, and relapsed vs. not relapsed) to produce a likelihood ratio. Continuous predictor variables including CRP, albumin and calcium were log-transformed to improve the assumption of normality. Categorical predictor variables included WHO stage (II-III vs. IV-V), substage (‘a’ vs. ‘b’), immunophenotype (B-cell vs. T-cell), site (generalised multicentric vs. not generalised multicentric), albumin (hypoalbuminaemic vs. not hypoalbuminaemic), calcium (hypercalcaemic vs. not hypercalcaemic) and CRP (≤30 mg/L vs. >30 mg/L). The significant variables from the univariate analysis were placed into the final model for multivariate logistic regression analysis.
Survival analysis was performed using a Kaplan-Meier product limit method. Overall survival time (OST) was defined as the number of days from first chemotherapy treatment to death, and time to first relapse (TFR) was defined as the number of days from first chemotherapy treatment to first episode of progressive disease (relapse of disease) in dogs that achieved partial or complete remission. Log-rank and Gehan-Breslow-Wilcoxon tests were used to determine whether the overall survival functions in two or more groups were equal. This was supplemented with a Cox proportional hazards model. Potential predictor variables included WHO Stage and substage, immunophenotype, albumin, and calcium. Survival curves were generated for the significant variables. Dogs were censored at the date of analysis if still alive.

To remove the effect on survival of the different treatment regimes and to homogenise the population, dogs that received any treatment other than a CHOP-type protocol were excluded from the analyses.
3 Results

3.1 Defining the final study population

To exclude any confounding variables, and to maximise the number of samples that could be used for final statistical analysis, the agreement between the different CRP assays was investigated along with the effects of repeat freeze-thaw cycles, of concurrent disease, and of previous glucocorticoid treatment. The results of this analysis determined the final population for analysis.

3.1.1 Testing the agreement between assays: paired samples

Due to modifications of the CRP assay over the study time period, many samples were run on more than 1 assay, providing paired CRP concentrations for time points from several dogs. The paired CRP concentrations from all dogs with LSA were assigned into 3 groups depending on the assays from which they were obtained: Pentra/Mira, Pentra/ELISA and Mira/ELISA. To allow visualisation of any patterns between the paired assays, time-series plots were created for each patient that had >5 sets of paired data (Appendix 2).

3.1.1.1 Comparison of the Pentra and Mira assays

Six dogs had >5 samples from several time points run on the Pentra and Mira assays. Taking each dog separately, statistical analysis using the WSR test showed a significant difference in median CRP concentration between assays in only 2 dogs (Table 2). Considering all paired samples for the Pentra and Mira assays together (n=95), there was no significant variance between them (Levene’s test p=0.17) and both assays significantly correlated (p<0.001), however this correlation was only moderate (rs =0.54). There was also no significant difference between the median CRP concentrations of the paired samples run on both these assays (MW test W=1853.0, p=0.11). In summary, the 2 assays showed good agreement in most statistical analyses except for the WSR test in 2 out of 6 dogs.
Table 2 Results of Wilcoxon signed-rank test for paired samples on Pentra & Mira assays

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>n</th>
<th>W-Stat</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>106.0</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>92.0</td>
<td>0.01*</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>22.0</td>
<td>0.62</td>
</tr>
<tr>
<td>61</td>
<td>11</td>
<td>26.0</td>
<td>0.56</td>
</tr>
<tr>
<td>62</td>
<td>15</td>
<td>57.0</td>
<td>0.89</td>
</tr>
<tr>
<td>67</td>
<td>10</td>
<td>55.0</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

* Indicates a significant difference

3.1.1.2 Comparison of the Pentra and ELISA assays

Seven dogs had >5 samples run on the Pentra and ELISA assays. For individual dogs, statistical analysis using the WSR test showed a significant difference between the median CRP concentrations between assays in 4 out of 7 dogs (Table 3). When considering all paired samples together (n=88), the CRP concentrations significantly correlated ($r_s=0.832$, $p<0.001$) however there was significant variance between both assays (Levene’s test $p=0.04$) indicating poor agreement. There was significant difference between the median CRP concentrations obtained from the Pentra and ELISA assay (MW test $W=474.0$, $p<0.001$). In summary, the statistical analyses indicated poor agreement between assays.

Table 3 Results of Wilcoxon signed-rank test for paired samples on Pentra & ELISA assays

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>n</th>
<th>W-Stat</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>100.0</td>
<td>0.54</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>88.0</td>
<td>0.003*</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>29.0</td>
<td>0.14</td>
</tr>
<tr>
<td>43</td>
<td>8</td>
<td>36.0</td>
<td>0.01*</td>
</tr>
<tr>
<td>44</td>
<td>5</td>
<td>15.0</td>
<td>0.06</td>
</tr>
<tr>
<td>47</td>
<td>25</td>
<td>325.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>52</td>
<td>12</td>
<td>78.0</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

* Indicates a significant difference
3.1.1.3 Comparison of the Mira and ELISA assays

Only 3 dogs had >5 samples run on the Mira and ELISA assays. For each individual, statistical analysis using the WSR test showed significant difference between the median CRP concentrations between assays in 1 dog only (Table 4). Considering all paired samples together (n=51), there was no significant difference in the variance between them (Levene’s test p=0.31), and there was significant correlation of the assays (p<0.001), however this was only moderate ($r_s =0.59$). There was no significant difference between the median CRP concentrations obtained from the Mira and ELISA assay (MW test $W=485.0$, $p=0.10$). In summary, the 2 assays showed good agreement except in the WSR test in 1 out of 3 dogs.

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>$n$</th>
<th>$W$-Stat</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>120.5</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>90.0</td>
<td>0.002*</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>31.0</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Indicates a significant difference

3.1.1.4 Bland-Altman analysis

Further comparison of the paired samples from the different assays was performed using the Bland-Altman method (Figure 4) where the difference between both CRP measurements on the y-axis was plotted against the average of the 2 CRP measurements on the x-axis. This analysis showed there was insufficient agreement between the ELISA and Pentra assays (bias = -7.06, 95% CI -33.31, 19.19) and the Mira and Pentra assays (bias = -4.09, 95% CI -58.32, 50.14). The agreement between the Mira and ELISA assays was more acceptable (bias = 1.8, 95% CI -11.9, 15.5).
Figure 3 Bland-Altman plot of paired CRP concentrations on i) ELISA & Mira assays; ii) ELISA & Pentra assays; iii) Mira & Pentra assays

These results confirmed that there was insufficient agreement between assays to be able to combine in a single data set the samples run on one assay with those run on a different assay. Therefore, as the Pentra analyser was the most current assay, and as the largest number of samples were run on this assay, the final population was taken from these samples. All dogs that had CRP concentrations obtained only on the ELISA or Mira assays were excluded from further statistical analysis.
3.1.2 Do multiple freeze-thaw cycles affect CRP?

Many of the samples were run over time on multiple assays, and ultimately on the Pentra analyser which we had previously determined to be the assay of choice. It was therefore important to determine that the repeat freeze-thaw cycles did not cause significant degradation of the CRP proteins and consequently result in artificially low serum CRP concentrations. All Pentra CRP concentrations available were placed into 2 groups: Group 1 included those samples that were run on the first freeze-thaw cycle (n=452) and Group 2 included those samples that were run after multiple freeze-thaw cycles (n=117). Table 5 displays the descriptive statistics and Figure 5 shows that the distribution of CRP concentrations in both groups is similar. There was no significant difference between the median CRP concentrations of groups 1 and 2 (MW test W=127417.5, p=0.38).

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>Range (mg/L)</th>
<th>Mean (95% CI) (mg/L)</th>
<th>SD</th>
<th>Median (95% CI) (mg/L)</th>
<th>IQR  (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>452</td>
<td>0.0-294.0</td>
<td>20.4 (17.1-23.8)</td>
<td>36.1</td>
<td>7.8 (6.7-9.4)</td>
<td>3.4-19.9</td>
</tr>
<tr>
<td>2</td>
<td>117</td>
<td>0.3-225.6</td>
<td>22.5 (16.0-29.0)</td>
<td>35.5</td>
<td>8.6 (6.0-12.3)</td>
<td>3.5-25.0</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>0.41-273.7</td>
<td>51.2 (37.1-65.3)</td>
<td>53.1</td>
<td>34.7 (24.9-49.2)</td>
<td>18.1-67.0</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>5.9-198.1</td>
<td>54.3 (27.0-81.6)</td>
<td>51.1</td>
<td>32.6 (22.2-67.9)</td>
<td>21.6-75.0</td>
</tr>
<tr>
<td>5</td>
<td>242</td>
<td>0.0-160.4</td>
<td>10.3 (8.3-12.3)</td>
<td>15.9</td>
<td>5.3 (4.8-6.3)</td>
<td>2.4-12.5</td>
</tr>
<tr>
<td>6</td>
<td>53</td>
<td>0.7-66.1</td>
<td>9.9 (6.4-13.3)</td>
<td>12.4</td>
<td>5.5 (4.1-6.8)</td>
<td>3.1-10.5</td>
</tr>
</tbody>
</table>
Figure 4 Boxplot of CRP in first thaw and multiple freeze-thaw cycles
a. All samples: Group 1 (all 1st thaw samples) vs. Group 2 (all multiple freeze-thaw samples);
b. High CRP concentration samples: Group 3 (pre-treatment 1st thaw samples) vs. Group 4 (pre-treatment multiple freeze-thaw samples); c. Low CRP concentration samples: Group 5 (complete remission 1st thaw samples) vs. Group 6 (complete remission multiple freeze-thaw samples)
The effect of multiple freeze-thaw cycles on different extremes of CRP concentration was also investigated, using pre-treatment samples (high CRP concentrations) and samples from dogs in CR (low CRP concentrations). A further 4 groups of Pentra CRP concentrations were allocated (Table 5): Group 3 included pre-treatment samples run on the first thaw cycle (n=57) and Group 4 included pre-treatment samples that were run on the Pentra assay following repeat freeze-thaw cycles (n=16). Group 5 included samples obtained from patients in CR following treatment and were run on the first freeze-thaw cycle (n=242) and Group 6 included samples from patients in CR which were run on the Pentra analyser following repeat freeze-thaw cycles (n=53). There was a similar distribution of CRP concentrations in groups 3 and 4 and groups 5 and 6 (Figure 5), with no significant difference in the medians of the 2 groups when analysed using the MW test (Group 3+4: W=2075.0, p=0.66; Group 5+6: W=35636.5, p=0.75).

These analyses indicated that it was acceptable to use samples that had undergone multiple freeze-thaw cycles.

**3.1.3 Does concurrent disease affect pre-treatment CRP?**

Of the 83 dogs that were included in the analysis, 54 dogs (65%) had no concurrent disease (NCD) at time of presentation and 29 (35%) had concurrent disease (CD). The 29 dogs with concurrent disease were split into 2 groups (Table 6): those with non-inflammatory concurrent disease (NICD, n=7, 24%), and those with inflammatory concurrent disease (ICD, n=22, 76%). Descriptive statistics for each group can be found in Table 7.
Table 6 List of concurrent diseases at time of diagnosis (non-inflammatory & inflammatory)

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease type</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-inflammatory</td>
<td>Heart disease - heart murmur</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- endocardiosis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- dilated cardiomyopathy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- mitral valve disease</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Chronic renal disease</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Protein-losing enteropathy</td>
<td>1</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Osteoarthritis</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Benign prostatic hyperplasia</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Biopsy wound</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Skin disease - atopic dermatitis</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- pruritus</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- sebaceous cyst</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Inflammatory bowel disease</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Otitis externa</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Chronic pancreatitis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Periodontitis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cystitis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 7 Descriptive statistics of CRP in the different concurrent disease groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>Range (mg/L)</th>
<th>Mean (95% CI) (mg/L)</th>
<th>SD</th>
<th>Median (95% CI) (mg/L)</th>
<th>IQR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCD</td>
<td>54</td>
<td>0.4-273.7</td>
<td>61.3 (43.5-79.1)</td>
<td>65.2</td>
<td>33.8 (25.6-61.1)</td>
<td>18.7-92.0</td>
</tr>
<tr>
<td>CD</td>
<td>29</td>
<td>2.4-94.4</td>
<td>34.2 (24.8-43.5)</td>
<td>24.7</td>
<td>30.6 (19.7-44.9)</td>
<td>11.6-53.7</td>
</tr>
<tr>
<td>NICD</td>
<td>7</td>
<td>10.3-94.4</td>
<td>39.5 (11.9-67.0)</td>
<td>29.8</td>
<td>30.2 (17.4-72.7)</td>
<td>20.0-64.8</td>
</tr>
<tr>
<td>ICD</td>
<td>22</td>
<td>2.4-72.7</td>
<td>32.5 (22.1-42.8)</td>
<td>23.4</td>
<td>31.7 (12.0-47.9)</td>
<td>9.8-52.9</td>
</tr>
</tbody>
</table>

NCD = no concurrent disease, CD = concurrent disease, NICD = non-inflammatory concurrent disease, ICD = inflammatory concurrent disease

Statistical analysis showed no significant difference between the median CRP concentration for dogs with NCD and those with CD (MW test W=2416, p=0.16). There was also no difference between the median CRP concentrations for the dogs with NCD, NCID and ICD (KW test H=2.23, d.f.=2, p=0.33).

Since CRP concentration did not differ between groups, it was concluded that common non-inflammatory and inflammatory conditions did not significantly
affect CRP in dogs with LSA, and as such all dogs were kept in the study even if they presented with concurrent disease.

