Investigation of serum clusterin as a suitable biomarker for canine multicentric lymphoma.

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Summary

Lymphoma (LSA) is a malignant neoplasm which arises from a clonal proliferation of lymphoreticular cells (Vail *et al.*, 2013). It is the most common canine haematopoietic neoplasm (Gavazza *et al.*, 2009; Vail *et al.*, 2013), and accounts for 7-24% of all canine malignancies (Greenlee *et al.*, 1990; Dobson *et al.*, 2002; Fournel-Fleury *et al.*, 2002). Multiple blood and tissue biomarkers have been investigated regarding potential prognostic significance for canine LSA, however currently there is no single marker which has proven to be more reliable than the established parameters such as substage, immunophenotype and grade.

Clusterin (CLU), also known as apolipoprotein J, is a heterodimeric glycoprotein which is widely expressed (Trougakos *et al.*, 2002, 2004) throughout the body. CLU is important in tumorigenesis, apoptosis, and immunoregulation (Trougakos *et al.*, 2002; Pucci *et al.*, 2004; Shannan *et al.*, 2006); its expression is regulated by cytokines and growth factors, and is increased in response to physiological stress (Trougakos *et al.*, 2002, 2004; Shannan *et al.*, 2006). In human medicine, CLU expression has been associated with various malignancies, including carcinoma, melanoma, colon, prostate and breast cancer (Saffer *et al.*, 2002; Pucci *et al.*, 2004; Shannan *et al.*, 2006; Frazzi *et al.*, 2011; Koltai, 2014). Cytoplasmic expression of CLU is also upregulated in cases of anaplastic large cell lymphoma and Hodgkin's lymphoma (HL) (Wellmann *et al.*, 2000; Saffer *et al.*, 2002; Shannan *et al.*, 2006; Frazzi *et al.*, 2011).

A previous study of the canine serum proteome identified CLU in serum from a dog with high grade multicentric lymphoma (MLSA), and the absence of CLU in serum from healthy controls (Atherton *et al.*, 2013a, 2013b). The objective of this current study was to determine whether CLU expression differed significantly between those dogs with MLSA and a healthy control population. It was hypothesised that serum CLU expression would be higher in dogs with MLSA in comparison to healthy controls and that serum CLU levels would subsequently decrease in affected animals, where clinical remission had been achieved following successful treatment.

In the current study, serum CLU levels in dogs with MLSA were compared to healthy control dogs using both western blot and enzyme linked immunosorbent assay (ELISA). The presence of CLU in dog sera was confirmed by western blot analysis, detected at the predicted molecular weight, and the relative levels detected via western blot correlated with those detected by ELISA. Serum CLU analysis by ELISA found treatment naïve dogs with MLSA had a significantly ($p<0.001$) lower serum CLU level in comparison to healthy controls. There was no significant difference in serum CLU concentrations between MLSA dogs prior to treatment and those in complete remission (CR).

Serum CLU concentrations showed wide variation across all canine samples, limiting the potential as a single candidate biomarker for MLSA. The potential for any prognostic predictive value of serum CLU concentrations has yet to be assessed.

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Declaration

I, Katie Anne McNaught, declare that the work in this thesis is original, and was carried out solely by myself or with due acknowledgements. It has not been submitted in any form for another degree or professional qualification. Replication of images or figures from the authors previous publications has been done with approval of copyright from the relevant sources.

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   European College of Internal Medicine – Companion Animal 28th Annual Congress, Rotterdam, September 2018.
   Oral abstract awarded ESVONC best oral abstract presentation.

2. Preliminary assessment of serum clusterin as a potential biomarker for canine lymphoma.
   McNaught, K.A, Morris, J.S, McLaughlin, M (2019).

   Veterinary and Comparative Oncology.

List of Abbreviations

AgNOR - Argyrophilic staining of nucleolar organising regions

AGP – Alpha-1 acid glycoprotein

ALKP – Alkaline phosphatase

ANOVA – Analysis of variance

APO-J – Apolipoprotein J

BCA – Bicinchoninic acid

BSA – Bovine serum albumin (protein standard)

CDR3 - Complementarity-determining region 3

cLBT – Canine lymphoma blood test

CLI – Complement cytolysis inhibitor

CLU – Clusterin

CNS – Central nervous system

CR – Complete remission

CRP – C-reactive protein

CSF – cerebrospinal fluid

DTT – 1M dithiothreitol

DFI – Disease-free interval

DLBCL – Diffuse large B-cell lymphoma

DNA – Deoxyribonucleic acid

ECL – Enhanced chemiluminescence

ECM – Extracellular matrix

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic reticulum

GP – 80 – Glycoprotein 80

GP III – Glycoprotein III

HG – High grade

HL – Hodgkin’s lymphoma

HRP - horseradish peroxidase

ICC – Immunocytochemistry

IHC – Immunohistochemistry

IV – Intravenous

kDa – Kilodaltons

LDH – Lactate dehydrogenase

LG – Low grade

LMR – Lymphocyte:monocyte ratio

LSA – Lymphoma/ Lymphosarcoma

mGPS – Modified Glasgow prognostic score

MES – 2(N-morpholino) ethanesulfonic acid

Mk – Molecular weight marker

MLSA – Multicentric lymphosarcoma

MMP – Matrix metalloproteinase

MRD – Minimum residual disease

MST – Median survival time

NHL – Non-Hodgkin’s lymphoma

NI – Neoplasia Index

NLR – Neutrophil:lymphocyte ratio

OST – Overall survival time

PARR – PCR for antigen receptor rearrangement

PCNA – Proliferating nuclear cellular antigen

PCR - Polymerase chain reaction

PD – Progressive disease

PFS – Progression free survival

PO – Per os

PR – Partial remission

PTH-rp – Parathyroid hormone related peptide

QC – Quality control

REAL – Revised European-American Classification of Lymphoma

RECIST – Response evaluation criteria in solid tumours

RNA - Ribonucleic acid

ROC – Receiver operating characteristic

RT – Radiation Therapy

SAA – Serum amyloid A

SD – Stable disease

SDS – sodium dodecyl sulfate

SDS PAGE – Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SGP-2 – Sulfated glycoprotein-2

SID – Once daily

SP-40,40 – Serum protein-40,40

T-TBS – Tris- buffered saline (pH 7.4) containing 0.5% Tween 20

TIFF – Tagged image file format

TK – Thymidine kinase

TMB – Tetramethylbenzidine

TRPM-2 – Testosterone repressed prostatic messenger 2

VEGF – Vascular endothelial growth factor

WHO – World Health Organization

1 Introduction

1.1 Lymphoma

Lymphoma (LSA) is a malignant neoplasm which arises from a clonal proliferation of lymphoreticular cells (Vail *et al.*, 2013). It is the most common canine haematopoietic neoplasm (Gavazza *et al.*, 2009; Vail *et al.*, 2013), and the third most common canine neoplasm (Greenlee *et al.*, 1990), accounting for 7-24% of all canine malignancies (Greenlee *et al.*, 1990; Dobson *et al.*, 2002; Fournel-Fleury *et al.*, 2002). Although usually occurring in lymphoid organs, including the lymph nodes, spleen and bone marrow, LSA can occur in any tissue in the body (Vail *et al.*, 2013).

1.1.1 Classification

Classification of canine LSA is based primarily on anatomical location, histological criteria and immunophenotypic characteristics (Vail *et al.*, 2013).

1.1.1.1 *Anatomical*

Multicentric lymphoma (MLSA) is the most common anatomical form of canine LSA, accounting for around 84% of all cases (Vail *et al.*, 2013, 2019), and primarily affecting the peripheral lymph nodes. Gastrointestinal lymphoma accounts for a further 5-7% of canine LSA diagnosis (Couto *et al.*, 1989; Vail *et al.*, 2013), and is the most common presentation of extranodal lymphoma, occurring as both focal and diffuse changes within the gastrointestinal tract. Mediastinal lymphoma accounts for approximately 5% of canine LSA (Rosenberg *et al.*, 1991; Vail *et al.*, 2019) with cutaneous lymphoma also noted with similar frequency (Moore *et al.*, 2009; Fontaine *et al.*, 2010; Vail *et al.*, 2019). Primary hepatosplenic (Fry *et al.*, 2003; Cienava *et al.*, 2004; Keller *et al.*, 2012), pulmonary (Berry *et al.*, 1990; Fitzgerald *et al.*, 1991), prostatic (Mainwaring, 1990; Winter *et al.*, 2006; Donato *et al.*, 2019), ocular and central nervous system (CNS) (Rosin, 1982; Couto *et al.*, 1984; Kim *et al.*, 2012; Siso *et al.*, 2016) LSA are less commonly reported forms of extranodal lymphoma.

1.1.1.2 *Morphological*

Canine LSA can be classified as high grade (HG), intermediate grade, or low grade/indolent (LG), depending on histology and morphological cellular characteristics. Large, immature cells, high mitotic rate, rapid progression and considerable malignant potential are features of HG LSA, with LG LSA exhibiting, a slow rate of growth, and smaller, more mature cells, with a low mitotic rate. Canine MLSA usually presents as diffuse and HG (Flood-Knapik *et al.*, 2013), with LG LSA less common, accounting for less than 30% of diagnosed cases (Teske *et al.*, 1994b, 1996; Fournel-Fleury *et al.*, 1997; Vail *et al.*, 2013).

1.1.1.3 *Immunophenotype*

At the cellular level, LSA arises from clonal proliferation of lymphoid cells, of B-cell or T-cell lineage (Vail *et al.*, 2013). Canine LSA most commonly arises from the B-cell lineage, with T cell LSA accounting for only around 30% of cases (Teske *et al.*, 1994b; Fournel-Fleury *et al.*, 1997, 2002; Modiano *et al.*, 2005; Valli *et al.*, 2013). Immunophenotyping is essential for an accurate diagnosis of LSA subtypes and is a fundamental part of LSA classification. Additionally, immunophenotype can be of prognostic value and alter treatment planning (Regan *et al.*, 2013; Vail *et al.*, 2019).

Immunohistochemistry and immunocytochemistry (IHC/ICC) determine immunophenotype by detection of cell surface antigens on cytology or histology specimens, utilizing antibodies against specific lymphocyte markers. Flow cytometry uses analysis of light scatter and fluorescence of cells in a fluid suspension in order to categorise cells via surface antigen expression, and size (Culmsee *et al.*, 2001). Antibodies against cell surface markers are utilised and cell heterogeneity assessed to determine lineage (Sözmen *et al.*, 2005; Tasca *et al.*, 2009; Comazzi *et al.*, 2011).

Molecules specific to B-lymphocytes include CD79a, CD20, CD21 and Pax5. Those specific to T-cells include CD3, CD4, and CD8, although some aberrant expression of markers has been documented in lymphoid malignancies (Wilkerson *et al.*, 2005; Seelig *et al.*, 2016; Vail *et al.*, 2019). Lymphomas which express neither CD3 nor CD79a are termed null cell lymphoma. These are rare, accounting for less than 1% of canine diagnoses (Ponce *et al.*, 2010).

Polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR) analysis detects lymphocyte antigen rearrangement at the DNA level, and thus determines B-cell and T-cell immunophenotype using specific primers for the T-cell receptor or B-cell immunoglobulin region (Keller *et al.*, 2016; Vail *et al.*, 2019). Immunophenotyping can be performed via PARR analysis (Thalheim *et al.*, 2013; Waugh *et al.*, 2016; Ehrhart *et al.*, 2019), with a high specificity (Thalheim *et al.*, 2013) and accuracy (Ehrhart *et al.*, 2019). While highly specific for determining immunophenotype, PARR is deemed inferior to flow cytometry due to a lower sensitivity, particularly for diagnosis of B-cell lymphoma (Thalheim *et al.*, 2013; Ehrhart *et al.*, 2019), although this can vary with different primer sets (Waugh *et al.*, 2016).

1.1.1.4 *Subtype*

Many classification systems for human Non-Hodgkin's lymphoma (NHL) have been described and adapted for canine LSA (Vail *et al.*, 2013, 2019), with the Revised European American Lymphoma (REAL) and World Health Organisation (WHO) systems the current accepted method of histological classification. These assess anatomic location, nodal architecture, cell morphology and immunophenotype to allow classification of specific LSA subtypes, with the aim of better understanding disease behaviour and progression (Vezzali *et al.*, 2010; Vail *et al.*, 2019). Nearly 50 individual subtypes of human NHL have been described, with many advocating to consider lymphoma a group of diseases rather than one distinct entity (Vail *et al.*, 2019). Using the WHO guidelines, 20 different canine subtypes have been identified (Seelig *et al.*, 2016). Based on these guidelines the majority of canine LSA can be classified into one of five subtypes; diffuse large B-cell lymphoma (DLBCL), the most common canine subtype, marginal zone lymphoma, peripheral T-cell lymphoma not otherwise specified, nodal T-zone lymphoma and T lymphoblastic lymphoma (Valli *et al.*, 2011; Seelig *et al.*, 2016).

1.1.2 Clinical Presentation of multicentric lymphoma

1.1.2.1 *Signalment*

LSA commonly occurs in older animals (Edwards *et al.*, 2003), with over 90% of cases diagnosed in dogs >5years old (Modiano *et al.*, 2005). The majority of dogs are middle aged at time of diagnosis, with a median age of 6 to 9 years (Teske, 1994; Edwards *et al.*, 2003; Modiano *et al.*, 2005). Prevalence varies between dog breeds, with certain breeds, particularly boxer dogs, bulldogs and bullmastiffs reported to have a high prevalence of lymphoproliferative disease (Teske, 1994; Teske *et al.*, 1994a; Edwards *et al.*, 2003; Modiano *et al.*, 2005; Villamil *et al.*, 2009; Vail *et al.*, 2013). Breeds with a lower relative risk include the dachshund, Pomeranian (Teske, 1994; Teske *et al.*, 1994a; Vail *et al.*, 2013) and Chihuahua (Modiano *et al.*, 2005). More recently LSA has been linked to body weight, with dogs >30kg having increased odds of diagnosis (Pittaway *et al.*, 2019). Gender and neutering status were previously not thought to affect prevalence (Edwards *et al.*, 2003; Modiano *et al.*, 2005). However recent reports indicate entire females at significantly lower risk (Villamil *et al.*, 2009; Vail *et al.*, 2013; Pittaway *et al.*, 2019), and neutered dogs at increased risk (Pittaway *et al.*, 2019), potentially indicating a protective hormonal component to the disease.

1.1.2.2 *Stage*

MLSA is grouped into stages based on the extent of lymph node or lymphoid tissue involvement. These stages are numbered I -V.

Clinical Stages of canine MLSA

I - Involvement limited to a single node or lymphoid tissue in a single organ, excluding bone marrow.

II - Involvement of many lymph nodes in a regional area, including tonsils.

III - Generalized lymph node involvement.

IV - Liver and/or spleen involvement, in addition to Stage III.

V - Manifestation in the blood and involvement of bone marrow and/or other organ systems, in addition to Stages I-IV.

1.1.2.3 *Substage*

Each MLSA stage is further classified into substage a or b, depending on the presence or absence of clinical signs (Owen, 1980), with substage b indicating the presence of concurrent, systemic signs e.g. diarrhoea, dyspnoea, hypercalcaemia (Owen, 1980; Vail *et al.*, 2013). The majority of dogs present with lymphadenopathy but are otherwise clinically well, i.e. substage a (Vail *et al.*, 2013). Dogs presented with T-cell LSA are more likely to present in substage b (Vail *et al.*, 2013) with clinical signs related to the anatomical location of their tumour burden, or concurrent paraneoplastic syndromes.

1.1.2.4 *Presenting signs*

Dogs typically present with a non-painful, peripheral lymphadenopathy (Greenlee *et al.*, 1990; Baskin *et al.*, 2000; Vail *et al.*, 2013), although clinical signs can vary with organs affected and extent of disease (Teske, 1994; Teske *et al.*, 1994a; Gavazza *et al.*, 2009; Vail *et al.*, 2013). Most dogs with MLSA, present with advanced disease, stages III-V (Greenlee *et al.*, 1990; Dobson *et al.*, 2001) but in substage a (Vail *et al.*, 2013). With progression of disease, different clinical signs can occur, including anorexia, weight loss, gastrointestinal signs, or signs relating to organ failure (Baskin *et al.*, 2000; Vail *et al.*, 2013). In addition dogs may present with, or develop, signs related to bone marrow infiltration, including anaemia, haemorrhage or infection secondary to pancytopenia (Vail *et al.*, 2013).

Paraneoplastic syndromes are frequently associated with canine LSA, with anaemia the most common, occurring in 30-43% (Madewell *et al.*, 1980; Abbo *et al.*, 2007; Miller *et al.*, 2009; Childress *et al.*, 2018; Bailey, 2019; Vail *et al.*, 2019). Other haematological disturbances (e.g. polycythaemia, eosinophilia) are less common but occur, particularly with T-cell LSA (Ozaki *et al.*, 2006; Tomiyasu *et al.*, 2010; Durno *et al.*, 2011; McNaught *et al.*, 2018; Vail *et al.*, 2019). Hypercalcaemia is also frequently seen, most commonly, although not exclusively, associated with T-cell LSA (Greenlee *et al.*, 1990; Rosenberg *et al.*, 1991; Bergman, 2012; Zandvliet, 2016; Bailey, 2019). Less common paraneoplastic syndromes include hypoglycaemia (Zhao *et al.*, 1993) hyperglobulinaemia/monoclonal gammopathies (Giraudel *et al.*, 2002; Tappin *et al.*, 2011; Bailey, 2019) and immune mediated diseases (e.g. immune mediated haemolytic anaemia, immune mediated thrombocytopenia (Keller, 1992), polymyositis (Evans *et al.*, 2004)).

1.1.3 Diagnosis

Diagnosis of MLSA, regardless of anatomic location or presenting signs, is made via morphological examination of neoplastic tissue and/or cells.

Cytology is frequently used for the diagnosis of canine LSA using morphological features of cells (Fournel-Fleury *et al.*, 2002; Sözmen *et al.*, 2005; Vail *et al.*, 2013). Cytology samples can also be used to determine immunophenotype, by applying suitable B and T cell antibodies. Misdiagnosis through cytology is possible and severe, reactive hyperplasia may be difficult to differentiate from LSA through cytology alone. In addition some LG small cell LSA may have subtle features of malignancy, making definitive diagnosis more difficult (Vail *et al.*, 2013).

Histology provides more information than cytology, as evaluation of nodal architecture and pattern of nodal infiltration, allows classification of LSA as either nodular, follicular or diffuse (Carter *et al.*, 1986; Seelig *et al.*, 2016). The majority of canine MLSA exhibit a diffuse pattern of proliferation, with neoplastic lymphocytes causing complete effacement of the normal nodal structure, often with capsular disruption (Carter *et al.*, 1986; Fournel-Fleury *et al.*, 1997; Valli *et al.*, 2011; Flood-Knapik *et al.*, 2013; Vail *et al.*, 2013). Histopathology of an entire lymph node is preferable for diagnosis and classification (Vail *et al.*, 2013). Based on histology characteristics numerous classification schemes to accurately categorise canine LSA have been reported, see section 1.1.1.4.

A complete diagnostic work up for MLSA usually involves physical examination, complete blood count, biochemistry profile and urinalysis. Thoracic and abdominal imaging, examination of peripheral blood smear and bone marrow sampling, are also usually performed to establish the extent of disease/clinical stage.

1.1.3.1 *Molecular diagnostics*

As lymphoid neoplasia derives from a clonal expansion of lymphocytes, the identification of a homogenous, monomorphic or clonal population of lymphoid cells supports a diagnosis of LSA.

Lymphocytes each have unique T-cell receptor and immunoglobulin genes, determined through recombination of variable gene regions, to encode for specific antigen-binding sites (Keller *et al.*, 2016; Vail *et al.*, 2019). Lack of variation of these specific complementarity-determining (CDR3) regions can be evaluated as marker of malignancy in a lymphoid population (Burnett *et al.*, 2003; Gentilini *et al.*, 2009; Waugh *et al.*, 2016). Clonality can be determined by isolating lymphoid DNA and using targeted PCR primers to amplify the individual variable regions (Avery, 2009). The PARR clonality assay is valuable diagnostic tool, and can be used in a variety of sample types, with high diagnostic accuracy (Ehrhart *et al.*, 2019).

Complete and thorough diagnosis of lymphoid neoplasia often requires ancillary testing and determination of immunophenotype, via immunohistochemistry/cytochemistry and/or flow cytometry, see section 1.1.1.3.

1.1.4 Treatment

Chemotherapy is the treatment of choice for MLSA, as it is considered a systemic disease. Current treatment aims to induce remission and prolong survival, and will rarely provide a clinical cure (Marconato, 2011; Vail *et al.*, 2013; Thamm, 2019). Multiagent chemotherapy is generally considered the most efficacious with regard to overall survival time (OST) and progression free survival (PFS) (Vail *et al.*, 2019). CHOP-based chemotherapy protocols are widely used, with cyclophosphamide (C), vincristine (O), doxorubicin (H) and prednisolone (P) given cyclically over 12-25 weeks (Keller *et al.*, 1993; Moore *et al.*, 2001; Simon *et al.*, 2006; Burton *et al.*, 2013; Curran *et al.*, 2015). The benefit of ongoing/maintenance treatment, following an initial chemotherapy course, has not been proven, and is rarely used (Chun *et al.*, 2000; Moore *et al.*, 2001; Garrett *et al.*, 2002; Simon *et al.*, 2008; Vail *et al.*, 2019). Multiagent CHOP-based protocols induce remission in 80-95% of cases, with median survival time (MST) of 10-12 months (Vail *et al.*, 2019) although 20% of patient may achieve survival times >2 years (Chun *et al.*, 2000; Garrett *et al.*, 2002; Burton *et al.*, 2013; Curran *et al.*, 2015; Thamm, 2019).

Single agent protocols, with the exception of doxorubicin, are used less frequently and do not result in significant or durable remission (Carter *et al.*, 1987; Mutsaers *et al.*, 2002; Lori *et al.*, 2010; Thamm, 2019; Vail *et al.*, 2019). Immunophenotype may dictate treatment, with T-cell MLSA responding less favourably to CHOP-based protocols (Beaver *et al.*, 2010), and the addition of alkylating agents thought to confer a survival advantage (Brodsky *et al.*, 2009; Goodman *et al.*, 2016; Moore, 2016; Brown *et al.*, 2018; Elliott *et al.*, 2019). Oral corticosteroids, have direct cytotoxic activity (Smith *et al.*, 2010), and when used as a sole treatment will frequently induce at least partial remission (PR), although disease free interval (DFI) is short (Thamm, 2019).

Other therapeutic options are used less frequently and may only be applicable to individual subtypes of LSA. Different treatment options may be appropriate for solitary or focal disease, and LG LSA, which may respond more favourably to less intensive therapy (Flood-Knapik *et al.*, 2013; Moore, 2016).

Lymphoid cells are radiosensitive and radiation therapy (RT) has been used both as a sole treatment and in combination with chemotherapy (Gustafson *et al.*, 2004; Williams *et al.*, 2004; Mayer *et al.*, 2005; Vaughan *et al.*, 2007; Lurie *et al.*, 2009), in naïve and relapsed LSA. As a systemic disease the indication for RT in the treatment of LSA is limited and the lack of evidence supporting a superior clinical outcome limits the use of RT for MLSA in clinical practice (Thamm, 2019; Vail *et al.*, 2019).

Immunomodulatory therapies have also been proposed as adjuvant treatment options for canine LSA. Various canine specific monoclonal antibodies, targeting both B and T-cells, have been evaluated (Impellizeri *et al.*, 2006; Rue *et al.*, 2015; Regan *et al.*, 2016; Weiskopf *et al.*, 2016; Klingemann, 2018; Thamm, 2019; Vail *et al.*, 2019). Several autologous and DNA vaccines have also been trialled, with reports suggesting improved outcome for indolent and HG B-cell LSA (Jeglum *et al.*, 1986, 1988; Turek *et al.*, 2007; Peruzzi *et al.*, 2010; Gavazza *et al.*, 2013; Marconato *et al.*, 2014, 2015a, 2019).

Regardless of modality or adjunct therapies, treatment is rarely curative. However, with appropriate therapy, complete clinical remission and extension of good quality life can be expected.

1.1.4.1 *Response to treatment*

Monitoring response to treatment can be subjective, through lymph node palpation, or objective, through lymph node measurement or biological detection of minimal residual disease (MRD). Standardisation of treatment response has been described (Vail *et al.*, 2010), recommending routine measurement of between one and five target lesions/lymph nodes, with response defined by the response evaluation criteria in solid tumours (RECIST) guidelines as:

- **Complete remission** (CR), no evidence of disease, all lymph nodes non-pathologic in size.
- **Partial remission** (PR), at least a 30% decrease in target lesions, but not complete resolution.
- **Stable disease** (SD), no significant increase or decrease in target lesions.
- **Progressive disease** (PD), at least a 20% increase in target lesions, and/or unequivocal progression of existing non-target lesions/the appearance of one or more new lesions.

Defining overall response takes into consideration target, non-target and new lesions. Accurate monitoring of response to treatment can aid detection of early relapse, or lack of response, allowing alterations to the treatment protocol.

