CHANGES IN THE SERUM PROTEOME IN CANINE LYMPHOMA

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SUMMARY

One dimensional (1D) serum protein gel electrophoresis (SPE) on agarose gels is a frequently used diagnostic tool for canine diseases; however, little is known regarding the precise composition of the different protein fractions in normal or diseased animals. To analyse the canine serum proteome in more detail we combined conventional 1D SPE with second dimension (2D) polyacrylamide gel electrophoresis (PAGE) and followed by tandem mass spectrometry (MS). One dimensional SPE was performed on the sera of 17 healthy dogs to establish normal reference ranges for the albumin and globulin sub-fractions. Two representative serum samples from the healthy dogs were further separated using a novel method of 2D PAGE, leading to the generation of 26 distinct bands across the six main sub-fractions, which were subjected to MS analysis. Thirty-two individual proteins were identified, most of which were found in both dogs. Twenty proteins belonged specifically to the species *Canis lupus familiaris*, with the remaining 12 proteins belonging to other mammalian species, likely reflecting incomplete sequencing knowledge of canine proteins. Two dimensional electrophoresis and MS allowed identification of canine serum albumin precursor, serpin peptidase inhibitor, kininogen-1, vitamin D binding protein, hemopexin, complement C4 and a variety of immunoglobulin class molecules and their localisation within their respective serum protein subfractions for the first time.

Sera from twenty-one dogs with high grade multicentric lymphoma underwent identical analysis and had significantly elevated α2 globulins on 1D SPE. Further separation of the serum proteins was performed on three patients using a 2D PAGE system. Thirty-eight separate protein bands were submitted for MS and 36 different proteins were identified. Most of the proteins were the same as those previously identified in the serum of healthy dogs, showing reproducibility of this novel proteomic technique. Haptoglobin was found in all three of the lymphoma dogs, having not previously been identified in any of the healthy samples, and could account for the increased α2 globulins. Several other proteins, including α-2 HS glycoprotein, α2 macroglobulin, α1 antichymotrypsin and inter-α-trypsin inhibitor were also present in dogs suffering from lymphoma. Clusterin, an anti-apoptotic protein, was identified for the first time in the serum of one dog

suffering from lymphoma. Kininogen, which is present in the serum of healthy dogs, was absent in all three dogs with lymphoma. This 2D electrophoresis technique has identified numerous changes in the serum proteome of dogs suffering from lymphoma and suggests a significant inflammatory component to the pathogenesis of this disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Plus Protein Standards All Blue, Bio-Rad) was run in first lanes of each of the 2 gels displayed here.

87

LIST OF ACCOMPANYING MATERIAL

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I would like to dedicate this thesis to my parents for their continued love, support and encouragement throughout my education.

AUTHOR'S DECLARATION

The work in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

PUBLICATIONS AND PRESENTATIONS

Part of the work in this thesis has been presented in the following written publications and as abstract form:

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CHAPTER I

REVIEW OF NORMAL CANINE SERUM PROTEINS AND CLINICOPATHOLOGIC ALTERATIONS IN CANINE LYMPHOMA

This thesis begins with a review of serum proteins, proteomics and clinicopathologic features of canine lymphoma. A novel method for analysis of the normal canine serum proteome is described before being applied to analyse differences in the proteome of dogs diagnosed with lymphoma. The thesis ends with a discussion regarding possible future applications of some potential biomarkers that have been reported here.

1.1 Structure and Physiologic Control of Proteins

Analysis of serum is one of the cornerstones of diagnostic clinicopathology across many species including the dog and the main constituent of serum is protein. Proteins have four different levels of structural formation, ranging from the initial primary structure which is determined by a unique combination of individual amino acids joined together by peptide bonds, up to the quaternary structure which involves interactions between multiple different protein subunits (Lehninger et al., 1993). As well as participating in protein to protein binding, proteins may also conjugate with other chemical species such as lipids, metallic ions, phosphate, carbohydrates and nucleic acid sequences. Proteins are synthesised within ribosomes and the synthesis of the appropriate polypeptide is determined by mRNA sequences that are transcribed from sequences of coding DNA. Translation of the mRNA results in chains of amino acids that ultimately form the primary structure of a protein (Lehninger et al., 1993). As there are up to 20 different amino acids there are several million possible primary structural combinations of canine proteins and further complexity can be added by the folding involved in the secondary and tertiary protein structures, a process which is facilitated by chaperones (Eckersall, 2008). Proteins are constantly being synthesized and catabolised by the body. As there is no capacity for storage of protein within the body these processes have to be carefully regulated, but alterations in nutritional and health status can result in alterations of the body's protein balance (Tvarijonaviciute et al., 2012). In carnivorous species such as the dog proteins can provide as much as 40-

50% of the energy from dietary intake as proteins can be converted into glucose or lipid or alternatively may serve as a substrates for the tricarboxylic acid cycle (Eckersall, 2008). The liver has a central role in dealing with nitrogenous waste, in particular ammonia which is produced alongside amino acids following digestion of food by the gastrointestinal tract and by certain bacteria within the gut. Ammonia is processed by the liver and ultimately forms urea by way of the urea cycle. In certain situations of hepatic insufficiency, toxic accumulations of ammonia and other substrates can manifest clinically as hepatic encephalopathy (Maddison, 1992). Urea, the end product of the urea cycle is excreted by the kidneys under normal healthy conditions (Eckersall, 2008).

The majority of plasma proteins are synthesized hepatically with the notable exception of immunoglobulins which are secreted by plasma cells which are mature cells of the B lymphocyte lineage. Serum differs from plasma by lacking the presence of fibrinogen amongst other clotting factors. Serum is often preferred for analysis as there can be various differences in clinical chemistry results between the two blood biproducts (Miles et al., 2004). In conditions of health albumin has the highest concentration of all the circulating serum proteins and albumin synthesis is controlled by alterations in the colloid oncotic pressure amongst other factors (Evans, 2002). Synthesis of immunoglobulins by the plasma cells is driven by the immune system often in response to pathogenic challenge to the host organism (Eckersall, 2008). Some circulating hormones such as ACTH are produced by specialist endocrine tissues such as the pituitary gland (Li et al., 1978) and plasma proteins can also be synthesized in other extrahepatic sites such as adipose tissue (Halleux et al., 2001). Plasma proteins are under close homeostatic regulation as they are vital for multiple different physiologic processes such as maintaining oncotic pressure, appropriate control of haemostasis, innate as well as acquired immunity, metabolite and drug transport, controlling metabolic pathways and preventing excessive tryptic activity (Eckersall, 2008).

1.2 Measuring, Separating and Identifying Serum Proteins

Multiple methods are available for measuring, separating and identifying different protein constituents within serum. Measurement of total protein concentration alongside concentrations of albumin and globulins are universally available and recommended as part of a serum biochemistry profile for assessment of numerous different pathologies in the canine species (McGrotty and Tennant, 2005). Globulins comprise a wide variety of different proteins with different molecular weights and charges. More sophisticated techniques are required to further separate this group of proteins and these include gel based protein electophoresis and immunoelectophoresis (McGrotty and Tennant, 2005). Definitive identification of proteins primarily utilises mass spectrometry preceded by fractionation of complex solutions of proteins into more refined individual proteins (Eckersall, 2008).

1.2.1 The Biuret and Bromcresol Green Methods

Total serum protein can be accurately assessed within the concentration ranges of 10-100 g/L by wet and dry automated biochemical analysers used by commercial and in house laboratories employing the Biuret technique (Eckersall, 2008). The Biuret technique for determination of serum proteins was first described in 1949 (Gornall et al.). This is a colourimetric test with the binding of the proteins to ionic copper in an alkaline solution resulting in the formation of a violet complex (Stockham and Scott, 2008).

Bromcresol green is a dye that preferentially binds to albumin, therefore allowing determination of serum albumin concentrations, again using a colourimetric technique. First described in 1971 (Doumas et al.) this test is now widely used by commercial clinical chemistry analysers throughout the

veterinary profession (Eckersall, 2008). Subtraction of the albumin concentration as determined by the bromcresol green method from the total protein concentration derived from the Biuret method generates the combined concentration of the serum globulins.

Normal protein concentrations are determined by each individual laboratory using their sample population. The University of Glasgow Laboratory reference intervals are 50-78 g/L, 29-36 g/L and 28-42 g/L for total protein, albumin and globulin respectively (McGrotty and Tennant, 2005).

1.2.2 One Dimensional Serum Protein Electrophoresis

Electrophoresis is the most widely used method for separating proteins found within serum (Eckersall, 2008). Serum is loaded onto a gel based medium usually under alkaline conditions and an electric current is applied across the gel. Proteins migrate to varying extent across the gel depending both on their size and electrical charge. Under standard conditions albumin is the most mobile protein and migrates towards the anode owing to its negative charge and small molecular mass (Eckersall, 2008); immunoglobulins are the least negatively charged and therefore tend to migrate towards the cathode. Once the serum has been electrophoresed across the gel, the gel is fixed and stained with solutions such as amido black or Coomasie brilliant blue allowing visualisation of protein bands. Scanning of the stained gel using a densitometer allows the generation of an electrophoretogram which is effectively a histogram representing the transmission of light through the stained gel (Stockham and Scott, 2008). Six major protein fractions can usually be identified from a serum protein electrophoretogram and these include albumin, α1, α2, ß1, ß2 and γ globulins (McGrotty and Tennant, 2005) (Fig. 1). The definitive concentrations of each protein fraction is calculated as a product of the relative fraction as determined by the electrophoretogram and the total protein concentration derived from the

Biuret method (Stockham and Scott, 2008). Electrophoresis of plasma as opposed to serum results in obscuration of the immunoglobulin fractions as fibrinogen is present in a large peak in between the ß and γ peaks (McGrotty and Tennant, 2005).