3.1.4 Does steroid treatment affect pre-treatment CRP concentration?

Of the 83 dogs that presented, 73 (88%) had received no previous steroid treatment (NPS) at time of presentation, and 10 (12%) had received previous glucocorticoid steroid treatment (PS) at a dose rate of 0.5-2 mg/Kg. The dogs who had received steroids were further split into 2 groups: previous steroid treatment for <7 days (PS<7d, n=6, 60%), and previous steroid treatment for >7 days (PS>7d, n=4, 40%). Table 8 shows the descriptive statistics for all groups of dogs.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Range (mg/L)</th>
<th>Mean (95% CI)</th>
<th>SD</th>
<th>Median (95% CI)</th>
<th>IQR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPS</td>
<td>73</td>
<td>0.4-273.3</td>
<td>51.9 (39.7-64.1)</td>
<td>52.3</td>
<td>34.3 (27.1-45.4)</td>
<td>19.1-273.7</td>
</tr>
<tr>
<td>PS</td>
<td>10</td>
<td>4.6-269.7</td>
<td>51.2 (-6.9-109.3)</td>
<td>81.2</td>
<td>24.0 (6.8-58.2)</td>
<td>7.0-53.3</td>
</tr>
<tr>
<td>PS&lt;7d</td>
<td>6</td>
<td>5.2-269.7</td>
<td>66.0 (-39.6-171.6)</td>
<td>100.6</td>
<td>32.9 (8.8-187.7)</td>
<td>12.7-97.5</td>
</tr>
<tr>
<td>PS&gt;7d</td>
<td>4</td>
<td>4.6-93.0</td>
<td>29.1 (-38.9-97.0)</td>
<td>42.7</td>
<td>9.4 (4.6-93.0)</td>
<td>5.3-93.0</td>
</tr>
</tbody>
</table>

NPS = no previous steroids, PS = previous steroids, PS<7d = steroids for <7 days, PS>7d = previous steroids for >7 days

There was no significant difference between the median CRP concentration for the NPS group compared to the PS group (MW test: W=3138.0, p=0.32). There was also no significant difference between the median CRP concentrations for the NPS group compared to the the PS<7d and PS>7d group (KW test: H=1.90, df=2, p=0.39).

Although there was no significant difference detected between groups, the median CRP concentration for the PS>7d group appeared very different from the other groups and it was felt there may have been too small a sample size in this group to detect significance. A decision was therefore made to exclude all 10 dogs that had received previous steroid treatment.
3.2 Demographic analysis

3.2.1 Comparison of the control and study cohorts

To determine whether the dogs included within the control group were similar to those included in the study group some basic statistics were performed.

3.2.1.1 Control dogs

Of the 59 dogs used as a normal control population, 15 breeds were represented (Figure 6), with greyhounds (n=25, 42%), Labrador retrievers (n=10, 17%), crossbreeds (n=5, 8%) and flatcoated retrievers (n=5, 8%) most prevalent. Thirty eight dogs (64%) were male (5 entire, 33 neutered) and 21 (36%) were female (5 entire, 16 neutered), making a male:female ratio of 1.8:1. The age of dogs ranged from 2-10 years with a median age of 6 years (mean 5.7 ± 1.9 years). Age was not normally distributed within this group (p<0.005) and was positively skewed.

![Figure 5 Chart of breeds for the Control & Lymphoma (LSA) populations](image)

Figure 5 Chart of breeds for the Control & Lymphoma (LSA) populations
3.2.1.2 Lymphoma dogs

Of the 73 dogs with LSA included for final statistical analysis, 26 breeds were represented (Figure 6), with crossbreeds (n=13, 18%), Labrador retrievers (n=12, 16%), boxers (n=6, 8%), golden retrievers (n=5, 7%), bullmastiffs, German shepherd dogs (GSD) and cocker spaniels (n=4, 5% respectively) most prevalent. Fifty dogs (68%) were male (21 entire, 29 neutered) and 23 (32%) were female (16 entire, 7 neutered), making a male:female ratio of 2.2:1. The age of the dogs ranged from 0.9-12.0 years with a median age of 7.3 years (mean 7 ± 2.6 years). Age was normally distributed within this group (p=0.49).

There was no statistical difference in the proportions of males and females between the control and LSA populations (chi-sq=0.25, d.f.=1, p=0.62). There was however a statistical difference in the distribution of ages between the control and LSA populations (chi-sq=23.5, d.f.=2, p<0.001), and a significant difference in the median ages (MW test: W=3167.5, p=0.0005), with the control group having a younger population of dogs.

3.3 Further demographics of the study population

3.3.1 Results of patient staging

3.3.1.1 Anatomical location

All dogs were clinically staged to determine the anatomical location of disease. The majority of dogs (n=60, 82%) presented with generalised multicentric disease. Other presentations included alimentary (n=4, 5%), mediastinal (n=3, 4%), hepatosplenic, nasal and bilateral submandibular lymph node (SMLN) involvement (n=2, 3% respectively).

Due to the low numbers of dogs in some groups, dogs were classified as being either ‘generalised multicentric’ (GM) which included those dogs with a true multicentric presentation (n=60) or ‘not generalised multicentric’ (NGM) which included those dogs with single site and extra-nodal presentation (n=12).
3.3.1.2 WHO stage and substage

On the basis of clinical staging, dogs were assigned a World Health Organisation (WHO) stage and substage. No dogs had stage I LSA and therefore only stages II-V were represented in the study population.

Five dogs (7%) were stage II (4 substage ‘a’, 1 substage ‘b’), 14 (19%) were stage III (10 substage ‘a’, 4 substage ‘b’), 28 (38%) dogs were stage IV (13 substage ‘a’, 15 substage ‘b’) and 26 (36%) were stage V (6 substage ‘a’, 20 substage ‘b’). More dogs presented with clinical signs and therefore substage ‘b’ (n=50) was more prevalent than substage ‘a’ (n=23). Figure 7 shows the distribution of substages ‘a’ and ‘b’ across the different WHO stages.

![Bar chart of distribution of WHO substage a + b across the different WHO stages](image)

3.3.1.3 Immunophenotyping

Immunophenotyping was available for 58 dogs (79%). Of these, 31 (53%) were T-cell, 25 (43%) were B-cell, and 2 (4%) were null cell.

3.3.1.4 Pre-treatment calcium

Hypercalcaemia is a common paraneoplastic syndrome in dogs with LSA. Pre-treatment serum total calcium concentration was available for 72 dogs (99%) and results were not available for 1 dog. Calcium concentrations ranged from 1.53-
4.46 mmol/L with a median concentration of 2.57 mmol/L [2.4-2.7 mmol/L]. The normal laboratory reference is 2.34-3.0 mmol/L.

A paraneoplastic hypercalcaemia was present in 10 dogs (14%), and the range of total calcium concentration for these dogs was 3.37-4.46 mmol/L (median 3.79 mmol/L). Hypocalcaemia was observed in 9 dogs (13%) with a serum calcium range of 1.53-2.33 mmol/L (median 2.30 mmol/L). Total calcium is bound to the protein in blood and as such hypoproteinaemia can result in a concurrent hypocalcaemia. All hypocalcaemic dogs in this group had a concurrent hypoalbuminaemia (albumin concentration range 10-28 g/L).

Ionised calcium (i.e. non-protein bound) was obtained for some patients that presented with hypercalcaemia; however this data was not consistently available to permit analysis.

### 3.3.1.5 Pre-treatment albumin

Pre-treatment serum albumin concentrations were available for 72 dogs (99%). The albumin concentration for all dogs ranged from 3-36 g/L with a median of 28 g/L [25-32 g/L]. The normal laboratory reference is 29-36 g/L.

Hypoalbuminaemia was present in 38 dogs (53%), with albumin concentrations ranging from 3-28 g/L (median 26 g/L). Albumin is a negative APP and concentrations decrease as the concentration of the positive APPs (e.g. CRP) increases. Of the hypoalbuminaemic dogs, 37 (97%) had CRP concentrations above the normal laboratory reference of 10.0 mg/L (range 14.0-273.7 mg/L, median 47.8 mg/L). The SR correlation test showed the negative correlation between albumin and CRP was significant (r = -0.39, p=0.02). General linear regression analysis of these 2 variables also showed a significant relationship (T=2.57, p=0.01), however there was poor goodness of fit of the regression line (R² = 8.6%, Figure 8) indicating that CRP accounted for only 8.6% of the variance in albumin concentration in this population of dogs with LSA.
The association between albumin and certain clinical parameters including WHO stage, substage and immunophenotype was investigated using the MW test. Dogs with WHO stage IV-V (n=53) had a significantly lower median albumin concentration (median 27 g/L) compared to dogs with WHO stage II-III (n=19, median 32 g/L; W=1014.5, p<0.001), and dogs with substage ‘b’ (n=39) had a significantly lower median albumin concentration (median 27 g/L) compared to dogs with substage ‘a’ (n=33, median 30 g/L; W=1453.0, p=0.005). No significant difference was noted between dogs with B-cell LSA (n=24, median 29 g/L) and dogs with T-cell LSA (n=31, median 28 g/L; W=655.0, p=0.78). These results indicate that albumin concentration is influenced by WHO stage and substage.

3.3.1.6 Initial treatment and relapse protocols

Of the 73 dogs in the lymphoma group, 65 (89%) were administered some form of drug treatment and 8 (11%) were not treated upon owner request. Chemotherapy was the most popular treatment choice with 60 dogs (92%) being given a standard protocol. The majority of dogs (n=51, 87%) received a CHOP or modified CHOP protocol, but 4 (7%) were given a lomustine-based protocol and 3 (5%) a LD-COP or modified LD-COP protocol. One dog was given a single dose of l-asparaginase, 1 dog received a chlorambucil-based protocol and the remaining 5 dogs (8%) were treated with prednisolone only. Appendix 1 describes the different chemotherapy protocols.
Of the dogs that started chemotherapy treatment, 44 (73%) relapsed at some point during or after treatment with the initial protocol. A ‘rescue’ chemotherapy protocol was used in an attempt to re-induce remission of disease in 36 (82%) relapsed dogs. Of these dogs, 14 (39%) re-started a CHOP or modified CHOP protocol, 12 (33%) started the ALP protocol and 5 (14%) were treated using another standard protocol (single-agent L-asparaginase n=2; LD-COP n=1; LMP n=1; DMAC n=1).

For the purpose of analysis, dogs were grouped according to the treatment they received:

1. No treatment/prednisolone only (No-tx/pred only).
2. CHOP-type protocol.
3. Any other protocol (including LD-COP, modified LD-COP, ALP, chlorambucil/pred, single-agent l-asparaginase).

3.3.1.7 Effect of chemotherapy on remission status

The effect of chemotherapy on the 60 dogs that were treated was examined and of specific interest was the remission status achieved by week 4 of treatment. Dogs were excluded if they died or stopped treatment at or before week 4 of chemotherapy or if no remission data was available. A total of 47 dogs met the inclusion criteria. The majority of these dogs (83%, n=39) achieved CR by week 4, 6 dogs (13%) achieved a PR and 2 dogs (4%) had PD.

3.3.1.8 Time to first relapse (TFR)

TFR ranged from 15-436 days, with a median of 105 days [55-241 days].

3.3.1.9 Overall survival time (OST)

At time of analysis, 8 dogs (11%) with LSA were censored as they were still alive. Sixty-five dogs (89%) in the study died or were euthanized due to LSA-related reasons. Of these, 52 (80%) had progressive disease and 8 (12%) had tumour- or
chemotherapy-related problems (3 had chemotherapy toxicosis, 2 had disseminated intravascular coagulation (DIC), 1 each had tumour-lysis syndrome, severe anaemia and severe disease-related symptoms including vomiting and diarrhoea). Three dogs (5%) died of other diseases (1 each from congestive heart failure, dilated cardiomyopathy and osteosarcoma). Two dogs (3%) died suddenly at home and for analysis purposes they were classified as having died from their LSA.

OST was determined for all dogs. Dogs that did not receive treatment were censored at 0 days survival. OST for all dogs ranged from 0-2089 days, with a median survival time of 136 days [18-356 days].

OST was determined for the different treatment groups (Table 9) and a boxplot showing the distribution was produced (Figure 9).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n (%)</th>
<th>OST Range (days)</th>
<th>Median OST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-tx/pred only</td>
<td>13 (18)</td>
<td>0-41</td>
<td>0</td>
</tr>
<tr>
<td>CHOP-type protocol</td>
<td>51 (70)</td>
<td>1-1720</td>
<td>198</td>
</tr>
<tr>
<td>Any other protocol</td>
<td>9 (12)</td>
<td>28-2089</td>
<td>273</td>
</tr>
</tbody>
</table>

Figure 8 Box-and-whisker plot of the distribution of overall survival time (OST) for the different treatment groups.
There was a significant difference between the median overall survival times for the dogs in the ‘no tx/pred only’ group compared to the ‘CHOP-type protocol’ group (MW test: $W=123.5$, $p<0.001$) and the ‘no tx/pred only’ group compared to the ‘any other protocol’ group (MW test: $W=95.0$, $p=0.0003$). There was no significant difference between the median OST for the ‘CHOP-type protocol’ group and the ‘any other protocol’ group (MW test: $W=1507.5$, $p=0.33$).