Definition and measurement of duration of response has also been standardised, to ensure a consensus for temporal response measurements, with common definitions defined below:

- **Median survival time (MST)**, measured from diagnosis or initiation of treatment to death, of 50% of the measured cohort.
- **Overall survival time (OST)**, measured from initiation of treatment to death, from any cause.
- **Lymphoma specific survival (LSS)**, measured from initiation of treatment, to disease progression or death, from LSA only.
- **Disease-free interval (DFI)**, assesses only those patients in CR, measured from documentation of response, to time of relapse, or death.
- **Progression-free survival (PFS)**, measured from initiation of treatment, to disease progression or death, from any cause.

As treatment for LSA is rarely curative and factors including owner finance and perceived quality of life can influence decisions for euthanasia, the preferred measurement of response is PFS (Vail *et al.*, 2010).

1.1.5 Prognosis

Prognosis for canine MLSA can be variable, due to the heterogeneity of the disease, and multiple factors can impact prognosis, both positively and negatively. Treatment with chemotherapy significantly improves prognosis, and survival time, when compared to no treatment, or treatment with corticosteroids only (Vail *et al.*, 2019). Additionally, response to treatment can be highly prognostic (Dobson *et al.*, 2001; Edwards *et al.*, 2003). The main prognostic factors are discussed below.

1.1.5.1 *Signalment*

Prevalence of LSA can be strongly linked to signalment, and many factors have been investigated regarding prognostic significance. While LSA is most common in older patients, age at diagnosis was not found to be independently prognostic (Greenlee *et al.*, 1990; Teske *et al.*, 1994c; Kiupel *et al.*, 1999; Dobson *et al.*, 2001; Jagielski *et al.*, 2002; Gavazza *et al.*, 2009). Numerous studies have linked prognosis to gender, with varied results. Both male (Siedlecki *et al.*, 2006; Zandvliet, 2016) and female (Keller *et al.*, 1993; Vail *et al.*, 2019) gender and neuter status, have been linked with an unfavourable prognosis, while other studies have indicated no prognostic association with sex or neuter status (Greenlee *et al.*, 1990; Teske *et al.*, 1994c; Kiupel *et al.*, 1999; Dobson *et al.*, 2001; Jagielski *et al.*, 2002; Gavazza *et al.*, 2009). Many breeds appear predisposed to LSA, however strong prognostic association with individual breeds has not been documented. Small-breed dogs may have a more favourable prognosis than large-breed (Zandvliet, 2016), with one study suggesting dogs weighing <18kg achieved longer DFI and OST (Garrett *et al.*, 2002). An unrelated study also linked body condition score to survival, with dogs underweight at time of diagnosis having shorter survival times (Romano *et al.*, 2016). The majority however, indicate no association between weight or breed, achieving remission, OST or DFI (Appelbaum *et al.*, 1984; Teske *et al.*, 1994c; Kiupel *et al.*, 1999; Dobson *et al.*, 2001). Overall, due to highly variable results, the prognostic significance of signalment appears minimal.

1.1.5.2 *Presenting signs*

The presence of clinical signs at diagnosis (WHO substage b (Owen, 1980)) has been consistently linked to poorer prognosis (Greenlee *et al.*, 1990; Hahn *et al.*, 1992; Keller *et al.*, 1993; Baskin *et al.*, 2000; Jagielski *et al.*, 2002; Romano *et al.*, 2016; Vail *et al.*, 2019), with clinical signs secondary to hypercalcaemia of malignancy often conferring a poorer prognosis (Greenlee *et al.*, 1990; Rosenberg *et al.*, 1991; Keller *et al.*, 1993; Marconato *et al.*, 2011).

Haematological abnormalities, particularly anaemia and thrombocytopenia at presentation, are negative prognostic indicators (Davies *et al.*, 2018; Vail *et al.*, 2019) associated with shorter OST (Abbo *et al.*, 2007; Miller *et al.*, 2009; Burton *et al.*, 2013; Romano *et al.*, 2016; Purzycka *et al.*, 2020), shorter PFS (Zemann *et al.*, 1998; Moore *et al.*, 2001; Burton *et al.*, 2013; Childress *et al.*, 2018; Davies *et al.*, 2018), and increased risk of incomplete remission (Childress *et al.*, 2018). Monocytosis and neutrophilia are also indicators of poor prognosis (Perry *et al.*, 2010; Childress *et al.*, 2018; Vail *et al.*, 2019; Purzycka *et al.*, 2020).

In addition to clinical signs and haematological abnormalities, prior treatment with steroids is a negative prognostic factor, with an unfavourable effect on survival time and poorer response to treatment (Price *et al.*, 1991; Dobson *et al.*, 1994; Gavazza *et al.*, 2009; Marconato *et al.*, 2011).

Anatomic location is also prognostic, with extra nodal LSA carrying a less favourable prognosis, and cutaneous, hepatosplenic, HG gastrointestinal and CNS LSA all individually associated with a poor prognosis (Vail *et al.*, 2019). Additionally, the presence of mediastinal lymphadenopathy was a negative prognostic factor for DFI and MST (Starrak *et al.*, 1997) in MLSA.

1.1.5.3 *Classification*

Prognosis for canine MLSA is strongly linked to classification, with clinical stage, histopathological grade and immunophenotype all holding varying degrees of prognostic significance.

Immunophenotype consistently holds prognostic significance for MLSA, with intermediate-high grade T-cell LSA responding less favourably to chemotherapy (Greenlee *et al.*, 1990; Beaver *et al.*, 2010) often with incomplete remission, shortened remission and survival times (Greenlee *et al.*, 1990; Teske *et al.*, 1994c; Kiupel *et al.*, 1999; Dobson *et al.*, 2001; Edwards *et al.*, 2003; Ponce *et al.*, 2004; Marconato *et al.*, 2011; Mutz *et al.*, 2013). The exception is T-zone LSA, which is typically low grade and confers a more favourable prognosis (Valli *et al.*, 2013; Avery *et al.*, 2014; Seelig *et al.*, 2014). Histological grade of malignancy is a reliable prognostic factor (Teske *et al.*, 1994c; Dobson *et al.*, 2001; Marconato *et al.*, 2011; Vail *et al.*, 2019), with intermediate and HG LSA tending to respond well to chemotherapy initially, but suffering early relapse, with OST poor. In comparison LG/indolent LSA is often associated with prolonged survival (Valli *et al.*, 2006, 2013; Flood-Knapik *et al.*, 2013; Vail *et al.*, 2019).

Various classification systems including the Kiel and National Cancer Institute Working Formulation show prognostic significance (Hahn *et al.*, 1992; Teske *et al.*, 1994c). Clinical stage, as determined by the WHO staging system (Owen, 1980), has been shown to hold prognostic significance, with higher stage associated with poor prognosis (Price *et al.*, 1991; Dobson *et al.*, 1994; Teske *et al.*, 1994c; Jagielski *et al.*, 2002; Marconato *et al.*, 2011). However a similar number of studies have shown no prognostic association with disease stage (Greenlee *et al.*, 1990; Rosenberg *et al.*, 1991; Hahn *et al.*, 1992; Kiupel *et al.*, 1999; Dobson *et al.*, 2001). This may, in part, be due to variation in diagnostic work/investigations carried out, either under or over-estimating the extent/stage of disease. Stage migration, due to the absence of imaging and cytological/histological confirmation of organ involvement, could render many accounts of clinical stage inaccurate.

1.1.5.4 *Proliferation markers*

Markers of proliferation also hold prognostic significance. Proliferation indices, including proliferating cell nuclear antigen (PCNA), Ki-67 staining and argyrophilic nucleolar organiser regions (AgNOR) are elevated in canine LSA (Hipple *et al.*, 2003; Bauer *et al.*, 2007). AgNOR and Ki-67 correlate with histological grade (Poggi *et al.*, 2015), and are prognostic for OST and DFI (Kiupel *et al.*, 1998; Kiupel, 1999; Poggi *et al.*, 2017).

1.2 Biomarkers

A biomarker is a substance, structure or process which can be measured to provide information regarding a biological condition or state, with medical biomarkers providing diagnostic or prognostic information, or information predicting response to therapy (Strimbu *et al.*, 2010; Chen *et al.*, 2011; Nalejska *et al.*, 2014; Bryan, 2016). Biomarkers should be objective, quantifiable and repeatable, as well as specific for the biological process of interest. Classified based on their function, biomarkers can be used to predict disease risk, act as diagnostic tools, at a clinical or subclinical level, or used to categorise disease severity, forecast future progression, and predict response to treatment or relapse (Srivastava *et al.*, 2002; Chen *et al.*, 2011). The most useful biomarkers have a high predictive accuracy, are easy to measure, non-invasive and reproducible (Srivastava *et al.*, 2002; Chen *et al.*, 2011).

Biomarkers measured in biological media, such as tissues, cells or fluid, have become widely used in the medical profession over the past few decades, for early disease detection, to monitor response to treatment, and also as a surrogate end point in clinical trials (Srivastava *et al.*, 2002; Strimbu *et al.*, 2010; Chen *et al.*, 2011). In comparison to other areas, biomarker use in neoplasia is relatively limited (Johann *et al.*, 2007) and, given the inherent heterogeneity of the population, and of individual tumours, panels of biomarkers are often preferred, giving greater insight than single markers alone (Johann *et al.*, 2007; Chen *et al.*, 2011). Biomarkers are particularly useful in human neoplasia, with regard to monitoring remission status (Zanatta *et al.*, 2003; Elliott *et al.*, 2011).

1.2.1 Biomarkers for canine lymphoma

The human literature on lymphoma related biomarkers is extensive and its potential relevance to veterinary medicine has generated wide-spread interest in biomarkers for canine LSA. Many markers found in tissue biopsies and blood (whole blood, serum and plasma) have been assessed for predictive or prognostic value and used to monitor response to treatment or remission status. Blood biomarkers are particularly useful in monitoring dogs with internal disease and without peripheral nodal involvement, since they avoid the need for repeat imaging/more invasive techniques.

1.2.1.1 *Thymidine Kinase*

Thymidine kinase (TK) is a cytoplasmic enzyme, involved in DNA synthesis. TK expression correlates with the fraction of cells in the "S phase" of the cell cycle, and as such can be used as an indicator of the proliferative activity of tumour cells (Von Euler *et al.*, 2004, 2011; Elliott *et al.*, 2011; Falk, 2018; Boyé *et al.*, 2019). In human literature serum TK levels are prognostic and can predict response to treatment in a variety of haematological neoplasia, including leukaemia, multiple myeloma, HL and NHL (Nakamu *et al.*, 1997; Von Euler *et al.*, 2004, 2008, 2011; Elliott *et al.*, 2011; Boyé *et al.*, 2019). In dogs, serum TK concentrations were significantly greater in patients with neoplasia (Selting *et al.*, 2015, 2016), with concentrations >4.9U/l reported as 73% sensitive and 84% specific for a diagnosis of neoplasia (Selting *et al.*, 2015), and both serum and plasma TK concentrations particularly elevated in LSA and leukaemia (Nakamu *et al.*, 1997; Von Euler *et al.*, 2004, 2011; Selting *et al.*, 2016).

Serum TK concentrations, as determined by radioenzyme assay and enzyme-linked immunosorbent assay (ELISA), were significantly elevated in dogs with LSA, in comparison to healthy controls (Von Euler *et al.*, 2004; Boyé *et al.*, 2019), and when compared to dogs with inflammatory diseases or non-haematologic tumours (Von Euler *et al.*, 2008; Boyé *et al.*, 2019). Although one study (Elliott *et al.*, 2011) found only 47% of dogs with LSA had elevations in serum TK at time of diagnosis.

In some studies (Von Euler *et al.*, 2004, 2008; Boyé *et al.*, 2019) serum TK concentrations correlate with WHO disease stage and substage, with higher TK levels associated with more advanced clinical stage, and substage b, although results have been conflicting (Elliott *et al.*, 2011). Additionally, TK levels are higher in dogs with B-cell LSA, in comparison to T-cell LSA (Elliott *et al.*, 2011; Boyé *et al.*, 2019), and are particularly elevated in DLBCL in comparison to other subtypes (Boyé *et al.*, 2019).

Only one study has found TK levels prognostic for outcome, with a pre-treatment serum TK >30U/l associated with significantly shorter survival times (Von Euler *et al.*, 2004). Any association with prognosis has been refuted by other studies, finding no link between elevations in TK, survival time, duration to first remission, or poorer prognosis (Elliott *et al.*, 2011; Falk, 2018). These conflicting results have limited the use of pre-treatment TK levels as a prognostic marker. However, serum TK levels have been shown to decrease, back to within normal range, in dogs achieving CR (Von Euler *et al.*, 2004, 2008, 2011; Boyé *et al.*, 2019), and will rise again, in the weeks prior to clinically detectable relapse (Von Euler *et al.*, 2004; Boyé *et al.*, 2019), indicating potential as a predictive biomarker or monitoring tool for disease remission.

1.2.1.2 *Lactate Dehydrogenase*

Lactate dehydrogenase (LDH) is a ubiquitous enzyme, with five separate isoforms identified across virtually all tissues. It is responsible for the conversion of lactate to pyruvate during anaerobic glycolysis and is often high in malignant cells, as these use anaerobic glycolysis as a primary mechanism of generating energy (Modiano, 2012). Serum LDH activity is also a marker of tissue necrosis or damage (Marconato *et al.*, 2009; Terragni *et al.*, 2016) as malignant or damaged tissues release LDH into the circulation (Marconato *et al.*, 2010; Terragni *et al.*, 2016). Elevations in LDH activity can be seen in a variety of conditions, including infarction, hepatopathies, myopathies and malignancy (Marconato *et al.*, 2009, 2010). LDH has been evaluated in various human neoplasms, including carcinomas and haematopoietic neoplasia (Zanatta *et al.*, 2003; Marconato *et al.*, 2009, 2010; Terragni *et al.*, 2016; Zandvliet, 2016), with serum levels showing some correlation to tumour burden (Marconato *et al.*, 2009, 2010). Serum LDH levels may also help differentiate between benign and malignant neoplasia in the female reproductive tract (Marconato *et al.*, 2009).

Serum LDH is commonly increased in HL, NHL, multiple myeloma and leukaemia (Marconato *et al.*, 2009, 2010; Terragni *et al.*, 2016), and is an important prognostic factor in human NHL, with increased LDH activity correlating with reduced survival times, disease progression and poor response to treatment (Zanatta *et al.*, 2003; Marconato *et al.*, 2010; Terragni *et al.*, 2016; Zandvliet, 2016).

In veterinary medicine, LDH has been evaluated in healthy dogs, dogs with non-neoplastic conditions and a variety of different neoplasms (Marconato *et al.*, 2009; Terragni *et al.*, 2016), with a reference range for LDH in canine serum and CSF

established (Kopanke *et al.*, 2018), facilitating future studies of canine LDH as a potentially useful biomarker.

Dogs with neoplasia had significantly higher mean LDH than healthy dogs, and those with non-neoplastic disease, although significant overlap was noted (Marconato *et al.*, 2009). Increased LDH was however, a poorly sensitive and non-specific marker of neoplasia, only elevated in around 45% of neoplasia, with nearly 30% of non-neoplastic conditions also having increased and no diagnostic cut out point established (Marconato *et al.*, 2009). Elevations in LDH have been specifically reported in both canine leukaemia and LSA (Greenlee *et al.*, 1990; Zanatta *et al.*, 2003; Marconato *et al.*, 2009), with mean LDH levels in canine LSA significantly higher than those with carcinoma or mast cell tumours, and over 78% of canine LSA having elevated LDH at presentation (Marconato *et al.*, 2009).

Correlation between serum LDH at time of LSA diagnosis and stage of disease has not been proven (Von Euler *et al.*, 2006; Marconato *et al.*, 2009), and reports disagree as to whether elevated serum LDH levels, in canine LSA, at diagnosis, are prognostic for survival, with only one study (Zanatta *et al.*, 2003) suggesting low LDH levels correlated with longer survival times. This is in contrast to other findings (Greenlee *et al.*, 1990; Von Euler *et al.*, 2006; Marconato *et al.*, 2010). These conflicting reports in the literature limit the utility of LDH as a prognostic biomarker.

While not useful as a diagnostic tool or prognostic biomarker for survival time, canine serum LDH activity may have prognostic merit in predicting LSA recurrence following treatment. Dogs with elevated serum LDH activity following chemotherapy, were more likely to relapse within 45 days (Marconato *et al.*, 2010). Thus, evaluation of LDH activity following treatment may predict early relapse, however the usefulness of this test is limited by poor sensitivity (Marconato *et al.*, 2010).

1.2.1.3 *C - reactive protein*

C-reactive protein (CRP) is an acute phase protein, produced by the liver in response to stress, inflammation or infection (Tecles *et al.*, 2005; Nielsen *et al.*, 2007; Nakamura *et al.*, 2008; Fontaine, 2013). Widely used as a surrogate marker of disease and disease response, in both human and veterinary medicine, CRP concentrations are elevated in a variety of inflammatory diseases, and particularly in cases of neoplasia or immune-mediated disease (Tecles *et al.*, 2005; Merlo *et al.*, 2007; Nielsen *et al.*, 2007;

Nakamura *et al.*, 2008; Fontaine, 2013; Kučer *et al.*, 2018). Serum CRP has been used as both a diagnostic and prognostic (Merlo *et al.*, 2007; Nielsen *et al.*, 2007; Fontaine, 2013; Alexandrakis *et al.*, 2017) marker, in human lymphoid neoplasia, with elevated CRP at time of diagnosis reflective of both disease burden and poor prognosis (Fontaine, 2013).

Serum CRP has potential as a highly sensitive, although non-specific serum biomarker for canine LSA (Merlo *et al.*, 2007; Mischke *et al.*, 2007; Nielsen *et al.*, 2007; Fontaine, 2013; Fontaine *et al.*, 2017), with CRP levels significantly elevated in dogs with LSA prior to treatment, in comparison to a healthy control population (Tecles *et al.*, 2005; Merlo *et al.*, 2007; Mischke *et al.*, 2007; Nielsen *et al.*, 2007; Nakamura *et al.*, 2008; Fontaine, 2013), and CRP levels above reference range in >85% of canine LSA (Fontaine, 2013). Despite high sensitivity, there remains an overlap with normal dogs, and many other inflammatory diseases, and serum CRP levels in a number of canine LSA patients remains within normal limits (Nielsen *et al.*, 2007; Fontaine, 2013). CRP levels may correlate with burden of disease and levels may be higher with HG LSA (Mischke *et al.*, 2007; Fontaine, 2013; Bryan, 2016), however no study has found individual CRP levels to hold prognostic value (Fontaine, 2013).

CRP levels reduce following induction of remission with chemotherapy, although may remain elevated in comparison to controls, and increase again at time of relapse (Merlo *et al.*, 2007; Nielsen *et al.*, 2007; Fontaine, 2013). Elevations in CRP levels do not appear to precede relapse (Merlo *et al.*, 2007; Fontaine, 2013; Bryan, 2016; Alexandrakis *et al.*, 2017), limiting the use of CRP as a monitoring tool during remission. Additionally CRP levels were not significantly different in CR, in comparison to PR or SD (Nielsen *et al.*, 2007; Fontaine, 2013), limiting the use of this biomarker to measure disease response to treatment.

1.2.1.4 *Combination Biomarkers*

Lack of specificity has limited the use of CRP as a biomarker for canine LSA. As such, studies have evaluated the use of multiple biomarkers, in combination with CRP, to improve disease specificity.

Using an algorithm combining serum CRP and haptoglobin levels, the canine lymphoma blood test (cLBT, Avacta Animal Health), was developed for diagnosis, screening, and remission monitoring (Mirkes *et al.*, 2014). The computer based

algorithm assessed CRP and haptoglobin levels, combined with age, sex and lymph node status, and was generated using data comparing confirmed LSA cases, healthy controls and controls with differing diseases (Mirkes *et al.*, 2014; Alexandrakis *et al.*, 2017).

Serum cLBT values at diagnosis were significantly associated with survival times, with low cLBT values correlating with improved survival (Bryan, 2016; Alexandrakis *et al.*, 2017), and elevated cLBT values accurately predicting relapse or progressive disease (Alexandrakis *et al.*, 2017). The algorithm claimed to detect relapse of LSA, via elevation of cLBT values, up to two months prior to the recurrence of clinical signs (Mirkes *et al.*, 2014), with both a high sensitivity (93.5%) and specificity (74.2%) for predicting disease (Alexandrakis *et al.*, 2017).

Despite these positive results in LSA prognostics and remission status monitoring, the canine lymphoma blood test was withdrawn from production in 2018.

Serum CRP has also been evaluated in conjunction with TK. The combination of the highly sensitive but non-specific acute phase protein, with the more disease specific kinase was used to calculate a value termed the Neoplasia Index (NI) (Selting *et al.*, 2015, 2016). The NI was significantly higher in neoplasia, particularly LSA, in comparison to healthy controls, although overlap existed (Selting *et al.*, 2016), with sensitivity and specificity, 82% and 91% respectively, for the detection of neoplasia where NI levels ≥5.8 (Selting *et al.*, 2015). Marketed as the "TK Canine Cancer Panel", by the VDI Laboratory in the USA, this combination of biomarkers can aid in the diagnosis of neoplasia, particularly LSA, where high values are associated with, although not specific for, disease (Selting *et al.*, 2016). While the NI may correlate with disease status and remission, any prognostic significance has not yet been proven (Bryan, 2016).

The modified Glasgow Prognostic Score (mGPS) uses a combination of pre-treatment serum CRP and albumin levels to generate a numerical score (Kim *et al.*, 2014). In human neoplasia, the mGPS has potential prognostic use in DLBCL (Kim *et al.*, 2014). Similarly evaluated in canine LSA (Fontaine *et al.*, 2017), the mGPS had prognostic potential and was inversely associated with survival time and progression free survival.

1.2.1.5 *Additional acute phase proteins*

Haptoglobin and albumin have both been used, as prognostic and diagnostic biomarkers in combination with other factors (Mirkes *et al.*, 2014; Alexandrakis *et al.*, 2017; Fontaine *et al.*, 2017). Additionally, they have been each been evaluated as separate entities.

Albumin is a negative acute phase protein, decreasing in response to stress and inflammation. Hypoalbuminemia is an independent negative prognostic factor in canine LSA and was significantly associated with higher stage and substage b (Fontaine *et al.*, 2017). Hypoalbuminemia at diagnosis was associated with reduced OST (Romano *et al.*, 2016) and PFS (Price *et al.*, 1991; Fontaine *et al.*, 2017).

Haptoglobin levels are significantly increased in neoplasia, particularly in dogs with acute lymphoid leukaemia and LSA, in comparison to healthy controls (Mischke *et al.*, 2007). A study (Atherton *et al.*, 2013a, 2013b) of the canine serum proteome identified haptoglobin in the serum of patients with LSA, and absent in healthy controls, with haptoglobin elevations greater with increased burden of disease (Bryan, 2016). While there is an inference that increased haptoglobin levels carry an unfavourable prognosis (Vail *et al.*, 2019), there are no studies evaluating the independent prognostic significance of haptoglobin.

Serum amyloid A (SAA) is considered a major canine acute phase protein, showing an increase in serum concentration in response to a variety of infectious, inflammatory and neoplastic diseases (Tecles *et al.*, 2005; Merlo *et al.*, 2008). When evaluated in combination, there was good correlation between CRP and SAA concentrations, however the high degree of individual variation for SAA concentrations limited utility (Tecles *et al.*, 2005). SAA was further evaluated as a biomarker for relapse of canine MLSA (Merlo *et al.*, 2008). While mean SAA concentrations were significantly higher in dogs with LSA prior to treatment in comparison to controls, there was no increase in SAA concentration at time of relapse, rendering SAA redundant as a biomarker for monitoring remission or LSA relapse.

Serum alpha 1-acid glycoprotein (AGP) is an acute-phase protein and serum marker of inflammation and neoplasia, with concentrations elevated in neoplasia, in comparison to healthy controls, although this was not specific for haematopoietic neoplasia (Ogilvie *et al.*, 1993). Serum AGP was also evaluated for use as a marker of LSA relapse (Hahn *et*

al., 1999); AGP concentrations were higher in LSA in comparison to healthy controls, and normalised following doxorubicin treatment. Additionally, mean serum AGP levels at the time of relapse, and 3 weeks prior to relapse were significantly higher than that measured while in remission, and not significantly different to pre-treatment concentrations (Hahn *et al.*, 1999). Thus, serum AGP levels could be a useful biomarker for predicting LSA relapse prior to clinically detectable disease, although this method is not been widely adopted in clinical practice.

1.2.1.6 *Vascular endothelial growth factor*

Vascular endothelial growth factor (VEGF) is a cytokine, with an important role in angiogenesis, lymphangiogenesis and metastasis (Dvorak, 2002; Gentilini *et al.*, 2005; Aresu *et al.*, 2012). Found to be a useful biomarker in human LSA (Gentilini *et al.*, 2005), studies have shown that serum and plasma VEGF are also elevated in canine LSA in comparison to healthy controls (Gentilini *et al.*, 2005; Aresu *et al.*, 2012). VEGF concentrations appear to correlate with other poor prognostic indicators, with VEGF higher in HG LSA, substage b, stage V and T-cell LSA (Gentilini *et al.*, 2005; Aresu *et al.*, 2012).