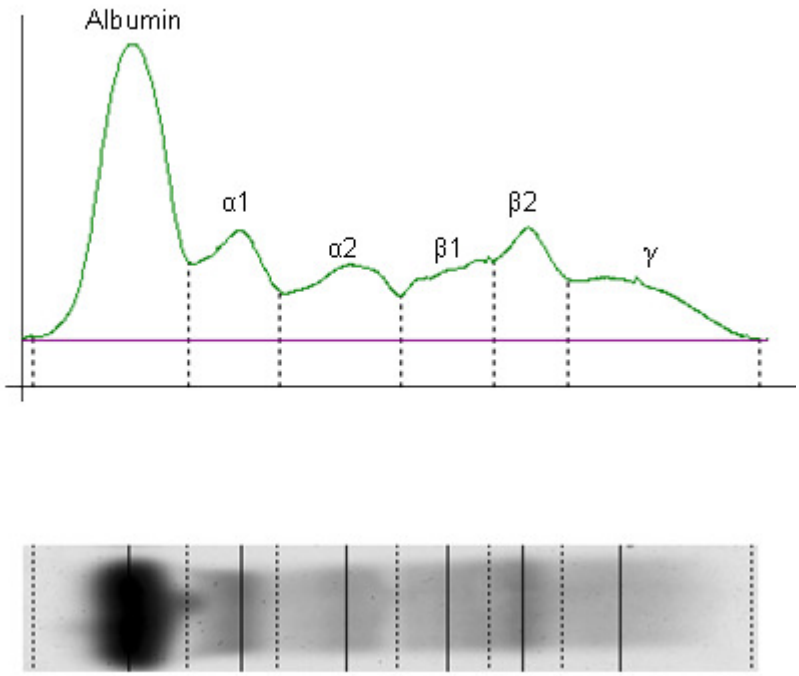


Figure 1 An electrophoretogram from a healthy two year old male neutered Flatcoated Retriever showing separation of proteins into six clearly defined bands. The top panel displays the electrophoretogram which is a graphical densitometric representation of the scanned gel lane seen in the corresponding bottom panel.

Agarose is now the most common gel material used within commercial laboratories and has largely superseded cellulose acetate due to its commercial availability and greater reproducibility in terms of the results generated (Eckersall, 2008). High resolution electrophoresis has also been performed on canine serum allowing for further separation of the serum protein fractions. This method has been combined with immunofixation resulting in the identification of some of the major proteins found within canine serum (Abate et al., 2000). Another method of electrophoresis namely capillary zone electrophoresis has also been used to fractionate canine serum proteins (Giordano and Paltrinieri, 2010). This technique does not require support media through which protein migrates and can result in rapid and reproducible results however, sample haemolysis and lipaemia may alter the morphology of the electrophoretic traces (Martínez-Subiela et al., 2002b). Capillary zone electrophoresis also requires more specialized equipment in comparison to the traditional gel based systems (Eckersall, 2008).

Previous studies have looked at establishing normal reference intervals for the various different serum protein fractions in dogs' sera (Fayos et al., 2005; Keay, 1982; Tappin et al., 2011). Factors other than disease can lead to alterations of the electrophoretogram and these can include breed, age, hormonal status and nutrition (Eckersall, 2008). A comparison of 1D agarose SPEs between 28 Greyhounds and 18 age and gender matched none-greyhound breeds found that Greyhounds had lower α and ß-globulin concentrations (Fayos et al., 2005). Although 1D-SPE is a widely used and easily accessible method of serum protein electrophoresis the resolution in terms of separation of the serum into constituent proteins is limited as each protein fraction contains multiple individual proteins. This was first recognised by Smithies and Poulik (1956) who devised a novel method of separating proteins within human serum using 2 dimensional electrophoresis.

1.2.3 Two Dimensional Polyacrylamide Gel Electrophoresis

Further separation and resolution of serum proteins can be used by staging the electrophoretic process into 2 dimensions. Conventionally the first dimension separates proteins on the basis of their charge or more specifically their pI. This is the pH at which a compound has no net electrical charge and in the setting of gel electrophoresis this protein will become stationary or "focused" (Eckersall, 2008). The second dimension is a further separation based on the molecular mass of the individual protein. Two dimensional electrophoresis was first described using filter paper in an alkaline buffer as a first dimension separating proteins on their free solution mobility. This first dimension was then applied perpendicularly to a starch-gel and was electrophoresed for a second time (Smithies and Poulik, 1956). Although this technique may be crude compared to modern day standards, more than 15 individual spots could be resolved from the serum of a healthy human. Since the original description of two dimensional electrophoresis this technique has continued to evolve and now enables high resolution separation allowing for the detection of hundreds of proteins on a single gel plate (Issaq and Veenstra, 2008). Isoelectric focusing is performed as a first dimension making use of immobilised pH gradients (IPGs) to help overcome issues of lack of reproducibility due to unstable pH gradients. The latter were encountered when in-house gels, incorporating reagents known as ampholytes, were used as the first dimension (Eckersall, 2008). IPGs are now available commercially as precast dry strips resulting in easier handling and better comparability of results (Görg et al., 2004). The second dimension of the electrophoresis takes place on a polyacrylamide gel (PAGE) in the presence of the detergent sodium dodecyl sulphate (SDS). SDS-PAGE systems further separate the proteins based on their mass with the gel acting like a "sieve" therefore impeding migration (Eckersall, 2008). Following completion of electrophoresis the gels are then stained with Coomasie brilliant blue stains, silver based stains or fluorescent agents allowing visualisation of the different protein spots (Eckersall, 2008). The amount of data produced can be vast meaning that computer programs are required in order to analyse the images acquired (Görg et al., 2004).

Reproducibility of the results generated by SDS-PAGE can be problematic as it is a technically demanding technique requiring advanced equipment and competent personnel to run such experiments (Issaq and Veenstra, 2008). The development of 2D differential in-gel electrophoresis (2D-DIGE) offers a novel method in which several samples can be compared on an individual gel. In 2D-DIGE each individual sample is covalently labelled with a unique cyanine fluorescent dye possessing its own excitation and emission wavelength allowing different samples to be run on one gel, facilitating direct comparison of the proteins present (Ünlü et al., 1997).

2D-PAGE techniques have allowed electrophoretic protein mapping in healthy dogs' sera (Presslmayer, 2002) as well as comparison of the serum proteome in healthy dogs before and after a controlled weight loss programme (Tvarijonaviciute et al., 2012). Proteomic mapping of healthy and diseased organs and tissues has also been performed using such methods for analysis of the canine heart (Dunn et al., 1997) and the comparison of normal lymph nodes with those of dogs diagnosed with B-cell lymphoma (McCaw et al., 2007).

Two dimensional electrophoresis provides a high resolution of protein separation allowing comparisons between different samples but in its own right it is unable to definitively identify the proteins responsible for such changes. As for 1D-SPE, where identification of proteins by immunofixation using antibodies has been described, specific stains can also be applied to PAGE gels to help determine the protein however, the advent of mass spectrometry has greatly advanced the ability to identify protein spots on PAGE gels (Eckersall, 2008). Despite the complexity and problems with reproducibility 2D-PAGE will likely remain an essential tool for the analysis of proteins for many years to come (Issaq and Veenstra, 2008).

1.2.4 Mass Spectrometry

Mass spectrometry (MS) allows the measurement of the mass of small molecules to a high degree of accuracy and in the case of proteomics is employed to help identify proteins and peptides (Eckersall, 2008). More specifically mass spectrometry precisely measures the mass-to-charge (*m/z)* ratio of ions in the gas phase (Han et al., 2008). In order to do this all mass spectrometers must have 3 basic features. These are a means of converting the analyte to gas-phased ions, a mass analyser to separate the ions based on their *m/z* ratio and a detector to quantify the number of ions at each particular *m/z* value (Han et al., 2008).

Recently mass spectrometry has become invaluable for the identification of proteins in proteomics and a significant contribution to facilitate this was the development of protein ionisation methods that led to the award of the Nobel Prize in Chemistry in 2002 (Aebersold et al., 2003). The two main methods to ionise proteins are electrospray ionisation (ESI) which is generally applied to liquid based separation methods such as gels, and matrix-assisted laser desorption/ ionisation (MALDI) which ionises samples out of a dry matrix. ESI-MS is more often used to separate complex mixtures of proteins whereas MALDI-MS is preferred for simpler solutions (Aebersold et al., 2003). As suggested by the name the measurement of mass is key to identification of the analytes in MS experiments and there are four basic types of mass analysers available including the quadrupole (Q), quadrupole ion trap (QIT), time-of-flight (TOF) mass analyser and the Fourier-transform ion cyclotron resonance (FTICR) mass analyser (Han et al., 2008). These analysers each have their own attributes and drawbacks and they can either function alone or can be arranged in tandem (Aebersold et al., 2003). Tandem MS (MS/MS) allows further structural information of substances to be gleaned by performing 2 MS experiments, the first of which is to select for a molecule of a specific *m/z* value. This filtered molecule is then subjected to collisional activation with an inert gas leading to the generation of fragments of this molecule that are then analysed using a second MS experiment (McLafferty, 1980). When MS/MS is applied to the

proteomic field, *de novo* protein sequence data can be acquired (Diamandis, 2004).

High throughput analysis of complex protein samples is usually performed using a "bottom-up" strategy where the proteins are subjected to enzymatic digestion, with trypsin commonly being used for such a purpose, prior to analysis with MS/MS. This "shotgun" approach leads to the generation of large datasets from complex mixtures of proteins (Han et al., 2008). "Top-down" proteomics involves gas ionisation of intact proteins followed by measurement of their respective *m/z* value, before direct fragmentation is performed to allow a 2nd MS experiment to be carried out. Theoretically this technique allows for more precise interrogation of the protein structure and will also account for post-translational modifications however, difficulties have been encountered in high through put systems and the analysis of large proteins (>50kDa) can also be problematic (Han et al., 2008).