Many dogs ($n=36$, 49%) in this study had a short survival time of only 0-3 months, with all untreated dogs appearing in this group (Figure 10). The next biggest survival group was those that survived >12 months ($n=15$, 21%). Ten dogs (14%) survived between <3 to 6 months, 5 (7%) survived >6 to 9 months and 7 (9%) survived >9-12 months.

![Figure 9 Bar chart of distribution of overall survival time (OST) categories for treated & untreated dogs](image)

### 3.4 CRP in normal dogs and dogs with LSA

The laboratory normal reference range used in this study was 0-10 mg/L.

#### 3.4.1 Descriptive statistics for the control and LSA dogs

CRP concentration demonstrated a high degree of variance in both the control dogs and those with LSA (25.7 and 2735.6 respectively), was positively skewed.
(Figure 11) and was not normally distributed in either group (p<0.005). CRP was within the normal reference range for 54 (92%) of the control dogs and 10 (14%) of the dogs with LSA. The range of CRP concentration in the control group was 0.1-30 mg/L, with the maximum CRP concentration being 3 times the upper limit of the normal reference range. Most dogs however (n=54, 92%) had CRP concentrations that were within reference. For the LSA dog group, the range of CRP concentrations was 0.4-273.7 mg/L, with the maximum CRP concentration being as much as 27 times the upper limit of the normal range. In this group only a small percentage of dogs (n=10, 14%) had serum CRP concentrations that were within normal reference. The majority of dogs with LSA (n=65, 89%) had CRP concentrations <100 mg/L (<10-fold increase). Outliers were identified for both groups: the control group had 5 outliers with CRP concentrations of 11.1, 12.5, 13.4, 16.5 and 30.5 mg/L, and the LSA group had 5 outliers with CRP concentrations of 171.5, 174.7, 184.9, 198.1 and 273.7 mg/L. Table 10 shows the descriptive statistics for the control dogs and the dogs with LSA.
3.4.2 Does CRP concentration differ between normal and LSA dogs?

There was a difference between the distribution of CRP in the control dogs and LSA dogs, as can be seen in Fig 12. The LSA group had a significantly higher median CRP (median 34.3 mg/L, W=2026.0, p<0.001) than the control group (median 2.5 mg/L).
3.4.2.1 Does the degree of CRP elevation differ between normal and LSA dogs?

As well as considering individual CRP concentrations, the degree of CRP elevation was also evaluated for normal dogs and dogs with LSA. CRP concentrations were placed into 4 different categories selected according to the degree of elevation and the distribution of concentrations (Table 11, Figure 13). Due to the low number of control dogs in some groups, analysis with the Chi-square test could not be performed; therefore a 2x2 analysis with the Fisher’s exact test was performed on the Normal/Mild and the Moderate/Marked groups. This showed a significant difference between control and LSA dogs, suggesting that LSA affects the degree of elevation of CRP concentration (p<0.001).

Table 11 Categories of CRP elevation with count and frequency in control & lymphoma (LSA) groups

<table>
<thead>
<tr>
<th>CRP Category</th>
<th>CRP range (mg/L)</th>
<th>Control dogs n (%)</th>
<th>LSA dogs n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>≤10</td>
<td>54 (91.5)</td>
<td>10 (13.7)</td>
</tr>
<tr>
<td>Mild increase</td>
<td>&gt;10-30</td>
<td>4 (6.8)</td>
<td>21 (28.8)</td>
</tr>
<tr>
<td>Moderate increase</td>
<td>&gt;30-80</td>
<td>1 (1.7)</td>
<td>29 (39.7)</td>
</tr>
<tr>
<td>Marked increase</td>
<td>&gt;80</td>
<td>0 / 0</td>
<td>13 (17.8)</td>
</tr>
</tbody>
</table>
3.4.3 The distribution of pre-treatment CRP concentration in the different treatment groups

Descriptive statistics for the CRP concentrations in the different treatment groups were generated (Table 12) and a boxplot showing the distribution of CRP concentration in the treatment groups was produced (Figure 14).

Table 12 Descriptive statistics for pre-treatment CRP concentration in the different treatment groups

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n (%)</th>
<th>CRP Range (mg/L)</th>
<th>Median CRP (95% CI) (mg/L)</th>
<th>IQR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-tx/pred only*</td>
<td>13 (18)</td>
<td>2.9-273.7</td>
<td>47.8 (19.4-172.5)</td>
<td>19.0-173.1</td>
</tr>
<tr>
<td>CHOP-type protocol</td>
<td>51 (70)</td>
<td>0.4-184.9</td>
<td>31.1 (26.1-44.3)</td>
<td>21.2-64.5</td>
</tr>
<tr>
<td>Any other protocol</td>
<td>9 (12)</td>
<td>2.4-136.0</td>
<td>18.9 (3.4-118.2)</td>
<td>4.3-108.9</td>
</tr>
</tbody>
</table>

* No-tx/pred only = no treatment/prednisolone only
3.5 Investigating which variables influence CRP in dogs with LSA

To investigate whether certain demographic and clinical parameters that are related to LSA influenced CRP concentrations, values at time of diagnosis (pretreatment) were investigated with regard to several patient variables including age, sex, breed, WHO stage and substage, site, immunophenotype, albumin and calcium.

To allow a comparison of median CRP concentrations, age was categorised into 3 groups (0-3 years, >3-7 years and >7 years), while others variables were categorised into 2 groups i.e. breed (pedigree and crossbreed), site (generalised-multicentric and not generalised-multicentric), albumin (hypoalbuminaemic and not hypoalbuminaemic) and calcium (hypercalcaemic and not hypercalcaemic). Certain variables had too few dogs to allow statistical comparison, therefore no analysis was performed on tumour grade, and only dogs that were B- and T-cell
were investigated for immunophenotype. Table 13 shows the descriptive statistics for each categorical variable.

Table 13 Descriptive statistics for CRP in the different patient variables

<table>
<thead>
<tr>
<th>Patient Variable</th>
<th>n=</th>
<th>Range (mg/L)</th>
<th>Mean (95% CI) (mg/L)</th>
<th>SD</th>
<th>Median (95% CI) (mg/L)</th>
<th>IQR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: 0-3 years</td>
<td>6</td>
<td>20.0-74.1</td>
<td>41.3 (16.6-66.0)</td>
<td>23.5</td>
<td>30.9 (2.4-72.0)</td>
<td>22.9-69.6</td>
</tr>
<tr>
<td>&gt;3-7 years</td>
<td>27</td>
<td>2.9-198.1</td>
<td>52.1 (31.1-73.2)</td>
<td>2.9</td>
<td>33.3 (18.2-65.0)</td>
<td>15.3-78.4</td>
</tr>
<tr>
<td>&gt;7 years</td>
<td>40</td>
<td>0.4-273.7</td>
<td>53.3 (35.5-71.0)</td>
<td>55.5</td>
<td>35.9 (25.1-55.2)</td>
<td>21.2-64.6</td>
</tr>
<tr>
<td>Breed: Pedigree</td>
<td>61</td>
<td>0.4-273.7</td>
<td>51.8 (37.8-65.8)</td>
<td>54.8</td>
<td>34.3 (26.7-42.1)</td>
<td>18.1-65.3</td>
</tr>
<tr>
<td>X-breed</td>
<td>12</td>
<td>20.4-133.4</td>
<td>52.3 (27.3-77.3)</td>
<td>39.4</td>
<td>43.3 (20.5-86.0)</td>
<td>20.4-86.2</td>
</tr>
<tr>
<td>Sex: Male</td>
<td>50</td>
<td>0.4-198.1</td>
<td>53.5 (39.5-65.5)</td>
<td>45.8</td>
<td>37.5 (27.0-55.1)</td>
<td>21.2-70.6</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>2.6-273.7</td>
<td>50.5 (22.3-78.8)</td>
<td>65.4</td>
<td>30.2 (12.9-50.4)</td>
<td>8.4-64.7</td>
</tr>
<tr>
<td>Stage: II</td>
<td>5</td>
<td>0.41-33.3</td>
<td>13.1 (-3.7-29.8)</td>
<td>13.5</td>
<td>5.9 (0.4-33.3)</td>
<td>3.01-26.7</td>
</tr>
<tr>
<td>III</td>
<td>14</td>
<td>2.4-136.0</td>
<td>32.0 (9.1-54.9)</td>
<td>39.7</td>
<td>18.6 (3.4-32.2)</td>
<td>3.4-39.7</td>
</tr>
<tr>
<td>IV</td>
<td>28</td>
<td>8.4-198.1</td>
<td>67.2 (46.7-87.8)</td>
<td>53.0</td>
<td>53.4 (35.7-71.4)</td>
<td>28.3-94.1</td>
</tr>
<tr>
<td>V</td>
<td>26</td>
<td>23.7-273.7</td>
<td>53.5 (30.6-76.4)</td>
<td>56.7</td>
<td>35.0 (25.8-58.2)</td>
<td>23.7-64.7</td>
</tr>
<tr>
<td>II-III</td>
<td>19</td>
<td>0.4-136.0</td>
<td>27.1 (10.0-44.1)</td>
<td>35.4</td>
<td>18.2 (5.3-28.9)</td>
<td>3.4-30.2</td>
</tr>
<tr>
<td>IV-V</td>
<td>54</td>
<td>2.9-273.7</td>
<td>60.6 (45.7-75.5)</td>
<td>54.7</td>
<td>38.5 (32.3-62.6)</td>
<td>25.0-73.0</td>
</tr>
<tr>
<td>Substage: a</td>
<td>33</td>
<td>0.4-136.0</td>
<td>36.0 (23.5-48.5)</td>
<td>35.2</td>
<td>25.4 (16.1-35.3)</td>
<td>9.3-56.8</td>
</tr>
<tr>
<td>b</td>
<td>40</td>
<td>2.9-273.7</td>
<td>65.0 (45.7-84.3)</td>
<td>60.4</td>
<td>38.5 (32.5-65.4)</td>
<td>24.1-77.3</td>
</tr>
<tr>
<td>Site: GM</td>
<td>60</td>
<td>2.4-273.7</td>
<td>55.9 (41.5-70.4)</td>
<td>56.0</td>
<td>32.9 (26.0-55.9)</td>
<td>19.0-72.0</td>
</tr>
<tr>
<td>NGM</td>
<td>13</td>
<td>0.4-78.4</td>
<td>33.1 (19.3-46.9)</td>
<td>22.8</td>
<td>34.3 (15.5-45.4)</td>
<td>13.0-46.0</td>
</tr>
<tr>
<td>IPT: B-cell</td>
<td>25</td>
<td>2.9-184.9</td>
<td>51.6 (33.0-70.1)</td>
<td>44.9</td>
<td>33.3 (24.6-60.4)</td>
<td>21.8-70.8</td>
</tr>
<tr>
<td>T-cell</td>
<td>31</td>
<td>0.4-136.0</td>
<td>40.1 (29.0-51.1)</td>
<td>30.1</td>
<td>30.2 (21.2-46.8)</td>
<td>19.4-64.8</td>
</tr>
<tr>
<td>Albumin: NHA</td>
<td>40</td>
<td>0.41-198.1</td>
<td>37.4 (24.2-50.7)</td>
<td>41.5</td>
<td>24.9 (18.5-32.4)</td>
<td>10.7-49.9</td>
</tr>
<tr>
<td>HA</td>
<td>32</td>
<td>3.1-273.7</td>
<td>69.1 (47.5-90.7)</td>
<td>3.1</td>
<td>53.7 (35.2-69.9)</td>
<td>28.3-87.3</td>
</tr>
<tr>
<td>Calcium: NHC</td>
<td>62</td>
<td>0.41-273.7</td>
<td>49.2 (36.2-62.3)</td>
<td>51.3</td>
<td>33.8 (25.1-44.9)</td>
<td>17.5-64.7</td>
</tr>
<tr>
<td>HC</td>
<td>10</td>
<td>18-198.1</td>
<td>65.5 (22.0-109.0)</td>
<td>60.8</td>
<td>33.6 (21.2-108.8)</td>
<td>21.2-105.0</td>
</tr>
</tbody>
</table>

X-breed = crossbreed, GM = generalised multicentric, NGM = non generalised multicentric, IPT = immunophenotype, NHA = not hypoalbuminaemic, HA = hypoalbuminaemic, NHC = not hypercalcaemic, HC = hypercalcaemic

Distribution of CRP concentrations for the categorical variables was normal for only 4 groups according to the AD test: 0-3 year olds (p=0.06), crossbreeds (p=0.25), Stage II (p=0.27) and the ‘not generalised-multicentric’ group (p=0.65). CRP concentration was positively skewed for all variables.
3.5.1 Do the median CRP concentrations for the different clinical parameters differ?

There was significant difference in the median CRP concentration for the different WHO stages, indicating that CRP was influenced by WHO stage (Table 14). Initial comparison of grouped stages i.e. II-III and IV-V showed a significant difference between these 2 groups (H=14.9, d.f.=3, p=0.002). This difference was maintained when comparing the medians of each separate stage (H=14.9, d.f.=3, p=0.002). Further analysis was performed to compare each stage with the other stages. This showed a significant difference in the median CRP concentrations between stage II and IV (W=27.0, p=0.004), stage II and V (W=33.0, p=0.01), stage III and IV (W=199.0, =0.007), and stage III and V (W=210.0, p=0.03). There was no significance when comparing stage II and III (W=43.0, p=0.55) and stage IV and V (W=845.0, p=0.20).