Serum VEGF concentrations have some prognostic significance, with one study (Gentilini *et al.*, 2005) finding high levels correlate negatively with DFI. Although VEGF levels did not correlate with likelihood of achieving remission or overall survival (Gentilini *et al.*, 2005; Aresu *et al.*, 2012), plasma VEGF concentrations did decrease in B-cell LSA following treatment, suggesting a possible predictive role in these cases.

1.2.1.7 *Matrix metalloproteinases*

Matrix metalloproteinases (MMP) are enzymes, important in the breakdown of extracellular matrix (ECM) proteins. Through the breakdown of cell-cell and cell-ECM adhesions, MMPs are integral to cell migration and differentiation, metastasis and angiogenesis (Shao *et al.*, 2013). MMP 2 and 9 are useful biomarkers in human LSA, associated with invasion, metastasis, cell migration and angiogenesis (Aresu *et al.*, 2012; Huang *et al.*, 2015). MMP's have a role in LSA pathogenesis and high levels are associated with an unfavourable prognosis in human NHL (Aricò *et al.*, 2013). Subsequent studies have shown that plasma and serum MMP 9 are significantly higher in dogs with LSA, in comparison to healthy controls (Gentilini *et al.*, 2005; Aresu *et al.*, 2012; Huang *et al.*, 2015).

Both MMP 2 and 9 were significantly higher in dogs with WHO substage b compared to substage a (Gentilini *et al.*, 2005), and higher in T-cell LSA in comparison to B-cell LSA (Aresu *et al.*, 2012; Huang *et al.*, 2015). Additionally MMP 9 was higher in stage V disease, in comparison to stage III-IV (Aresu *et al.*, 2012). While no prognostic significance could be attributed to pre-treatment levels of serum or plasma MMPs (Gentilini *et al.*, 2005; Aresu *et al.*, 2012; Huang *et al.*, 2015), MMP 9 decreased following treatment (Aresu *et al.*, 2012; Huang *et al.*, 2015), suggestive of a potential benefit as a predictive marker.

1.2.1.8 *Serum biochemistry parameters*

In addition to specific biomarkers, as biochemistry is frequently assessed as part of routine staging, there has been interest in the potential prognostic value of various biochemical parameters.

Alkaline phosphatase (ALKP) is a membrane-bound glycoprotein, responsible for the hydrolysis of phosphate esters (Johnston, 2017). Abnormalities in serum ALKP are common biochemical findings (Comazzi *et al.*, 2004), with canine serum ALKP increasing in response to necrosis, inflammation, neoplasia and hepatobiliary disease (Johnston, 2017). ALKP has been evaluated with regard to prognosis in canine LSA, but was not a significant indicator of response or progression free survival (Wiedemann *et al.*, 2005).

Cobalamin (vitamin B12) is required for the synthesis of nucleic acids, essential haematopoiesis and a cofactor for multiple enzyme systems. Dogs are unable to synthesize cobalamin and thus reliant on dietary absorption. Hypocobalaminaemia is associated with small intestinal disease and is an independent poor prognostic factor for chronic canine enteropathies (Allenspach *et al.*, 2007). Hypocobalaminaemia has also been recorded, although not evaluated for significance, in LG gastrointestinal LSA (Lane *et al.*, 2018). While relatively uncommon in canine MLSA, hypocobalaminaemia was associated with a poor outcome and shorter OST (Cook *et al.*, 2009), suggesting that serum cobalamin concentrations may be prognostic.

Hypercalcaemia of malignancy is a common paraneoplastic syndrome, frequently associated with T-cell LSA (Zandvliet, 2016; Bailey, 2019), with hypercalcaemia at diagnosis independently associated with a poor prognosis (Marconato *et al.*, 2011; Burton *et al.*, 2013; Zandvliet, 2016; Vail *et al.*, 2019). Calcium levels can be used as a

biomarker to monitor response to therapy in LSA and other neoplasia, with recurrence of hypercalcaemia, following successful treatment, frequently a marker of relapse (Vail *et al.*, 2019).

1.2.1.9 *Haematology parameters*

Similarly, studies have evaluated the use of routine haematological parameters as prognostic or predictive biomarkers.

Anaemia, usually consistent with an anaemia of chronic disease, is one of the most common haematological abnormalities found in canine LSA (Vail *et al.*, 2019). Anaemia at presentation is a negative prognostic indicator (Davies *et al.*, 2018; Vail *et al.*, 2019) associated with shorter OST (Abbo *et al.*, 2007; Miller *et al.*, 2009; Romano *et al.*, 2016) and PFS (Childress *et al.*, 2018; Davies *et al.*, 2018). Anaemia at time of diagnosis was also associated with an increased risk of incomplete remission (Childress *et al.*, 2018).

Thrombocytopenia is thought to occur in 30-50% of canine LSA diagnosis (Grindem *et al.*, 1994; Zemann *et al.*, 1998; Vail *et al.*, 2019). Although rarely causing clinically significant haemorrhage or petechiation, thrombocytopenia is associated with incomplete remission (Childress *et al.*, 2018), decreased progression free interval (Zemann *et al.*, 1998; Moore *et al.*, 2001; Burton *et al.*, 2013; Childress *et al.*, 2018), decreased survival times (Burton *et al.*, 2013), and generally a poor overall outcome (Childress *et al.*, 2018; Vail *et al.*, 2019) in canine LSA patients. A small number of studies have, however, associated thrombocytopenia with increased survival times (Garrett *et al.*, 2002; Rebhun *et al.*, 2011), and made a tentative link between thrombocytopenia and T-cell immunophenotype (Rebhun *et al.*, 2011).

Individual blood leucocyte counts have also been evaluated, with neutrophilia associated with poor response rates (Purzycka *et al.*, 2020), reduced PFS (Childress *et al.*, 2018) and described as an unfavourable prognostic factor (Childress *et al.*, 2018; Vail *et al.*, 2019). Monocyte and neutrophil numbers were both found to be significantly elevated in dogs with LSA (Perry *et al.*, 2010), in comparison to healthy controls, and elevated neutrophil and monocyte counts were both found to be predictive of reduced DFI (Perry *et al.*, 2010), even when values remained within reference ranges.

Ratios of leucocytes have also been evaluated for prognostic significance. The neutrophil:lymphocyte ratio (NLR) compares the inflammatory response to the host immune response (Mutz *et al.*, 2013). Studies have identified a potential prognostic significance, for both PFS and OST, although a consensus is lacking (Mutz *et al.*, 2013; Davies *et al.*, 2018). Similarly, the value of the lymphocyte:monocyte ratio (LMR) has also been assessed, with low LMR levels predictive of shorter time to disease progression, and OST (Marconato *et al.*, 2015b) and LMR > 1.43 predictive of longer overall survival (Davies *et al.*, 2018). The LMR was also significantly associated with incomplete remission (Childress *et al.*, 2018).

1.2.2 Summary

While multiple biomarkers have been assessed, there is no individual marker, or combination of markers which have become mainstream biomarkers used in clinical practice. Limitations regarding the sensitivity and specificity of individual markers for diagnosis, disparity between studies and individual variation has hindered the use of single biomarkers for diagnostic, prognostic or predictive purposes.

1.3 Clusterin

Clusterin (CLU), is a glycoprotein, also known as apolipoprotein J (APO-J). CLU was initially identified in the fluid of ram rete testes, and can be seen to elicit aggregation, or clustering, of a variety of different cells (Fritz *et al.*, 1983). The CLU protein can be identified in virtually all tissues and body fluids (Flach *et al.*, 1995; Bettuzzi, 2009; Rizzi *et al.*, 2009b), and is well conserved across species (Michel *et al.*, 1997; Calero *et al.*, 1999; Wilson *et al.*, 2000). Investigated and described under multiple names, across numerous different species, many species specific homologues of the CLU protein have been identified (Jenne *et al.*, 1992; Rosenberg *et al.*, 1995; Jones *et al.*, 2002) (**Table 1**).

Table 1)

Synonyms/homologues of the CLU protein

Name	Source: species/tissue	Identification/function	Reference
Apolipoprotein J (Apo-J)	Human, serum	High density lipoprotein/apolipoprotein, involved in lipid transport	(de Silva *et al.*, 1990; De Silva *et al.*, 1990)
Complement cytolysis inhibitor (CLI)	Human, serum (liver)	Part of the complement complex, complement modulator Also identified in semen.	(Jenne *et al.*, 1989)
pADHC-9	Human, brain	Found in the hippocampus of an Alzheimer's patient	(May *et al.*, 1989, 1990)
pTB-16	Human, brain	Found in glioma and expressed in epileptic foci	(Danik *et al.*, 1991)
Serum protein-40,40 (SP-40,40)	Human, serum	Complement modulator, component in immune deposits in glomerular disease.	(Murphy *et al.*, 1988; Kirszbaum *et al.*, 1989)
Glycoprotein 80 (Gp-80)	Dog, renal	Glycoprotein secreted from the apical surface of renal epithelial cells	(Urban *et al.*, 1987; Hartmann *et al.*, 1991)
Glycoprotein III (Gp-III)	Cow, adrenal	Component of chromaffin secretory granules	(Fischer-Colbrie *et al.*, 1984; Palmer *et al.*, 1990)
Clusterin (CLU)	Sheep, Ram rete teste fluid	Protein from testis fluid, functions in reproduction, elicits cell aggregation activity	(Blaschuk *et al.*, 1983; Fritz *et al.*, 1983)
Sulfated glycoprotein-2 (SGP-2)	Rat, Sertoli cell	Sertoli cell secretion product, found on sperm membranes, functions in reproduction	(Collard *et al.*, 1987; Bettuzzi *et al.*, 1989; Sylvester *et al.*, 1991)
Testosterone repressed prostatic messenger-2 (TRPM-2)	Rat, prostate	Found during involution of prostate following androgen withdrawal, involved in apoptosis	(Leger *et al.*, 1987; Buttyan *et al.*, 1989)

Synonyms/homologues of the CLU protein found in a variety of different tissues, across different species (Jenne *et al.*, 1992; Fritz *et al.*, 1993; Bettuzzi, 2009).

Human CLU was first described in 1989 (Jenne *et al.*, 1992) as a complement cytolysis inhibitor (CLI) found in serum, with other synonyms including APO-J, sulfated glycoprotein-2 (SGP-2), serum protein-40,40 (SP-40,40), testosterone repressed prostatic messenger 2 (TRPM-2), and glycoprotein 80 (Gp-80) (Jenne *et al.*, 1992; Bettuzzi, 2009; Foster *et al.*, 2019), all of which have been identified as proteins produced from the same gene. A single-copy gene, the human *CLU* gene comprises 9 exons and is located on chromosome 8 **(Figure 1**), with the canine *CLU* gene, identified on chromosome 25 (Slawin *et al.*, 1990; Tobe *et al.*, 1991; Jones *et al.*, 2002; Foster *et al.*, 2019; NCBI, 2019).

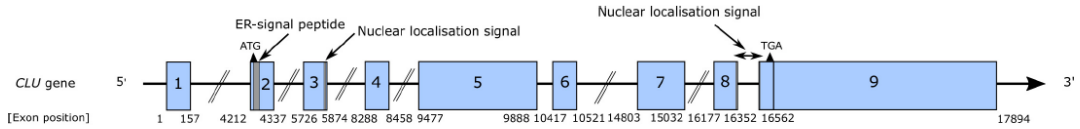


Figure 1:

CLU gene structure.
Secretory CLU is derived from mRNA transcript NM_001831.3, with translation beginning in exon two, producing the immature CLU precursor

(Adapted from Foster, 2019).

Gene expression is tightly controlled (Foster *et al.*, 2019), however upregulation of CLU mRNA and protein are widespread phenomena in developmental and pathophysiological states, suggesting that control of expression levels is dynamic (Jones *et al.*, 2002). Numerous factors have been suggested as regulatory for *CLU* gene expression, including nuclear factor-κβ, growth factors, stress and ionising radiation, with cell death and apoptotic signalling also thought to play key roles (Trougakos *et al.*, 2009b; Foster *et al.*, 2019).

1.3.1 Clusterin structure/form

Although a single gene, the *CLU* gene codes for different protein products; nuclear, cytoplasmic and secretory, each with different biological functions (Rizzi *et al.*, 2009b, 2010). Thus CLU could be thought of as a group of protein products as opposed to one single protein (Bettuzzi, 2009).

The structure of the CLU protein shows little variation between species (Michel *et al.*, 1997; Calero *et al.*, 1999; Wilson *et al.*, 2000). In humans, the mature form of secretory CLU is a heterodimeric glycoprotein of 75–80kDa (Jenne *et al.*, 1992; Bettuzzi, 2009; Naponelli *et al.*, 2017), which tends to dissociate into non identical subunits, the alpha and beta chains, each of approximately 40kDa (**Figure 2**) (Jenne *et al.*, 1992; Bettuzzi, 2009). The structure of the protein itself, with coiled/helical domains, allows CLU to interact in a stable manner, with both lipids and other proteins (Michel *et al.*, 1997). The ability of CLU to bind to a variety of different ligands facilitates the protein's diverse biological function (Calero *et al.*, 1999).

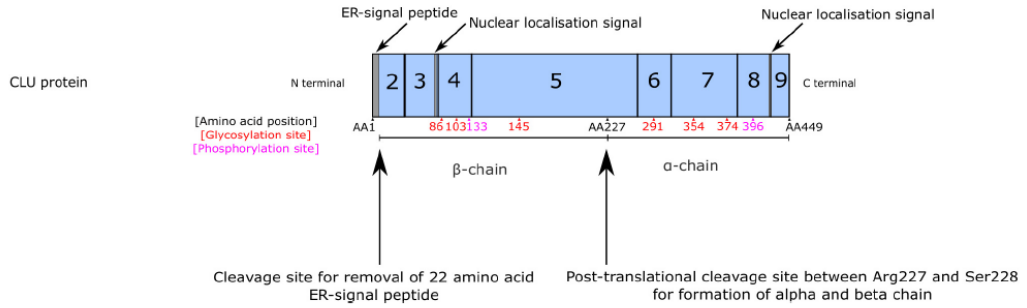


Figure 2:

CLU protein structure.
The immature CLU precursor protein is present as a non-glycosylated protein, of around 60-64kDa, which undergoes processing in the endoplasmic reticulum and maturation within the Golgi apparatus.

The protein is cleaved and modified, by glycosylation and phosphorylation, to produce the mature secretory CLU which comprises of two protein monomers, termed α and β, which are linked via five disulphide bonds. The resultant protein is a highly glycosylated heterodimer, of 75-80kDa, comprised of two approx. 40kDa subunits/chains.

(Urban, 1987; Federica Rizzi, 2009; Figure adapted from Foster, 2019).

Secretory CLU, formed in the Golgi apparatus, is exported from the cell and enters the extracellular compartment as a glycosylated protein (Bettuzzi, 2009). Intracellular CLU can be cytoplasmic and/or nuclear, with CLU staining commonly detected concurrently, in intact, fixed cells (Bettuzzi, 2009).

1.3.2 Clusterin function

Often termed an enigmatic and ubiquitous protein, the biological and pathogenic functions of CLU have been poorly defined (Bettuzzi, 2009). CLU has been implicated in many different roles, including cell-cell interaction, transportation and regulation (Wilson *et al.*, 2000). However, the exact nature and function of the protein *in vivo* are elusive.

From the extracellular compartment CLU binds to a number of serum proteins including albumin (Wyatt *et al.*, 2010) and complement factors. CLU is heavily glycosylated, a feature which can affect both the nature of the protein itself, and its binding properties. Widely expressed throughout the body (Flach *et al.*, 1995; Jones *et al.*, 2002; Trougakos *et al.*, 2002, 2004; Bettuzzi, 2009), CLU acts as an extracellular chaperone protein (Wilson *et al.*, 2000), acting to stabilise protein folding. CLU will aid in correction or facilitate degradation of misfolded proteins and assist in the clearance of proteins from extracellular spaces (Humphreys *et al.*, 1999; Wilson *et al.*, 2000, 2017; Trougakos *et al.*, 2009a; Wyatt *et al.*, 2009, 2010). Functioning similar to the small heat-shock proteins (Humphreys *et al.*, 1999; Wyatt *et al.*, 2009; Wilson *et al.*, 2017), an increase in CLU expression, regulated by cytokines and growth factors, has been shown to correlate with physiological stress (Michel *et al.*, 1997; Humphreys *et al.*, 1999; Trougakos *et al.*, 2002, 2004; Shannan *et al.*, 2006; Wyatt *et al.*, 2009; Wilson *et al.*, 2017). The chaperone function of secretory CLU is widely described; protecting cells from physical stress and neutralizing cellular insult, CLU also facilitates clearance and disposal of toxic molecules from within the cell (Wilson *et al.*, 2000, 2017). CLU has been linked to multiple biological processes; including apoptosis, tissue remodelling, membrane recycling, cell cycle regulation and immune system regulation (Wilson *et al.*, 2000; Jones *et al.*, 2002; Trougakos *et al.*, 2002; Pucci *et al.*, 2004; Shannan *et al.*, 2006).

In addition to a physiological role, CLU has also been implicated in a number of pathological processes including tumorigenesis and regulation of cell death (Trougakos *et al.*, 2002; Shannan *et al.*, 2006; Wilson *et al.*, 2017). As may be expected, given the role of CLU and cell cycle regulation and death, CLU has also been implicated in the aging process. In humans, a relationship has been evaluated between CLU expression and age, with CLU expression increasing from gestation to adulthood (Mondello *et al.*, 1999; Trougakos *et al.*, 2002, 2006), although significant individual variation exists.

1.3.2.1 *Clusterin in neoplasia*

Dysregulation of CLU has been associated with various human malignancies (Saffer *et al.*, 2002; Shannan *et al.*, 2006; Rizzi *et al.*, 2010; Frazzi *et al.*, 2011). Multiple studies identified positive IHC staining for cytoplasmic and nuclear CLU, as well as increases in serum CLU (Rodriguez-Pineiro *et al.*, 2006; Nafee *et al.*, 2012; Guo *et al.*, 2014), in a variety of epithelial neoplasia (Steinberg *et al.*, 1997; Redondo *et al.*, 2000; Kang *et al.*, 2004; Pucci *et al.*, 2004; Yom *et al.*, 2009), including prostatic (Steinberg *et al.*, 1997; Rizzi *et al.*, 2010) and mammary carcinoma (Redondo *et al.*, 2000; Krüger *et al.*, 2007; Yom *et al.*, 2009; Rizzi *et al.*, 2010), oesophageal squamous cell carcinoma (Guo *et al.*, 2014), hepatocellular carcinoma (Nafee *et al.*, 2012) and colorectal neoplasia (Pucci *et al.*, 2004; Rodriguez-Pineiro *et al.*, 2006). CLU has been implicated both as a tumour suppressor (Bettuzzi *et al.*, 2009; Trougakos *et al.*, 2009a) and a modifier of tumour promotion (Trougakos *et al.*, 2009a). Some human studies have suggested that down regulation, and subsequent loss of the CLU chaperone function, could play a role in the development of neoplasia (Scaltriti *et al.*, 2004; Shannan *et al.*, 2006; Rizzi *et al.*, 2008, 2010), with others suggesting that dysregulation of CLU expression, both upregulation or downregulation, can have an impact on tumorigenesis.

1.3.2.2 *Clusterin in human lymphoid neoplasia*

Cytoplasmic expression of CLU has been positively identified, via IHC, in cases of anaplastic large cell LSA (Wellmann *et al.*, 2000; Lae *et al.*, 2002; Saffer *et al.*, 2002; Nascimento *et al.*, 2004). Positive staining is less common in other forms of T-cell and B-cell LSA (Wellmann *et al.*, 2000; Saffer *et al.*, 2002; Nascimento *et al.*, 2004), with Saffer et al. (2002) identifying cytoplasmic CLU staining in 35% of lymphoid neoplasia assessed; and present in only a small subset (12%) of DLBCL cases.

CLU has also been identified *in vitro* (Coletta *et al.*, 2012), in the Toledo DLBCL cell line. Very low levels of CLU were identified, via fluorescence microscopy and real-time PCR, in the LSA cell line in comparison to B lymphocytes from healthy human blood. With regard to lab based cell lines, it has been shown that doxorubicin treatment upregulates secretory CLU isoforms (Frazzi *et al.*, 2011; Coletta *et al.*, 2012), this has also been shown *in vivo*, with serum CLU expression upregulated in a subset of patients following treatment for HL (Frazzi *et al.*, 2011).

1.3.3 Canine clusterin

Canine CLU has been evaluated as a marker in various inflammatory and disease states. Studies evaluating serum and plasma of overweight dogs identified CLU overexpression, with levels decreasing in line with weight loss (Tvarijonaviciute *et al.*, 2012, 2013). Urinary (García-Martínez *et al.*, 2012; Zhou *et al.*, 2014) and renal tissue CLU (Tsuchiya *et al.*, 2005) were increased in response to renal damage, with urinary CLU levels significantly increased following intravenous (IV) contrast agent administration (Fowlie *et al.*, 2017). Additionally, evaluation of CSF found CLU levels to be higher in canine degenerative myelopathy and intervertebral disc disease, in comparison to other neurological diseases (Shafie *et al.*, 2014). These changes indicate potential for urinary and CSF CLU as early biomarkers for renal or spinal cord disease. Thus far little has been reported regarding CLU expression in canine malignancies.

1.3.3.1 *Canine clusterin in lymphoma*

In 2013, Atherton et al. characterised the serum proteome of dogs with LSA, via serum protein electrophoresis and mass spectrometry. In this study multiple canine proteins, which were not previously identified in healthy dogs, were identified in dogs with LSA. One of these proteins was a CLU precursor, present in the alpha-2 band of the serum proteome of a dog with high grade MLSA and absent in serum from two healthy controls (Atherton *et al.*, 2013a). This led to speculation that CLU may have an anti-apoptotic, pro-neoplastic role in canine LSA (Atherton *et al.*, 2013a). The CLU protein was also identified in a separate study (Fonghem *et al.*, 2017), investigating the proteomic profiles of canine LSA, although it was not identified as significantly elevated or decreased in LSA in comparison to a small control population.

1.4 Aims

The objectives of this study were to determine if:

i) serum CLU expression differed significantly between those dogs with high grade MLSA, and a healthy control population and therefore had potential as a useful diagnostic tool.

ii) serum CLU expression differed significantly in individual patients following successful treatment and induction of remission and therefore had potential as a monitoring tool, as a marker of remission, early relapse or treatment failure.

It was hypothesised that serum CLU levels would be higher in those patients with MLSA in comparison to healthy controls and may return to "normal" levels following successful treatment.

1.4.1 Subsequent aims

1.4.1.1 *Prognostic*

A subsequent aim was to determine whether disease stage, substage or immunophenotype was linked to alterations in CLU expression, and subsequently whether CLU expression could be linked to prognosis, particularly time to relapse or treatment failure.

2 Materials & Methods

2.1 Sample Selection

Archived serum samples were selected from canine patients who presented to the Small Animal Hospital between 2013 and 2018, with a confirmed cytological or histopathological diagnosis of high grade MLSA. Samples from dogs with higher stages of disease, as determined by WHO classifications (Owen, 1980), were preferentially included. Retrospective analysis of medical records was performed to obtain information pertaining to signalment, WHO stage (I-V) and substage (a = no clinical signs, b = clinical signs) (Owen, 1980; Vail *et al.*, 2013), confirmation of diagnosis and treatment. Exclusion criteria included severely haemolysed or lipemic samples, concurrent infectious or systemic inflammatory disease, prior treatment and failure to achieve CR.

As the study was retrospective, detailed analysis of the medical records was performed to obtain case information, particularly WHO stage and substage. Investigations for staging were not standardised, and differed between patients, based on historical clinician preference. Investigations included complete blood count, serum biochemistry, urinalysis, cytology of lymph node, liver, spleen and bone marrow, thoracic imaging, either radiographs or computed tomography, and abdominal ultrasound scan. However not all investigations were performed for each case. Information regarding immunophenotype/genotype and clonality was included where performed, either following IHC, flow cytometry, PARR analysis or a combination of these techniques. Due to the retrospective nature of the study, and the lack of standardized staging investigations, stage migration could not be excluded.

The study was performed with full ethical approval from the Ethics and Welfare committee (Ref 56a/15) at the University of Glasgow, with owner consent (**Appendix 1**) obtained for the retention of spare serum. All serum samples were surplus blood, from samples collected for diagnostic and treatment purposes. Clotted serum samples were centrifuged at 9000rpm for three min, prior to serum extraction and freezing. All serum samples were stored frozen, at -20 °C, prior to transfer for storage at -80 °C, for future analysis.

2.1.1 Sample categories

Samples were grouped into three broad categories: -

1. Pre-treatment: Samples from dogs with MLSA taken at the time of diagnosis, prior to any corticosteroid or chemotherapy treatment
2. Complete remission (CR): Samples from dogs with MLSA in CR, as determined by physical examination and lymph node palpation, at week 4-6 of a (CHOP-based) chemotherapy protocol (**Appendix 2**), by a qualified veterinary surgeon experienced in veterinary oncology.
3. Control samples: Serum samples from healthy dogs were obtained from the Pet Blood Bank UK. These serum samples were acquired from the dogs for screening and monitoring purposes at the time of blood donation. Control samples were stored at -20 °C prior to transport and subsequently transported frozen to Glasgow. Samples were then stored in the same conditions as the MLSA samples, at -80 °C, for future analysis.