Using the data generated from MS experiments the identification of proteins is made possible by comparison of the generated *m/z* values with known protein and gene databases (Eckersall, 2008). Complex statistical algorithms, such as that described by Nesvizhskii and co-workers (2003), can be employed by computer search engines to rapidly generate lists of potential candidate proteins for the peptide sequences identified. Latterly, sequencing of the canine genome performed by Lindblah-Toh et al.(2005) means that even without a full protein database for the domestic dog, novel proteins may be identified from their predicted structure as determined by their genetic code.

1.3 The Serum Proteome of the Human and Other Species

Application of the above proteomic techniques to serum has led to the characterisation of the proteome in man as well as the horse and cow. In humans, plasma is the most complex derived proteome as it contains proteins from many other tissues (Anderson and Anderson, 2002). Moderate and low abundance human serum proteins were identified using an immunoaffinity subtraction procedure in combination with liquid chromatography, to allow the generation of a list of 325 distinct proteins as identified by MS performed on 2D electrophoresis gels from 2 healthy adult males (Pieper et al., 2003). Serum from a healthy cow separated into 47 spots on PAGE and MS allowed the identification of 21 proteins (Wait et al., 2002). Problems were encountered when analysing the bovine serum proteome as the genome was not completely characterised and full separation of serum proteins was difficult due to the high relative concentrations of certain serum proteins such as albumin (Wait et al., 2002). Similarly in the horse, 2D electrophoresis was performed on healthy sera from a stallion and several mares and 25 protein spots or spot groups were identified using mass spectrometry, generating a list of 29 different gene products. Interestingly proteins such as albumin and apoliprotein-A1 were present in multiple different spots inferring complete separation from other proteins is not possible or that these proteins may fragment into smaller subsets of peptide chains (Miller et al., 2004). One dimensional electrophoresis in combination with tandem MS was performed in a population of healthy cats and compared to a group of cats with lymphoma. In the cats with lymphoma an increase in the ß-globulin group was apparent however, healthy cats had higher albumin concentrations (Gerou-Ferriani et al., 2011). Further preliminary information on the components of the various fractions was provided using mass spectrometry although the lack of further separation using a second dimensional electrophoresis technique limited the amount of data gained regarding the feline proteome from this study.

There is no published work which aims to map and identify the different constituents of the canine serum proteome using a combination of gel-based

electrophoresis techniques and mass spectrometry in healthy dogs. One dimensional high resolution electrophoresis in combination with immunofixation (utilising specific goat anti-dog antibodies when available, to detect canine complement C3 and transferrin for example) and substrate specific staining (such as Sudan black staining for detection of lipoprotein) has been performed. This method allowed identification of albumin in the albumin band. α1 lipoprotein was found as a vaguely defined band in the α1 region; haptoglobin and α2 macroglobulin were identified in the α2 band. Complement C3 and transferrin were localised to the ß bands with only IgG being found in the γ band (Abate et al., 2000). When two dimensional gel electrophoresis was performed on serum from healthy dogs before and after enforced weight loss, a consistent significant difference in three of the protein spots was identified in the dogs' serum after losing weight. Serum retinol-binding protein 4 and clusterin precursor were both upregulated following weight loss but α1-antitrypsin was down regulated (Tvarijonaviciute et al., 2012). Although this last study used a combination of 2D-electrophoresis and mass spectrometry to identify proteins only three proteins were identified. Little definitive information is known about the normal or diseased canine serum proteome and the interpretation of alterations in the electrophoretogram relies on the assumption that canine proteins will migrate in a similar manner to human proteins (Stockham and Scott, 2008).

1.4 Causes and Consequences of Dysregulated Serum Proteins

In states of disease alterations of plasma proteins may be directly responsible for clinical signs and can be seen secondary to both insufficient and excessive circulating protein concentrations. Factor VII is a vitamin K dependent clotting factor required for normal coagulation in dogs (Macpherson et al., 1999). Absence of factor VII is a good example of how decreased protein concentrations can lead to manifestations of disease. In this particular instance deficiency can be recognised through prolonged pro-thrombin (PT) bleeding time and can

manifest clinically as a coagulopathy (Macpherson et al., 1999). Factor VII deficiency is thought to be a hereditary defect and can be recognised in certain breeding colonies such as the Beagles reported by Mustard et al. (1962). Whilst decreased serum proteins can lead to problems the reverse is also true. Hyperviscosity syndrome can result from excess serum proteins amongst other causes and may manifest both as bleeding tendencies such as spontaneous epistaxis or alternatively neurological dysfunction secondary to cerebrovascular accidents due to resultant hypertension or thrombo-embolic disease (Rylander, 2010). The presentation of dogs with multiple myeloma with signs of hyperviscosity secondary to a monoclonal gammopathy has previously been reported (Finnie and Wilks, 1982). A review of eighteen dogs with monoclonal gammopathies indicated these dogs frequently presented for bleeding diatheses considered secondary to hyperviscosity and coagulopathy due to thrombocytopathia (Giraudel et al., 2002). The most common cause of canine monoclonal gammopathy in this case series was multiple myeloma but other lymphoid neoplasms alongside non-neoplastic diseases such as leishmaniasis and ehrlichiosis can also be responsible for monoclonal gammopathies (Giraudel et al., 2002). Biclonal gammopathies secondary to multiple myeloma have also been reported but occur less frequently than monoclonal gammopathies (Ramaiah et al., 2002) (Fig. 2).

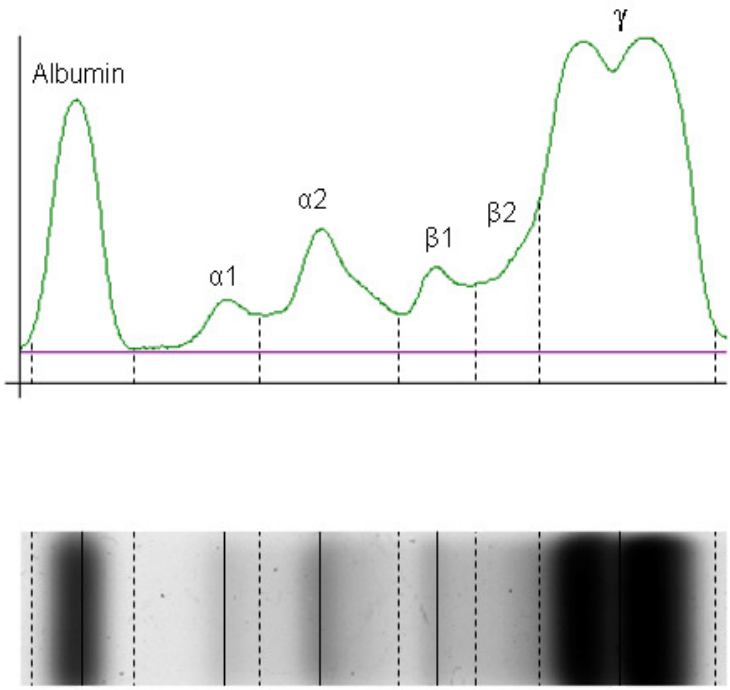


Figure 2 An electrophoretogram from an 11 year old female entire cross bred dog diagnosed with multiple myeloma at the Small Animal Hospital, University of Glasgow. Note the biclonal peaks in the γ fraction. The top panel displays the electrophoretogram which is a graphical densitometric representation of the scanned gel lane seen in the corresponding bottom panel.

More subtle changes within the serum proteome may occur secondary to various diseased states and in such cases systemic signs of illness may not always be a direct sequel to the alterations. Nevertheless alterations in individual protein concentrations and electrophoretograms can give clinicians valuable insight towards the diagnosis of various different diseases. In people it has long been known that the production of various plasma proteins may be induced or inhibited by trauma (Neuhaus et al., 1966) forming part of a so called acute phase response. Changes in the canine serum protein electrophoretogram can be caused by many different disease classes including renal disease, hepatic disease, nutritional disease, gastrointestinal disease and infectious disease amongst others (Eckersall, 2008). In a review of electrophoretograms from 147 sick dogs 140 of these had traces outside of the group's normal reference range however, this population was likely biased as the clinicians chose to perform SPE's as they believed there would be a dysproteinaemia present (Tappin et al., 2011). In the same study the most common documented cause of altered serum protein concentrations was inflammatory or infectious disease. Eight monoclonal gammopathies were also recorded and as in previous work these were most frequently encountered in myeloma patients (Tappin et al., 2011).

1.5 Acute Phase Reactants

The acute phase response is a series of specific physiological reactions in response to tissue damage during which a number of proteins known as the acute phase proteins (APPs) will have altered serum concentrations (Baumann and Gauldie, 1994). Increased liver production of certain α-globulins in response to trauma was documented in humans many years ago (Neuhaus et al., 1966). Although many proteins are increased in the acute phase, the so called negative acute phase proteins circulate in decreased concentrations. In people such proteins include albumin, transferrin (Tf) and transthyretin (Ritchie et al., 1999) and an analogous situation exists for many domestic animals (Eckersall, 2008). Acute phase proteins have been used as biomarkers in human disease for many

decades but their utility in veterinary diagnostics is less well established. Many recent studies however, have looked at their response to various different conditions of disease in multiple domestic species (Eckersall and Bell, 2010).