Other variables which influenced CRP concentration significantly included WHO substage and albumin, where dogs that were substage ‘b’ had a significantly higher median CRP concentration compared to those that were substage ‘a’ (W=976.0, p=0.007), and where those dogs with hypoalbuminaemia had a significantly higher median CRP concentration compared to those dogs that were not hypoalbuminaemic (W=1175.0, p=0.001).

No other patient clinical parameters were found to influence CRP significantly as can be seen in Table 14. Figure 15 shows the distribution of CRP in the significant variable groups.
<table>
<thead>
<tr>
<th></th>
<th>Median (mg/L)</th>
<th>H-stat</th>
<th>d.f.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kruskal-Wallis analysis variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (0-3/3-7/&gt;7 years)</td>
<td>30.9/33.3/35.9</td>
<td>0.07</td>
<td>2</td>
<td>0.97</td>
</tr>
<tr>
<td>Stage (II/III/IV/V)</td>
<td>5.9/18.6/53.4/35.0</td>
<td>14.9</td>
<td>3</td>
<td>0.002*</td>
</tr>
<tr>
<td><strong>Mann-Whitney analysis variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>37.5/30.2</td>
<td>751.0</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Breed (pedigree/crossbreed)</td>
<td>34.3/43.3</td>
<td>2227.0</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Stage (II-III/IV-V)</td>
<td>18.2/38.5</td>
<td>419.0</td>
<td>0.0004*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(II/III)</td>
<td>5.9/18.6</td>
<td>43.0</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>(II/IV)</td>
<td>5.9/53.4</td>
<td>27.0</td>
<td>0.004*</td>
</tr>
<tr>
<td></td>
<td>(II/V)</td>
<td>5.9/35.0</td>
<td>33.0</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>(III/IV)</td>
<td>18.6/53.4</td>
<td>199.0</td>
<td>0.007*</td>
</tr>
<tr>
<td></td>
<td>(III/V)</td>
<td>18.6/35.0</td>
<td>210.0</td>
<td>0.03*</td>
</tr>
<tr>
<td></td>
<td>(IV/V)</td>
<td>53.4/35.0</td>
<td>845.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Substage (a/b)</td>
<td>25.4/38.5</td>
<td>976.0</td>
<td>0.007*</td>
<td></td>
</tr>
<tr>
<td>Site (GM/NGM)^</td>
<td>32.9/34.3</td>
<td>2282.0</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Immunophenotype (B-cell/T-cell)</td>
<td>33.3/30.2</td>
<td>756.0</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Albumin (non-/hypoalbuminaemic)</td>
<td>24.9/53.7</td>
<td>1175.0</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td>Calcium (non-/hypercalcaemic)</td>
<td>33.8/33.6</td>
<td>2207.0</td>
<td>0.37</td>
<td></td>
</tr>
</tbody>
</table>

^GM = generalised multicentric. NGM = non-generalised multicentric disease. * indicates significant variables.
Figure 14 Box-and-whisker plots of CRP concentration
a. WHO stage (II, III, IV & V); b. WHO substage (a & b); c. albumin status (not hypalbuminaemic & hypoalbuminaemic)
3.5.2 Is there a linear relationship between CRP concentration and the different clinical parameters?

As well as comparing the effect of clinical parameters on CRP concentration by categorical methods, the effects of different patient variables on pre-treatment CRP were also investigated using general linear regression, as shown in Table 15. To improve the assumption of normality, the CRP concentrations were log transformed (logCRP) prior to analyses. Univariate analysis showed WHO stage, substage and albumin to be significant variables and Figure 16 shows the fitted line plots and regression equations for these (the residual plots for all significant variables can be seen in Appendix 3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coef</th>
<th>95% CI</th>
<th>T-stat</th>
<th>F-stat</th>
<th>R^2 (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site (GM/NGM)^a</td>
<td>-0.11</td>
<td>-0.05, 0.27</td>
<td>1.40</td>
<td>1.97</td>
<td>2.70</td>
<td>0.17</td>
</tr>
<tr>
<td>WHO Stage (II-III/IV-V)</td>
<td>-0.28</td>
<td>-0.40, -0.15</td>
<td>-4.52</td>
<td>20.43</td>
<td>22.34</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>WHO Substage (a/b)</td>
<td>-0.17</td>
<td>-0.29, -0.06</td>
<td>-3.03</td>
<td>9.17</td>
<td>11.44</td>
<td>0.003*</td>
</tr>
<tr>
<td>Immunphenotype (B-/T-cell)</td>
<td>0.06</td>
<td>-0.07, 0.19</td>
<td>0.95</td>
<td>0.90</td>
<td>1.64</td>
<td>0.35</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>-0.03</td>
<td>-0.05, -0.01</td>
<td>-3.36</td>
<td>11.32</td>
<td>13.92</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

^aGM = generalised-multicentric, NGM = not generalised-multicentric; * indicates significant variables

Both WHO stage and substage were positively correlated with logCRP and as stage migrated from II-III to IV-V and substage migrated from ‘a’ to ‘b’ the logCRP increased. Albumin was negatively correlated and its concentration decreased as logCRP increased.
Figure 15 Fitted-line plots and regression equations for log-transformed CRP (logCRP) vs. a) WHO stage, b) WHO substage, c) albumin
Multivariate analysis was performed using the significant variables from the univariate analysis and only WHO stage retained significance (p=0.006, T=-2.86, F=8.19, $R^2$ 28.3%). The low $R^2$ value for the final model indicates that WHO stage only accounts for some of the elevation in CRP concentration and is not the only contributing factor.

### 3.5.3 Investigating the strength of association between the clinical variables and CRP concentration

The association of clinical variables with CRP was investigated as a binary outcome (≤30 mg/L and >30 mg/L) using logistic regression. The 3 predictors which were statistically significant were:

1. WHO stage IV-V compared to stage II-III was 6.09 times (95% CI 1.89-19.67) more likely to result in a CRP concentration >30 mg/L (p=0.002).

2. WHO substage ‘b’ compared to substage ‘a’ was 3.17 times (95% CI 1.20-8.32) more likely to result in a CRP concentration >30 mg/L (p=0.02).

3. Hypoalbuminaemia compared to non-hypoalbuminaemia was 2.74 times (95% CI 1.05-7.18) more likely to result in a CRP concentration >30 mg/L (p=0.04).

These 3 variables were tested in a multivariate model (with CRP categorised as the same binary outcome) and WHO stage retained significance, where stage IV-V compared to stage II-III was 4.07 times (95% CI 1.11-14.99) more likely to result in a CRP concentration >30 mg/L (p=0.03).

### 3.6 The effect of LSA remission status on CRP concentration

The test whether CRP concentration could act as a potential biomarker for remission status in dogs receiving chemotherapy for LSA, pre-treatment (Pre-TX) CRP concentration was used as baseline as it represented the CRP concentrations at the time of diagnosis. The remission statuses investigated included CR, PR and PD.
3.6.1 Does CRP concentration normalise following treatment with chemotherapy?

The effect of chemotherapy on CRP concentration at week 4 of chemotherapy was investigated. Dogs were excluded from analysis if they died or had PD at week 4 of chemotherapy, or if no further follow-up CRP concentrations were available after the Pre-Tx value. CRP concentrations were excluded if they were obtained at times of chemotherapy toxicity, and where possible, the next available CRP concentration was used. 41 dogs met the inclusion criteria, and of these dogs, 39 (95%) achieved CR, and 2 (5%) achieved PR by week 4 of treatment with chemotherapy. Of the dogs that achieved CR, 32 (82%) had CRP concentrations that were within the normal reference range by week 4 (range 0.2-44.9 mg/L, median 3.7 mg/L). Of the dogs that achieved a PR, both had CRP concentrations within the normal reference range (range 4.4-8.5 mg/L, median 6.5 mg/L) by week 4. Of the dogs that achieved CR, the CRP concentration at time of diagnosis was normal in 5 dogs (13%) and elevated in 34 dogs (87%). The range of CRP concentration for all dogs in this group was 0.4-184.9 mg/L and the median CRP concentration was 28.7 mg/L. The elevation in CRP concentration in the pre-treatment group was mild in 15 dogs, moderate in 13 dogs and marked in 6 dogs. Of the dogs that only achieved PR by week 4 of treatment, both had a CRP concentration that was elevated at time of diagnosis (range 22.5-78.4 mg/L, median 50.5 mg/L). The elevation in CRP concentration in this group was mild in 1 dog and moderate in the second dog. Figure 17 shows a boxplot of the Pre-Tx CRP concentrations vs. the CRP concentrations at week 4 post treatment for the dogs in CR and PR.
3.6.2 How does the CRP concentration differ between the different remission status groups

A remission status was recorded at every patient visit. Where a CRP concentration was available, this was pooled into the appropriate remission status group. CRP concentrations were excluded if they were obtained at times of concurrent disease or chemotherapy toxicity. The remission groups included the results from all 73 patients with LSA, and descriptive statistics were generated (Table 16).
Table 16 Descriptive statistics for CRP in the different remission status groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>Range (mg/L)</th>
<th>Mean (95% CI) (mg/L)</th>
<th>SD (mg/L)</th>
<th>Median (95% CI) (mg/L)</th>
<th>IQR (mg/L)</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Tx</td>
<td>73</td>
<td>0.4-273.7</td>
<td>51.2 (39.7-64.1)</td>
<td>52.3</td>
<td>34.3 (27.1-45.4)</td>
<td>19.1-67.0</td>
<td>2736</td>
</tr>
<tr>
<td>CR</td>
<td>311</td>
<td>0.0-160.4</td>
<td>10.0 (8.4-11.7)</td>
<td>14.8</td>
<td>5.5 (5.0-6.3)</td>
<td>2.8-10.9</td>
<td>219</td>
</tr>
<tr>
<td>PR</td>
<td>62</td>
<td>0.0-95.5</td>
<td>9.7 (6.2-13.1)</td>
<td>13.6</td>
<td>4.6 (3.3-9.4)</td>
<td>2.2-13.5</td>
<td>185</td>
</tr>
<tr>
<td>PD</td>
<td>118</td>
<td>0.2-294.0</td>
<td>36.6 (27.2-46.0)</td>
<td>51.7</td>
<td>14.3 (11.8-21.6)</td>
<td>5.8-49.1</td>
<td>2672</td>
</tr>
</tbody>
</table>

CRP was positively skewed for all groups and was not normally distributed (AD test p<0.005). A boxplot of each group showed there was a wider spread of CRP concentrations and higher medians in the Pre-Tx and PD groups. Each group had outliers (Figure 18).

![Box-and-whisker plot of serum CRP concentrations of the control dogs and dogs with lymphoma in different remission statuses](image)

**Figure 17 Box-and-whisker plot of serum CRP concentrations of the control dogs and dogs with lymphoma in different remission statuses**

### 3.6.3 Does the absolute CRP concentration differ with remission status?

The KW test was performed to compare the median CRP concentrations of the control group with that of the LSA dogs at each different remission status (using all CRP concentrations available for each remission status). This analysis confirmed there was a significant difference between the groups (H=128.3,
d.f.=3, p<0.001). To determine where the precise differences occurred, the MW test was performed looking at each pair of remission status groups (Table 17).

Table 17 Results of analysis with the Mann-Whitney-U test comparing median CRP concentrations of the control group and the groups of different remission statuses

<table>
<thead>
<tr>
<th>Analysis Groups</th>
<th>Median CRP (mg/L)</th>
<th>W-stat</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/CR</td>
<td>2.5/5.5</td>
<td>7006.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Control/PR</td>
<td>2.5/4.6</td>
<td>2903.0</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Control/PD</td>
<td>2.5/14.3</td>
<td>2704.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Pre-Tx/CR</td>
<td>34.3/5.5</td>
<td>22546.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Pre-Tx/PR</td>
<td>34.3/5.5</td>
<td>6709.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Pre-Tx/PD</td>
<td>34.3/14.3</td>
<td>8336.5</td>
<td>0.0003*</td>
</tr>
<tr>
<td>CR/PR</td>
<td>5.5/4.6</td>
<td>60016.0</td>
<td>0.68</td>
</tr>
<tr>
<td>CR/PD</td>
<td>5.5/14.3</td>
<td>60055.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>PR/PD</td>
<td>4.6/14.3</td>
<td>4028.5</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

* indicates a significant result

This analysis showed there were significant differences in median CRP of all groups except between the CR and PR group (p=0.68). Since CRP differed significantly between the remission statuses for all dogs in the study population, changes in CRP concentration for individual dogs over the course of treatment (i.e. assessment of within-dog variation) were also investigated.

### 3.6.4 Does the CRP concentration differ with remission status within individual patients?

#### 3.6.4.1 Pre-treatment compared to complete remission

For 43 dogs, statistical analysis was repeated using WSR test comparing each dogs’ Pre-Tx CRP concentration (range 0.405-269.7 mg/L, median 30.21 mg/L) with their first reported CRP concentration in CR (range 0.0-44.9 mg/L, median 3.73 mg/L). This analysis showed a significant difference in the median CRP concentration between these 2 groups (W=902.0, p<0.001) which confirmed the results for the whole population in section 3.6.2.
3.6.4.2 Complete remission compared to partial remission

To determine whether a difference between CR and PR could be detected on an individual dog basis, the WSR test was repeated for 26 dogs comparing a CRP concentration in PR (range 0.0-147.5 mg/L, median 5.96 mg/L) with their first subsequent CRP concentration in CR (range 0.0-91.0 mg/L, median 5.11 mg/L). This analysis showed that there was no significant difference between PR and CR (W=210.0, p=0.28) for each individual dog, which was consistent with the results for the whole population in section 3.6.2.