Full sample lists are documented in **Appendix 3**.

2.1.2 Sample groups

All samples were selected, from the previously identified pool of suitable samples and controls, to generate distinct sample groups.

A pilot group of samples, consisting of dogs with MLSA, pre-treatment (n=20) and healthy control dogs (n=20) (**Table 2**), was initially used for optimisation and refinement of technique. Latterly, samples were divided into three samples groups for analysis and comparison of CLU levels, a small number of samples matched criteria, and were included in, multiple sample groups (as outlined in **Appendix 3**). When creating these sample groups, preference was given to samples with shorter storage history, samples with larger sample volume, and samples with higher burden of disease (i.e. Stage III or above, as per the WHO staging system).

Sample group one consisted of 3 cohorts; dogs diagnosed with MLSA, pre-treatment (A, n=12), unrelated dogs with MLSA, in CR (B, n=12) and healthy control dogs (C, n=12), (**Table 4**).

Sample group two consisted of 2 cohorts; dogs diagnosed with MLSA, pre-treatment (n=18), and in CR (n=18), i.e. paired samples from the same individuals, (**Table 5**).

Sample group three consisted of 2 cohorts; dogs diagnosed with MLSA, pre-treatment (n=18) and healthy control dogs (n=18). Samples for group three were intentionally selected to form age matched cohorts, (**Table 6**).

2.2 Sample storage and dilution

Selected samples were thawed in an ice bath. An aliquot of serum (100µl) was removed from the sample, labelled and stored as neat serum. A dilution was performed for portion of each serum sample, with 10µl of sample diluted; 1:20 in purified water (Milli-Q^{TM}) and diluted samples divided into aliquots. Aliquots were stored at -20 °C and individual aliquots thawed as required. This was in order to reduce the number of freeze/thaw cycles for each individual sample. The impact of repeated freeze/thaw cycles on the protein content of canine serum, and the stability of the CLU protein in canine serum is as yet unknown.

2.3 Protein Quantification

Total protein concentrations of the canine serum samples were determined using the bicinchoninic acid (BCA) protein assay system (Thermo Life Science Ltd, UK).

2.3.1 Optimisation

Initial protein assays were performed on 96 well plates, with protein standards and four serum samples. Protein standards (BSA 2mg/ml stocks from Thermo LifeScience.uk) of varying concentrations (0.05 – 0.8µg/ml) were run, in duplicate, to provide reference points and allow generation of a standard curve. The standard curve then facilitated the quantification of protein concentration within the samples.

Serum samples (diluted 1:20) were loaded onto the 96 well plates at volumes of 2.5µl, 5µl, 75µl, 10µl, 15µl, and 20µl. All samples were run in duplicate and the varying volumes used in order to determine optimal sample volume for the protein assay.

Initial assays for optimization of sample volume established that the volume of 2.5µl of sample/well was optimal for determination of protein concentration.

2.3.2 Protein Assays

Diluted samples were thawed, and reagents brought to room temperature. Samples (2.5µl) and standards (25µl) were added to the appropriate wells on the microplate, along with 200µl of buffer solution. Buffer solution was created by mixing Pierce BCA protein assay sample Reagent A (10ml) with Reagent B (200µl) prior to adding to each well. The microplate was agitated at 350rpm on an orbital microplate shake for 30s and incubated for 30 min at 37.0°C. After incubation, the plate was read immediately at 562nm on a microplate reader, using Ascent software (Multiskan Ascent, MTX Lab Systems). Data was analysed using Microsoft Excel, (2010) and GraphPad Prism software, version 5 for Windows (GraphPad Software, San Diego, California, USA), with a calibration curve generated using protein standards to determine the protein concentrations of each sample.

2.4 Assessment of serum clusterin levels

Serum CLU levels in individual samples were assessed using two separate techniques:- western blot was performed, to confirm the detected protein had the predicted molecular weight, using a previously validated (Shafie *et al.*, 2013, 2014) commercial anti-human antibody, predicted to have cross species reactivity with dog (at 93%). Initial experiments were performed to confirm the specificity of the antibody (a comparison of a western blot with the CLU primary antibody and secondary antibody only) and sensitivity of the method (signal intensity of CLU versus increased amount of protein loaded onto the gel). An ELISA was also performed using a commercial (BioVendor Ltd) kit, to increase the throughput of samples in larger quantities. The assay was previously validated, by BioVendor, using canine serum, and validation was not specifically repeated during these investigations.

2.4.1 Western Blot

Western blot analysis was performed as previously described (Shafie *et al.*, 2013, 2014). Initially, protein concentrations of the serum samples were determined using the BCA protein assay system (Thermo Life Science Ltd, UK) as described in section 2.3.

2.4.1.1 *Gel electrophoresis*

Serum sample aliquots (10µg protein, as determined by optimisation experiments, described in section 3.1) were diluted with purified water (Milli-Q^{TM}) to give a sample volume of 10µl. Five microliters of 3x denaturing buffer (BioRad Ltd, UK) containing freshly added dithiothreitol (DTT) to give a final concentration of 50mM was added to each of the sample tubes, to facilitate denaturation of the proteins, prevent disulphide bond reformation and attach negatively charged sodium dodecyl sulfate (SDS) and allow electrophoretic separation of the proteins. Samples were centrifuged for six seconds at 9000rpm, incubated at 90°C for four min, then loaded on to the gel.

Samples were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS – PAGE) using Criterion 18 or 26 well gels (BioRad Ltd, UK). Samples from diseased and control dogs were loaded in alternate wells, with protein molecular weight standards (BioRad Ltd, UK) run in the first and last well of each gel. Electrophoresis was performed in 5% MES (2(N-morpholino) ethanesulfonic acid) buffer, with a voltage ranging between 130-145 V, for 45-60 min.

Following electrophoresis samples were transferred to nitrocellulose membranes using the iBlot^{TM} system (Invitrogen, Thermo Fisher, UK). Initial gels were run and stained with Coomassie Brilliant Blue (**Figure 3**), to allow visualisation of remaining individual protein bands and confirm equal loading across the sample lanes. When outliers were apparent, the protein assay and SDS-PAGE process was repeated.

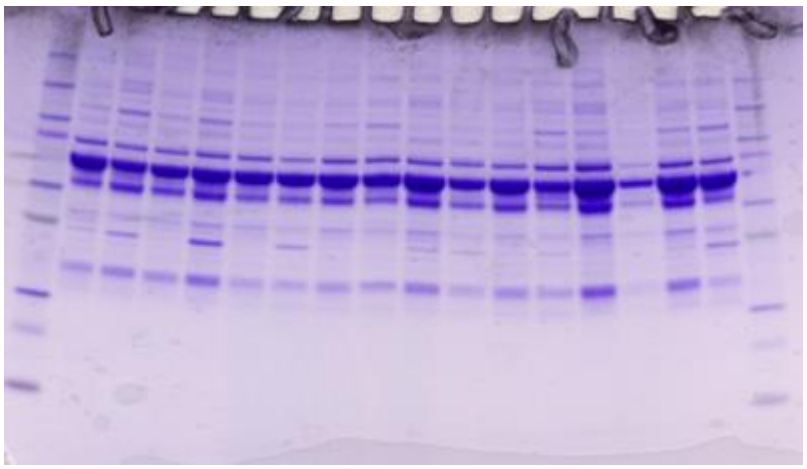


Figure 3:

Gel electrophoresis of serum samples showing separation of protein by molecular weight.

2.4.1.2 *Antibody incubation*

Following transfer, the membranes were blocked in 5% semi skimmed milk solution, with Tris-buffered saline (pH 7.4) containing 0.5% Tween 20 (T-TBS), at room temperature, at 300rpm on an orbital microplate shaker. Blots were washed, three times with 0.5% T-TBS solution and incubated overnight at 4°C, with a goat primary antibody against anti-human CLU (Antibody 104652, Abcam Ltd, UK), diluted 1:20,000, with gentle shaking at 300rpm on an orbital microplate shaker.

Two separate commercial, primary antibodies were initially analysed to assess which generated the cleanest signal with minimal nonspecific background, with the canine samples. The primary antibodies, raised in goat against a human CLU protein, were compared; Abcam anti-clusterin antibody 39991 (991) and anti-clusterin antibody 104652 (652), (**Figure 4A**). More consistent staining, at the correct molecular weight (37kDa), was seen with antibody 652 and all further western blots were performed with this antibody.

To determine optimal primary antibody concentration, dilutions of the primary antibody were made in 5% semi skimmed milk solution with (T-TBS) to 1:10,000, 1:20,000 and 1:50,000. Protein band density and background interference were analysed on radiographic films after performing electrophoresis. Whilst a 1:10,000 dilution was initially used for the pilot samples, comparison of dilutions showed that a 1:20,000 dilution of the primary Abcam anti-clusterin antibody 104652 provided the best signal, with minimal background interference and was therefore used for ongoing analysis.

After incubation with the primary antibody, the blots were washed with 0.5% T-TBS solution, repeated three times. Membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam Ltd, UK), diluted to 1:10,000, at room temperature. After incubation the blots were washed with 0.5% T-TBS solution, repeated three times.

2.4.1.3 *Enhanced Chemiluminescence and film development*

Immunocomplexes were detected using the enhanced chemiluminescence (ECL) reagent (Pierce ECL, Thermo Fisher, UK) and the protein bands visualised via radiographic film. Equal volumes of ECL reagents were constituted as per manufacturer guidelines. Nitrocellulose membranes (blot) were immersed in ECL solution and the solution agitated. Excess ECL reagent was removed by gentle blotting with 3MM paper, the blot was then enclosed in Saran wrap and images acquired using radiography film and standard darkroom development techniques for chemiluminescence. Exposures of between 30s to 2 min were found to produce optimal signal intensity for CLU. The radiographic films were then scanned and stored digitally as TIFF files.

To determine optimal exposure times for radiographic films, different exposure times were selected and trialled. Films were exposed to the blot, following ECL contact, for 30s, 1 minute, 3 minutes and 5 minutes. Protein band density and background interference were compared on radiographic films to determine the optimal exposure times. Initial pilot studies were performed with exposure times of 5 minutes to generate radiographic images. However, elimination of background interference and clarity of CLU bands was deemed best at 30s exposures although a range of exposure times were collected due to the subtle variations that are encountered with this method.

2.4.1.4 *Data analysis*

Data pertaining to protein band density was quantified using Image-J software (Schneider *et al.*, 2012), generating numerical values equivalent to the protein signal intensity of each sample.

2.4.2 ELISA

CLU concentrations were measured using a sandwich enzyme immunoassay (BioVendor Ltd, UK) for quantitative measurement of canine CLU. This assay was previously validated, by BioVendor, to have low within-run imprecision and an intra-assay coefficient of variance (n = 8) of 4.3%, and an acceptable between-run comparability, with inter-assay coefficients of variance (n = 8) of 6.2%. A normal canine serum CLU concentration range has not yet been established.

The samples were thawed, and reagents brought to room temperature. Standards, buffers and washes were constituted as per manufacturer guidelines (**Appendix 4**). Samples were diluted 1:2500 dilution buffer [to facilitate the ELISA]. The ELISA was run as per manufacturer guidelines, using dilution and wash solutions provided.

Quality control reference samples and CLU standards supplied with the ELISA kit were included with each run, along with the diluted samples (100µl) and added to the appropriate wells. The plate was incubated for 1h at room temperature with gentle shaking, at 300rpm on an orbital microplate shaker. After incubation the plate was washed with 350µl of wash solution per well, repeated three times.

After the final wash, the plate was inverted and blotted with paper towel. Biotin labelled antibody (100µl) was added to each well and the plate incubated for one hour at room temperature, with gentle shaking. After incubation the plate was washed with 350µl of wash solution, repeated three times; the plate was then inverted and blotted.

Streptavidin-HRP conjugate (100µl) was then added to each well and the plate was incubated for 30min at room temperature with gentle shaking. After incubation the plate was washed with 350µl of wash solution, repeated three times; the plate was then inverted and blotted.

Tetramethylbenzidine (TMB) Substrate Solution (100µl) was then added to each well and the plate incubated for 10min, at room temperature. Finally, 100µl hydrogen peroxide stop solution was added to the wells. The plate was read immediately at 450nm and 650nm on a microplate reader, using Ascent software (Multiskan Ascent, MTX Lab Systems).

2.4.2.1 *Data analysis*

Data was analysed with Microsoft Excel, (2010) and GraphPad Prism software, version 5 for Windows (GraphPad Software, San Diego, California, USA). A standard curve was generated, using results from the supplied CLU standards, to allow numerical absolute values to be generated for the CLU concentrations in each sample.

2.5 Statistical analysis

Analyses were performed using GraphPad Prism, version 5 for Windows (GraphPad Software, San Diego, California, USA) and Minitab 19 (Minitab® Statistical Software) with statistical significance set at $P \leq 0.05$. The serum CLU concentrations were analysed using parametric and non-parametric testing, depending on normality of data distribution, with student t- test, Mann–Whitney U test, analysis of variance (ANOVA) and Wilcoxon signed rank tests. Group characteristics were compared using Mann–Whitney U test, ANOVA, Chi squared and Fisher's exact tests.

3 Results

3.1 Optimisation of western blot technique

Initial optimisation of technique was performed using two canine serum samples, one control sample and one MLSA pre-treatment sample. Gel electrophoresis was performed, western blots were run, and radiographic films created, as described in section 2.4.1.

Results of the optimisation for the primary antibody, as documented in section 2.4.1.2, identified a more consistent staining, at the correct molecular weight (37kDa) with antibody 652 (**Figure 4A**).

To confirm specificity of the 652 anti-clusterin antibody, western blots were run for the serum samples, one blot was incubated with the primary goat antibody against CLU; the other was incubated without the primary antibody. The presumed CLU protein band, visible at approximately 37kDa, was only identified following incubation with the primary antibody. This indicated that the CLU antibody detected a protein of the correct molecular weight. The absence of a protein band at this weight, without the primary antibody, implied specificity of the antibody to canine CLU, (**Figure 4B**).

For each of the antibody optimisation experiments, both samples were run in quadruplicate, at differing protein concentrations. In order to determine optimal concentrations, aliquots of serum containing 2.5µg, 5µg, 10µg, and 20µg of protein, as determined via previous protein assay, were prepared and run as per section 2.4.1. Western blots were run, and examination of the resultant radiographic films revealed an optimum protein concentration for sample handing and band density, of 10µg of sample, (**Figure 4C**).

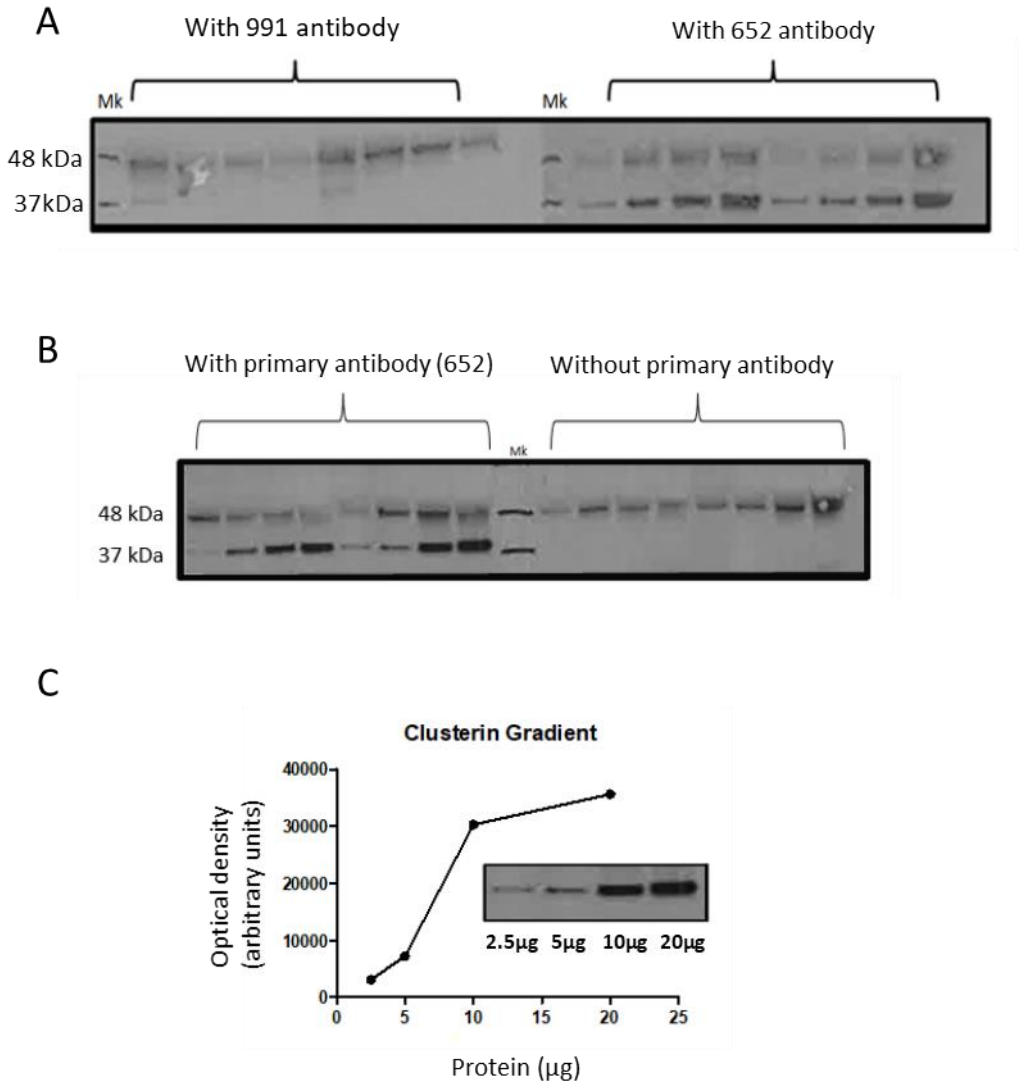


Figure 4:

A) *Western blot of control and MLSA pre-treatment serum samples, comparing different antibodies are shown for anti-clusterin antibodies, 991 and 652, both at 1:10,000 dilutions. The position of the molecular weight marker (Mk) is indicated at 48 and 37 kDa. A stronger, clearer protein band is seen with the CLU 652 antibody, at the predicted molecular weight of approximately 37kDa.*

B) *Western blot of control and MLSA pre-treatment serum samples, incubated with and without primary anti-clusterin antibody 652, at a 1:10,000 dilution confirming CLU antibody 652 specificity. The position of the molecular weight marker (Mk) is indicated at 48 and 37 kDa. A clear protein band can be seen at approx. 37kDa in the presence of the primary anti-clusterin antibody and not present without the primary antibody.*

C) *The intensity of the CLU signal is proportional to the amount of protein loaded onto the gel. Analysis of the protein intensity by Image J is shown graphically and there is indication of saturation with 20ug of protein, 10ug of protein was used in subsequent experiments.*

3.2 Clusterin expression in pilot samples

Samples were initially selected from a bank of archived serum samples, using the selection criteria described in section 2.1. Samples consisted of; dogs with MLSA pre-treatment (n=20) and healthy controls (n=20), (**Table 2**). Eight samples from each cohort were selected for western blot analysis, (**Table 3**) before experiments were expanded to include all 20 samples from each cohort.

Table 2)

Signalment of pilot samples

	Pre-treatment MLSA (n=20)	Controls (n=20)	p values
Age (years) Median Range	 7 2 - 12	 4 0.75 - 7	 Mann Whitney U Test $p < 0.05$
Sex: Male entire Male neutered Female entire Female neutered	 9 6 2 3	 4 8 1 7	 Chi squared Fisher's exact $p > 0.05$
Stage: III IV V	 5 8 7	 N/A	 -
Substage: a b	 6 14	 N/A	 -
Immunophenotype: B T Null	 8 2 1	 N/A	 -
Breed: Airedale terrier Border collie Border terrier Boxer Bullmastiff Cairn terrier Cocker spaniel Crossbreed Doberman Flat-coated retriever German shepherd Golden retriever Gordon setter Greyhound Labrador retriever Patterdale terrier Springer spaniel Tibetan terrier	 1 3 1 3 1 1 1 2 0 0 1 0 1 0 1 1 2 1	 0 0 0 0 0 0 0 1 1 1 2 4 0 2 8 0 1 0	 -

Table 3)

Signalment of partial pilot group

	Pre-treatment MLSA (n=8)	Controls (n=8)	p values
Age (years) Median Range	 6.5 2 – 10	 3 0.75 – 7	 Mann Whitney U Test p > 0.05
Sex: Male entire Male neutered Female entire Female neutered	 6 2 0 0	 3 4 0 1	 Chi squared Fisher's exact p > 0.05
Stage: III IV V	 4 2 2	 N/A	 -
Substage: a b	 3 5	 N/A	 -
Immunophenotype: B T	 4 1	 N/A	 -
Breed: Border collie Boxer Cocker spaniel Crossbreed Flat-coated retriever German shepherd Golden retriever Labrador retriever Springer spaniel Tibetan terrier	 1 1 1 2 0 1 0 0 1 1	 0 0 0 1 1 2 1 3 0 0	 -

3.2.1 Western blot on 18 well gel.

Serum CLU concentrations of dogs with MLSA, pre-treatment (S, n=8) were compared to healthy controls (C, n=8), via western blot analysis, (**Figure 5A**). Single samples were loaded onto the gel in an alternating pattern, control sample then MLSA sample, repeated from left to right. Molecular weight protein markers were run in the first and last well of each gel. The median protein band density of the pre-treatment serum samples was 6697 (range 1874 - 8043), compared to 6901 (range 4773 - 8736) for control samples, (**Figure 5B**). There was no statistically significant difference between the two groups (p=0.51, Mann Whitney U test).

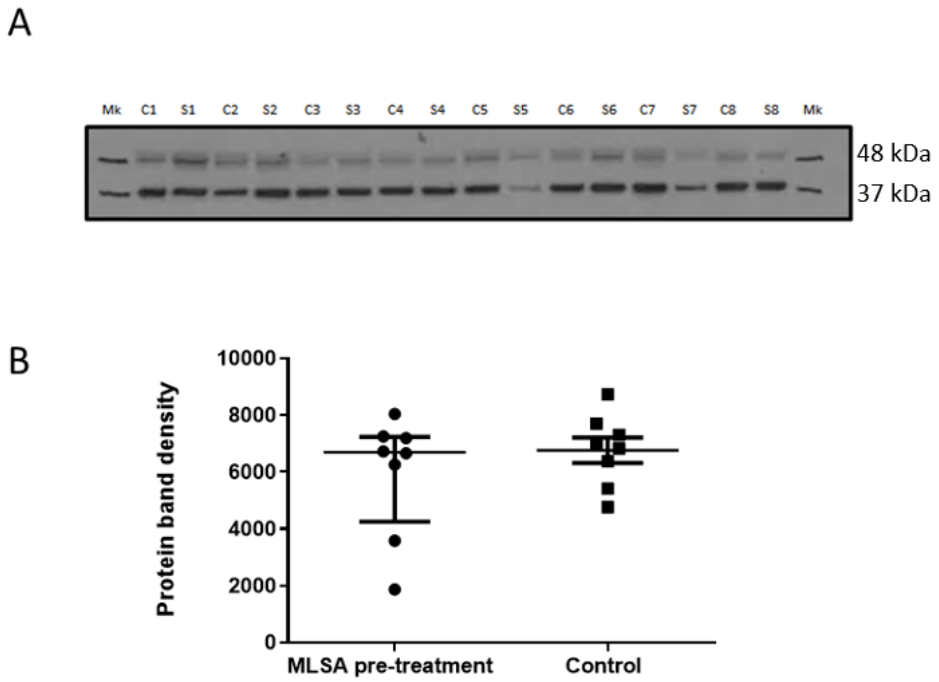


Figure 5:

A) *Western blot analysis comparing serum CLU in pilot samples from dogs with MLSA pre-treatment (S1-S8) and healthy controls (C1-C8).*
CLU 652 antibody at a 1:10,000 dilution.
Mk denotes annotation of molecular weight protein marker, labelled at 37kDa.

B) *Scatter graphs comparing protein band density, depicting serum CLU levels, in pilot samples from dogs with MLSA pre-treatment (n=8) and healthy controls (n=8).*
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between MLSA pre-treatment and healthy controls.

3.2.2 Western blots run across two 26 well gels.

Serum CLU concentrations of dogs with MLSA, pre-treatment (S, n=20) were then compared to healthy controls (C, n=20), via western blot analysis, (**Figure 6**). To facilitate the number of samples, two BioRad gels were run concurrently. Single samples were again loaded onto the gel in an alternating pattern, control sample then MLSA sample, repeated from left to right. Molecular weight protein markers were run in the first and last well of each gel. Eight samples chosen at random, four MLSA pre-treatment and four control samples were run in duplicate, appearing once on each gel. These samples were to serve as between gel control, due to the inherent variability than can occur between western blots.