The acute phase proteins are highly sensitive but often poorly specific markers of disease and the conditions that induce them and the magnitude in which their concentrations alter are highly dependent on the species (Eckersall, 2008). Positive acute phase proteins are classified either as major, moderate or minor responders on the basis of the magnitude of elevation in the face of a stimulating process, with the major protein concentrations increasing up to 1000 fold under appropriate conditions (Eckersall, 2008). In the dog α1 acid glycoprotein (AGP), ceruloplasmin (Cp), C-reactive protein (CRP), fibrinogen (Fb), haptoglobin (Hp) and serum amyloid-A (SAA) are all positive acute phase proteins with CRP being a major reactor and the remainder being moderate reactors (Murata et al., 2004). In people induction of the acute phase proteins is secondary to the action of pro-inflammatory cytokines such as IL-1, IL-6 and TNF manufactured by activated macrophages, in the liver. This results in increased transcription of the acute phase genes as part of an orchestrated response to tissue injury, infection or inflammation (Moshage, 1997). Following local muscle inflammation in dogs increased levels of IL-6 precede increased concentrations of CRP and AGP as well as decreases in serum albumin with normalisation of the protein concentrations 14 days after the initial insult (Yamashita et al., 1994). CRP which is the major acute phase protein in the dog, is able to bind to pathogenic antigens activating the classical complement pathway required for opsonisation. CRP is also able to facilitate phagocytosis of various different pathogens and can have other immunomodulatory roles (Eckersall, 2008; Murata et al., 2004). The other positive canine acute phase reactants listed above also play various roles in host defence, being important elements of the non-specific innate immune response, as well as helping limit host tissue injury during inflammatory processes (Murata et al., 2004). A group of reactants known as the protease inhibitors (PIs), which include α-antichymotrypsin and α2 macroglobulin, limit damage to the host organism when proteases are released as part of the inflammatory response (Murata et al., 2004).

In dogs various different diseases lead to the induction of acute phase proteins and these include tissue trauma secondary to surgery (Conner et al., 1988), leishmaniasis (Martínez-Subiela et al., 2002a) and leptospirosis (Caspi et al., 1987) amongst others. Acute phase protein concentrations also correlate with the severity of some disease conditions such as lymphoid neoplasia. The greatest increases in haptoglobin and CRP concentrations in dogs were observed with acute lymphoblastic lymphoma, followed by malignant lymphoma then chronic lymphocytic leukaemia, multiple myeloma and finally the healthy control set of dogs (Mischke et al., 2007). In canine steroid responsive meningitis the acute phase proteins AGP, CRP, haptoglobin and SAA were useful in the diagnosis of the condition but also, with the exception of haptoglobin, provided further information regarding remission status of this disease and therefore proved valuable in the management of this condition with prednisolone (Lowrie et al., 2009). Acute phase proteins tend to change their concentration secondary to pathologic processes however, they can also be induced iatrogenically in certain situations. Haptoglobin concentrations are increased by the exogenous administration of corticosteroids (Martínez-Subiela et al., 2004) and a similar increase can also be seen in naturally occurring corticosteroid excess in dogs diagnosed with hyperadrenocorticism (McGrotty et al., 2005). In recent years much more knowledge has been gained regarding the role of acute phase proteins in the diagnosis, prognosis and management of various canine disease processes but it is thought that this area is still in its infancy with much more scope to increase the application of such changes in veterinary medicine (Eckersall and Bell, 2010).

Table 1 The most common acute phase proteins in domestic animals (adapted from Murata et al., 2004)[1].

APP	Chicken	Dog	Horse	Pig	Ruminant
AGP	Moderate	Moderate	Minor-moderate	Moderate	Moderate
Cp	Moderate	Moderate	Minor	Minor	Minor
CRP	Unknown	Major	Moderate	Moderate	Moderate
Fb	Minor	Moderate	Minor-moderate	Minor-moderate	Moderate
Hp	Unknown	Moderate	Moderate	Moderate	Major
PIs	Unknown	Unknown	Minor	Unknown	Minor-moderate
SAA	Moderate	Moderate	Major	Moderate	Moderate-major
Tf	Moderate	Unknown	Minor negative	Minor negative	Minor negative

[1] APP- acute phase protein, AGP- α1 acid glycoprotein, Cp- ceruloplasmin, CRP- C-reactive protein, Fb- fibrinogen, Hp- haptoglobin, PIs- protease inhibitors, SAA- serum amyloid-A, Tf- transferrin. Major, moderate and minor reflect the magnitude of protein concentration elevation in response to inflammatory stimulation.

1.6 Prevalence and Presentation of Canine Lymphoma

Lymphoma is the most common canine haematopoietic neoplasm accounting for 83% of all haematopoietic tumours in this species (Moulton and Harvey, 1990) and representing between 7% and 24% of all canine cancer cases (Vail and Young, 2007). Canine lymphoma is relatively common in the UK with a recently reported incidence rate of 114 per 100,000 dogs at risk per year (Dobson et al., 2002). In the UK the peak occurrence of lymphoma is at around 10 years of age with boxers, bulldogs and bull mastiffs all over represented (Edwards et al., 2003). Basset hounds, Saint Bernards, Scottish and Airedale terriers are also predisposed to this disease (Vail and Young, 2007). Both male and female dogs can be affected by this disease process with no sex predilection recorded (Vail and MacEwen, 2000).

At a cellular level lymphoid tumours arise from cells of lymphoreticular origin, commonly found within the lymph nodes but also present in the bone marrow and thymus where they are produced. The spleen also contains large amounts of lymphoid tissue in health and disease. Lymphocytes and hence lymphoid neoplasia can be found in almost any organ system within the body and despite their uniform appearance there are many diverse sub-populations with varying functions vital to both the innate and acquired immune system under conditions of health (Tizzard, 2004).

Lymphoma may be classified on the basis of anatomic location as well as histologic, cytologic and immunophenotypic characteristics. In descending order the most common sites for presentation of lymphoma in the dog are multicentric, craniomediastinal, gastrointestinal and cutaneous (Vail and Young, 2007) however multiple other extranodal sites have also been documented and these can either exist as an extension from a multicentric form or may be primary neoplasms. Peripheral and superficial lymphadenopathy is present in 80% of dogs diagnosed with lymphoma and this is usually painless (Theilen and Madewell, 1987).

Dogs diagnosed with lymphoma are more commonly asymptomatic but up to 44% of affected dogs may present with clinical signs including lethargy, pyrexia, weight loss and inappetance (Keller et al., 1993). The paraneoplastic syndrome of hypercalcaemia may be present in between 10% and 40% of dogs suffering from lymphoma (Vail and Young, 2007) and a strong correlation exists with hypercalcaemia and the T-cell immunophenotype (Greenlee et al., 1990). In a series of 37 hypercalcaemic dogs diagnosed with lymphoma 41% had craniomedistinal involvement (Rosenberg et al., 1991) which is concordant with the majority of dogs suffering from mediastinal involvement having a T-cell immunophenotype (Lana et al., 2006). As well as hypercalcaemia dogs with the T-cell phenotype are more likely to display other clinical signs at presentation as demonstrated by a study analysing 46 dogs with T-cell lymphoma (Fournel-Fleury et al., 2002). Signs at presentation may directly result from specific organ involvement such as cranial nerve deficits in dogs suffering with primary CNS lymphoma (Long et al., 2001). Ocular involvement is also recognised with canine lymphoma and in a retrospective study of 102 dogs presented for investigation of uveitis 17 animals were diagnosed with lymphoma (Massa et al., 2002). Dogs may also present for other paraneoplastic disorders such as peripheral neuropathies (Presthus and Teige, 1986), cancer cachexia (Vail et al., 1990) and hyperviscosity secondary to monoclonal gammopathies (Gentilini et al., 2005).

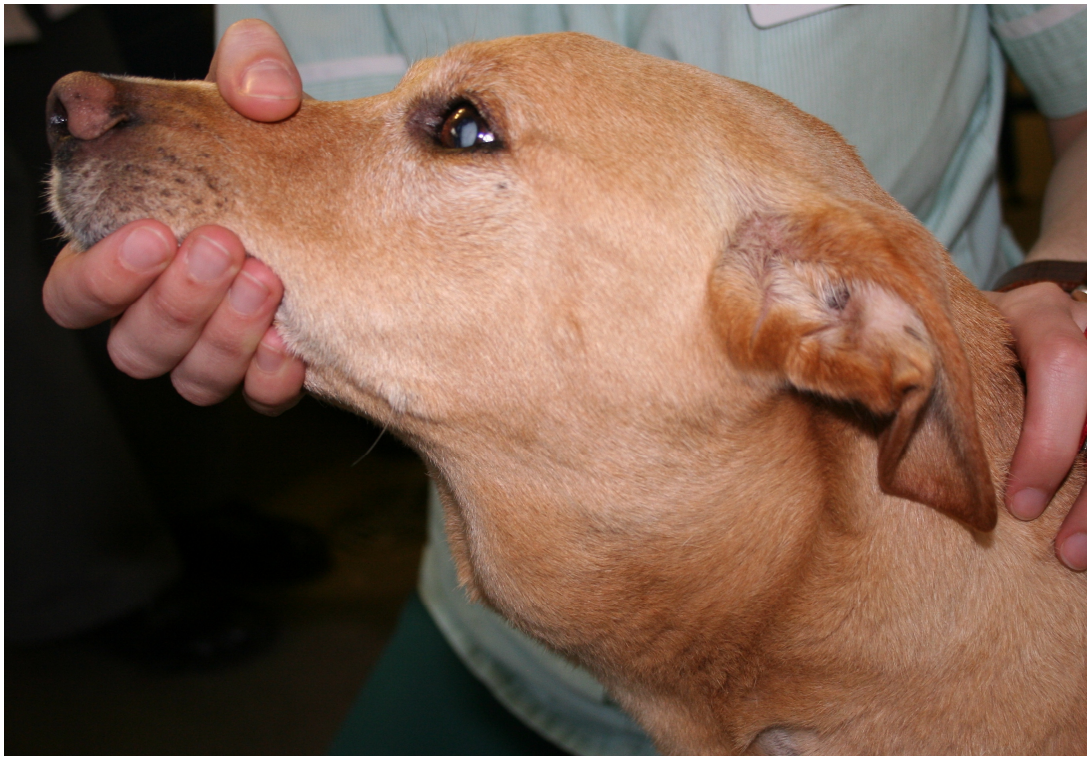


Figure 3 Submandibular lymphadenopathy present in an eight year old male neutered cross bred dog diagnosed with stage IVa B-cell lymphoma at the Small Animal Hospital, University of Glasgow.