3.6.4.3 Pre-treatment compared to progressive disease

To determine whether the CRP concentration differed at time of relapse (PD) compared to Pre-Tx when considering individual dogs (Fig. 19), the WSR test was repeated for 57 dogs.

Figure 18 Box-and-whisker plot of CRP concentration for individual dogs at pre-treatment (Pre-Tx) and time of first relapse (PD).

The CRP concentration at Pre-Tx (range 0.4-184.9 mg/L, median 27.5 mg/L) was compared with the CRP concentration at their first subsequent episode of PD (range 0.2-294.0 mg/L, median 12.7). This analysis confirmed that there was a significant difference between Pre-Tx and PD CRP concentration (W=1172.0,
p=0.006) for each individual dog which agreed with the results for the whole population. The degree of change was determined for each individual dog and most dogs (n=41, 72%) had a lower CRP concentration at PD compared to the CRP concentration at time of diagnosis (median % difference -49.2%, range -98.7-956.1%). Sixteen dogs (28%) dogs however had a higher concentration i.e. they exhibited a positive % difference between PD and Pre-Tx.

3.7 Determining a Diagnostic Threshold for CRP

Although a normal reference range for CRP is available, an AUROC curve was generated using the control dogs and dogs with LSA to determine an upper limit of CRP concentration (cut-off value) which could potentially be used as a diagnostic threshold for LSA (Figure 20).

The area under the ROC curve was 0.94 (95% CI 0.9-1.0, p<0.001) indicating that CRP has a good ability to discriminate between individuals with LSA and those without. The current recognised upper reference limit for CRP is 10 mg/L and
the closest cut-off point in this AUROC curve was 9.5 mg/L. This cut-off had a sensitivity of 86.3% (95% CI 76.3-93.2%) and a specificity of 91.5% (95% CI 81.3-97.2%), with a likelihood ratio of 10.2. A CRP cut-off of 13.7 mg/L gave the best balance between sensitivity and specificity based on the AUROC curve results. At this threshold sensitivity was 83.6% (95% CI 73.1-91.2%) and specificity was 96.6% (95% CI 88.3-99.6%), with a likelihood ratio of 24.7. The likelihood ratio reached its maximum value of 46.9 at a CRP cut-off of 17.3 mg/L, and at this threshold although the sensitivity was reduced to 79.5% (95% CI 68.4-88.0%) the specificity was increased to 98.3% (95% CI 90.9-100.0%) indicating there would be fewer false positives if this cut-off was used. There was 100% specificity at a CRP cut-off of 30.7 mg/L.

### 3.8 Using CRP as a predictor of relapse in LSA

The ideal biomarker for LSA would become elevated prior to relapse of the disease i.e. before it was clinically apparent so that earlier therapeutic changes could be implemented and the overall survival time for the patient improved. The use of CRP as an early predictor of relapse was investigated using both a paired t-test and an AUROC curve.

#### 3.8.1 Does CRP concentration become elevated prior to relapse?

To determine whether CRP concentration became elevated immediately prior to an episode of relapse in individual dogs in our population, the CRP concentrations at 2 weeks prior to relapse (PD-2), 1 week prior to relapse (PD-1) and at time of relapse (PD) were examined. These data were only available for 29 dogs (Table 18). To improve the Gaussian distribution and to facilitate the use of the parametric paired t-test, CRP was log-transformed (logCRP).

<table>
<thead>
<tr>
<th>Impending relapse group</th>
<th>n</th>
<th>LogCRP range (mg/L)</th>
<th>Mean (mg/L)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-2</td>
<td>28</td>
<td>-0.32 to 1.41</td>
<td>0.67</td>
<td>0.45</td>
</tr>
<tr>
<td>PD-1</td>
<td>29</td>
<td>-0.96 to 2.21</td>
<td>0.82</td>
<td>0.68</td>
</tr>
<tr>
<td>PD</td>
<td>29</td>
<td>0.22 to 2.47</td>
<td>1.21</td>
<td>0.62</td>
</tr>
</tbody>
</table>

PD-2 = 2 weeks prior to relapse, PD-1 = 1 week prior to relapse, PD = time of relapse.
There was a general trend of CRP concentration becoming elevated over the 3 weeks, as can be seen in the boxplot in Figure 21. As might be expected, there was a significant difference between the mean logCRP concentrations of PD-1 and PD ($T=-3.92, p=0.001$) indicating that CRP concentration becomes elevated at the point of clinical relapse. However, there was no significant difference between the mean logCRP concentration between PD-2 and PD-1 ($T=-1.46, p=0.16$). These results indicate that CRP does not become elevated before the point of relapse (i.e. within the time period of 1-2 weeks before relapse) and only elevates at the point when relapse is already clinically apparent.

![Box-and-whisker plot of CRP concentration at 2 weeks (PD-2) and 1 week (PD-1) prior to relapse, and at relapse of disease (PD).](image)

To further investigate whether CRP concentration could guide clinical decision-making with regards impending relapse, an AUROC curve was generated using the CRP concentrations at PD and the CRP concentrations at time of CR as a control group (Figure 22). The area under the ROC curve was 0.73 (95% CI 0.67-0.78, $p<0.0001$) which indicates a moderate ability for CRP to discriminate between dogs in PD compared to those in CR. The cut-off value with the best balance between sensitivity and specificity was 11.9 mg/L. This level would not be particularly useful from a clinical perspective however as the sensitivity was only 61.3% (95% CI 51.6-70.4%) and specificity was only 77.7% (95% CI 72.5-82.3%). The results of this AUROC curve suggest that there is not enough difference between CRP concentrations in PD and CR to provide a useful cut-off
that could help guide clinical decision making with respect to whether a patient may be relapsing.

![AUROC curve of CRP concentration at progressive disease (PD) and complete remission (CR)](image)

### Figure 21 AUROC curve of CRP concentration at progressive disease (PD) and complete remission (CR)

<table>
<thead>
<tr>
<th>Area under the ROC curve</th>
<th>0.7248</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>0.02993</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.02993</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.6661 to 0.7834</td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Data</td>
<td></td>
</tr>
<tr>
<td>Controls (PD No)</td>
<td>296</td>
</tr>
<tr>
<td>Patients (PD Yes)</td>
<td>111</td>
</tr>
</tbody>
</table>

### 3.9 The prognostic significance of CRP concentration in dogs with LSA

To determine whether Pre-Tx CRP held any prognostic significance in dogs with LSA, both logistic regression and survival analysis was performed. To remove the effect on survival of different treatments and to homogenise the population, only the 51 dogs that were given a CHOP or modified CHOP protocol were included in the analysis.

### 3.9.1 Which patient variables affect OST in dogs with LSA?

To determine which parameters might affect survival time, different patient variables were examined at a univariate level using a logistic regression model with different binary outcomes (survival time ≤6 months vs. >6 months, dead vs.
alive, death due to LSA vs. death due to other reasons and relapsed vs. not relapsed). Continuous variables examined included log-transformed CRP, albumin and calcium. Categorical variables included WHO stage (II-III/IV-V), substage (a/b), immunophenotype (B-/T-cell), site (multicentric/not-multicentric), albumin (hypalbuminaemic/not hypoalbuminaemic), calcium (hypercalcaemic/not hypercalcaemic) and CRP (≤30 mg/L/>30 mg/L).

The only significant findings for survival were:

- WHO substage ‘b’ when compared to substage ‘a’ was 0.22 times (95% CI 0.07-0.73) more likely to result in an OST ≤6 months (p=0.013).

- T-cell immunophenotype when compared to B-cell was 0.28 times (95% CI 0.08-0.99) more likely to result in an OST ≤6 months (p=0.05).

Multivariate logistic regression analysis using the significant variables from the univariate analyses was performed and WHO substage retained significance with substage ‘b’ being 0.13 times (95% CI 0.03-0.56) times more likely to result in an OST ≤6 months (p=0.006).

Kaplan-Meier (KM) survival curves were generated for both patient variables significant on univariate analysis, and confirmed the significant differences in overall survival time (OST) for both immunophenotype and substage for those dogs treated with a CHOP-type protocol (Figure 23). Dogs were censored at the date of analysis if they had not died. Dogs with B-cell LSA had a median OST of 330 days compared to just 136 days for dogs with T-cell LSA (LR test p=0.0008, GBW test p=0.003), and the hazard ratio was 0.31 (95% CI 0.15-0.61). Dogs with substage ‘a’ had a median OST of 297 days compared to just 92 days for dogs with substage ‘b’ (LR test p=0.004, GBW test p=0.002), and the hazard ratio was 0.53 (95% CI 0.29-0.98).
3.9.2 Which patient variables affect time to first relapse (TFR) in dogs with LSA?

Further analysis of variables affecting period of remission revealed that the TFR was significantly different between the dogs with B-cell and T-cell immunophenotypes that were treated with a CHOP-type protocol (Figure 24). Dogs were excluded if they had died before a first relapse was reached and dogs were censored at the date of analysis if they had not reached the first relapse point. The median TFR was 270 days for dogs that had B-cell LSA and was 126 days for dogs that had T-cell LSA (LR test p=0.03). The hazard ratio was 0.41 (95% CI 0.18-0.93). No other variables significantly affected TFR.
Although absolute pre-treatment CRP concentration did not significantly affect survival by logistic regression, the different CRP elevation categories (normal, mild, moderate and marked) were also examined with respect to OST using KM survival curves (Figure 25). The outcome was days to death, and dogs were censored at time of analysis if they were still alive. The median survival times for each group differed (Table 19), but comparison of the curves with the LR test showed there was no statistically significant difference between the groups (p=0.71).

Table 19 Median survival times from the Kaplan-Meier survival curves of the different CRP elevation categories

<table>
<thead>
<tr>
<th>CRP elevation group</th>
<th>n=</th>
<th>Median survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>198</td>
</tr>
<tr>
<td>Mild</td>
<td>17</td>
<td>249</td>
</tr>
<tr>
<td>Moderate</td>
<td>24</td>
<td>123</td>
</tr>
<tr>
<td>Marked</td>
<td>5</td>
<td>328</td>
</tr>
</tbody>
</table>

**Median time to relapse:**
B-cell 270 days
T-cell 126 days

**Comparison of curves:**
Log-rank test p=0.03
Hazard ratio 0.41 (95% CI 0.18-0.93)
To increase the number of dogs in the groups, the KM survival curves were repeated by combining the dogs in the normal and mild CRP categories and those in the moderate and marked CRP categories (Figure 26). This resulted in 2 groups of dogs for analysis: those with pre-treatment CRP concentrations ≤30 mg/L (n=22) and those with pre-treatment CRP concentrations >30 mg/L (n=29).

Figure 24 Kaplan-Meier survival curves of overall survival time of CRP elevation categories for dogs on a CHOP-type protocol

Figure 25 Kaplan-Meier survival curve of overall survival time of dogs on a CHOP-type protocol with a CRP concentration 0-30 mg/L and >30 mg/L
The dogs in the ≤30 mg/L CRP group had a longer median survival time of 243 days compared to the dogs in the >30 mg/L CRP group who had a median survival time of 153 days, however significance was not reached when comparing the 2 curves using the LR test (p=0.91) and the GBW test (p=0.36).

### 3.9.4 CRP elevation categories and time to first relapse

To determine if any significant differences existed between the 2 CRP groups (CRP ≤30 mg/L [n=17], CRP >30 mg/L [n=20]) with regards TFR, KM survival curves were repeated for 37 dogs (Figure 27) with the outcome being the time in days from the start of treatment to when the patient relapsed. Patients were censored if they had not reached a first relapse by the time of analysis and dogs that died prior to relapsing were excluded from analysis.

![Figure 26 Kaplan-Meier survival curve for time to first relapse for dogs with a CRP concentration 0-30 mg/L and >30 mg/L](image)

The dogs in the ≤30 mg/L CRP group had a shorter median time to first relapse of 146 days compared to the dogs in the >30 mg/L CRP group (median TFR 242 days), however, significance was not reached when comparing the 2 curves using the LR test (p=0.42) or GBW test (p=0.70).
4 Discussion

4.1 Study aims

The aim of this longitudinal study was to investigate whether the acute phase protein CRP was a useful serum biomarker in dogs with LSA. The following points were examined and are discussed in more detail below:

1. How CRP concentration differs between dogs with LSA and normal healthy dogs.

2. The clinical parameters which affect CRP concentration at time of diagnosis in dogs with LSA.

3. How CRP concentration changes in dogs with LSA once treatment with chemotherapy is initiated.

4. How CRP concentration changes with different remission statuses and whether CRP can be used to predict relapse of LSA.

5. The prognostic significance of pre-treatment CRP concentration in dogs with LSA that have been treated with a CHOP-type chemotherapy protocol.