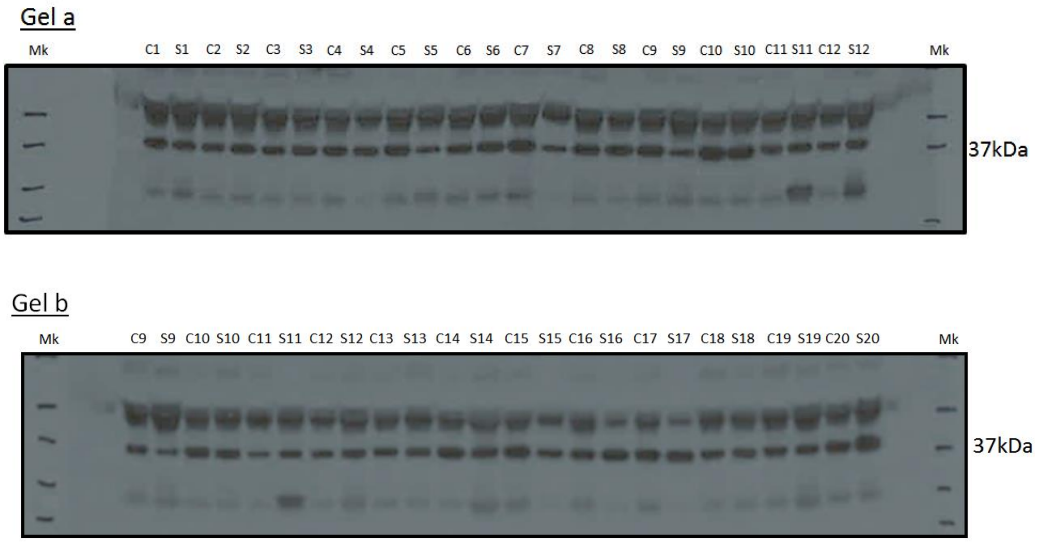


Figure 6:

W*estern blots comparing serum CLU in pilot samples from dogs with MLSA pre-treatment (S1-S20) and healthy controls (C1-C20), CLU 652 antibody at a 1:20,000 dilution.*
Mk denotes annotation of molecular weight protein standard marker, labelled 37kDa.
Four MLSA pre-treatment samples, S9-S12 and four control samples C9-C12 appear in duplicate, once on each gel.

Comparison between western blots is frequently problematic, due to potential differences in the final product. Experimental technique, due to human factors, equipment and environment could alter the final radiographic quality and hence the ultimate density of protein bands. To take this into account, the samples which were run in duplicate were used to produce a correction factor (×). The mean protein band density from duplicated samples on Gel a was divided by the mean protein band density from duplicated samples on Gel b to create correction factor ×. The remaining protein band density results from Gel b were each individually multiplied by × to give a corrected protein band density for those eight controls and eight MLSA samples.

When the correction value was applied to protein band density results from Gel b, all 40 samples could be directly compared. The median protein band density of the MLSA pre-treatment serum samples was 2697 (range 778.6 - 5347), the median protein band density of the healthy control samples was 2589 (range 197 - 5377), (**Figure 7**).

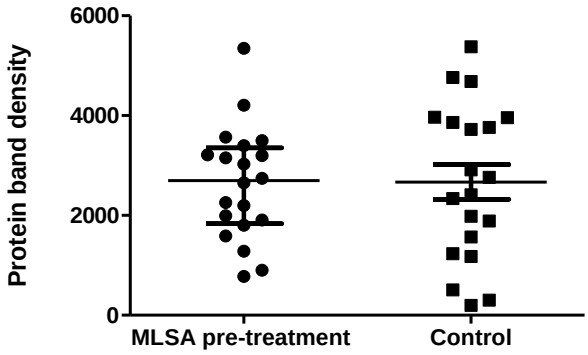


Figure 7:

Scatter graphs comparing protein band density, depicting serum CLU levels, comparing pilot samples from dogs with MLSA pre-treatment (n=20) and healthy controls (n=20). Vertical scatter graphs showing data presented with median and interquartile range. Serum CLU was not significantly different between MLSA pre-treatment and healthy controls.

There was no statistically significant difference in protein band density between MLSA pre-treatment samples and healthy controls (p=0.88, Mann Whitney U test).

Although using a correction factor allowed comparison between blots and accounted for individual experiment variation, it was deemed imprecise and as such not a reliable method of experimentation. Further direct comparisons were therefore confined to intra-blot data, which limited sample numbers.

3.3 Clusterin - sample group one (unrelated samples)

All samples were selected, using selection criteria as described in section 2.1, to create the first sample group, and generate three distinct sample cohorts.

Sample group one consisted of 3 cohorts; dogs diagnosed with MLSA, pre-treatment (A, n=12), unrelated dogs with MLSA, in CR (B, n=12) and healthy control dogs (C, n=12).

3.3.1 Group signalment

Signalment of the dogs in sample group one is summarised in **Table 4**.

Of the pre-treatment MLSA samples (cohort A) median age was 8 years (range 5-12). Nine dogs were male (four entire, five neutered) and three were female (three entire). There were eight breeds represented, with border collie the most common, representing 25% of the population; boxer and Labrador retriever followed, representing 16.7% each, of the group population. Eight dogs had stage V disease and four had stage IV disease; there were three dogs in substage a and nine in substage b, as determined by the WHO TNM classifications(Owen, 1980; Vail *et al.*, 2013). Immunophenotype was known for 8/12 (66.6%), there were seven dogs with B-cell LSA (87.5%) and one with T cell LSA (12.5%).

Of the samples in CR (cohort B) median age was 7.5 years (range 4-12). Seven dogs were male (three entire, four neutered) and five were female (five neutered). There were eleven breeds represented, with border collie again the most common, representing 16.7% of the group population. Two dogs had stage V disease, 6/12 had stage IV disease, 3/12 stage III disease and 1/12 stage II disease. There were five dogs in substage a and seven in substage b. Immunophenotype was known for 10/12 cases (83.3%), with all dogs having B-cell LSA.

Of the healthy control dogs (cohort C), median age was 4 years (range 1-7). Five dogs were female (one entire, four neutered) and seven were male (three entire, four neutered). There were seven breeds represented, with Golden retriever and Labrador retriever the most common, each representing 25% of the control population.

Signalment of the dogs with untreated and treated MLSA was similar. Median age did not differ significantly between cohort A (8 years) and B (7.5 years) groups, however the median age of the control dogs (4 years) was significantly younger than both MLSA, pre-treatment and in CR ($p<0.05$, ANOVA, Bonferroni). All cohorts were predominantly male, with no significant difference in sex or neuter status between the three ($p>0.05$, Chi-squared). Stage of disease at initial presentation (untreated) was higher in cohort A, with significantly more stage V disease in comparison to the cohort B ($p<0.01$, Fisher's exact); there was no significant difference in substage. Immunophenotype was known in 75% of cases overall (75%), with a higher proportion of B-cell LSA in cohort B, although this was not statistically significant ($p>0.05$, Fisher's exact).

Table 4)

Signalment of sample group one

	Pre-Treatment MLSA (A, n=12)	CR MLSA (B, n=12)	Control (C, n=12)	p values
Median Age (years) Range	8 5 - 12	7.5 4 - 12	4 1 - 7	ANOVA $p < 0.05$
Sex: Male entire Male neutered Female entire Female neutered	 4 5 3 0	 3 4 0 5	 3 4 1 4	 Chi squared $p > 0.05$
Stage: II III IV V	 0 0 4 8	 1 3 6 2	 N/A	 Chi squared $p < 0.05$
Substage: a b	 3 9	 5 7	 N/A	 Fisher's exact $p > 0.05$
Immunophenotype: B T	 7 1	 10 0	 N/A	 Fisher's exact $p > 0.05$
Breed: Beagle Border collie Border terrier Boxer Cairn terrier Cavalier King Charles spaniel Cocker spaniel Crossbreed Dachshund Doberman pinscher Flat coated retriever German shepherd Golden retriever Gordon setter Greyhound Hungarian visla Jack Russel terrier Labrador retriever Tibetan terrier Whippet	 0 3 1 2 1 1 0 1 0 0 0 0 0 1 0 0 0 0 2 0 0	 1 2 0 0 0 0 1 1 1 0 0 1 1 0 0 1 1 0 0 1 1	 0 0 0 0 0 0 0 1 0 1 1 2 3 0 1 0 0 3 0 0	-

3.3.2 Western Blot analysis

Serum CLU concentrations, of the three cohorts, were initially compared by western blot analysis, on three separate western blots. Intra-blot comparison of protein band density was carried out.

3.3.2.1 *Comparison of dogs with MLSA pre-treatment and healthy controls*

Serum samples from dogs with MLSA pre-treatment (A) were compared to control samples (C), via western blot analysis, (**Figure 8A**). Single samples were loaded onto the gel in an alternating pattern, MLSA sample then control sample, repeated from left to right. Molecular weight protein markers were run in the first and last well of each gel.

The median protein band density of the MLSA pre-treatment serum samples was 11074 (range 2885-19590) compared to 14699 (range 7324-20622) for control samples, (**Figure 8B**).

There was no statistically significant difference between the two groups (p=0.07, student's t-test).

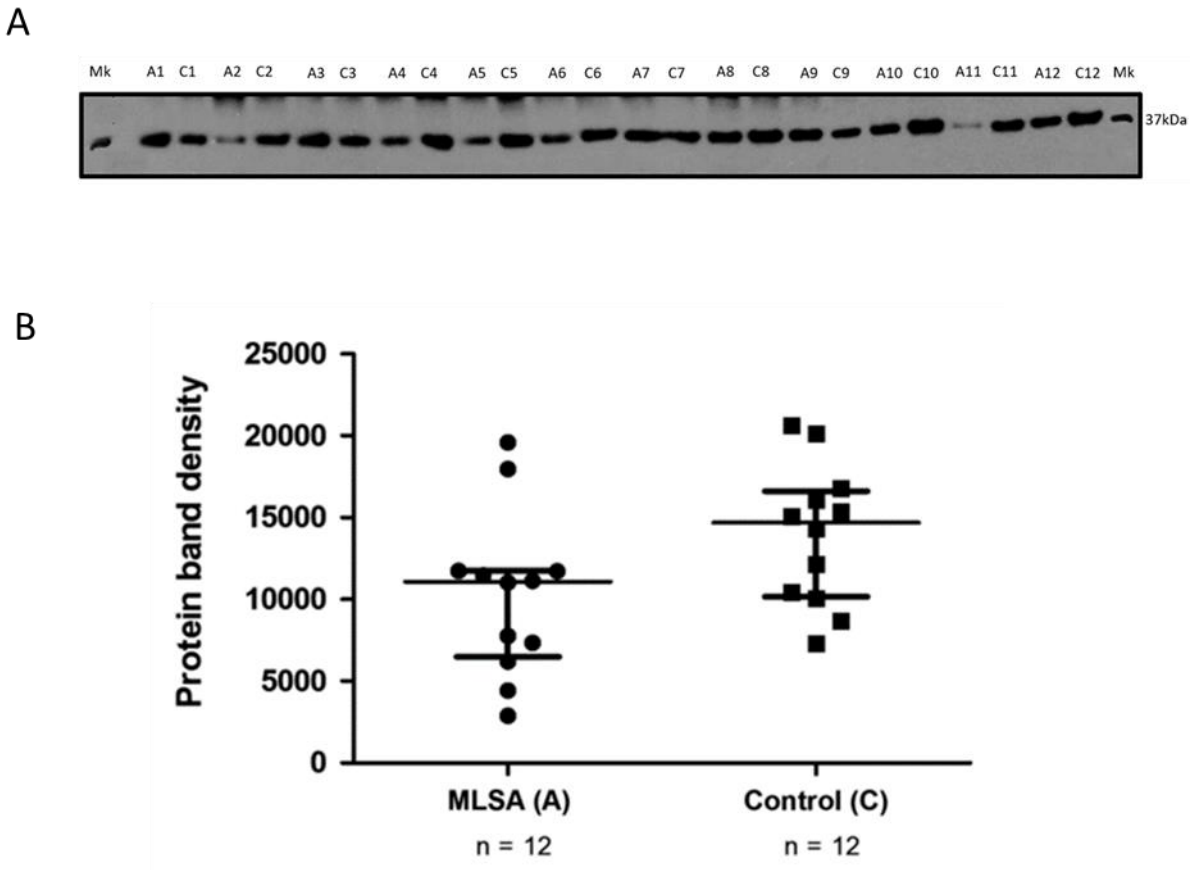


Figure 8:

A) *Western blot analysis comparing serum CLU in dogs with MLSA pre-treatment (A1-A12) and healthy controls (C1-C12).*
Mk denotes annotation of molecular weight protein standard marker, labelled at 37kDa.

B) *Scatter graphs comparing protein band density, depicting serum CLU levels, comparing dogs with MLSA pre-treatment (A, n=12) and healthy controls (C, n=12).*
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between MLSA pre-treatment and healthy controls.

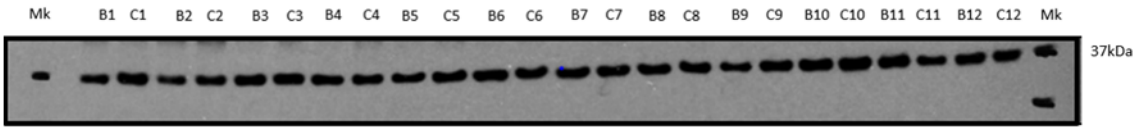
3.3.2.2 *Comparison of dogs with MLSA in CR and healthy controls*

Serum samples from dogs with MLSA in CR (B) were compared to control samples (C), via western blot analysis, (**Figure 9A**). Single samples were loaded onto the gel in an alternating pattern, MLSA CR sample then control sample, repeated from left to right. Molecular weight protein markers were run in the first and last well of each gel.

The median protein band density of the serum samples from dogs in remission was 12654 (range 10620-14756) compared to 13977 (range 9947-15863), for serum of control samples, (**Figure 9B**).

There was no statistically significant difference between the two groups ($p=0.15$, student's t-test).

A



B

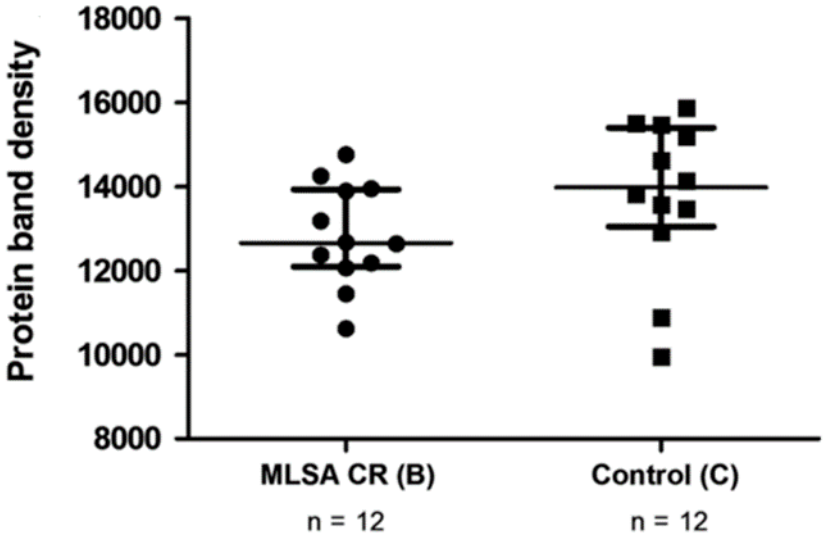


Figure 9:

A) *Western blot analysis comparing serum CLU in dogs with MLSA in CR (B1-B12) and healthy controls (C1-C12).*
Mk denotes annotation of molecular weight protein standard marker, labelled at 37kDa.

B) *Scatter graphs comparing protein band density, depicting serum CLU levels, comparing dogs with MLSA in CR (B, n=12), and healthy controls (C, n=12). Vertical scatter graphs showing data presented with median and interquartile range.*
Serum CLU was not significantly different between MLSA CR and healthy controls.

3.3.2.3 *Comparison of MLSA pre-treatment and CR*

Serum samples from dogs with MLSA pre-treatment (A) were compared to unrelated dogs with MLSA in CR (B), via western blot analysis, (**Figure 10A**). Single samples were loaded onto the gel in an alternating pattern, MLSA sample then CR sample, repeated from left to right. Molecular weight protein markers were run in the first and last well of each gel.

The median protein band density of the pre-treatment serum samples (A) was 6503 (range 2040-18247) compared to 11252 (range 6109-13343), for dogs in CR (B), (**Figure 10B)**.

There was no statistically significant difference between the two groups (p=0.38, student's t-test).

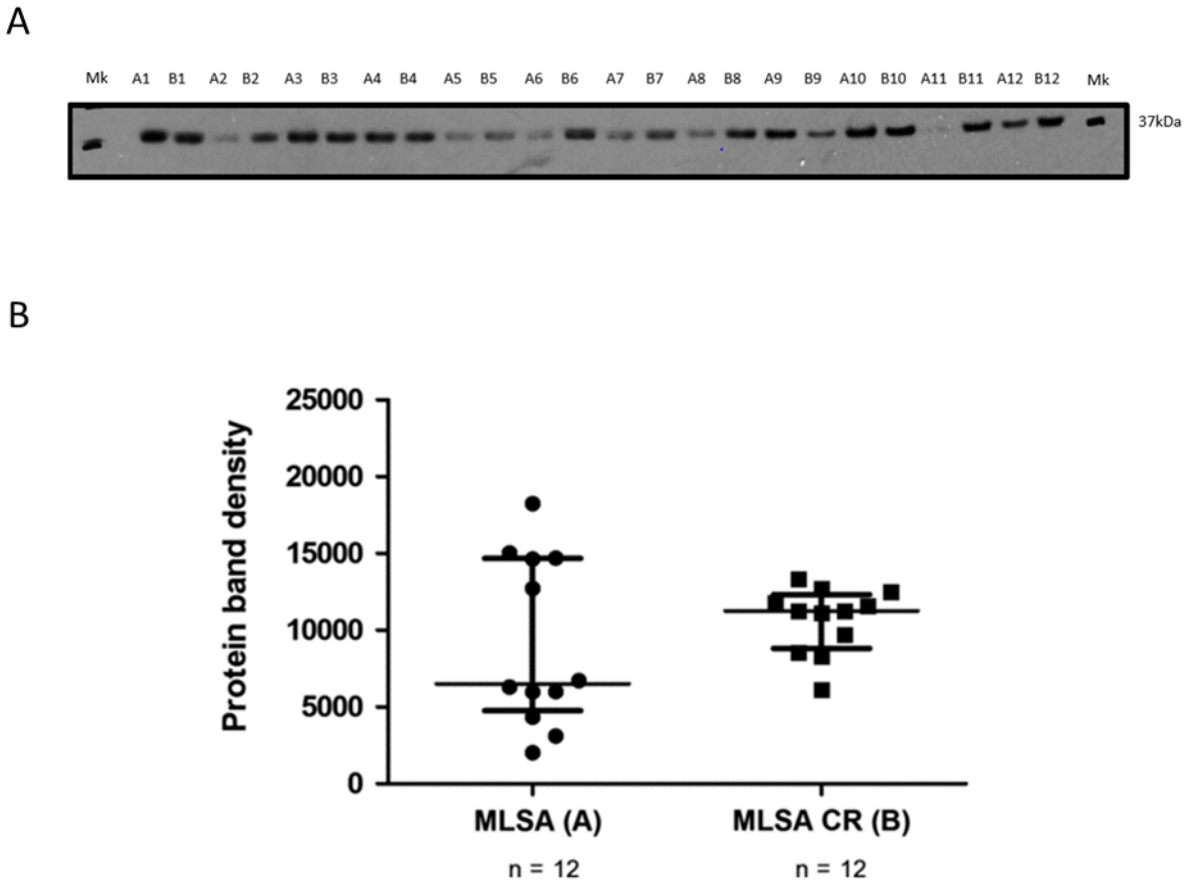


Figure 10:

A) *Western blot analysis comparing serum CLU in dogs with MLSA pre-treatment (A1-A12) and unrelated dogs with MLSA in CR (B1-B12)*
Mk denotes annotation of molecular weight protein standard marker, labelled at 37kDa.

B) *Scatter graphs comparing protein band density depicting serum CLU levels, comparing dogs with MLSA pre-treatment (A, n=12) with unrelated MLSA dogs, in CR (B, n=12).*
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between MLSA pre-treatment and CR.

3.3.2.4 *Western blot results summary*

The three western blots were analysed independently, each comparing 12 diseased samples to 12 control samples. Intra-blot comparison of protein band density from the western blots comparing diseased and control samples (section 3.3.2.1 and 3.3.2.2) were suggestive of a trend for a lower CLU level in dogs with MSLA in comparison to controls. When performing statistical testing on results data however, there was no statistically significant difference between sample cohorts on any of the three western blots ($p>0.05$, student's t-test).

Inter-blot analysis was not performed due to the difficulties with interpretation and configuration of data (as discussed in section 3.2.2). However, each sample was run in duplicate, singly, on two separate western blots. The two separate protein band density results, one from each western blot, of all 36 samples were compared and a positive correlation between western blot results was found (Pearson's r = 0.43), (**Figure 11**). While the numerical values of the samples from different gels are not directly comparable, the positive correlation gives confidence in the repeatability of results and highlighted the robust nature of the technique.

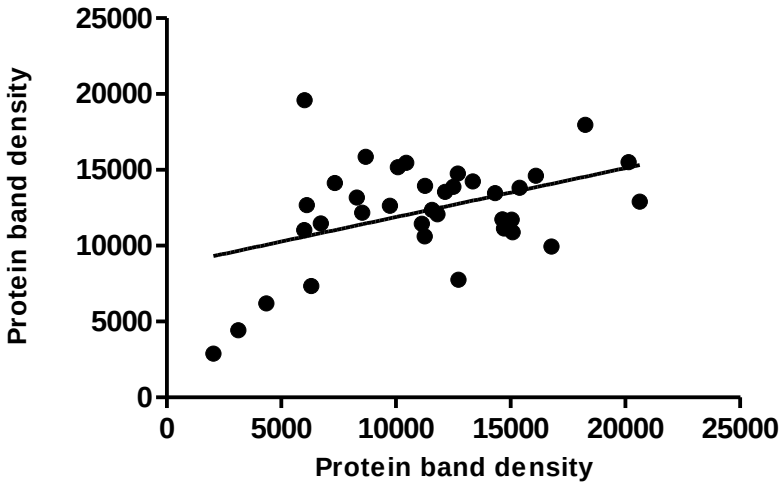


Figure 11:

Scatter graph with line of best fit, depicting correlation of samples on two separate western blots.
Pearson's r = 0.43

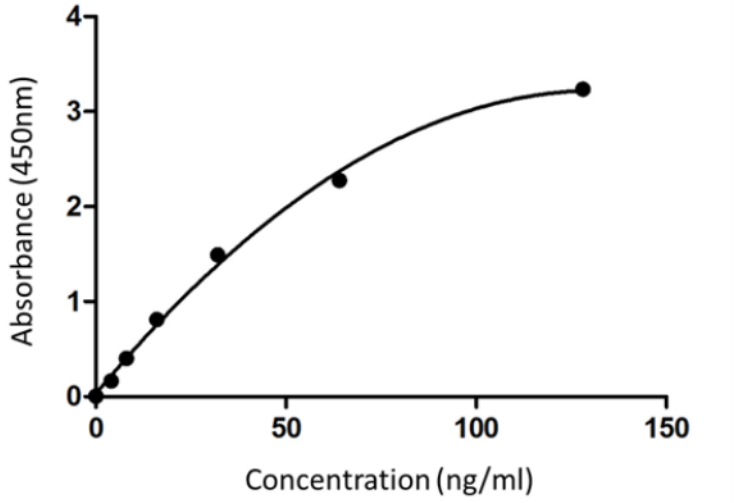
3.3.3 ELISA Analysis

For a more reproducible way of assessing CLU concentration, serum CLU was measured again, in the same 36 samples (Cohorts A, B and C) using a previously validated ELISA assay, as described in section 2.4.2, (**Figure 12A**).

3.3.3.1 *Clusterin concentration*

Median serum CLU concentration in MLSA dogs, pre-treatment (A) was 53.8µg/ml (range 15.9 – 101.1), compared to dogs in CR (B) 67.0µg/ml (49.3-116.8), and 93.6µg/l (70.1- 120.1) in control samples (C), (**Figure 12B**).