1.7 Clinical Evaluation of the Canine Lymphoma Patient

1.7.1Diagnosis and Staging

Lymphoma may be diagnosed clinically both cytologically and histologically. Cytologic diagnosis requires the acquisition of samples using fine needle aspirates and is easily performed on peripheral lymph nodes, with ultrasound guidance facilitating aspiration from intra-abdominal lymph nodes, viscera and in some scenarios intra-thoracic structures (Vail and Young, 2007). Cytology is rapid and convenient as well as being minimally invasive in comparison to lymph node biopsy or extirpation. Overall cytologic assessment of canine lymph nodes affected by lymphoma correlates well with histologic findings (Teske and Van Heerde, 1996) however, cytology does not permit assessment of architecture of the lymph node nor breech of the lymph node capsule and in cases in which the disease is caused by a proliferation of lymphocytes with a mature appearance histology of the node is more informative. Whereas cytology is accurate in the diagnosis of high-grade lymphomas there is a risk that certain lower grade lymphomas such as follicular B-cell lymphoma and some indolent T-cell lymphomas may well be overlooked without histopathology (Fournel-Fleury et al., 1997).

Staging of canine lymphoma is the process by which the clinical extent of the disease is defined after the establishment of a diagnosis. In order to stage a patient, a clinical history and full physical examination are prerequisite alongside a variety of other investigations performed routinely in clinics, including thoracic radiographs, abdominal ultrasound, image guided aspirates and procurement of bone marrow aspirates assessed in context of haematological anomalies (Vail and Young, 2007). Other advanced diagnostic imaging modalities such as MRI (magnetic resonance imaging) (Palus et al., 2012) and CT (computed tomography) (Yoon et al., 2004) have assisted staging

procedures for intracranial and mediastinal lymphoid neoplasia. More recent approaches to staging address the fusion of anatomic imaging with functional imaging using PET (positron emission tomography) and this has facilitated whole body staging in a canine patient suffering from lymphoma (Leblanc et al., 2009). In human medicine PET combined with CT provides accurate staging information for aggressive forms of lymphoma and is considered standard of care in many institutes. PET-CT may also be useful in monitoring response to treatment and when used correctly can positively influence clinical outcome (Hutchings and Barrington, 2009).

The information accrued from the patient's history, clinical examination and diagnostic imaging modalities can be applied to the WHO staging scheme to assign a patient with a defined stage of disease (Owen, 1980) (Table 2). This universally applied staging system takes into account the anatomic location alongside a 5 tier system which reflects the extent of the disease; dogs are further substaged to either *a* without systemic signs or *b* with systemic signs.

Table 2 A five tier staging system for canine multicentric lymphoma (adapted from Owen, 1980).

Stage	**Organ System**
I	Single lymph node or lymphoid tissue in one organ
II	Multiple lymph nodes in a single region
III	Generalised lymph node involvement (multicentric)
IV	Hepatic and splenic involvement and stage III
V	Manifestation in blood, bone marrow or other organ system and stage III/IV

1.7.2 Haematologic and Biochemical Abnormalities

Anaemia is a frequent finding in dogs with lymphoma and is the most common haematologic abnormality identified in canine patients. Approximately 30%- 50% of patients are anaemic at diagnosis and most commonly the anaemia is non-regenerative being normocytic and normochromic (Gavazza et al., 2009; Miller et al., 2009). In one study anaemia was found to be a negative predictor for patient survival in canine lymphoma when compared to patients with normal red cell indices. In this same study anaemia was more prevalent in patients suffering from lymphoid neoplasia in comparison to a control population of dogs diagnosed with osteosarcoma (Miller et al., 2009). In people, cancer related anaemia is also a known phenomenon and treatment of such anaemia in conjunction with the neoplastic disorder can translate to improved clinical outcomes (Birgegard et al., 2005). The reasons for such anaemia is thought to be due to complex interactions between tumour cells and the patient's homeostatic control of erythrocyte manufacture and metabolism mediated via inflammatory cytokines (Birgegard et al., 2005). Other investigators have documented the presence of anaemia inducing factors within the plasma of patients suffering from advanced malignancies (Honda et al., 1995). In a retrospective study incorporating clinicopathologic abnormalities of canine lymphoma patients, anaemia was found to be the most common haematologic disturbance but leukocytosis was observed in 32% of cases with half of these being due to a neutrophilia, monocytosis, eosinopenia and lymphopenia with these changes being attributed to a chronic inflammatory state (Gavazza et al., 2009). The same study documented circulating blasts in 18% of patients with diminished circulating white cells in 8% of patients and diminished platelet counts in 26% of patients. Alterations to circulating cell lines is an indication for assessment of bone marrow function via aspiration or biopsy if not performed as part of routine clinical staging, however absence of abnormalities to haematologic profiles does not exclude a leukaemic process. In a series of 53 dogs diagnosed with lymphoid neoplasia 30 dogs in total were shown to have bone marrow infiltration but only 15 of these had circulating abnormalities (Raskin and Krehbiel, 1989). Thrombocytopenia is also documented in dogs with multicentric lymphoma and in a retrospective analysis of over 2000 dogs diagnosed with cancer, 10% were

thrombocytopenic, with the most common neoplastic histotype being lymphoma (Grindem et al., 1994). Causes for thrombocytopenia in cancer patients included myelophthisis, immune mediated destruction secondary to an underlying neoplastic trigger, platelet sequestration and consumption.

Biochemical abnormalities are frequently seen with canine lymphoma and in certain cases will reflect disturbances to the organ systems infiltrated by the disease. As already mentioned hypercalcaemia, as a paraneoplastic syndrome, may be seen in 10%- 40% of newly diagnosed cases of canine lymphoma (Vail and Young, 2007). Azotaemia may occur secondary to hypercalcaemia but marked elevations in serum urea and creatinine have also been documented secondary to direct renal infiltrate (Batchelor et al., 2006). Presumed primary hepatic lymphoma will frequently lead to elevations of the liver transaminases as well as hyperbilirubinaemia and hypoalbuminaemia with the latter being associated with a poor prognosis in this subset of lymphoma (Dank et al., 2011).

Hypoproteinaemia primarily due to hypoalbuminaemia can occur in around three quarters of dogs with primary gastrointestinal lymphoma and around 30% of these dogs may also suffer from elevations of their liver enzymes which may be secondary to hepatic infiltrate or prior treatment with corticosteroids (Couto et al., 1989). Hyperproteinaemia secondary to monoclonal gammopathies is most commonly caused by lymphoproliferative disease in dogs. It is sometimes found in lymphoma patients but is more frequently seen in multiple myeloma, a neoplastic expansion of plasma cells (Giraudel et al., 2002). In a survey of 120 dogs diagnosed with lymphoma including 114 dogs with multicentric disease, over 60% of the patients had biochemical abnormalities including alterations to hepatic markers as well as azotaemia and abnormal serum protein concentrations (Gavazza et al., 2009). In this same study, protein electrophoresis was performed on the sera of 52 of the patients with 28 of these cases having increased ß-globulin concentrations most likely secondary to chronic inflammation. Interestingly 15% of patients were seropositive for ehrlichiosis and leishmaniasis, organisms which are known to alter the serum electrophoretogram.

1.7.3 Cytologic, Histopathologic and Immunophenotypic Characterisation

Classification schemes for canine lymphoma were originally based on morphologically equivalent forms of human non-Hodgkin lymphoma by histologic or cytologic examination of specimens using light microscopy (Valli et al., 2011). One of the earliest methods for classifying lymphoma used cell size and differentiation (devised by Henry Rappaport (1966)) in the days prior to the division of lymphocytes into the B and T-cell lineages. Further histologic classification required information regarding immunophenotypic characterisation alongside morphologic attributes and two systems were developed in the 1970's with the Lukes-Collins system (Lukes and Collins, 1974) being adopted in North America and the Kiel system being applied in Europe (Lennert et al., 1975). A further major classification was developed in North America in the 1980's known as the working formulation ("National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas" 1982) whilst the Kiel system was revised but continued to be applied to lymphoma throughout Europe. The revised European-American classification of lymphoid neoplasms (REAL) (Harris et al., 1994) sought to unify the differences across the Atlantic between pathologists and clinicians and relieve frustrations regarding variations between the identification of the various forms of lymphoma. Most recently the REAL system was approved and subsequently adopted by the WHO making all other systems of classification in human histology obsolete (Harris et al., 1999).

Many of the human schemes including the Rappaport, Luke-Collins, Kiel, working formulation and REAL have been assessed and used in multiple studies on canine lymphoma (Carter et al., 1986; Greenlee et al., 1990; Ponce et al., 2010). The WHO system has also been applied to dogs with good concordance between pathologists including those who were not deemed to be specialists in the subfield of haematopathology (Valli et al., 2011). Using a combination of immunophenotyping and histologic examination of hematoxylin and eosin stained sections an accuracy of around 85% was obtained between 20 pathologists. Eighty percent of canine lymphomas in this study were made up of 6 types of

lymphoid disease out of a total of 30 different entities. These 6 included diffuse large B-cell lymphoma, marginal zone lymphoma, peripheral T-cell lymphomas not otherwise specified, T-zone lymphoma, T-cell lymphoblastic lymphoma and a subset being reclassified as diseases other than lymphoma (these included pathologies such as follicular hyperplasia). This consensus group believe that the WHO system should currently be applied to the diagnosis of canine lymphoma allowing further information regarding the prognoses of the subtypes defined by it to be gleaned in the future. Preliminary evaluation of some data showed that dogs with indolent/ low grade lymphoma have normal appetite and activity even at advanced stages of disease (Valli et al., 2011) and ongoing evaluation in future work is hoped to give more prognostic information.