4.2 Discussion of results

4.2.1 CRP concentration in dogs with LSA

4.2.1.1 LSA dogs compared to healthy controls

This study of dogs with LSA confirmed that the median CRP concentration is elevated in this group of animals when compared to a healthy control group. As previously reported (Nielsen et al., 2007, Tecles et al., 2005), there was marked variability in CRP concentration in the dogs with LSA. The majority of control
dogs (92%, 54/59) had serum CRP concentrations within the normal reference (0-10mg/L) however the majority of dogs with LSA (86%, 63/73) had serum CRP concentrations above the normal reference. The CRP concentration can become markedly elevated in dogs with LSA, and a maximum 27-fold increase (273.7 mg/L) above reference was observed in this study, though most dogs (89%, 65/73) had less than a 10-fold increase which was consistent with earlier studies (Tecles et al., 2005, Nielsen et al., 2007, Mischke et al., 2007). As observed previously (Mischke et al., 2007, Tecles et al., 2005, Nielsen et al., 2007), not all dogs with LSA exhibited an elevation in CRP concentration and 14% (10/73) of dogs in this study had pre-treatment serum CRP concentrations that were within the normal reference range. The dogs with normal CRP concentrations presented with obvious gross disease and a variety of clinical signs, and all WHO stage and substage groups were represented. It was therefore unclear as to why these dogs did not have an elevated CRP concentration like the other dogs. CRP is a sensitive marker of inflammation and tissue injury and levels can fluctuate in response to insult within hours (Caspi et al., 1984). This study suggests however that the mechanism of inflammation in LSA is not simple and that CRP expression is very variable in dogs with this disease. It is currently unclear why some dogs do not appear to mount an inflammatory response despite the presence of gross disease and I propose that there may be a relationship between absolute gross tumour volume and the inflammatory response, and that there may be a critical threshold which must be exceeded before CRP concentration increases. The elevations in CRP may be as a direct consequence of the ischaemic damage to local tissues by lymphadenomegaly or may also be a consequence of the release of interleukin-6 (IL-6) from neoplastic B-cells (Kato et al., 1996). IL-6 concentration is elevated in human patients with LSA prior to treatment, and the degree of elevation is associated with the presence of clinical symptoms in this disease, a poorer response to treatment and reduced TFR and OST (Preti et al., 1997, Wieland et al., 2003, Legouffe et al., 1998, Gaiolla et al., 2011). It would be reasonable to suspect that elevated IL-6 causes similar consequences in the canine patient with LSA. Further investigation including measuring the levels of the cytokines that are known to drive CRP production such as IL-1, IL-6 and TNF-α may determine whether it is their levels which are contributing to the magnitude of the APR and hence the variability of serum CRP concentration. It should be noted however that recent investigation into TNF-α in dogs with LSA
has suggested that this cytokine has limited value as a tumour marker (Hofer et al., 2011).

The 5 dogs from the LSA population that were identified as being outliers with extreme elevations in CRP concentration were all diagnosed as having WHO Stage IV-V substage b multicentric LSA. Four of the 5 dogs (80%) had marked hepatic involvement with LSA, and indeed euthanasia was recommended for one patient due to the degree of hepatic damage that was present. Interestingly only 1 of the outlier dogs went on to receive chemotherapy treatment, with the owners of 3 of the dogs opting for euthanasia at time of diagnosis and one owner opting for palliative prednisolone followed rapidly by euthanasia. This may suggest that these dogs with extremely elevated CRP presented with worse clinical signs than other patients which encouraged clients to choose euthanasia over treatment, however other reasons for euthanasia could have included financial constraints or personal ethical beliefs of using chemotherapy to treat animals. The presence of such marked CRP production in the face of hepatic damage shows that the APR can still occur in dogs with infiltration of the liver, and perhaps that the liver is not the only site of APP production in these patients.

There was a large degree of variation in serum CRP concentration observed not only in the dogs with LSA (both at time of diagnosis and throughout the course of treatment) but also within the group of healthy control dogs which has been previously reported in dogs with LSA (Nielsen et al., 2007) and in client-owned healthy dogs (Carney et al., 2011). Despite most laboratory populations showing CRP concentrations within the normal reference range (Kuribayashi et al., 2003, Merlo et al., 2007), very variable concentrations of CRP have been reported exceeding the upper reference limit in at least one study involving a controlled laboratory population of dogs (Otabe et al., 1998). The elevation observed in our client-owned dogs may be due to the fact that, unlike laboratory animals, these dogs do not live in a strictly regulated environment and are exposed to a multitude of environmental insults which could stimulate the APR such as sub-clinical trauma, gastro-intestinal disturbances and infections. A relationship between increased serum CRP and exercise has been shown (Wakshlag et al., 2010) though this study looked at sled-dogs that were exposed to extreme levels of exercise and it is unclear what effect more modest levels of exercise would
have had on CRP production in our population of control dogs. These findings suggest that a standardised reference range may not be suitable, as clearly dogs can both exhibit an increased CRP concentration when there is no detectable disease present and exhibit a normal CRP concentration when there is a known disease present. Due to the variability of serum CRP, it has been suggested that an individual patient-centred baseline may be more appropriate and that multiple measurements of CRP concentration over time may prove more clinically relevant than a single absolute value (Kjelgaard-Hansen et al., 2003b). I believe that the degree of change in CRP concentration within a patient rather than the absolute change may be significant, however a much larger study cohort with greater homogeneity with regards clinical presentation and pathological diagnosis would be required to investigate this hypothesis.

4.2.1.2 The use of CRP concentration to aid diagnosis of LSA

The ability of CRP concentration to aid diagnosis of LSA was investigated by means of an AUROC curve (generated using the CRP concentrations from the control group and from the dogs with LSA at time of diagnosis). The AUROC curve indicated that CRP concentration has a good ability to discriminate between individuals with LSA and those without, and 13.7 mg/L was the cut-off CRP concentration with the best balance between sensitivity (83.6%) and specificity (96.6%). This cut-off is only just above the published upper reference limit for CRP (Eckersall et al., 1989) and reflects the fact that many of the dogs with LSA in our study had normal or only mild elevations in CRP concentration at time of diagnosis. To test the cut-off value obtained from the AUROC curve it should have been possible to calculate the positive and negative predictive values using CRP concentrations obtained from other control dogs and dogs known to have LSA or other diseases. However, this was not possible within the limits of this study due to a lack of availability of further control samples and samples from dogs with known diseases, including LSA.

Other researchers have found CRP useful in diagnosing LSA. CRP is currently included in a commercial proteomic assay which has been developed using chromatography and mass spectrometry to identify the serum protein fingerprint of dogs that have LSA (PetScreen Ltd, UK). During the development of this assay
13 protein peaks were identified that differed significantly between dogs with and without LSA, with 2 of these peaks specifically capable of differentiating between the 2 groups of dogs. One of these protein peaks has since been identified as being the positive APP haptoglobin (Hp); the second is still under investigation. To improve the performance of a single biomarker alone, the PetScreen Ltd assay combines different biomarkers, currently the APPs Hp and CRP. Results from 194 test samples have provided encouraging results with an assay sensitivity of 75-85% and a specificity of 91-93%, with a negative predictive value of 88-93% and a positive predictive value of 80-86% (Ratcliffe et al., 2009, PetScreen Ltd, 2012). Although this assay has been marketed as an early diagnostic test which should be used when a dog first presents with enlarged lymph nodes, the assay does not have 100% sensitivity and specificity and therefore a suspected diagnosis should always be confirmed by cytological or histopathological methods. These allow a definitive diagnosis to be made with specific classification of the type of LSA, and can offer information regarding the grade and immunophenotype which may guide therapeutic options and provide prognostic information (Ponce et al., 2004, Marconato, 2011, Marconato et al., 2011, Williams et al., 2008).

4.2.2 The relationship between CRP concentration and LSA patient variables

4.2.2.1 Pre-treatment albumin

The concentration of the negative acute phase protein albumin had a modest but significant inverse correlation with log-transformed CRP (logCRP) concentration in our group of dogs with LSA. Similar findings have been reported in humans with lymphoid neoplasia and have been shown to have prognostic significance (Elahi et al., 2005, Nakamura et al., 2008). The median albumin concentration was significantly lower in dogs with WHO stage IV-V and substage ‘b’ compared to dogs with WHO stage II-III and substage ‘a’, however it is worth noting that the median albumin concentration of dogs with stage IV-V and substage ‘b’ was only just below the lower reference limit of 29 g/L. Univariate analysis using logistic regression with CRP concentration as a binary outcome (≤30 mg/L and >30 mg/L) showed that hypoalbuminaemia was a significant
predictor and was 2.74 times (95% CI 1.057-7.18) times more likely to result in a CRP concentration >30 mg/L when compared to non-hypoalbuminaemic dogs with LSA. Hypoalbuminaemia can arise in dogs with LSA for a myriad of reasons including as a direct consequence of intestinal or renal involvement resulting in protein loss (Dossin and Lavoué, 2011, Bryan et al., 2006), from chronic anorexia causing malnutrition and protein catabolism (McMillan, 2009), and from hepatic involvement causing reduced production of the plasma proteins (Dank et al., 2011, Hoskins, 2005, Balkman, 2009, Gruys et al., 2005). In humans, it has recently been hypothesised that the reduction in the negative APPs, which includes albumin, is affected more greatly by the APR than the nutritional state (Fuhrman et al., 2004, McMillan, 2009). Most of the 15 dogs that had a more moderate to marked hypoalbuminaemia (defined here as an albumin concentration ≤24 g/L) presented either with chronic diarrhoea, hepatic infiltration including marked hepatic disease as observed by elevated liver enzymes, and/or a history of anorexia and weight loss. The significant negative correlation with logCRP suggests there is an inverse relationship between the negative APP albumin and the positive APP CRP, likely due to the activation of the APR which causes altered mobilisation of the amino acids by the liver (Kushner, 1982, Gabay and Kushner, 1999, Ebersole and Cappelli, 2000, Nakamura et al., 2008). However, the low $R^2$ (13.92%) for the model implies that CRP is not the only driver of albumin levels in these dogs, and it would seem reasonable that the reduction in albumin concentration in our population was also as a direct consequence of the disease.

4.2.2.2 Clinical stage of LSA

The association between cancer and the APR has been widely investigated and a positive correlation between CRP concentration and both the clinical stage of lymphoid neoplasia and the presence of clinical signs at time of diagnosis has been reported for humans (Wieland et al., 2003, Herishanu et al., 2007, Legouffe et al., 1998, Khalifa et al., 2008). However, to date this has not been reported for dogs. Univariate analysis with linear regression revealed a significant positive relationship between logCRP concentration at time of diagnosis and WHO stage and substage, though this relationship was only present when grouped stages II-III were compared to stages IV-V, with no significant
difference determined when comparing the 2 lower stages (II and III) to each other and the 2 higher stages (IV and V) to each other. Following multivariate analysis, WHO stage was the only variable which retained significance with stages IV-V having a significantly increased concentration of logCRP compared to stages II-III. Logistic regression using CRP concentration as a binary outcome (≤30 mg/L and >30 mg/L) instead of an absolute concentration, also revealed WHO stage and substage to be significant categorical predictors, and once again on multivariate analysis WHO stage retained significance with stages IV-V 4.07 times (95% CI 1.11-14.99) more likely to result in a CRP concentration >30 mg/L when compared to stages II-III. The significant relationship between WHO stage, and to a lesser degree substage, in this population of dogs with LSA suggests that in this disease the APR is influenced by clinical status of the animal rather than merely just the underlying primary disease. Dogs with advanced WHO stages of disease tend to have heavier tumour burden and grossly enlarged lymph nodes which cause ischaemic damage to surrounding tissue with localised inflammation. The rapid growth of the tumour results in high cell turnover and constant death of neoplastic cells which release cytokines. Tumour cells can also produce chemokines that attract local inflammatory cells such as macrophages and neutrophils (Coussens and Werb, 2002). These inflammatory cells secrete cytokines including interleukins IL-1, IL-6 and TNF-α into the blood which induce the hepatocytes to secrete the APPs, such as CRP (Coussens and Werb, 2002, Gruys et al., 2005). This study only had a small number of dogs in the lower WHO stage groups and didn’t include any dogs with stage I disease; also the exact quantity of tumour burden was not available. A larger cohort containing more dogs with lower WHO stages, and an accurate assessment of tumour burden using advanced imaging modalities such as computed tomography would be required to determine if there were significant differences between the individual WHO stages and whether tumour burden had a significant relationship with serum CRP concentration.
4.2.3 Changes in CRP concentration in dogs receiving chemotherapy

4.2.3.1 CRP concentration following treatment initiation

The change in CRP concentration between week 0 (pre-treatment) and week 4 of treatment was investigated in 41 dogs that exhibited some degree of response to treatment with chemotherapy (i.e. had achieved PR or CR). Of these dogs, 34 (87%) had a pre-treatment CRP above reference. Following induction of a chemotherapy protocol, the majority of dogs (83%) that had had a response to treatment had a CRP concentration within the normal reference limit.

These results suggest that treatment with chemotherapy causes normalisation of CRP concentration in most dogs by week 4 of treatment. The effect on CRP concentration by the actual chemotherapy agents administered, and from the glucocorticoids that were also included in the protocols, could not be quantified in these dogs due to the variability in chemotherapy protocols used and the timing of samples. Previous studies investigating the effect of chemotherapy and prednisolone on healthy dogs have suggested that these agents do not affect serum APP concentrations (Ogilvie et al., 1993, Merlo et al., 2007, Martínez-Subiela et al., 2004) and it would be reasonable therefore to suspect that the reduction of CRP in these dogs was directly related to the reduction in tumour size rather than being treatment-mediated.

4.2.3.2 CRP concentration and remission status

There was marked variation in CRP concentration and overlap of the range of concentrations at time of diagnosis (Pre-Tx) and at each remission status, which supports the current literature (Nielsen et al., 2007). The widest spread of CRP concentrations was found for Pre-Tx and progressive disease (PD) indicating the results were most variable for dogs in these states. When examining the differences that existed between the remission statuses with the pooled samples from all dogs, median CRP concentration in the control population and at time of diagnosis (Pre-Tx) was significantly different when compared to CR, PR and PD. Median CRP concentration also differed between CR and PD, and PR and PD.
There was no significant difference between the median CRP concentration of dogs in CR and PR.