A



B

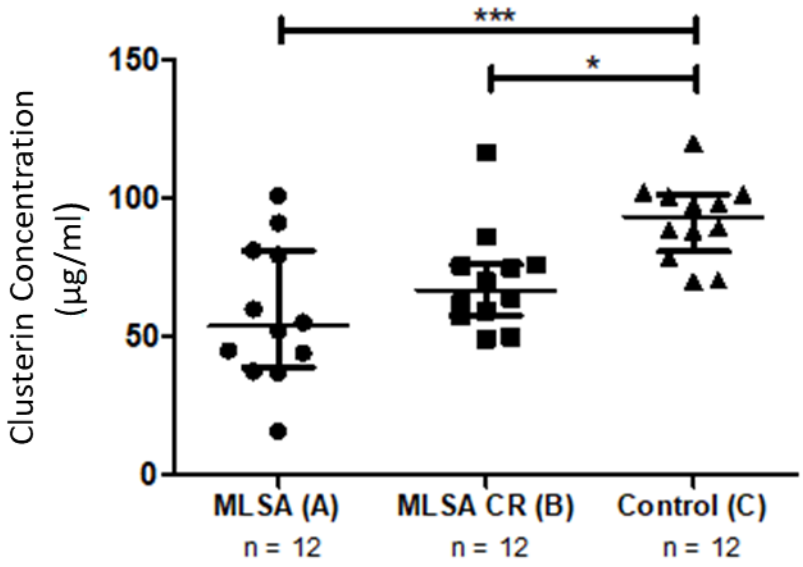


Figure 12:

A) *Representative example of standard curve for ELISA data, generated using results from protein standards (4-128ng/ml).*

B) *ELISA of serum CLU concentrations, comparing samples from three cohorts; dogs with MLSA pre-treatment (A, n=12), unrelated dogs with MLSA in CR (B, n=12) and healthy controls (C, n=12).*
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was significantly lower in MLSA pre-treatment in comparison to controls, and MLSA CR in comparison to controls.
**** p <0.001, * p<0.05, ANOVA.*

There was a significant difference in the median serum CLU concentrations between the three groups, (ANOVA, p=0.0008). Bonferroni's multiple comparison test showed a significant difference when individual cohorts were also compared. Serum CLU was significantly lower (p=0.0006) in MLSA pre-treatment compared with controls, and significantly lower (p=0.0036) in MLSA in CR, in comparison to controls. When looking at the two MLSA cohorts, there was no significant difference in serum CLU levels between dogs with MLSA, prior to treatment and in remission.

3.3.4 Comparison of Western Blot and ELISA

Expression data from the 36 samples run by western blot analysis and the ELISA concentrations were directly compared and showed a significant correlation (Pearson's r = 0.47), (**Figure 13)**. This suggested that the two methods were comparable and gave confidence in the perceived repeatability of results.

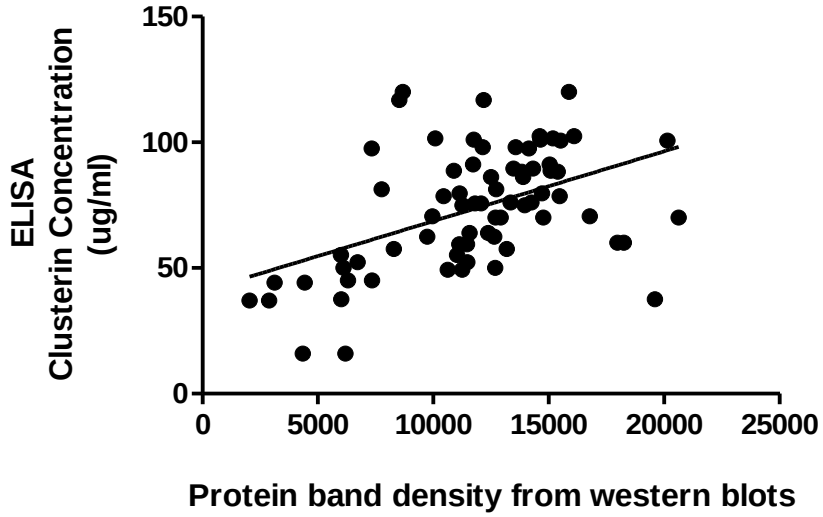


Figure 13:

Scatter graph with line of best fit, depicting correlation of samples from western blots and ELISA.
Pearson's r = 0.47

3.4 Clusterin - sample group two (paired samples)

To account for individual variation between dogs with MLSA, serum CLU concentrations were compared in a further 18 dogs. Samples were again selected using selection criteria in 2.1, and the ELISA performed as described in section 2.4.2. The serum CLU concentrations from paired samples, from the same dog, at time of diagnosis (pre-treatment) and again in CR, were measured and compared.

Sample group two consisted of 2 cohorts; dogs diagnosed with MLSA, pre-treatment (n=18), and in CR (n=18), i.e. paired samples from the same individuals.

3.4.1 Group signalment

Signalment of the dogs in sample group two is summarised in **Table 5**.

Of the eighteen dogs the median age was 9 years (range 5-14). Nine dogs were female (two entire, seven neutered) and nine males (four entire, five neutered). There were thirteen breeds represented, with golden retriever the most common, representing 37.5% of the population, border collies and Labradors followed, representing 11% each of the group population.

Three dogs (16.6%) had stage III disease, nine (50%) stage IV and six (33.3%) stage V. Eleven (61%) dogs were substage a and seven (39%) substage b, as determined by the WHO classification (Owen, 1980; Vail *et al.*, 2013). Immunophenotype was known for 66% of cases, with ten cases (83%) of B cell LSA and two cases of T cell LSA (16.6%).

Group signalment and MLSA phenotype were similar to that of the previously assessed samples although direct statistical comparisons between groups were not performed.

Table 5)

Signalment of sample group two - paired samples

	Paired Samples (n=18)
Median Age (years) Range	9 5 - 14
Sex: Male entire Male neutered Female entire Female neutered	 4 5 2 7
Stage: II III IV V	 0 3 9 6
Substage: a b	 11 7
Immunophenotype: B T	 10 2
Breed: Border collie Cocker spaniel Crossbreed German shepherd Golden retriever Jack Russel terrier Labrador retriever Parsons terrier Shih Tzu Spinone Tibetan terrier West Highland White terrier Yorkshire terrier	 3 1 1 1 3 1 2 1 1 1 1 1 1

3.4.2 Clusterin concentration

Median serum CLU concentration in samples from dogs prior to treatment was 50.4µg/ml (range 36.1 –118.1). Median serum CLU concentration from the same dogs in CR was 51.3µg/ml (34.0 – 141.1), (**Figure 14**).

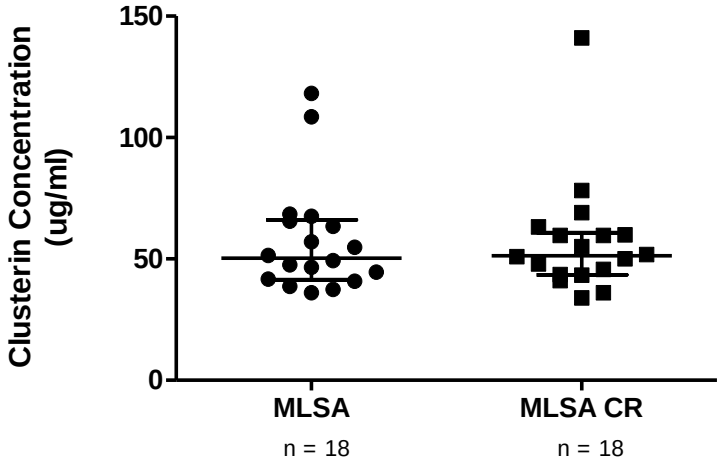


Figure 14:

ELISA of serum CLU concentrations, comparing paired samples from dogs with MLSA pre-treatment (n=18) and at time of CR (n=18).
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between MLSA pre-treatment and CR.

3.4.3 Statistical analysis

There was no significant difference in serum CLU concentrations between the two cohorts, prior to treatment and in CR (p=0.83, Wilcoxon signed rank test).

Subjective assessment of results showed no consistent trends in individual serum CLU concentrations; 7/18 (38.9%) dogs had increased CLU concentrations, 7/18 (38.9%) decreased and 4/18 (22.2%) remained relatively static when dogs achieved remission (**Figure 13**).

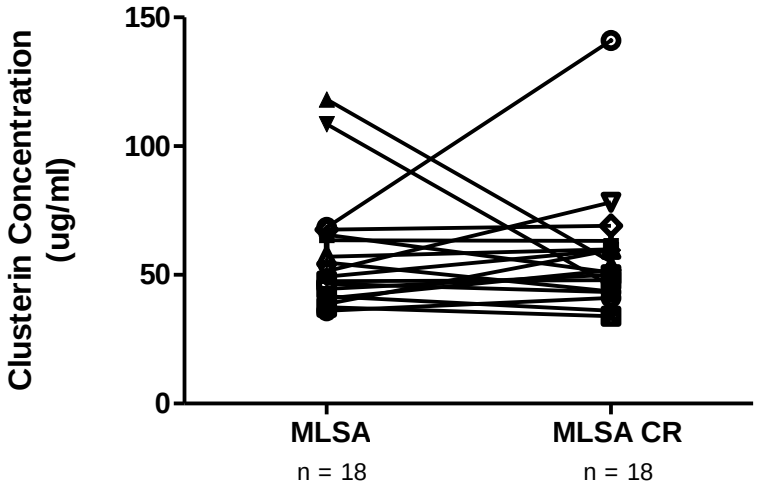


Figure 15:

Line graph illustrating changes in individual CLU concentration when CR is achieved, showing no significant trend for increasing or decreasing levels.
Serum CLU was not significantly different between MLSA pre-treatment and CR.

3.5 Clusterin - sample group three (age matched)

There was a significant difference in age between dogs with MLSA and controls, in the initial samples. In an attempt to eliminate bias from the results, a further cohort of serum samples was selected. Using selection criteria in section 2.1, samples were preferentially chosen to represent a younger cohort of dogs diagnosed with MLSA, pre-treatment. Further samples were also selected for the control population, with preference for control samples given to samples from dogs over four years old. ELISA analysis was performed, as described in section 2.4.2, on the new cohorts of samples.

Sample group three consisted of 2 cohorts; dogs diagnosed with MLSA, pre-treatment (n=18) and healthy control dogs (n=18). Samples for group three were intentionally selected to form age matched cohorts.

3.5.1 Group signalment

Signalment of the dogs in sample group three is summarised in **Table 6**.

Eighteen treatment naïve dogs with MLSA were compared to 18 healthy controls. The median age of the dogs with MLSA was 6 years (range 3-7). Six dogs were female (one entire, five neutered) and 12 males (four entire, eight neutered). There were thirteen breeds represented, with golden retriever and border collie the most common, each representing 16.7% of the population.

There were seven dogs (38.8%) with stage III disease, eight (44.4%) with stage IV and three (16.6%) with stage V disease. Seven (39%) dogs were classified in substage a and eleven (61%) substage b, as determined by the WHO TNM classifications (Owen, 1980; Vail *et al.*, 2013). Immunophenotype was known for 77.7% of cases, with five cases (35.7%) of B cell LSA and nine T cell LSA (64.3%).

Of the healthy control dogs, median age was 5 years (range 4-7). Nine dogs were female (one entire, eight neutered) and nine were male (four entire, five neutered). There were seven breeds represented, with greyhound the most common, accounting for 44% of the control population.

The age, sex and neuter status were not significantly different between the two groups.

Table 6)

Signalment of sample group three - age matched samples

	Pre-treatment MLSA (n=18)	Controls (n=18)	p values
Age (years) Median Range	 6 3 - 7	 5 4 - 7	 Mann Whitney U Test $p > 0.05$
Sex: Male entire Male neutered Female entire Female neutered	 4 8 1 5	 4 5 1 8	 Chi squared Fisher's exact $p > 0.05$
Stage: II III IV V	 0 7 8 3	 N/A	 -
Substage: a b	 7 11	 N/A	 -
Immunophenotype: B T	 5 9	 N/A	 -
Breed: Bernese mountain dog Border collie Boxer Bullmastiff Cocker spaniel Crossbreed Doberman Dogue de Bordeaux Flat-coated retriever Golden retriever Greyhound Jack Russell terrier Labrador retriever Lurcher Shetland sheepdog Springer spaniel Tibetan terrier Weimaraner	 1 3 1 1 2 1 0 1 1 3 0 1 0 1 1 0 1 0	 0 0 0 0 0 1 1 0 0 2 8 0 4 0 0 1 0 1	 -

3.5.2 Clusterin concentration

The median serum CLU concentration in pre-treatment MLSA dogs was 58.3μg/ml (range 30.5 – 137.4) compared to 98.9μg/ml (range 73.2 – 141.0) from healthy controls, (**Figure 16**).

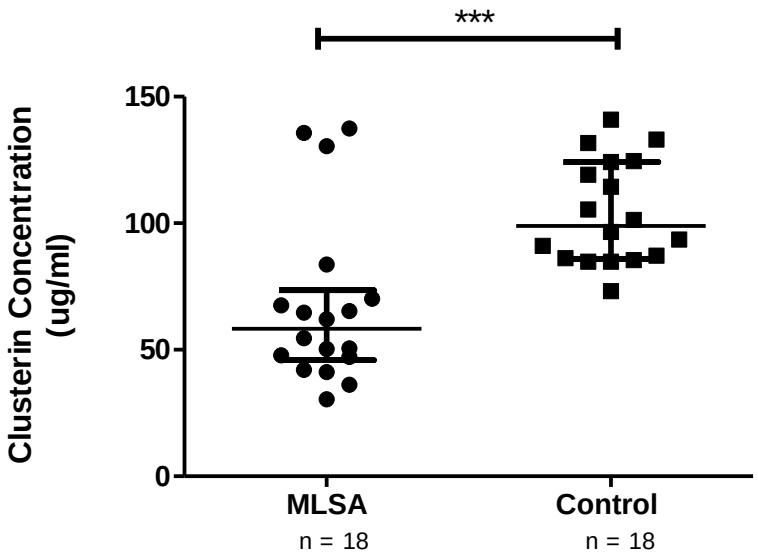


Figure 16:

ELISA of serum CLU concentrations, comparing age matched samples from dogs with MLSA pre-treatment (n=18) and healthy controls (n=18).
Vertical scatter graphs showing data presented with median and interquartile range.
*Serum CLU was significantly lower in MLSA pre-treatment in comparison to controls ***p<0 .001, Mann Whitney U test.*

3.5.3 Statistical analysis

There remained a significant difference in the CLU concentration between dogs with MLSA, pre-treatment, and healthy controls. The median serum CLU concentration in pre-treatment MLSA dogs was significantly lower than healthy controls (p=0.0002, Mann Whitney U test).

3.6 Clusterin - combined ELISA data

The CLU concentrations were determined using a commercial immunoassay (BioVendor Ltd, UK). The assay was previously validated, internally by BioVendor and independently as part of an investigation into urinary CLU (García-Martínez *et al.*, 2012). Internal validation, through BioVendor, identified an acceptable between-run comparability, with an inter-assay coefficient of variance (n = 8) of 6.2%. Whilst external validation also showed an acceptable inter-assay comparability with inter-assay coefficient of variance of 14.2-16.1% (García-Martínez *et al.*, 2012).

All three ELISA plates were run following the same protocol, in the same conditions (section 2.4.2 and **Appendix 4**), at separate times. Since the CLU assay is a commercially validated assay (BioVendor), the individual CLU concentrations across all ELISAs should be directly comparable. Additionally, reference quality controls for intra-assay assurance (high and low, supplied with each ELISA kit) were also run on each ELISA plate and gave values consistent with the concentrations reported for these controls (see section 4.3). Inter-assay coefficient of variance for the quality control samples varied from 8.9-16.7%.

3.6.1 Duplicated ELISA samples

During sample selection (section 2.1) some samples, from dogs with MLSA pre-treatment, were found to fulfil criteria for multiple sample groups. Six individual samples were deliberately included in both sample group two and sample group three, thus two separate CLU concentrations were generated for each of these samples, on separate ELISA plates.

CLU concentrations were significantly higher ($p<0.05$, Wilcoxon signed rank test) in the six samples when run for the second time, as part of sample group three (on ELISA plate three). The inter-run percentage difference between the mean CLU concentrations from the two plates varied from 2% to 40.1%. When the individual sample results were analysed, the coefficient of variance between the results from the two ELISA plates varied from 5.9-19.4% with only one of the six samples reporting a coefficient of variance less than 6.2%, the inter-assay coefficient of variance, reported by BioVendor (**Figure 17**).

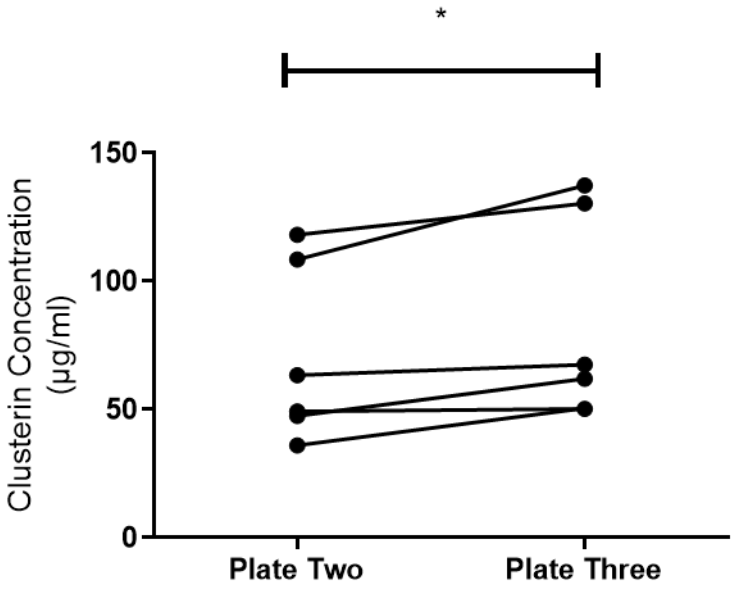


Figure 17:

Line graph of ELISA data from two separate ELISA plates, run at different time points. Graph illustrates CLU concentrations from six individual samples run once on each ELISA plate.
Serum CLU was significantly higher when run on ELISA plate 3
**p<0.05 Wilcoxon signed rank test.*

Unexpectedly, based on the ELISA kit data provided, there was a significant difference in CLU concentrations when run on separate ELISA plates. Three samples had an acceptable degree of variation, with 5.9%, 6.3% and 11.5% variation, two samples had a higher coefficient of variance at 14.2% and 16%, with one sample set resulting in a coefficient of variance of 19.4%. Although these differences are greater than anticipated, there were only a small number of samples which appeared on two ELISA plates allowing for analysis. It was assumed that any drift between plates would be consistent, influencing both MLSA samples and healthy controls. Thus, notwithstanding these limitations, it was still considered of value to assess all ELISA results in a combined analysis.

Results from all sample groups were therefore combined to assess serum CLU concentrations in 42 individual dogs with MLSA pre-treatment and 30 individual control samples. For any samples run on more than one ELISA, only the initial CLU values from the first ELISA analysis were used.

3.6.2 Group signalment

Signalment of the dogs in the combined sample group is summarised in **Table 7**.

The median age of the 42 pre-treatment samples was 7 years (range 3-14). There was a male preponderance with fifteen female dogs (five entire, 10 neutered) and 27 male dogs (10 entire, 17 neutered). There was a wide range of breeds represented, with border collie accounting for 19% of samples (8/42). Labrador and golden retriever each accounted for 9.5% of samples, with four dogs from each breed. There were three each of crossbreed and boxer dog (7%), and two cocker spaniels (4.8%). There were seven terriers, one each of Parsons, border, West Highland, Yorkshire, Cairn, Jack Russell terrier and Tibetan. And one each of German shepherd, shih tzu, cavalier King Charles spaniel, Italian Spinone, flat-coated retriever, bullmastiff, Shetland sheepdog, Bernese mountain dog, lurcher, Gordon setter and Dogue de Bordeaux.

Eight of the 42 dogs were stage III (19%), 19 (45%) stage IV and 15 (36%) stage V. Seventeen (40.5%) dogs were substage a and 25 (59.5%) substage b. Immunophenotype was known for 69% of cases, with 19 cases (65.5%) of B cell and 10 cases of T cell LSA (34.5%).

The median age of the 30 healthy control dogs was 5 years (range 1-7), significantly lower than the diseased population ($p<0.05$ Mann Whitney test). Sex was more evenly distributed in the control population with 14 female dogs (two entire, 12 neutered) and sixteen male dogs (seven entire, nine neutered). The sex and neuter status of the two groups did not differ significantly ($p>0.05$, Chi-squared, Fisher's exact). There were nine breeds represented with nine greyhounds, seven Labradors and five golden retrievers representing the majority of the population. There were also two from each of crossbreeds, Doberman pinschers and German shepherd, and one each of Weimaraner, springer spaniel and flat-coated retriever. While not compared statistically, the breed distribution of the two groups was notably different, due to the control population comprising of blood donors.

Table 7)

Signalment of combined sample group

	Pre-treatment MLSA (n=42)	Controls (n=30)	p values
Median Age (years) Range	7 3 - 14	5 1 - 7	Mann Whitney U Test $p < 0.01$
Sex: Male entire Male neutered Female entire Female neutered	 10 17 5 10	 7 9 2 12	 Chi squared Fisher's exact $p > 0.05$
Stage: III IV V	 8 19 15	 N/A	 -
Substage: a b	 17 25	 N/A	 -
Immunophenotype: B T	 19 10	 N/A	 -
Breed: Bernese mountain dog Border collie Border terrier Boxer Bullmastiff Cairn terrier Cavalier King Charles Spaniel Cocker spaniel Crossbreed Doberman Dogue de Bordeaux Flat-coated retriever German shepherd Golden retriever Gordon setter Greyhound Jack Russell terrier Labrador retriever Lurcher Parsons terrier Shetland sheepdog Shih Tzu Spinone Springer spaniel Tibetan terrier Weimaraner West Highland White terrier Yorkshire terrier	 1 8 1 3 1 1 1 2 3 0 1 1 1 4 1 0 1 4 1 1 1 1 1 0 1 0 1 1	 0 0 0 0 0 0 0 0 2 2 0 1 2 5 0 9 0 7 0 0 0 0 0 1 0 1 0 0	 -

3.6.3 Clusterin concentration

Median serum CLU concentration from samples taken from dogs with MLSA prior to treatment was 51.9μg/ml (range 15.9 – 135.6). Median serum CLU from healthy control samples was 97.1μg/ml (range 70.1 – 141.0), (**Figure 18**). There was a significant difference noted in serum CLU concentrations in samples from diseased and control dogs, with serum CLU significantly lower in dogs with MLSA pre-treatment in comparison to healthy controls ($p<0.0001$, Mann Whitney U test).

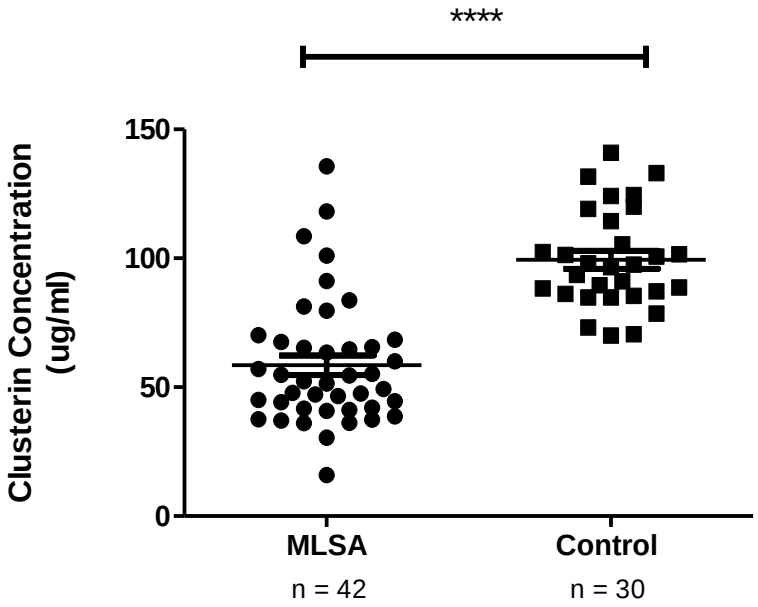


Figure 18:

Combined ELISA data, of serum CLU concentrations comparing dogs with MLSA pre-treatment (n=42) and healthy controls (n=30).
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was significantly lower in MLSA pre-treatment in comparison to controls
*****p<0 .0001, Mann Whitney U test.*

Serum CLU concentrations from dogs with MLSA were further separated into stage, substage and by immunophenotype. Median serum CLU concentration in samples from dogs with stage III disease was 47.4µg/ml (36.2 - 65.3). Median serum CLU concentrations from dogs with stage IV disease was 52.3µg/ml (36.1 – 135.6) and from stage V disease, 55.2µg/ml (15.9-118.1), (**Figure 19**). There was no significant difference noted in CLU concentrations between dogs in different stages of disease ($p>0.05$, Kruskal-Wallis).

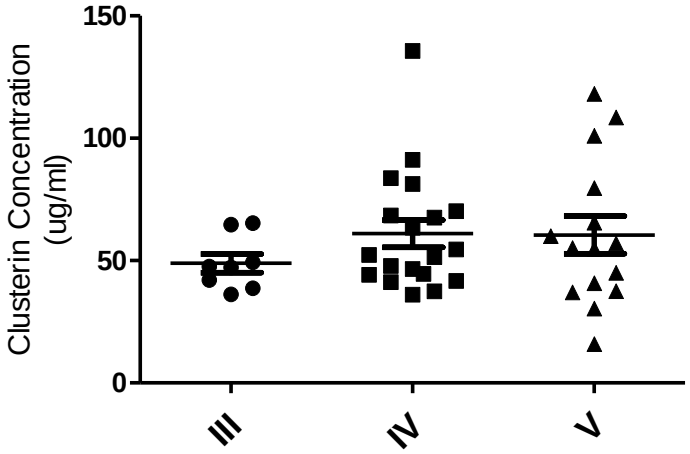


Figure 19:

Combined ELISA data, comparing pre-treatment serum CLU concentrations in dogs with MLSA in stage III, IV and V disease.
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between different stages of MLSA pre-treatment.