Cytological assessment of specimens acquired via fine needle aspiration allows rapid diagnosis of lymphoma and a good correlation between histologic and cytologic classification when using the modified-Kiel system (Teske and Van Heerde, 1996), however there is a risk of not detecting some of the indolent lymphomas as cytology is unable to assess tissue architecture (Marconato, 2011). When the modified-Kiel system is applied to cytologic interpretation of aspirates from dogs with lymphoma a high or a low grade is able to be assigned with the majority (73.9%) of patients falling into the former category (Fournel-Fleury et al., 1997). As with histologic assessment the modified-Kiel system for cytology relies on the presence of immunophenotyping for full classification (Fournel-Fleury et al., 1997) and is able to assign the neoplastic process into 1 of over 20 separate categories.

There are many ways to determine the immunophenotype of canine patients with lymphoma including immunohistochemistry (IHC), immunocytochemistry (ICC), flow cytometry (FC) and clonality assays (Vail and Young, 2007). Certain morphologic features may be of use for determining the phenotype (i.e. T vs B-cell) in lymphoma however these can be misleading as seen in a case series of dogs diagnosed with T-cell lymphoma despite having a plasmacytoid appearance more typical of B-cell neoplasia (Ponce et al., 2003). Monoclonal antibodies raised against the CD 3 and CD 79a cell surface markers (cluster differentiation

molecules) and have been used in immunohistochemical staining techniques to differentiate T-cell from B-cell lymphoma respectively for over 15 years (Milner et al., 1996). These markers have also been described for immunocytochemistry as have the additional molecules of CD 3, CD 4, CD 8 and CD 21 amongst others (Caniatti et al., 1996). Even when using extended panels of markers however, it may not be possible to classify certain neoplasms as T or B-cell lineage and a separate null cell entity which is negative for both T and B-cell CD markers has been documented (Ruslander et al., 1997).

Flow cytometry offers an alternate way to determine CD marker status from neoplastic lymphocytes and is considered the gold standard for immunophenotyping people with haematopoietic malignancies (Rothe et al., 1996). Flow cytometry has been applied in conjunction with cytologic analysis to define T or B-cell status of canine lymphoma (Sözmen et al., 2005). It can also be used to further separate groups of lymphoma patients based on co-expression of CD markers for example CD 3+, CD 4+,CD 8- T-cell lymphomas, as well as finding co-expression of T and B-cell markers by some cell lines for example CD 3+,CD 79a+ lymphoma (Wilkerson et al., 2005). Decreases in certain CD markers such as CD-45 and CD-18 can also be assessed using flow cytometry and these may add further weight to the diagnosis of lymphoid neoplasia (Comazzi et al., 2006). Finally, assays of clonality based on utilising PCR (polymerase chain reaction) may be used to aid in the diagnosis of haematolymphoid neoplasms in dogs as clonality is one of the fundamental characteristics of neoplasia (Vail and Young, 2007). Clonal re-arrangements of the T-cell receptor gene (TCR) for T cell neoplasms and the immunoglobulin (Ig) variable gene for B-cell tumours have both been described for dogs (Burnett et al., 2003). Another benefit of PCR is the high diagnostic sensitivity as PCR is able to identify small neoplastic populations undetectable by histology and flow cytometry, as detected by the presence of minimal residual disease in dogs with multicentric lymphoma that were clinically in complete remission after being treated with multi-agent chemotherapy (Yamazaki et al., 2008).

Using the techniques described above we are now able to classify canine lymphomas accurately into one of many subgroups of this disease. Although currently not possible, the ultimate aim of much of the further research performed into canine lymphoma will be to tailor treatment protocols for specific subsets of lymphoma based on morphologic and phenotypic data alongside other information derived from cytogenetic and proteomic studies (Marconato, 2011).

Table 3 Immunophenotypic markers used to discern canine T-cell from B-cell lymphoma[2].

Method	T-cell marker	B-cell marker	Reference
IHC	CD 3	CD 79a	Milner et al., 1996
ICC	CD 3, CD 4 and CD 8	CD 21	Caniatti et al., 1996
FC	CD 3, CD 4 and CD 8	CD 21 and CD 79a	Sözmen et al., 2005
FC	CD 3, CD 4 and CD 8	CD 21 and IgM	Wilkerson et al., 2005
FC	CD 3	CD 21 and CD 79a	Comazzi et al., 2006
PCR	TCR rearrangement	Ig rearrangement	Burnett et al., 2003
PCR	TCR rearrangement	Ig rearrangement	Yamazaki et al., 2008

[2] CD- cluster of differentiation, IHC- immunohistochemistry, ICC- immunocytochemistry, FC- flow cytometry, PCR- polymerase chain reaction, TCR- T-cell receptor, Ig- immunoglobulin.

1.7.4 Prognostic Indicators

Multiple factors that are gained from staging, morphologic and phenotypic characterisation as well as more specialised testing confer prognostic information on dogs diagnosed with lymphoma (Vail and Young, 2007). Without treatment most dogs will die of their disease within 4-6 weeks, and systemic combination chemotherapy is therefore the treatment of choice for most cases of canine lymphoma (Vail and Young, 2007).The 6 month CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) protocol is used by many oncologists to treat multicentric lymphoma with remission rates expected to be around 90% and median survival times around 1 year (Garrett et al., 2002).

Certain elements of the staging process give further information regarding prognosis. The WHO stage is prognostic in some studies (Teske et al., 1994) with stage I carrying the best prognosis and stage V the worst (Jagielski et al., 2002) but this information is not always reproducible. Substage is a much more consistent prognostic factor in canine lymphoma with multiple studies documenting decreased survival times in animals with systemic signs (i.e. substage *b*) (Greenlee et al., 1990; Jagielski et al., 2002; Keller et al., 1993). Anatomic site also affects prognosis with poorer outcomes expected in diseases such as primary hepatic lymphoma (Dank et al., 2011) and gastrointestinal lymphoma whether as a primary entity or part of a multicentric disease (Rassnick et al., 2009).

The value of histologic grade in predicting outcome for multicentric lymphoma is currently questionable as in certain studies, higher grade forms of the disease are associated with improved outcomes in terms of response to chemotherapy and overall survival times (Greenlee et al., 1990; Teske et al., 1994). This may in part be due to ambiguity or inaccuracy of classification relating to older histologic schemes as a recent paper reviewing survival data from 75 dogs all affected by indolent lymphoma and with the majority (69 dogs) presenting with multicentric disease found the overall median survival time to be 22.1 months (Flood-Knapik et al., 2012). This study used the WHO grading scheme and it is likely as more pathologists apply this system to the grading of lymphoma further

information regarding survival times for specific subtypes will become available. The role of immunophenotype in prognostication for canine lymphoma is more clear cut with multiple studies showing a tendency for high grade T-cell lymphoma to have a poorer prognosis in comparison with the B-cell phenotype (Dobson et al., 2001; Greenlee et al., 1990; Ruslander et al., 1997). It should be noted however, that small cell variants of the T-cell phenotype can carry a relatively good prognosis and some B-cell variants (such as Burkitt-like lymphoma) can be associated with poor outcomes (Ponce et al., 2004) therefore immunophenotype needs to be interpreted in light of other histologic and clinical findings. Further classification of canine lymphomas using flow cytometry has already found new prognostic indicators such as down regulation of MHCII (class II major histocompatibility complex) in B-cell lymphoma predicting shorter survival times (Rao et al., 2011). In certain phenotypes of lymphoid neoplasia with circulating lymphocytosis the presence of CD 34+ cells, cell size and absolute lymphocyte number as determined flow cytometrically have also been associated with patient outcome (Williams et al., 2008).

In a group of dogs treated with multi-agent chemotherapy suffering from non-Hodgkin's lymphoma (Modiano et al., 2007) inactivation of retinoblastoma (a tumour suppressor gene) was associated with a poor prognosis. A median survival time of 4 months was reported in dogs with inactivation of the gene compared to 24 months in dogs with normal retinoblastoma function. Loss of the RB1 locus leading to decreased levels of retinoblastoma has been documented in dogs with chronic lymphocytic leukaemia (Breen and Modiano, 2008). Levels of expression of the proto-ongogene gene BCL6 vary in some forms of diffuse B-cell lymphoma in people, with high expression correlated to longer overall survival times when these patients are treated with anthracycline based chemotherapy (Lossos et al., 2001). Whilst it is also possible to separate canine patients into those expressing high and low levels of BCL6 there is no survival advantage between the 2 groups suggesting that although there may be genetic similarities between the diseases, response to therapy in light of such changes is not always analogous (Sato et al., 2012). Higher levels of survivin (a protein known to be an inhibitor of apoptosis) within neoplastic tissue of people suffering from diffuse large B-cell lymphoma is considered an unfavourable prognostic factor (Adida et al., 2000). Similarly in dogs increased survivin expression detected by

immunohistochemistry of lymph node biopsies predicted earlier treatment failure in comparison to those nodes with low immunoreactivity scores (Rebhun et al., 2008). Amongst many other factors hypothesized to relate to prognosis two studies have found a role for inflammation worsening outcomes in canine lymphoma. In a retrospective study of 120 dogs with lymphoma chronic inflammation documented haematologically and by use of serum protein electrophoresis was associated with shorter remission and survival times (Gavazza et al., 2009). Another study found the presence of pre-existing diseases such as otitis externa and atopy, characterised by inflammation to the affected organ (in these examples the ear and skin), to increase the risk of disease relapse by a factor of 3.23 at any time point (Baskin et al., 2000).