To determine if these differences still existed when considering individual dogs, comparison of the CRP concentrations at Pre-Tx with their first subsequent CRP concentration at CR (after week 4 of treatment), and comparison of the CRP concentration at PR with their first subsequent CRP concentration at CR was performed on matched samples from individual dogs. The results from these analyses were in agreement with the results from the analysis of the pooled samples.

The lack of significant difference between the median CRP concentration for CR and PR was interesting. Dogs in PR are still showing signs of gross clinical disease and by definition ‘PR’ indicates that the tumour has shrunk by >30% but <100% (Eisenhauer et al., 2009). One would therefore expect there to be a higher CRP concentration in dogs in PR compared to those in CR (where there is no gross clinical disease present). This finding suggests that despite the presence of disease in PR, the tumour burden has decreased enough to reduce the degree of local inflammation and ischaemia. Consequently, there is minimal activation of the APR and reduced production of CRP.

The results of the pooled and paired analyses suggest that there are significant differences present between the median CRP concentrations of the different remission groups (except between CR and PR) which are in agreement with the current literature (Nielsen et al., 2007). However, there are often only subtle differences as observed by the overlap of the ranges of CRP concentration in the different groups. This indicates that CRP would not be a useful biomarker to discriminate between the different remission statuses and could not aid clinical decision making with regard to categorising patients following treatment with chemotherapy.

### 4.2.3.3 CRP concentration at time of relapse of LSA

The pooled analysis revealed a significant difference between the median CRP concentrations in the Pre-Tx group compared to PD group. Comparison of individual dogs’ pre-treatment CRP concentration with their first subsequent
relapse (PD) CRP concentration again revealed that a significant difference exists. In the majority of dogs (72%), the CRP concentration was lower at the point of relapse compared to the time of diagnosis.

The fact that CRP concentrations at time of diagnosis were not the same as those obtained at time of relapse could be because relapse is often detected much more rapidly than when the disease is initially diagnosed, as patients are being more closely monitored. Consequently, the degree of tumour burden at this time is often much less than it is at the time of diagnosis. The fact that 28% (16/57) of dogs had a higher relapse (PD) CRP concentration compared to their Pre-Tx value however suggests that the response of individual dogs is very variable. There is also the possibility that for these dogs with elevated CRP concentration at relapse, in addition to clinically detectable peripheral disease, there may have been internal gross disease. This degree of disease is usually not quantified by diagnostic imaging techniques due to financial constraints and the fact that it generally would not change the clinical outcome. These findings again suggest that there may be a relationship between tumour burden and CRP concentration which warrants further investigation and indeed, it has been suggested in patients with NHL that tumour burden could be used as a prognostic factor (Gobbi et al., 2004). I propose that there may be a minimum threshold for tumour volume which must be exceeded before the APR is activated which would account for the variance in CRP concentration at time of diagnosis and relapse.

4.2.4 CRP as a predictor of relapse

CRP has a moderate ability to discriminate between dogs with CR and PD, and a cut-off value of 10.7 mg/L was deemed suggestive of relapse following AUROC curve analysis. This cut-off value was only just above the upper reference limit for CRP concentration, and there was poor balance between sensitivity (60.7%) and specificity (73.3%), suggesting it would not be useful in a clinical setting.

The role of CRP as a predictor of relapse was more closely examined using matched samples from individual dogs. The ranges of CRP concentration at 3 time points (2 weeks prior to relapse, 1 week prior to relapse, and point of clinical relapse) showed marked overlap, and the only significant difference
between these 3 time points occurred between the mean logCRP concentrations at 1 week prior to relapse and time of relapse. This suggests that the CRP concentration becomes elevated at the point of relapse but does not increase significantly in the weeks immediately leading up to that point, which adds further weight to the suggestion that CRP production is mediated directly by the tumour and that a certain volume of gross disease is required before an elevation in this biomarker is apparent. These findings imply that CRP is not a useful biomarker for the early prediction of relapse of disease in LSA which agrees with the earlier findings by Nielsen et al (2007).

4.2.5 CRP concentration and prognosis of LSA

In human medicine the presence of a systemic inflammatory response and the magnitude of that response, as observed by CRP elevation, has been shown to predict the duration of cancer-specific and non-cancer disease survival in a variety of solid tumours and lymphoid neoplasia (McMillan et al., 2001, Elahi et al., 2005, Shimada et al., 2003, Falconer et al., 1995, Beer et al., 2008, Karakiewicz et al., 2007). In this study CRP was not predictive of survival or TFI, neither as a continuous variable (i.e. log-transformed CRP), nor as a categorical variable (i.e. CRP concentration ≤30 mg/L or >30 mg/L). The median survival time was shorter for those dogs in the CRP >30mg/L group compared to those in the ≤30 mg/L group; however this difference was not significant. These initial findings suggest that a difference in survival may exist between dogs with moderately and markedly elevated CRP concentrations and it is possible that the lack of significance in survival reflects the small number of dogs in each of these groups. Further analysis with a larger sample size is needed to confirm or refute this suspicion.

There are many human studies that have shown survival advantages for those patients that present with a normal CRP concentration over those that present with an elevated CRP concentration in a variety of tumours (Beer et al., 2008, Crozier et al., 2007, Shimada et al., 2003, Hashimoto et al., 2005, Yoshida et al., 2008); however there is currently no veterinary literature with similar findings. Ideally, any future studies looking at CRP concentration with regards to OST in dogs with LSA, would include enough dogs with pre-treatment CRP
concentrations in the normal range to allow for comparison with dogs with elevated CRP concentrations.

**4.2.6 Negative prognostic indicators for LSA in this study**

The presence of clinical signs at diagnosis (i.e. being classified as WHO substage ‘b’) and the T-cell immunophenotype were shown to be significant negative predictors of OST in this study of dogs with LSA. Other patient variables including WHO stage, site of disease, albumin concentration and calcium concentration were not found to have any prognostic significance.

It is generally accepted that WHO substage and immunophenotype carry prognostic significance (Keller et al., 1993, Kiupel et al., 1999, Baskin et al., 2000, Dobson et al., 2001, Marconato et al., 2011). However, there is a degree of disparity in the literature as to what other prognostic factors are significant, with some authors suggesting that increased WHO stage, the presence of hypercalcaemia at time of diagnosis and previous treatment with steroids carries a poorer prognosis (Jagielski et al., 2002, Gavazza et al., 2009, Kaiser et al., 2007).

In human medicine, the traditional primary method for assessing prognosis for NHL was to consider the Ann Arbour stage, which is similar to the WHO staging system used in dogs with LSA. This system alone was found to be inadequate at predicting survival and therefore the International Prognostic Index (IPI) was developed. This considers a range of clinical parameters to predict patient outcome more accurately including age, sex, Ann Arbour stage, LDH concentration, performance status and the number of extranodal sites of disease. Using a point system for each of the risk factors, the sum of the points assigned to each risk factor correlates with a risk group which provides a predicted 5 year survival rate (The International Non-Hodgkin's Lymphoma Prognostic Factors Project, 1993, Hermans et al., 1995). Some veterinary researchers are also moving towards considering a panel of different patient variables to predict more accurately remission period and survival time (Marconato et al., 2011).
4.2.7 Other possible serum biomarkers for canine LSA and their clinical relevance

4.2.7.1 Serum biomarkers reported in the veterinary literature

The ability of other serum biomarkers to predict disease relapse has been investigated in dogs with LSA. Those shown to have the greatest clinical potential include serum TK, AGP and LDH. TK is a cytoplasmic enzyme which catalyses the phosphorylation of thymidine to thymidine monophosphate and exists in two forms: cytoplasmic thymidine kinase 1 (TK-1) and mitochondrial thymidine kinase 2 (TK-2). TK-1 is associated with cellular proliferation and may be of clinical relevance in dogs with LSA as this disease is characterised by a very high rate of proliferation which is reflected in the levels of TK-1 (von Euler and Eriksson, 2011). Serum TK levels are elevated in dogs with LSA, and concentration normalises once remission of disease is achieved (von Euler et al., 2004, Nakamura et al., 1997, Elliott et al., 2011). AGP is a glycosylated protein which is a positive APP which behaves in a similar way to CRP in dogs with LSA. Its concentration is often elevated at time of diagnosis and at relapse of disease, and normalises once remission has been achieved (Ogilvie et al., 1993, Hahn et al., 1999). Serum concentrations of both serum TK and AGP have been shown to become elevated at least 3 weeks prior to relapse of disease becoming clinically evident (Hahn et al., 1999, von Euler et al., 2004). LDH is a glycolytic enzyme which catalyses glycolytic metabolism by converting lactate to pyruvate, and is expressed as 5 isoenzymes. LDH is elevated in dogs with LSA, specifically the isoenzymes LDH2 and LDH3 (Zanatta et al., 2003, Nakamura et al., 1997, Marconato et al., 2009, Marconato et al., 2010). The predictive ability of LDH was revealed in one study, where dogs with LSA that had elevated LDH concentrations at the end of treatment or 1 month after completion of chemotherapy were more likely to relapse within a 45 day period than those dogs with a normal LDH concentration at the same time points (Marconato et al., 2010). Showing similarities to CRP, serum TK and LDH concentrations show marked variance in dogs with LSA, and their concentrations are not elevated in all dogs (Elliott et al., 2011, Nakamura et al., 1997). This suggests that the mechanism for the expression of these enzymes is not fully understood in this complex disease.
Some of the biomarkers that may be useful in predicting early relapse of LSA may also have prognostic significance, and it has been hypothesised that elevated concentrations of serum TK and LDH at time of diagnosis result in reduced disease-free interval (DFI) and OST. However, there is currently disparity in the literature and further investigation is required (Elliott et al., 2011, von Euler et al., 2004, Zanatta et al., 2003, Marconato et al., 2010). It has been suggested by several authors that the presence of anaemia (described as a haematocrit count ≤35-40%) at time of diagnosis is a negative prognostic indicator in dogs with LSA (Abbo and Lucroy, 2007, Marconato et al., 2011, Miller et al., 2009). Unlike serum TK and LDH, the haematocrit is routinely measured at initial presentation as part of the clinical staging of the patient and may prove an easy and inexpensive biomarker to use in general practice. This evidence would suggest that these serum biomarkers may be useful in the prediction of patient outcome and disease monitoring in dogs with LSA, however further validation is required before serial monitoring would be implemented into routine clinical practice.

Some serum biomarkers which have been shown to have some clinical relevance in human patients with LSA are also currently under investigation in veterinary science. These include the pro-angiogenic molecules matrix metalloproteinases (MMPs), and the cytokine, vascular endothelial growth factor (VEGF). Angiogenesis plays an important role in the pathophysiology of solid tumours and haematologic malignancies such as LSA, and circulating levels of MMP 2 and 9 and VEGF have been shown to be overexpressed in dogs with LSA. Increased levels of these factors correlate with poor response to treatment and a poor prognosis, both with respect to DFI and OST (Gentilini et al., 2005, Aresu et al., 2012). VEGF also appears to correlate with the degree of malignancy of LSA in dogs (Zizzo et al., 2010). High mobility group box 1 (HMGB1) protein is a nuclear chromosomal protein found in nearly all cells. It is a cytokine which is secreted by activated macrophages and monocytes, and mediates the response to injury, infection and inflammation (Lotze and Tracey, 2005). Overexpression of HMGB1 is associated with the hallmarks of cancer, including angiogenesis, evasion of programmed cell death, and limitless replicative ability (Tang et al., 2010). HMGB1 concentration is significantly higher in dogs with LSA when compared with control dogs. Its concentration reduces in dogs with LSA which are treated
with chemotherapy, and early results indicate that levels may be lower in dogs which have achieved CR compared to those which have achieved PR (Meyer et al., 2010). Monocyte chemotactic protein 1 (MCP1) stimulates myeloid cells from the bone marrow and is a cytokine which recruits monocytes, and other cells such as neutrophils and NK cells, to sites of inflammation, injury and infection (Yadav et al., 2010, Melgarejo et al., 2009). A recent study by Perry et al (2010) investigating this protein, showed that serum levels are significantly elevated in dogs with LSA when compared to a control group. It has been suggested by the authors that an increased concentration of MCP1 in conjunction with elevated circulating concentrations of monocytes and neutrophils may be associated with poorer DFI for dogs receiving cytotoxic therapy. Finally, circulating tumour-derived DNA has been quantified in the plasma of dogs with LSA and the levels were significantly increased in this disease when compared to healthy dogs (Schaefer et al., 2007). The levels of plasma DNA have a potential prognostic significance and it has been shown that those dogs with higher concentrations have shorter remission periods than those dogs with lower concentrations (Schaefer et al., 2007).

Despite promising early results for these serum biomarkers, they are currently only available for quantification in research laboratories, and therefore can not currently be applied in general practice.