Median serum concentrations from serum samples from dogs in substage a was 47.6µg/ml (36.1 - 91.2) and substage b, 55.2µg/ml (15.9 - 135.6), (**Figure 20**). There was no significant difference in CLU concentrations in different substage of disease ($p>0.05$, Mann Whitney U test).

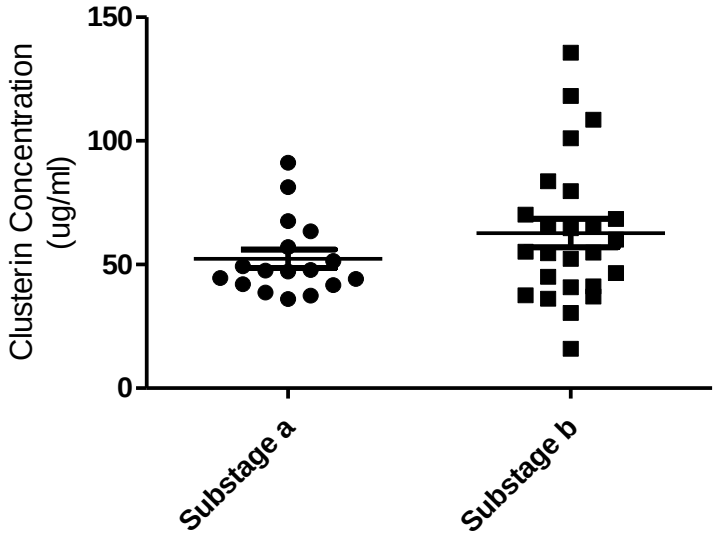


Figure 20:

Combined ELISA data, comparing pre-treatment serum CLU concentrations in dogs with MLSA in substage a and substage b.
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between different substages of MLSA pre-treatment.

Median serum CLU concentration for samples from dogs with B cell LSA was 51.5µg/ml (15.9 – 101.1), and T cell LSA 65.0µg/ml (30.5 – 135.6), (**Figure 21**). There was no significant difference in serum CLU concentrations based on immunophenotype, ($p>0.05$, student's t-test).

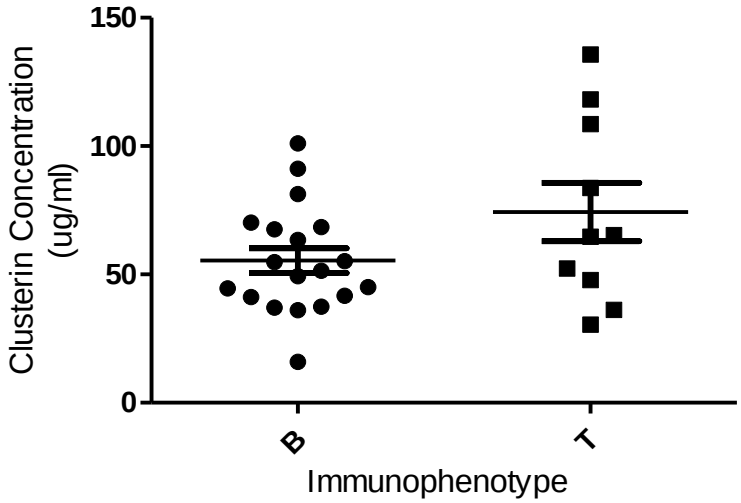


Figure 21:

Combined ELISA data, comparing pre-treatment serum CLU concentrations in dogs with B-cell LSA and T-cell LSA.
Vertical scatter graphs showing data presented with median and interquartile range.
Serum CLU was not significantly different between different immunophenotypes of MLSA pre-treatment.

3.6.3.1 *CLU concentration in controls*

The median serum CLU concentration in healthy control samples was 97.1µg/ml (range 70.1 – 141.0), significantly higher than dogs with MLSA pre-treatment ($p<0.0001$, Mann Whitney U test). When data from all ELISA plates was combined, there remained a significant difference in age between the control and MLSA populations, with control dogs significantly younger ($p<0.05$, Mann Whitney U test).

When considering the control population alone, serum CLU concentrations did not vary significantly in relation to age ($p=0.742$, univariate analysis). And when comparing the control and MLSA population, on multivariate analysis, when disease status is taken into account, CLU did not vary significantly with regard to age ($p=0.33$, multivariate analysis).

4 Discussion

In 2013, the serum proteome of three dogs with LSA, was characterised via serum protein electrophoresis and mass spectrometry (Atherton *et al.*, 2013a), identifying a CLU precursor in the alpha-2 band of the proteome of one dog, which was absent in two healthy controls. This finding led to speculation that CLU may have a role in canine LSA. The research undertaken in this thesis identified the presence of the CLU protein, using Western Blot analysis and ELISA, in all canine serum samples tested.

4.1 Clusterin

The primary aim of the thesis was to evaluate whether serum CLU expression differed significantly between those dogs with HG MLSA, and a healthy control population, and therefore had potential as useful diagnostic tool. Serum CLU levels in MLSA samples both pre-treatment and in CR, were consistently lower than that of a healthy control population, although statistically significant differences were detected only with the ELISA method. Although marked individual variation in CLU levels existed, results were suggestive of a possible down regulation in CLU activity in canine MLSA, however the degree of overlap with the healthy control population limits the potential of serum CLU measurement as a diagnostic tool.

4.1.1 Serum clusterin in MLSA

Serum CLU concentrations were significantly lower in dogs with MLSA, pre-treatment, in comparison to healthy controls. While this finding was unexpected, compared to the original hypothesis based on a previous pilot study, multiple investigations, using different experimental techniques, demonstrated repeatable results. Additionally, even where potential differences were not statistically significant, there was a trend for lower CLU in MLSA samples than healthy controls.

Atherton et al. (2013a) previously identified a CLU precursor in the serum of a dog with high grade multicentric lymphoma (MLSA), and the absence of CLU in serum from two healthy controls, as determined by mass spectrometry (Atherton *et al.*, 2013a, 2013b). While western blot analysis and ELISA identified serum CLU at varying concentrations in all samples, MLSA and healthy controls. The discordance in results may be due to the small number of samples originally assessed through mass spectrometry. Serum CLU was highly variable across individuals, both with MLSA and controls, and type II error may have led to a non-representative sample cohort, when initial mass spectrometry data consisted of samples from five dogs. Additionally, while present in all samples assessed via ELISA, CLU concentrations were low, measured in μg/ml. When analysing the serum proteome, with median total protein values of 64g/L, it is possible that CLU and CLU precursors were overshadowed by an abundance of other proteins.

Since the original observation was based on a single MLSA case and two controls, using a qualitative rather than quantitative proteomic approach (Atherton *et al.*, 2013a), the current findings, using two different methods, western blot analysis and ELISA, can be considered more reliable.

CLU is an extracellular chaperone protein, important in stabilising protein folding, correcting misfolded proteins, and facilitating protein clearance (Humphreys *et al.*, 1999; Wilson *et al.*, 2000, 2017; Trougakos *et al.*, 2009a; Wyatt *et al.*, 2009, 2010). Multiple studies (Michel *et al.*, 1997; Humphreys *et al.*, 1999; Wyatt *et al.*, 2009; Wilson *et al.*, 2017) describe an increase in CLU expression in response to cell stress or insult. But reduced levels of CLU and subsequent loss of the chaperone function may play an important role in tumourigenesis (Scaltriti *et al.*, 2004; Shannan *et al.*, 2006; Rizzi *et al.*, 2008, 2010).

CLU levels have previously been assessed in human lymphoid neoplasia, most frequently via IHC. While not directly comparable, due to different methodologies used, the low CLU levels detected in MLSA serum, are consistent with human literature reports of low or absent CLU levels in B-cell and T-cell LSA tissues, with the exception of anaplastic large cell LSA (Wellmann *et al.*, 2000; Saffer *et al.*, 2002; Nascimento *et al.*, 2004). Saffer et al. (2002) identified cytoplasmic CLU expression on IHC in 35% of lymphoid neoplasia assessed; but only present in a small subset (12%) of DLBCL, and 7% of peripheral T-cell LSA cases. Additionally CLU levels were low in the Toledo DLBCL cell line when compared to healthy B lymphocytes (Coletta *et al.*, 2012).

While immunophenotype was only known in 69% of the MLSA population, the majority (65%) were B-cell, presumed DLBCL, although definitive classification was not performed since histopathology was not obtained for most cases. When comparing to human studies, it is to be expected that CLU levels would be low in canine DLBCL. In the current study serum CLU did not differ significantly with immunophenotype, although sample sizes were small. Since most cases were diagnosed cytologically, immunophenotyping was performed primarily via PARR analysis, rather than gold standard IHC or preferred flow cytometry, however this is unlikely to have impacted on the results. While not considered to be gold standard, PARR is highly specific for identification of B or T cell gene rearrangements, thus false positive results are unlikely (Thalheim *et al.*, 2013; Waugh *et al.*, 2016).

Given that serum CLU was significantly lower in canine MLSA than healthy controls, there were considerations as to whether a higher tumour burden/WHO stage of disease may have even lower serum CLU concentrations. No significant difference with WHO stage was detected, however, the lack of significant difference between different stages of disease may represent a type II error, due to the small sample numbers, and only 8 dogs with stage III disease. Additionally, as staging was not standardised, and did not always include cytology of the liver, spleen or bone marrow, there would be the possibility of stage migration within the sample population. Similarly, there was no significant difference between MLSA patients presenting with clinical signs (substage b), and those without clinical signs, (substage a).

When directly comparing all MSLA pre-treatment samples with healthy controls, a serum CLU concentration of <69.2µg/ml had 100% specificity for disease (**Appendix 6**). However, over 20% of the MLSA population had CLU serum concentrations >69µg/ml (sensitivity 78% for detection of disease at CLU concentrations <69.2µg/ml). The receiver operating characteristic curve, (ROC curve) identified the highest likelihood of disease (likelihood ratio, 24.29), at CLU concentrations <70.42µg/ml, with this cut off having diagnostic sensitivity of 81% and specificity of 96.7%.

Individual CLU concentrations showed wide variation within the MLSA cohorts. Serum CLU in pre-treatment MLSA, across separate ELISA plates, ranged from 15.9µg/ml to 135.6µg/ml, and significantly overlapped with healthy controls which ranged from 70.1-141.0µg/ml. This overlap and relative lack of sensitivity in detecting disease (in comparison to currently available diagnostics), limits the use of CLU as a diagnostic biomarker for MLSA.

4.1.2 Serum clusterin in MLSA, at time of CR

The second aim of this thesis was to determine whether serum CLU expression differed significantly following successful treatment and induction of remission, and therefore had potential as a monitoring tool, as a marker of remission, early relapse or treatment failure. This was attempted firstly by comparing MLSA dogs in CR to healthy controls and secondly pre-treatment MLSA dogs to those in CR.

4.1.2.1 *Serum CLU in MLSA(CR) in comparison to healthy controls*

Serum CLU concentrations were still significantly lower in dogs with MLSA, at time of CR, in comparison to healthy controls. It was hypothesised, that having been significantly lower in MLSA at time of diagnosis, CLU levels would normalise following successful treatment and may not differ from control dogs. Serum CLU levels were assessed in MLSA patients following 4-6 weeks of a CHOP based chemotherapy. While patients were evaluated as having achieved CR on the basis of clinical examination, no molecular testing or cytological evaluation for MRD was carried out. MRD testing has been shown to reach a nadir, following multidrug chemotherapy, between 4 and 17 weeks, and remains low until the end of the chemotherapy protocol at week 25 (Yamazaki *et al.*, 2010). It is possible that patients were not in complete molecular remission, or that re-evaluation was performed too early. While 4-6 weeks should have been sufficient to assess remission on the basis of clinical signs, it could not be excluded that some patients continued to carry a burden of disease, thus causing the low CLU levels which were noted. Evaluation at additional points throughout treatment, including the end of the chemotherapy protocol and at time of relapse would better determine the relationship between CLU levels and disease status. With additional time, it is possible that CLU levels in MLSA patients would increase, with the achievement of clinical remission, to the level to healthy controls.

The CLU protein has a relatively short half-life (Rizzi *et al.*, 2009a), it is possible that the ongoing inflammatory state caused by disease and concurrent chemotherapy treatment facilitated increased protein turnover, reducing the circulating CLU levels in the CR patients. MLSA patients show low levels of serum CLU at time of diagnosis, and downregulation of the CLU protein, which may have facilitated the initial tumourigenesis, may not be not rectified by chemotherapy treatment. It could be postulated that patients will continue to show low serum CLU levels following a diagnosis of MLSA, regardless of treatment protocol, time from treatment or remission status.

4.1.2.2 *Comparing serum CLU in MLSA pre-treatment and CR*

Serum CLU concentrations were not significantly different between dogs with MLSA, prior to treatment, and those in CR, following 4-6 weeks of chemotherapy. Due to the wide variation in individual serum CLU levels, paired samples from the same dog with MLSA prior to treatment and once in CR were also evaluated, with no significant difference in individual serum CLU levels following treatment, and no significant trend for increasing or decreasing serum CLU.

Doxorubicin has been shown to upregulate CLU, produced by HL and DLBCL (Toledo) cell lines, *in vitro* (Frazzi *et al.*, 2011; Coletta *et al.*, 2012). Most patients received doxorubicin as part of their chemotherapy protocol (week 4 of CHOP), with substitutions for mitoxantrone made if cardiac pathology was noted on echocardiography scanning. Patients who received mitoxantrone were not separately evaluated. This may have impacted results, as the effects of mitoxantrone chemotherapy on CLU levels is unknown. Additionally, there were a portion of patients, also not separately evaluated, deemed in CR at week 4 evaluation, prior to their doxorubicin chemotherapy. These patients would not have received doxorubicin at the time of their serum sampling. These discrepancies within the study population may account for the lack of significant difference in CLU levels between the MLSA and MLSA CR samples.

The increased CLU in the Toledo cell line (Coletta *et al.*, 2012) occurs in conjunction with an inhibition of cell growth, and causing death through necrosis and apoptosis, thus it could be hypothesised that CLU increases following cell death. However, the same study showed an increase in CLU following Rituximab administration, which did not induce apoptosis. CLU was shown to increase in a dose dependent fashion (Frazzi *et al.*, 2011), thus it may be that a single dose of doxorubicin, at a 30mg/m^2 dose (as per the CHOP protocol, **Appendix 2**) was insufficient to impact CLU levels. However these *in vitro* studies (Frazzi *et al.*, 2011; Coletta *et al.*, 2012) would indicate that treatment regime could influence the levels of circulating CLU at specific time points.

Individual serum CLU concentration in MLSA patients following treatment was variable, with increased, decreased and static CLU concentrations noted following CR. Similar variation in serum CLU has been reported in a small subset of human patients with HL following treatment with chemotherapy (Frazzi *et al.*, 2011). In that study of 18 HL patients, CLU levels increased in 39%, decreased in 28%, and remained static in 33%. There are similarities to the population of this thesis, where there was no trend for increasing or decreasing serum CLU levels in dogs who achieved remission following treatment. In the HL study, PET2 analysis performed following two cycles of chemotherapy appeared to correlate with CLU levels i.e. those patients PET2 negative and free from detectable disease, had decreased CLU detected in peripheral blood samples, while CLU levels in those who were PET2 positive, with an ongoing disease burden, increased (Frazzi *et al.*, 2011), although CLU levels in some patients remained static following treatment, regardless of PET2 status (Frazzi *et al.*, 2011).

There was not a significant difference in CLU levels in MLSA pre-treatment and CR, thus CLU levels cannot be used as a marker for remission or relapse. However, it cannot be excluded that a relative change in individual CLU level could act as a prognostic or predictive marker for remission, relapse or prognosis following chemotherapy. Evaluation of this would require larger sample numbers, standardised treatment protocols to mitigate the effects of chemotherapeutics on CLU levels, and definitive evaluation of remission status *via* repeat staging and detection of MRD.

4.2 Population factors

Serum CLU was identified, in varying quantities, in both MLSA populations and the healthy control samples, conflicting with previous reports of the characterisation of the canine proteome (Atherton *et al.*, 2013a, 2013b; Fonghem *et al.*, 2017). While implicated in tumourigenesis, CLU has also been identified in a wide array of normal physiological and pathological processes, including cell stress, regulation of cell death and the aging process (Trougakos *et al.*, 2002; Shannan *et al.*, 2006; Wilson *et al.*, 2017). Thus, the presence of CLU in healthy canine serum, while not found in the initial characterisation of the canine proteome, could be explained through normal cell turnover and the aging process.

In humans CLU expression increases with age (Mondello *et al.*, 1999; Trougakos *et al.*, 2002, 2006), although, similar to the canine population in this thesis, significant individual variation exists. The possible impact of age on CLU levels prompted further evaluation in this study, using age matched MLSA and control samples, for sample group three. When cohorts were age matched there remained a highly significant difference in serum CLU levels, with serum CLU significantly lower in MLSA in comparison to controls. Additionally, when evaluating the combined data, multivariate analysis showed that the only factor which held significance for CLU concentration was MLSA disease status. Within the healthy control population serum CLU concentrations also did not vary significantly in relation to age, although this may in part be due to small sample size and overall narrow age range (1-7 years).

The CLU protein is described as well conserved (Michel *et al.*, 1997; Calero *et al.*, 1999; Wilson *et al.*, 2000), with similar variants across species. Occasional studies refer to levels of other apolipoproteins (e.g. A, C and E) varying with race and ethnicity (Heiss *et al.*, 1984; Brouwer *et al.*, 2000). One study evaluating CLU determined coding variants and polymorphisms within exons of the CLU gene, which were more frequent in African-American than Hispanics and rare in Caucasians (Tycko *et al.*, 1996). While the MLSA population had a wide variety of breeds, predominated by dogs predisposed to LSA (Edwards *et al.*, 2003; Dobson, 2012), the control population was heavily weighted towards large breed dogs. Greyhounds and retrievers accounted for over 70% of the control population, with greyhounds comprising 30% of all healthy controls. It is known that the haematological and biochemical parameters of greyhounds can vary significantly (Zaldívar-López *et al.*, 2011) from that of the general canine population. With no known canine serum CLU reference range, it is not possible to know if greyhounds could have aberrant serum CLU levels. The lack of breed heterogeneity within the control population may have adversely impacted results.

Additionally, since the control population were blood donors, the mean body weight of the control group was higher than that of the MLSA population. Studies have shown that both human and canine circulating CLU levels are positively correlated with body mass index and obesity (Won *et al.*, 2014), with decreasing CLU correlating with weight loss (Tvarijonaviciute *et al.*, 2012, 2013). Whether higher serum CLU levels would be expected in dogs with a higher lean body weight is unknown. The effects of obesity on CLU levels may be due to the chronic inflammatory state which obesity conveys (Barić Rafaj *et al.*, 2017), rather than directly linked to lean body mass. The body condition scores of both MLSA and control dogs were not available for analysis.

The sample population in the present study was varied and representative of a portion of patients referred to Glasgow University Small Animal Hospital. However, samples may not be representative of the wider population. Samples were selected based on sample age, storage conditions, sample quality and stage of disease, rather than selected at random, thus unconscious bias may have impacted the results. It remains possible that results of this study may represent type I error, a false positive result and rejection of a true null hypothesis. However, notwithstanding these potential factors and while the sample size was small, there was a statistically significant difference in CLU concentration between dogs with MLSA prior to treatment and healthy controls, although this does not rule out the possibility of an aberrant population.

4.3 Techniques

All of the techniques used in this thesis were subject to the variations that are normally encountered by multi-step methodologies associated with protein analysis. Typically, these may include, sample handling, pipetting accuracy and repeatability, washing regimes for western blots and ELISAs, and sample integrity due to freeze/thaw cycles. In order to limit variability and maintain an empirical consistency, the author of this thesis was directly responsible for sample handling and execution for all experiments with the inclusion of appropriate inter and intra experimental controls where appropriate.

Ultimately comparison between separate western blots only occurred when evaluating the pilot samples and not in the main sample groups. This was due to variation in signal density between blots that was difficult to standardise. While attempts were made to introduce a constant/correction factor (×), in order to correct for blot variation, and allow inter-blot comparison, this technique ultimately was not pursued, as it was deemed imprecise. Thus, statistical comparisons were only made on data accrued within individual experiments, i.e. intra-blot comparison, rather than inter-blot comparison.

Another option, to allow comparison between blots would have been to run known standard samples on each gel. Additionally, there could be the option of using computer software to correct for signal variance and exposure on each blot. These options were not pursued, as ultimately numerical values for CLU in the canine samples were generated via ELISA.

Numerical values for CLU concentrations were generated using a commercially available ELISA kit for canine CLU (BioVendor Ltd). Each sample on the ELISA plate was run in duplicate, with final results an average of the two sample values, this allowed for some pipetting error. Additionally, standard quality control (QC) samples were included with each individual ELISA run, with the predicted values provided for each kit. These provided an intra-assay control of the experimental technique itself, unrelated to sample quality or disease status. ELISA manufacturing data reports an intra-assay variance of 4.3%, which gave confidence in the comparisons across individual experiments. Furthermore, with each ELISA run, the QC values matched the reported range and the standards generated an appropriate standard curve for the samples. In addition, the positive correlation between Western blot values (where CLU is visualised at the correct molecular weight) and ELISA protein concentrations provided strong evidence that the ELISA method was indeed detecting the CLU protein.

Inter-assay comparison was also carried out, with the aim of allowing comparison of a larger number of samples. The ELISA plates should have generated reliable, and comparable data. However, despite inter-assay variance reported at 6.2% (BioVendor) our own data showed a more marked discrepancy between samples run on separate ELISA plates. With coefficient of variance for individual samples ranging from 5.9-19.4%, more in line with independent validation of the ELISA (García-Martínez *et al.*, 2012) which found inter-assay coefficient of variation to range from 14.2-16.1%, with higher concentrations found to be more variable, both on inter-assay and intra-assay validation. Canine serum CLU concentrations, as described in section 3.6.1, were significantly higher in samples run on ELISA plate three, in comparison to results from ELISA plate two, (**Figure 19**) with percentage increases in mean CLU concentrations ranging from 2% - 40.1%.

Based on the ELISA kit information provided, regarding repeatability and comparability, this degree of variability between individual samples was unexpected. There were only a small number of samples available which were run on multiple ELISA plates, thus sample quality, sample handling and experimental technique, may have influenced these results. Each ELISA plate was run with individual master standards, used to generate a standard curve. An increase in CLU levels between the two ELISA plates may also have been due to standard variability.

Regardless of variability in results between different western blots or ELISA plates, the outcome of each experiment revealed the same results or trend towards results. Across multiple western blots, and ELISAs, the CLU levels in dogs with MLSA were consistently (although not always significantly) lower than those of healthy controls. This gives confidence, even if results were not directly comparable, or repeatable within a 5% variable, and carried a degree of imprecision. All data trends, regardless of technique used, show that CLU levels were lower in diseased samples.

4.4 Study limitations and considerations

There are limitations associated with this study that merit discussion. Since the study was partly retrospective nature, many limitations resulted from reliance on patient records and previously collected, stored samples.

Patient records were occasionally incomplete, and samples frequently lacked histological evaluation. Lack of histopathology limited MLSA classification beyond cytological grade and immunophenotype, meaning the sample group consisted of a relatively heterogenous population. Previous studies (Saffer *et al.,* 2002) have shown variable CLU levels different subtypes of human lymphoid neoplasia. It is possible that different subtypes of canine LSA could have varying serum CLU levels, and the lack of precise classification could be limiting the understanding of CLU in canine MLSA.

While every precaution was taken to ensure the integrity of the cases and samples selected, there were multiple samples stored, at -80°C, for over a year. Previous studies of sample stability have shown no alteration in human CLU levels in plasma following >6 years stored at -80°C (Morgan *et al.*, 2017), with storage of serum samples for up to a year also having no effect on CLU levels (Morrissey *et al.*, 2001), although studies for stability in stored serum over longer periods of time were not found. As samples were selected from a bank of stored samples the exact freeze/thaw cycle history was not always known. However, previous studies have shown no change in CLU levels following five freeze/thaw cycles (Aguilar-Mahecha *et al.*, 2009), a number which was not exceeded in the current investigations. Protocol for sample storage was to have samples stored frozen within 24 hours of collection, due to the retrospective nature of this study, it is not possible to know the history of every sample. This may be of importance, as although stable when frozen or refrigerated, a study of CLU stability in CSF (Shafie *et al.*, 2013), showed CLU levels significantly reduced when incubated at room temperature for 48 hours.

Additionally, the ELISA technique used showed a higher degree of inter-assay variability than expected from the manufacturer guidelines. Ideally samples would have been run across multiple ELISA plates, and repeated multiple times to ensure optimum technique and minimal variation. This was not possible due to a lack of various resources, including time, sample volume and finances, with each ELISA kit a considerable expense.