1.8 Current Biomarkers in Canine Lymphoma

Biomarkers are defined as cellular indicators of the physiological state, and also of change during a disease process (Srinivas et al., 2001). Biomarkers are of interest to oncology patients in general as they may give information pertinent to diagnosis, staging, prognosis and monitoring of remission status; all of these traits are desirable in biomarkers for dogs diagnosed with lymphoma (Henry, 2010).

Only one group has investigated tissue (as opposed to serum) in the search for biomarkers in canine lymphoma patients. When lymph nodes from healthy dogs were compared to lymph nodes from dogs with B-cell lymphoma, using PAGE and MALDI/ TOF MS, 3 proteins (prolidase, triosephosphatase isomerise and glutathione S-transferase) were down regulated in dogs with lymphoma whilst macrophage-capping protein was up regulated showing that these proteins may be of use as diagnostic markers (McCaw et al., 2007).

Two recent studies used combinations of serum fractionation via chromatographic techniques followed by MS to try and differentiate the sera of dogs diagnosed with lymphoma from healthy controls. Using healthy dogs' sera as reference the first study generated a classification tree composed of a group of biomarkers which when used simultaneously gave a sensitivity of 97% and

specificity of 91% for diagnosing B-cell lymphoma (Gaines et al., 2007). The second study, developed a classification and regression tree (CART) algorithm with a sensitivity of 75%, a specificity of 91%, a positive predictive value of 80% and a negative predictive value of 88% when analysing a combination of 2 particular biomarkers to differentiate lymphoma sera to that from healthy dogs (Ratcliffe et al., 2009) . Both of these studies revealed the respective *m/z* values of the biomarkers in question but neither published the identification of these proteins. As well as assessing the presence or absence of distinct proteins, MS if used in combination with specific proteolysis, peptide labelling and fractionation techniques can also characterise certain post translational protein modifications such as glycosylation of proteins. Fucosylation (a specific type of glycosylation) of proteins is increased in the sera of dogs with B-cell lymphoma, as well as transitional cell carcinoma of the urinary bladder and analysis of their respective serum peptide profiles allowed discrimination of the cancer patients from healthy animals as well as separating the two types of malignancy (Wilson et al., 2008).

Several investigations have been performed to define the role of acute phase proteins as possible biomarkers for canine lymphoma. A preliminary study evaluating possible changes of APPs in dogs with various neoplasms and other inflammatory diseases concluded that elevations of haptoglobin, ceruloplasmin, serum amyloid A and C-reactive protein could be caused by these pathologies and that a decrease in the concentrations of these molecules could be associated with a favourable outcome (Tecles et al., 2005). When CRP and haptoglobin were measured in dogs with lymphoid neoplasia they were significantly elevated relative to healthy patients moreover the degree of elevation appeared to reflect the severity of the disease with the greatest increases seen in dogs diagnosed with acute lymphoid leukaemia followed by multicentric lymphoma then chronic lymphocytic leukaemia and finally multiple myeloma (Mischke et al., 2007). Some of these findings were echoed in a study documenting increased CRP concentrations in dogs diagnosed with multicentric lymphoma however this study also concluded that CRP was not a useful marker in monitoring for relapse of lymphoma and that chemotherapy did not directly cause increases in the concentration of CRP (Merlo et al., 2007). Serum amyloid

A was not of value as a diagnostic or monitoring tool in dogs suffering from lymphoma (Merlo et al., 2008).

Serum thymidine kinase (TK) is an enzyme involved in the salvage pathway of DNA synthesis and therefore its level is correlated to tumour proliferation (Hallek et al., 1992). It is possible to measure TK in the canine sera using a non-radiometric assay (Von Euler et al., 2006) and more recently a fully automated non-radiometric assay has also been developed (Von Euler et al., 2009). Comparison of TK levels in dogs with lymphoma found that such patients had higher TK concentrations relative to both healthy patients and those diagnosed with non-haematologic neoplasms. TK concentrations were also able to help predict survival times as well as relapses of lymphoma proving the worth of TK as a biomarker in malignant canine lymphoma (Von Euler et al., 2004).

1.9 Aims of this Thesis

Many disease processes may lead to changes in the serum proteome of dogs but without precise knowledge of what comprises the proteome of healthy dogs comparisons between health and disease are not possible. At the time of writing most of the data available for normal serum proteins are derived from other species. The main objective of this thesis was to evaluate any possible differences in the proteomes of healthy dogs compared with dogs diagnosed with high grade multicentric lymphoma in the hope of being able to further understand its pathogenesis as well as revealing possible surrogate biomarkers for this disease. This was achieved by numerous minor aims as follows:

1. To define normal reference ranges of the concentrations of albumin and globulins from a population of healthy dogs.

2. To further separate the proteins of a subset of these dogs (selected on their values being closest to the mean or median values of the different protein fractions from the entire population) using a technically simple and reproducible PAGE technique.

3. To identify the major proteins separated using the PAGE technique with MS thus compiling a list of the most abundant serum proteins in the canine serum proteome.

4. To apply this technique to dogs diagnosed with high grade multicentric lymphoma in order to assess the reproducibility of this method and identify possible differences.

5. To measure haptoglobin and CRP concentrations in dogs with high grade multi-centric lymphoma in order to assess the possible inflammatory nature of this disease.

CHAPTER II

MATERIALS AND METHODS

2.1 Case Selection

2.1.1 Selection of Control Patients

To establish reference ranges for albumin and globulin fractions on 1D-SPE and to establish a control population to have its proteome characterised using 2D-PAGE and MS, blood samples were obtained from 60 healthy dogs between March 2005 and July 2009. The 60 dogs were from a group of pet dogs volunteered by their owners to be used as blood donors. Criteria for blood donation included unremarkable physical examination, no known current illness, no medication for 2 weeks prior to venipuncture and were fasted for at least 10 hours. Matching haematology and biochemistry were also performed as part of the screening process for eligibility to the donor program, and dogs with no significant abnormalities on these profiles were identified for further analysis. Serum samples were selected from 17 dogs (numbered 1-17) without significant abnormalities on haematology and biochemistry and comprised 4 flatcoated retrievers, 4 Labrador retrievers, 3 cross-breeds, 2 greyhounds and one each of Boxer, Italian Spinone, British bulldog and Leonberger. There were 10 neutered males, two entire males, one neutered female and four entire females, with a median age of 3 years (range 1-7 years).

Blood for this study was collected at the same time as the haematology and biochemistry samples and placed into plain serum tubes (Sarstedt AG & Co), allowed to clot, then separated by centrifugation (Minispin, Eppendorf) at 12,100 *g* for 3 minutes. The serum samples were stored at -80 °C and were thawed prior to use. The use of these samples was approved by the Ethics and Welfare committee, College of Medical, Veterinary and Life Sciences, University of Glasgow.

2.1.2 Selection and Staging of Lymphoma Patients

Blood samples were obtained from 21 dogs with untreated lymphoid neoplasia between October 2005 and November 2009. Blood was collected, as part of the clinical staging process, into plain serum tubes (Sarstedt AG & Co, Germany) and allowed to clot, then separated by centrifugation (Minispin Eppendorf AG, Germany) at 12,100 *g* for 3 minutes. The serum samples were stored at -80 °C and were thawed prior to use. The use of these samples was approved by the Ethics and Welfare committee, College of Medical, Veterinary and Life Sciences, University of Glasgow.

The lymphoid neoplasia group of dogs were selected from cases referred to the Small Animal Hospital for diagnosis, staging and treatment as appropriate for the disease. The diagnosis was confirmed either cytologically or histopathologically and immunophenotyping was also performed in some cases. Only dogs with cytologically or histologically high grade lymphoma were included and dogs with low grade/ indolent lymphomas were excluded. Dogs had to have multi-centric disease to be eligible for study and only dogs with WHO (Owen, 1980) stage III or higher were included. Animals were excluded if pretreated with any cytotoxic agents or more than seven days of corticosteroids prior to referral or if concurrent illness or hyperproteinaemia were present. All patients underwent full pre-treatment staging including haematologic and biochemical profiles, 3 view thoracic radiographs, abdominal ultrasound, urinalysis and in some cases bone marrow aspiration and or/ biopsy. Further investigative techniques were performed at the discretion of the attending clinician. The serum used in the study was taken at the time of pre-treatment staging with dogs having been fasted for at least 10 hours. Twenty-one dogs met the inclusion criteria. Breeds represented included 8 Labrador Retrievers, 2 Boxers, 2 Weimeraners and 1 of each of the following: German Shepherd Dog, Newfoundland, Springer Spaniel, Golden Retriever, Cocker Spaniel, Doberman, Bull Mastiff, Great Dane and a Cavalier King Charles Spaniel. There were 6 neutered males, 8 entire males, 2 neutered females and 5 entire females with a median age of 6 years (range 2-12).

2.2 Protein Electrophoresis

2.2.1 One Dimensional Agarose Gel Electrophoresis

Prior to electrophoresis total protein was measured with the biuret method using an automated analyser (Olympus AU 640, Olympus) and calibrated using a protein prepared from human serum (Olympus System calibrator 66300, Olympus).

Agarose 1D electrophoresis was carried out using 2 different agarose systems. Initially serum protein electrophoresis on serum of all 17 healthy and all 21 lymphoma samples was performed on an agarose gel system (The Paragon SPE Kit, Beckman Coulter). Subsequently sera from 2 healthy dogs and 3 dogs with lymphoma had SPE on a second type of agarose gel system performed (Hydragel Protein(E) K20, Sebia) since by this time the Paragon gels were no longer available. To minimise the influence of inter-user variation, all the electrophoresis experiments were performed by one person (MA).