4.2.7.2 Serum biomarkers warranting future investigation

In human medicine, the human leukocyte antigen (HLA) class 1 molecule beta-2 microglobulin (B2M) has been investigated in patients with LSA. B2M is a primitive immunoglobulin which is involved in the immune response. Serum B2M is elevated in people with LSA, and its concentration drops following treatment with chemotherapy (Child et al., 1980, Bien and Balcerska, 2009, Johnson et al., 1993). It has been proposed that persistent elevations reflect resistant or partially responsive disease (Child et al., 1980). B2M appears to have prognostic significance with levels of this molecule allowing the accurate separation of NHL patients into low-, medium- and high-risk categories (Avilés et al., 1992). Findings from another study showed that there was a greater proportion of patients with a 6-year remission rate who had a normal concentration of B2M at
time of diagnosis when compared to those with elevated concentrations of β2M (Johnson et al., 1993). This molecule has been examined in conjunction with other serum biomarkers including CRP and LDH, and the presence of increased concentrations across multiple markers have been found to be correlated with the presence of bulky disease, b-symptoms and advanced stage and hepatic infiltration (Johnson et al., 1993, Bien and Balcerska, 2009). To date, there are no studies examining this biomarker in canine LSA and unfortunately, there is no commercial assay currently available.

4.3 Study limitations

All dogs in this study had been referred from other veterinary practices, and as such, there may have been a bias towards those dogs that had more aggressive and advanced disease, and that were exhibiting worse clinical signs of disease. This would explain the lack of WHO stage I dogs and the paucity of dogs with WHO stage II disease in this cohort. The T-cell immunophenotype was over-represented and may explain why our median survival times for dogs treated with a CHOP-type protocol was less than the established literature (Chun, 2009, Garrett et al., 2002, Baskin et al., 2000). There may also have been variability in the classification of remission status during the monitoring of dogs on treatment. Remission is objectively assessed, and multiple staff members were often involved in the treatment of any one case. The classification of remission status was made in accordance with the RECIST guidelines v1.1 (Eisenhauer et al., 2009); however the decision-making process routinely involved the palpation and measurement of peripheral lymph nodes only. Abdominothoracic imaging was generally only performed to confirm remission status if there was no peripheral disease present at time of initial presentation. This may have resulted in a potential bias towards more dogs being classified as being in CR when they were in fact only in PR. It is generally accepted that there is disparity between the classifications of remission status obtained from the measurement of peripheral gross tumour volume using calipers when compared to more objective assessment techniques such as flow cytometry of lymph nodes to assess minimum residual disease (Williams et al., 2005). Currently however, gross evaluation of dogs to determine the response to treatment is the standard practice within veterinary medicine as repeated imaging is expensive and often
difficult to justify to clients for a patient that is clinically well, especially when the findings do not often facilitate a change in the treatment protocol.

A complicating factor in this study was that several dogs with LSA had concurrent disease at time of diagnosis. It is not uncommon for dogs with LSA to present in this way as many are mature animals and chronic diseases such as osteoarthritis and degenerative heart disease are common in older age. No difference was noted in pre-treatment CRP concentration in dogs with LSA that presented with a variety of inflammatory compared to non-inflammatory concurrent diseases. The elevation of CRP is greater in infectious conditions and haematologic neoplasms (including LSA) compared to other chronic conditions (Nakamura et al., 2008, Kjelgaard-Hansen et al., 2003a). No dogs with concurrent disease were excluded unless they presented with active infection as it was felt that the resultant population of dogs with LSA was very typical of the general canine LSA caseload and would provide a more accurate investigation of the use of CRP as a biomarker in a true clinical setting. Dogs were excluded from this study if they had received previous corticosteroid therapy despite initial analysis suggesting no significant difference between the median CRP concentrations in those dogs on steroids compared to those that were not. A study by Martínez-Subiela, Cerón and Ginel (2004) also found no significant difference in the CRP concentration in dogs prior to and after administration of immunosuppressive doses of prednisolone. However, those dogs were healthy and as glucocorticoids are known to cause apoptosis of neoplastic lymphoid cells which could result in the release of inflammatory mediators (Schwartzman and Cidlowski, 1994, Moalli and Rosen, 1994) it was deemed more robust to exclude these dogs from this study.

The major limitations of this study were that it was retrospective in design and that there was a large variation in sample numbers obtained from each patient with LSA due to the use of different chemotherapy protocols. Also, samples were not obtained at every chemotherapy time point nor at consistent time points between patients, and there was no control sample for every time point. Another limitation was that many samples had to undergo multiple freeze-thaw cycles over several years due to the ongoing modification of the CRP assay used. The stability of CRP at -70°C and its ability to withstand multiple freeze-thaw cycles has been previously investigated (Aziz et al., 2003, Macy et al., 1997) and
our comparison of CRP concentrations over the different freeze-thaw cycles confirmed that it was unlikely that significant degradation to the protein had occurred which would have resulted in lower values than previously obtained.

Despite this being the biggest study to be performed investigating serum CRP concentration in dogs with LSA, the numbers were still relatively small. The lack of homogeneity within the population was also a limitation, however in order to increase numbers within the study to facilitate statistical analysis, dogs from all WHO stages and substages, sites, grades and immunophenotypes had to be included, and indeed it was of interest to see if any of these clinical variables had any bearing on CRP concentration.

### 4.4 Conclusions

This study has demonstrated that serum CRP concentration is significantly higher in dogs with LSA compared to a healthy control population. Serum CRP concentration returns to normal in most dogs following initiation of a chemotherapy protocol, however a significant difference remains between the median CRP concentration in dogs in CR, as judged by LN palpation, and the control dogs. The median CRP concentration differs in patients in PD compared to those in CR and PR. There is too much variability in CRP concentration within each remission group and too much overlap of concentrations to allow it to be used to monitor response to treatment or to predict disease relapse. More objective quantification of lymph node size using advanced imaging techniques such as CT or PET-CT at time of diagnosis and throughout treatment may help determine if there is a relationship between CRP concentration and gross tumour volume, which may help explain the variability in CRP concentration. These techniques, along with other techniques for assessing minimum residual disease such as flow-cytometry or PCR of lymph node aspirates, may also facilitate more accurate assessment of remission status and reveal those patients that have recrudescence of disease or have refractory disease.

A significant relationship has been revealed between the CRP concentration in dogs with LSA and WHO stage, substage and albumin concentration. A significant relationship between pre-treatment albumin and WHO stage and substage has
also been revealed. Pre-treatment CRP concentration does not appear to have any prognostic significance, though there is a trend for the median survival time for dogs with CRP concentrations ≤30 mg/L to be longer than for those with a CRP concentration >30 mg/L. These results warrant further investigation with a larger cohort of dogs.

The findings in this study suggest that there are potential clinical applications for monitoring serum CRP concentrations in dogs with LSA. Serum CRP concentration at time of diagnosis may be of some value, though its use for serial monitoring throughout treatment is more questionable due to the variability of CRP concentration across the different remission statuses. Rather than focusing purely on CRP it would seem reasonable to suggest that future work would include the investigation of a panel of serum biomarkers known to be useful in LSA such as albumin, haematocrit, TK, AGP and LDH. The use of multiple markers may improve the utility of any single biomarker alone. Analysis of this panel at different time points including at diagnosis and serially throughout treatment may provide greater prognostic information and give earlier evidence of impending relapse of disease. This could facilitate treatment modifications which would improve the time to first relapse and overall survival time for dogs with LSA.
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### Appendix 1: Chemotherapy protocols

#### Table 20 CHOP protocol

| Week | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Drug | *V | C | ^D/E/M | #P | *V | C | ^D/E/M | #P | *V | C | ^D/E/M | #P | *V | C | ^D/E/M | #P | *V | C | ^D/E/M | #P | *V | C | ^D/E/M | #P |
|      | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

* Protocol can be modified to include administration of l-asparaginase (400 iu/Kg or 1,000 iu/m^2^) up to 1 week prior to week 1 vincristine.

*V = vincristine (0.7 mg/m^2^); ^C = cyclophosphamide (250 mg/m^2^); ^D/E/M = doxorubicin (30 mg/m^2^)/epirubicin (30 mg/m^2^)/mitoxantrone (5.5 mg/m^2^); #P = prednisolone (2 mg/Kg SID week 1, 1.5 mg/Kg SID week 2, 1 mg/Kg SID week 3, 0.5 mg/Kg SID week 4).

#### Table 21 ALP protocol

<table>
<thead>
<tr>
<th>Drug</th>
<th>Week</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-asparaginase (400 iu/Kg or 10,000 iu/m^2^)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Lomustine (60-90 mg/m^2^)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Prednisolone^</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^ Prednisolone given as a tapering dose: 2 mg/Kg SID for 2 weeks then 1 mg/Kg SID for 2 weeks then 1 mg/Kg EOD for 2 weeks

#### Table 22 Low dose (LD) COP protocol

<table>
<thead>
<tr>
<th>Phase</th>
<th>Drug</th>
<th>Induction</th>
<th>Maintenance (after 2 months)</th>
<th>Maintenance (after 6 months)</th>
<th>Maintenance (after 12 months)</th>
<th>Maintenance (after 18 months)</th>
<th>Maintenance (after 21 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Drugs given 1 week in 2</td>
<td>Drugs given 1 week in 3</td>
<td>Drugs given 1 week in 4</td>
<td>Drugs given 1 week in 5</td>
<td>Drugs given 1 week in 6</td>
<td></td>
</tr>
<tr>
<td>*V</td>
<td>0.5 mg/m^2^</td>
<td>0.5 mg/m^2^</td>
<td>0.5 mg/m^2^</td>
<td>0.5 mg/m^2^</td>
<td>0.5 mg/m^2^</td>
<td>0.5 mg/m^2^</td>
<td></td>
</tr>
<tr>
<td>^C</td>
<td>50 mg/m^2^ EOD</td>
<td>50 mg/m^2^ EOD</td>
<td>50 mg/m^2^ EOD</td>
<td>50 mg/m^2^ EOD</td>
<td>50 mg/m^2^ EOD</td>
<td>50 mg/m^2^ EOD</td>
<td></td>
</tr>
<tr>
<td>^P</td>
<td>40 mg/m^2^ SID for 7 days then 20 mg/m^2^ EOD</td>
<td>20 mg/m^2^ EOD</td>
<td>20 mg/m^2^ EOD</td>
<td>20 mg/m^2^ EOD</td>
<td>20 mg/m^2^ EOD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^ Protocol stops at 24 months.

*V = vincristine; ^C = cyclophosphamide; ^P = prednisolone.
Table 23 DMAC protocol

<table>
<thead>
<tr>
<th>Drug</th>
<th>Week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytarabine (200-300 mg/m²)</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycin-D (0.75 mg/ m²)</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melphalan (20 mg/m²)</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Dexamethasone (0.23 mg/Kg)</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

*Continue alternating drugs weekly until patient relapses

Table 24 Chlorambucil and prednisolone protocol for low grade lymphoma

<table>
<thead>
<tr>
<th>Drug</th>
<th>Week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td></td>
<td>2-4 mg/m² SID</td>
<td>2-4 mg/m² SID</td>
<td>2-4 mg/m² EOD</td>
<td>2-4 mg/m² EOD</td>
</tr>
<tr>
<td>Prednisolone</td>
<td></td>
<td>40 mg/m² SID</td>
<td>20 mg/m² EOD</td>
<td>20 mg/m² EOD</td>
<td>20 mg/m² EOD</td>
</tr>
</tbody>
</table>

*The dose and frequency of chlorambucil and prednisolone is dictated by the response to treatment. Treatment is usually given for 6 months after which the dose and frequency of the drugs are tapered off.

Table 25 LMP protocol

<table>
<thead>
<tr>
<th>Drug</th>
<th>Week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td></td>
<td>20 mg/m²</td>
<td>20 mg/m²</td>
<td>20 mg/m²</td>
<td>20 mg/m²</td>
</tr>
<tr>
<td>Methotrexate</td>
<td></td>
<td>2.5-5 mg/m² 2-3 times/week</td>
<td>2.5-5 mg/m² 2-3 times/week</td>
<td>2.5-5 mg/m² 2-3 times/week</td>
<td>2.5-5 mg/m² 2-3 times/week</td>
</tr>
<tr>
<td>Prednisolone</td>
<td></td>
<td>20-25 mg/m² EOD</td>
<td>20-25 mg/m² EOD</td>
<td>20-25 mg/m² EOD</td>
<td>20-25 mg/m² EOD</td>
</tr>
</tbody>
</table>

* Protocol continues until relapse occurs.
Appendix 2: Time-series graphs and boxplots comparing serum CRP concentrations obtained on multiple assays

1.1 ELISA v’s Mira: Time-series graphs

**Dog ID 1**

![Time-series graph for Dog ID 1](image)

**Dog ID 3**

![Time-series graph for Dog ID 3](image)
1.2 ELISA v’s Mira: Boxplots
2.1 ELISA v’s Pentra: Time-series graphs

Dog ID 1

Dog ID 3

Dog ID 8
Dog ID 43

Dog ID 44

Dog ID 47
2.2 ELISA v’s Pentra: Boxplots

Boxplot of Dog ID 1

Boxplot of Dog ID 3
Boxplot of Dog ID 8

Boxplot of Dog ID 43

Boxplot of Dog ID 44
3.1 Mira v’s Pentra: Time-series graphs

Dog ID 1

Dog ID 3

Dog ID 8
3.2 Mira v’s Pentra: Boxplots

**Boxplot of Dog ID 1**

**Boxplot of Dog ID 3**

**Boxplot of Dog ID 8**
Appendix 3: Plot of residuals

i) Log-transformed CRP vs. WHO Stage II-III and IV-V

![Residual Plots for LogCRP](image1)

ii) Log-transformed CRP vs. WHO substage ‘a’ and ‘b’

![Residual Plots for LogCRP](image2)
iii) Log-transformed CRP vs. albumin (g/L)