As previously mentioned, it could not be excluded that the results of this study represent type I error, and rejection of a true null hypothesis due to a non-representative sample population. In addition, the small sample numbers could also be a limiting factor, representing a type II error particularly when evaluating the difference in CLU levels within the pre-treatment MLSA samples and between pre-treatment and CR samples. When evaluating power calculations, due to the wide individual variation CLU levels, sample groups of 400-500 would have been required to further assess statistical significance when comparing pre-treatment and CR MLSA samples.

Due to the retrospective nature of the study, treatment of the MLSA cohorts was not standardised, other than the first 4-6 weeks of the CR cohort. Some patients did not pursue chemotherapy, others chose less intense/single agent regimes thus it was not appropriate to assess MST with regard to CLU levels or attempt to attribute prognostic value to pre-treatment CLU levels.

These limitations are typical for a study of this nature that relies on the collection of material from cases over an extended period of time. However, when giving consideration to these factors, it can be concluded that that despite limitations, studies of this nature are important and have the potential to identify biomarkers that may prove to be of diagnostic and prognostic value.

4.5 Further research

The significant variation in serum CLU levels suggests it is unlikely to be of use as a diagnostic biomarker. While not useful as a stand-alone biomarker, CLU may prove of use in combination with other, emerging or established markers. Investigation of larger sample cohorts could help to develop a reference range for CLU in the healthy adult dog, and aid in determining accurate and meaningful diagnostic cut off values for serum CLU. Additionally, expanding the MLSA sample population to a larger size would also allow better evaluation of CLU in relation to immunophenotype, stage and substage of MLSA and potentially enable evaluation of CLU as a prognostic biomarker.

Larger cohorts would increase statistical power and reduce the likelihood of type II error. This would be of use particularly in the comparison of pre-treatment and CR samples, where alterations in serum CLU could prove predictive for remission or early relapse. Prospective assessment of serum CLU concentration, with standardised treatment protocols, would also enable evaluation of MST and prognostic value of pre or post treatment CLU levels.

In addition, further investigations to evaluate the cytoplasmic CLU expression via IHC in canine MLSA tissue, in tandem with the circulating serum CLU levels, would be of interest. Establishing any association between circulating serum CLU levels and tissue CLU could help to clarify the relationship between CLU and canine MLSA and determine whether IHC could be of more value than serum analysis of CLU.

Alternatively CLU has also been widely evaluated as a biomarker excreted in the urine (Aulitzky *et al.,* 1992; García-Martínez *et al.,* 2012; Fowlie *et al.,* 2017). Evaluation of urinary CLU in canine MLSA patients has yet to be investigated but may hold potential as a non-invasive monitoring tool for detection of disease or early relapse.

4.5.1 Complement component three (C3)

During protein electrophoresis of the pilot samples an additional protein was noted, expressed preferentially in dogs with MLSA, in comparison to healthy controls. Since this could represent a potential MLSA biomarker, two protein bands were excised from the agarose gel and the protein content identified via mass spectrometry, (**Appendix 5**).

Complement C3 was identified in both samples analysed, as determined by protein sequences to an established database. While likely to be raised in a variety of conditions, and not specific to canine MLSA, complement C3 was recognised as a potential marker and may warrant further investigation.

5 Conclusion

In the current study, serum CLU was significantly lower in patients with MLSA, although significant individual variation existed. The repeatability and marked degree of individual variation, leading to a wide range of results within sample populations, suggests that serum CLU would have limited benefit as a stand-alone diagnostic biomarker, or marker of remission. However, the potential prognostic value of pre-treatment or post-treatment serum CLU has not been investigated. Additional studies, including concurrent immunohistochemical evaluation of CLU in lymph nodes/tissue, would be required to fully evaluate CLU expression in canine MLSA.

6 Bibliography

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7 Appendices

7.1 Appendix 1 – Hospital consent form

Small Animal Hospital
University of Glasgow Veterinary School
464 Bearsden Road, Bearsden
Glasgow G61 1QH
Telephone: 0141-330-5848
Fax: 0141-330-3663
Email: sah-reception@glasgow.ac.uk

CONSENT FOR VETERINARY CARE

Case Number: 248600
Animal Name: Marmite **Age:** 8 Years Old **Sex:** Male Neutered **Species/Breed:** Feline/Domestic Shorthair
Owner: Ms Katie McNaught
Address:
Owners phone number:
Today's Date: Monday, 22 June 2020

Treatments/procedures. I, the owner or agent of the above animal, hereby request and give permission that this animal receives such examination and treatment as may be required. This may include the administration of anaesthetic drugs, the act of surgery and treatment with drugs that may only be licensed for use in other species. The nature and effect of this examination and treatment have been explained to me. I understand and accept that all diagnostic, therapeutic, anaesthetic and surgical procedures carry some risk to the life and health of my animal, and that while reasonable care will be taken, such measures are carried out at my sole risk. This risks exist even in apparently healthy animals and I have discussed my concerns with the veterinarian. In the event that the veterinary surgeon is unable to contact me on the numbers provided, I understand the veterinary surgeon will act in the best interests of my animal. No assurance has been given that the treatment will be carried out by a particular veterinary surgeon.
Teaching. I understand that students, acting under appropriate supervision, may be involved in the examination and care of my animal.
Ownership of records and samples. I give my permission for the retention and use of all clinical data/records, pictures (including digital diagnostic images), samples (urine, blood, faeces, DNA, biopsies, autopsies) for teaching purposes and for future studies to help human and animal welfare. All such material will be used anonymously.

Data Protection. Any information that you supply via this form will be entered into a filing system and will only be accessed by authorised persons of the University of Glasgow or its agents. By supplying such information you consent to the University storing the information for the stated purposes. The information is processed by the University in accordance with the provisions of the Data Protection Act 2018. For full details of the Small Animal Hospital privacy policy see our website: www.gla.ac.uk/schools/vet/sah/

I agree [ ] I disagree [ ]

Signed .....

Estimate £ (inc VAT)

Payment. I also realise that the estimated costs of treatment can only be approximate and do not include any emergency procedures, complications, follow up treatment or investigation. I accept that I am liable for all costs incurred in the treatment of my animal and agree to pay in full upon collection of my animal. I agree to the University's Terms and Conditions for the provision of services.

Signed .....
I confirm that I have explained to the owner or agent the nature and effects of the examination and treatment to be carried out on the above named animal.

Signed ..... (MRCVS / VN)
Print name Dr. Katie McNaught

7.2 **Appendix 2 – CHOP protocol**

WEEK	DRUGS
1	Vincristine 0.7mg/m^2 IV Prednisolone 2mg/Kg or 40mg/m^2 PO SID
2	Cyclophosphamide 250mg/m^2 PO Prednisolone 1.5mg/kg or 30mg/m^2 PO SID Furosemide 1-2mg/Kg PO
3	Vincristine 0.7mg/m^2 IV Prednisolone 1mg/kg or 20mg/m^2 PO SID
4	Doxorubicin 30mg/m^2 IV (+ 0.9% NaCl) Prednisolone 0.5mg/kg or 10mg/m^2 PO SID Chlorphenamine 2-10mg total dose IM Maropitant 1mg/Kg SC
6	Vincristine 0.7mg/m^2 IV
7	Cyclophosphamide 250mg/m^2 PO Furosemide 1-2mg/Kg PO
8	Vincristine 0.7mg/m^2 IV
9	Doxorubicin 30mg/m^2 IV (+ 0.9% NaCl) Chlorphenamine 2-10mg total dose IM Maropitant 1mg/Kg SC
11	Vincristine 0.7mg/m^2 IV
13	Cyclophosphamide 250mg/m^2 PO/IV (+ NaCl) Furosemide 1-2mg/Kg PO
15	Vincristine 0.7mg/m^2 IV
17	Doxorubicin 30mg/m^2 IV (+ 0.9% NaCl) Chlorphenamine 2-10mg total dose IM Maropitant 1mg/Kg SC
19	Vincristine 0.7mg/m^2 IV
21	Cyclophosphamide 250mg/m^2 PO Furosemide 1-2mg/Kg PO
23	Vincristine 0.7mg/m^2 IV
25	Doxorubicin 30mg/m^2 IV (+ 0.9% NaCl) Chlorphenamine 2-10mg total dose IM Maropitant 1mg/Kg SC

7.3 Appendix 3 - Sample Lists

*indicates samples also used in pilot sample group

7.3.1 Control samples

7.3.1.1 *Pilot study*

Sample number	Age(years)	Breed	Sex
1	2	Flat-coated Retriever	ME
2	7	Labrador Retriever	MN
3	2	Golden Retriever	MN
4	7	Labrador Retriever	FN
5	0.75	German Shepherd	ME
6	4	German Shepherd	ME
7	2	Crossbreed	MN
8	5	Labrador Retriever	MN
9	7	Greyhound	FN
10	4	Doberman	FN
11	1	Golden Retriever	FE
12	5	Golden Retriever	FN
13	1	Golden Retriever	MN
14	7	Springer Spaniel	MN
15	5	Labrador Retriever	FN
16	4	Labrador Retriever	FN
17	4	Greyhound	FN
18	3	Labrador Retriever	MN
19	3	Labrador Retriever	MN
20	3	Labrador Retriever	ME

7.3.1.2 *Sample Group One – Cohort C*

Sample number	Age(years)	Breed	Sex
C1*	2	Flat-coated Retriever	ME
C2*	7	Labrador Retriever	MN
C3*	2	Golden Retriever	MN
C4*	7	Labrador Retriever	FN
C5*	0.75	German Shepherd	ME
C6*	4	German Shepherd	ME
C7*	2	Crossbreed	MN
C8*	5	Labrador Retriever	MN
C9*	7	Greyhound	FN
C10*	4	Doberman	FN
C11*	1	Golden Retriever	FE
C12*	5	Golden Retriever	FN

7.3.1.3 *Sample Group Three – age matched samples*

Sample number	Age(years)	Breed	Sex
Ec13 *	4	Labrador Retriever	FN
Ec14 *	4	Greyhound	FN
Ec15	4	Weimaraner	ME
Ec16	4	Labrador Retriever	ME
Ec17	4	Greyhound	MN
Ec18 *	5	Labrador Retriever	FN
Ec19	5	Greyhound	FN
Ec20	5	Greyhound	MN
Ec21	5	Crossbreed	FN
Ec22	5	Greyhound	FN
Ec23	5	Greyhound	MN
Ec24	5	Greyhound	MN
Ec25	6	Golden Retriever	FN
Ec26	6	Greyhound	FN
Ec27 *	7	Springer Spaniel	MN
Ec28	7	Doberman	ME
Ec29	7	Golden Retriever	FN
Ec30	7	Labrador Retriever	ME

7.3.2 MLSA pre-treatment samples

7.3.2.1 *Pilot study*

Sample number	Age (years)	Breed	Sex	Stage	Substage	Immunophenotype
1	7	Boxer	MN	III	b	-
2	2	Cocker spaniel	ME	III	b	-
3	6	German shepherd	ME	III	a	-
4	6	Crossbreed	ME	V	a	B
5	10	Border collie	ME	V	b	B
6	4	Tibetan terrier	MN	IV	b	B
7	8	Springer spaniel	ME	III	b	T
8	10	Crossbreed	ME	IV	a	B
9	9	Cairn terrier	MN	IV	a	-
10	6	Border collie	ME	IV	b	-
11	10	Boxer	MN	V	b	B
12	7	Airedale terrier	MN	III	a	-
13	6	Bullmastiff	FN	IV	b	B
14	12	Border collie	ME	V	b	B
15	3	Patterdale terrier	FE	V	b	-
16	8	Labrador retriever	FN	IV	a	null
17	3	Springer spaniel	FN	IV	b	-
18	7	Gordon setter	FE	IV	b	T
19	7	Boxer	MN	V	b	-
20	10	Border terrier	ME	V	b	B

7.3.2.2 *Sample Group One – cohort A*

Sample number	Age (years)	Breed	Sex	Stage	Substage	Immunophenotype
A1*	10	Crossbreed	ME	IV	a	B
A2*	9	Cairn terrier	MN	IV	a	-
A3*	10	Border terrier	ME	V	b	B
A4	6	Labrador retriever	FE	IV	a	B
A5*	10	Border collie	ME	V	b	B
A6*	10	Boxer	MN	V	b	B
A7*	12	Border collie	ME	V	b	B
A8 *	7	Boxer	MN	V	b	-
A9	7	Labrador retriever	MN	V	b	-
A10	5	Cavalier King Charles spaniel	FE	V	b	-
A11	6	Border collie	MN	V	b	B
A12 *	7	Gordon setter	FE	IV	b	T

7.3.2.3 *Sample Group Two – paired samples*

Sample number	Age (years)	Breed	Sex	Stage	Substage	Immunophenotype
D1	14	Parsons terrier	ME	V	b	-
D2	11	Crossbreed	FN	III	a	-
D3	6	Golden retriever	FN	V	b	T
D4	3	Cocker spaniel	ME	V	b	T
D5	5	Golden retriever	ME	III	a	B
D6	10	Border collie	FE	IV	b	-
D7	9	West Highland terrier	FN	IV	a	B
D8	8	Golden retriever	MN	V	a	-
D9	12	Yorkshire terrier	FN	IV	a	B
D10	12	Border collie	FN	IV	a	B
D11	9	Labrador retriever	MN	V	b	-
D12	8	German shepherd	ME	V	b	B
D13	5	Border collie	MN	IV	a	B
D14	9	Shih tzu	MN	IV	a	B
D15	9	Labrador retriever	FN	IV	b	B
D16	6	Jack Russel terrier	ME	III	a	-
D17	5	Tibetan terrier	FN	IV	a	B
D18	10	Spinone	FN	IV	a	B

7.3.2.4 *Sample Group Three – age matched samples*

[+] indicates samples also used in sample group two

Sample number	Age (years)	Breed	Sex	Stage	Substage	Immunophenotype
E1	3	Flat-coated retriever	ME	IV	b	B
E2	4	Boxer	FN	IV	b	T
E3	5	Bullmastiff	FN	III	b	T
E4	5	Cocker spaniel	ME	III	b	T
E5	6	Golden retriever	MN	V	b	T
E6	6	Bernese mountain dog	MN	IV	b	B
E7	6	Shetland sheepdog	MN	III	b	T
E8	6	Lurcher	MN	III	a	-
E9	6	Crossbreed	FN	III	a	-
E10	7	Border collie	MN	IV	a	T
E11	7	Dogue de Bordeaux	MN	IV	b	-
E12	7	Border collie	MN	IV	b	T
E13[+]	6	Golden retriever	FN	V	b	T
E14[+]	3	Cocker spaniel	ME	V	b	T
E15[+]	5	Golden retriever	ME	III	a	B
E16[+]	5	Border collie	MN	IV	a	B
E17[+]	6	Jack Russell terrier	ME	III	a	-
E18[+]	5	Tibetan terrier	FN	IV	a	B

7.3.3 MLSA CR samples

7.3.3.1 *Sample Group One – cohort B*

Sample number	Age (years)	Breed	Sex	Stage	Substage	Immunophenotype
B1	10	Golden retriever	FN	IV	b	B
B2	7	Crossbreed	MN	IV	b	-
B3	12	Crossbreed	MN	IV	b	B
B4	4	Jack Russel terrier	FN	III	a	B
B5	12	Whippet	MN	V	b	B
B6	8	Cocker spaniel	ME	III	a	B
B7	4	Hungarian vizsla	ME	III	a	B
B8	5	German shepherd	FN	IV	b	B
B9	8	Dachshund	FN	IV	a	B
B10	9	Beagle	ME	II	b	-
B11	6	Border collie	MN	V	b	B
B12	5	Tibetan terrier	FN	IV	a	B

7.3.3.2 *Sample group two - paired samples*

Samples from the same cohort in section 7.2.2.3, were also taken at time of CR, as determined by physical examination and lymph node palpation, at week 4-6 of a (CHOP-based) chemotherapy protocol, by a qualified veterinary surgeon experienced in veterinary oncology.

7.4 Appendix 4 – ELISA protocol

7.4.1 Serum sample preparation

Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles and using haemolysed or lipemic samples.

Serum samples: Dilute samples just prior to perform the test 2500x with Dilution Buffer in two steps as follows -

Dilution A (50x): Add 5 µl of sample into 245 µl of Dilution Buffer and mix well (not to foam). Vortex is recommended.

Dilution B (50x): Add 5 µl of Dilution A into 245 µl of Dilution Buffer to prepare final dilution 2 500x. Mix well (not to foam). Vortex is recommended.

Stability and storage: Samples should be assayed immediately after collection or should be stored at -20°C, or preferably at -70°C for long-term storage.

7.4.2 Assay procedure

1. Pipet 100 µl of diluted standards, quality controls, dilution buffer and samples, into the appropriate wells, preferably in duplicate.

2. Incubate the plate at room temperature (25°C) for 1 hour, at 300 rpm on an orbital microplate shaker.

3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

4. Add 100 µl of Biotin Labelled Antibody solution into each well.

5. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.

6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

7. Add 100 µl of Streptavidin-HRP Conjugate into each well.

8. Incubate the plate at room temperature (25°C) for 30 min, shaking at 300 rpm on an orbital microplate shaker.

9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

10. Add 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight.

11. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.

12. Stop the colour development by adding 100 µl of Stop Solution.

13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 – 650 nm). Subtract readings at 630 nm (550 - 650 nm) from the readings at 450 nm. The absorbance should be read within 5 minutes following step 12.

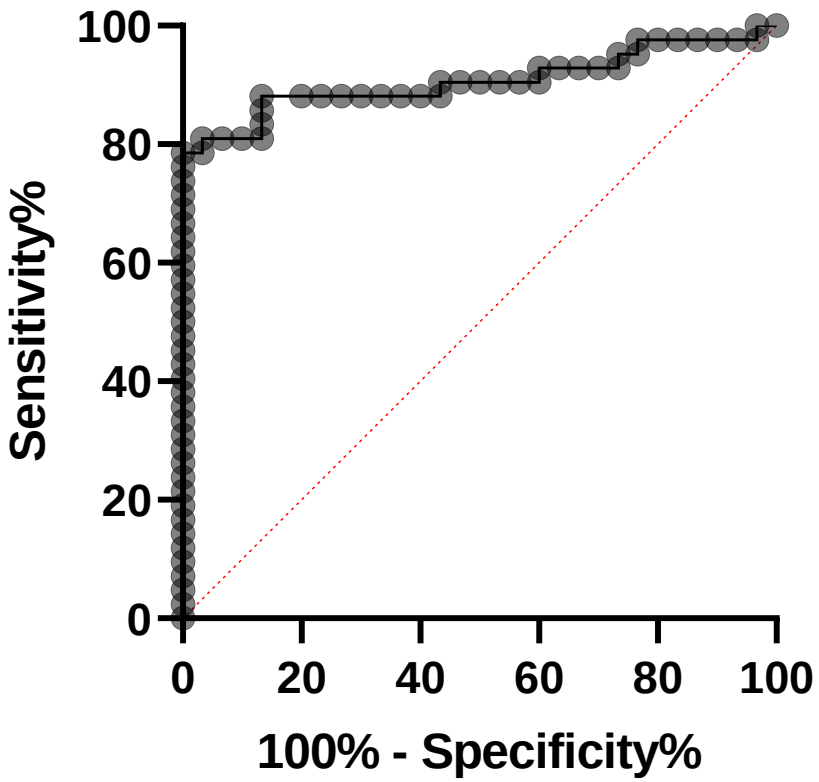
7.5 Appendix 5 - Mass Spectrometry for Complement 3

Mascot Search Results
Search title: Orbitrap NCBI Dog Search
Database: NCBInr 20160830 (93482448 sequences; 34454162062 residues)
Taxonomy: Canis lupus familiaris (Dog) (41680 sequences)
Timestamp: 11 Jan 2017 at 10:52:42 GMT
Enzyme: Trypsin
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass values: Monoisotopic
Protein Mass: Unrestricted
Peptide Mass Tolerance: ± 10 ppm (# 13C = 1)
Fragment Mass Tolerance: ± 0.5 Da
Max Missed Cleavages: 1
Instrument type: ESI-FTICR
Number of queries: 4178

Protein hits with Mascot score >100 listed:
gi|545535669 PREDICTED: complement C3 [Canis lupus familiaris]
Mass: 181723 Score:1564 Matches: 42(42) Sequences: 36(36)
gi|545520919 PREDICTED: serum albumin isoform X1 [Canis lupus familiaris]
Mass: 70558 Score: 1538 Matches: 34(34) Sequences: 23(23)
gi|55742764 serum albumin precursor [Canis lupus familiaris]
Mass: 70556 Score: 1277 Matches: 31(31) Sequences: 22(22)
gi|123511 Haptoglobin alpha chain; Haptoglobin beta chain
Mass: 36890 Score: 868 Matches: 28(28) Sequences: 16(16)
gi|75043394 Keratin, Cytokeratin-10
Mass: 57847 Score: 747 Matches: 14(14) Sequences: 10(10)
gi|928167632 PREDICTED: serotransferrin [Canis lupus familiaris]
Mass: 80222 Score: 602 Matches: 14(14) Sequences: 13(13)
gi|3915605 Apolipoprotein E
Mass: 35332 Score: 426 Matches: 8(8) Sequences: 7(7)
gi|928150787 PREDICTED: complement C4-A [Canis lupus familiaris]
Mass: 199301 Score: 414 Matches: 10(10) Sequences: 9(9)
gi|545552242 PREDICTED: complement factor I isoform X1 [Canis lupus familiaris]
Mass: 69506 Score: 366 Matches: 7(7) Sequences: 3(3)
gi|3915607 Apolipoprotein A-I
Mass: 30178 Score: 346 Matches: 9(9) Sequences: 8(8)
gi|50979272 keratin, type II cytoskeletal 1 [Canis lupus familiaris]
Mass: 63922 Score: 320 Matches: 7(7) Sequences: 6(6)
gi|560879429 zinc-alpha-2-glycoprotein precursor [Canis lupus familiaris]
Mass: 36060 Score: 303 Matches: 6(6) Sequences: 6(6)
gi|345791833 PREDICTED: keratin, type II cytoskeletal 3 [Canis lupus familiaris]
Mass: 62762 Score: 243 Matches: 4(4) Sequences: 4(4)
gi|75062694 Keratin, type II cytoskeletal 2 epidermal
Mass: 64812 Score: 239 Matches: 6(6) Sequences: 5(5)
gi|17066526 immunoglobulin gamma heavy chain B [Canis lupus familiaris]
Mass: 52553 Score: 228 Matches: 7(7) Sequences: 6(6)
gi|345791904 PREDICTED: keratin, type II cytoskeletal 5 [Canis lupus familiaris]

Mass: 63006 Score: 209 Matches: 4(4) Sequences: 4(4)
gi|50979240 clusterin precursor [Canis lupus familiaris]
Mass: 52327 Score: 205 Matches: 4(4) Sequences: 4(4)
gi|928172771 PREDICTED: Ig lambda chain V-I region BL2 isoform X1 [Canis lupus familiaris]
Mass: 25286 Score: 197 Matches: 4(4) Sequences: 3(3)
gi|345791839 PREDICTED: keratin, type II cytoskeletal 6A isoform X2 [Canis lupus familiaris]
Mass: 63174 Score: 186 Matches: 4(4) Sequences: 4(4)
gi|928186325 PREDICTED: inter-alpha-trypsin inhibitor heavy chain H4 isoform X1 [Canis lupus familiaris]
Mass: 105582 Score: 169 Matches: 4(4) Sequences: 4(4)
gi|73960685 PREDICTED: C4b-binding protein beta chain [Canis lupus familiaris]
Mass: 29379 Score: 163 Matches: 5(5) Sequences: 4(4)
gi|545544683 PREDICTED: Ig lambda chain V-I region BL2 isoform X42 [Canis lupus familiaris]
Mass: 25081 Score: 149 Matches: 3(3) Sequences: 3(3)
gi|345778397 PREDICTED: complement factor B [Canis lupus familiaris]
Mass: 87635 Score: 135 Matches: 3(3) Sequences: 3(3)
gi|359279911 keratin 14 [Canis lupus familiaris]
Mass: 52621 Score: 129 Matches: 3(3) Sequences: 3(3)
gi|73996461 PREDICTED: keratin, type II cytoskeletal 78 [Canis lupus familiaris]
Mass: 56429 Score: 118 Matches: 2(2) Sequences: 2(2)
gi|73956164 PREDICTED: microfibril-associated glycoprotein 4 [Canis lupus familiaris]
Mass: 28775 Score: 117 Matches: 2(2) Sequences: 1(1)
gi|545553759 PREDICTED: alpha-2-HS-glycoprotein [Canis lupus familiaris]
Mass: 40021 Score: 108 Matches: 1(1) Sequences: 1(1)
gi|57106717 PREDICTED: keratin, type II cuticular Hb4 [Canis lupus familiaris]
Mass: 64358 Score: 101 Matches: 2(2) Sequences: 2(2)

7.6 Appendix 6 – ROC curve for combined CLU data.



ROC curve for serum CLU concentrations using combined data from MLSA pre-treatment and control samples across three ELISA plates.

Area under the ROC curve – 0.9063

$p<0.0001$