For the first gel system, 5 uL of patient serum diluted 1:4 with 1.2% W/V 5,5 diethylbarbituric acid (B-2 Barbital Buffer, Beckman Coulter) was loaded onto preformed agarose gels. A control serum, (Pathonorm H, SERO AS) allowed assessment of protein migration between each gel. Protein electrophoresis was achieved using a constant voltage of 100 V for 25 minutes in 1.2% W/V 5,5 diethylbarbituric acid (B-2 Barbital Buffer, Beckman Coulter). The electrophoresed gels were fixed in acid-alcohol (20% acetic acid and 30% methanol, Fisher Scientific) for 3 minutes before they were dried for 16-30 hours in an incubator. Dry gels were immersed in (Paragon Blue Stain (0.5% w/v solution)Beckman Coulter) for 3 minutes before destaining in 5% acetic acid solution (Fisher Scientific) for 2 minutes followed by 2 minutes in acid alcohol solution and a further 2 minutes in 5% acetic acid solution. The gels were then left to air-dry completely.

Two of the control patients and 3 of the lymphoma had 1D electrophoresis performed again using the Sebia system. Ten uL of serum was loaded onto lane 1 and lanes 3-7 inclusively (Fig. 4a). A total of 5 gels were run and each gel contained the serum from a single individual. Gels were again electrophoresed according to manufacturer's instructions at a constant voltage of 100V in 0.092% W/V barbital, 0.515% W/V sodium barbital and 0.01% sodium azide solution (Tris-Barbital Buffer, Sebia), for 20 minutes then fixed in acid alcohol for 3 minutes before being left to dry for 16-30 hours in an incubator at 37 °C. Once the gels were completely dry, lane 1 was dissected away from the other remaining 5 lanes containing serum and was stained using amido black (Amidoblack, Sebia) for 4 minutes before destaining in acid alcohol solution. Visual inspection of the stained lanes identified the separate albumin, α1, α2, ß1, ß2 and γ fractions. This stained lane was then used as a guide to dissect out each of the corresponding bands from the replicated non-stained 5 lanes of each gel. These lanes were combined in order to amplify protein levels to be run as a second PAGE electrophoresis.

Stained gels from both agarose gel methods were digitally captured using a flatbed scanner (UMAX PowerLock III, UMAK UK Ltd) and saved as grayscale TIF files. Densitometric traces were obtained using computer software (TotalLab Life Science Analysis Essentials, Nonlinear Dynamics). The electrophoretograms were analysed by three people (MA, JSM and PDE) and the visual boundaries of each protein fraction were established by consensus. Using the analysis software relative protein fraction values (%) were obtained and then absolute values could be calculated by multiplying the total protein measure for each sample by the relative protein fraction value.

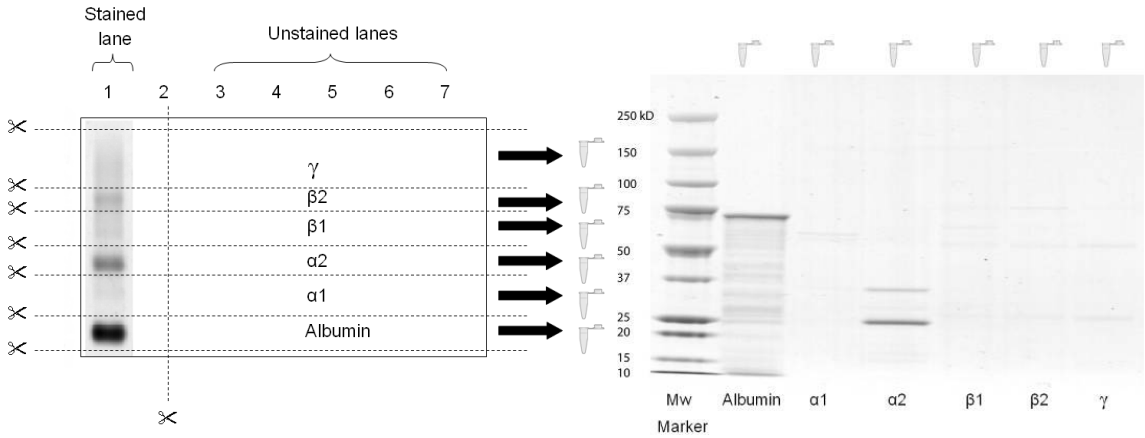
2.2.2 Novel Second Dimension Polyacrylamide Gel Electrophoresis

For polyacrylamide gel electrophoresis (PAGE), protein was extracted from the non-stained gel fractions which had been dissected from the Sebia agarose gel system. For each individual patient, combined (albumin, α1, α2, β1, β2 and γ) unstained bands from each separate fraction were cut into small fragments and placed in 0.5 ml Eppendorf tubes (Fisher Scientific) (Fig. 4a). To extract protein 30 uL sample buffer (Bio-Rad XT Sample Buffer 20X, Bio-Rad Inc), 44 uL distilled water and 6 uL of reducing agent (Bio-Rad XT Reducing Agent 4X, Bio-Rad Inc) were added to each of the gel bands which were then heated at 95 °C for 5 minutes. Gel fragments were then left for 12 hours, before being heated for a further 5 minutes at 95 °C to complete protein extraction from the gel fragments. These samples were then stored frozen at -80 C until they were run on a second dimension gel.

Ten uL of the extracted protein solution from each of the individual bands of the respective patients were added to prefabricated wells of a precast polyacrylamide gel (Bio-Rad Precast Criterion XL Gel, Bio-Rad Inc) alongside control ladder (Precision Plus Protein Standards All Blue, Bio-Rad Inc) (Fig. 4b). Gels were electrophoresed for 50 minutes each at a constant voltage of 200V in a solution of 5% running buffer (Bio-Rad XT MOPS Running Buffer, Bio-Rad Inc), then stained in Coomasie Blue stain (Biosafe Coomassie Blue, Bio-Rad Inc) for 1 hour using gentle agitation before being rinsed in acid alcohol solution (20% methanol and 7.5% acetic solution) and destained for 12 hours in acid acohol again using gentle agitation. Distilled water replaced the acid alcohol solution whilst the gels were stored at room temperature for 48 hours.

Digital images of the second dimension gels were captured using a flat bed scanner (UMAX PowerLock III, UMAK UK Ltd) and individual protein bands were then identified. The individual bands were then dissected out of the polyacrylamide gel using a scalpel in a laminar flow air cupboard to minimise

contamination. Protein bands were then stored individually in 0.5 ml Eppendorf tubes at -80 C.



4a 4b

Figure 4 Combined one dimensional (1D) agarose electrophoresis and second dimension (2D) polyacrylamide gel electrophoresis (PAGE). (a) Diagram of 1D agarose gel illustrating how the stained lane 1 was used as a guide to cut and combine, in polypropylene tubes, unstained bands from each of the protein sub-fractions in lanes 3-7. (b) The combined protein sub-fractions in the polypropylene tubes were then separated further within each fraction by PAGE. A molecular weight (Mw) control marker (Precision Plus Protein Standards All Blue, Bio-Rad Inc) was run in lane 1.

2.3 Mass Spectrometry

The excised gel bands were washed (with shaking) in 100 mM ammonium bicarbonate (GE Healthcare) for 1 hour at room temperature, followed by a second wash in 50% acetonitrile/100mM ammonium bicarbonate (GE Healthcare). Proteins were reduced with 3mM dithiothreitol in 100mM ammonium bicarbonate (GE Healthcare) for 30 min at 60°C, followed by alkylation with 10 mM iodoacetamide (GE Healthcare) for 30 min in the dark at room temperature. The gel pieces were washed with 50% acetonitrile/100mM ammonium bicarbonate, shaking for 1 hour at room temperature, then dehydrated by incubation with 0.1 mL acetonitrile for 10 min at room temperature. Gel pieces were dried to completion under vacuum, then rehydrated with a sufficient volume of trypsin (Promega sequencing grade trypsin, 20 mg/mL in 25mM ammonium bicarbonate, Promega Ltd) to cover the gel pieces. Digestion was performed at 37°C overnight. The liquid was then transferred to a fresh tube, and gel pieces washed for 10 min with a similar volume of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried to completion.

Tryptic peptides were solubilized in 0.5% formic acid and fractionated on a nanoflow uHPLC system (Thermo RSLCnano) before being analysed by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min at a flow rate of 0.2 ml / min. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to five MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120s.

MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein

identifications were assigned using the Mascot search engine[3] to interrogate mammalian protein sequences in the National Centre for Biotechnology Information (NCBI) database, allowing a mass tolerance of 0.3 Da for both MS and MS/MS analyses. For all searches, carbamidomethylation of cysteines was assumed and oxidation of methionine was considered. Mascot MOWSE (Molecular Weight Search) scores of 64 or greater indicated identity or extensive homology ($p<0.005$). When proteins matched sequences from multiple species, only the species with the highest MOWSE score was included in the results unless a match with a MOWSE score ≥64 within the species *Canine lupus familiaris* was noted, when this was included instead.

2.4 Haptoglobin and C-Reactive Protein Assays

Five uL of stored serum from each dog with lymphoma was used to measure C-reactive protein by an immunoturbimetric assay (Eckersall et al.,1991) modified by a commercial laboratory[4]. Antibody- antigen turbidity was measured using a spectrophotometer in a biochemical analyser (ABX Pentra 400, Horiba). Seven and a half uL of stored serum was used to measure haptoglobin concentrations by colourimetric methodology based on haptoglobin- haemoglobin (Eckersall et al., 1999) and modified by a commercial laboratory[4]. Colour change was analysed using a spectrophotometer in a biochemical analyser (ABX Pentra 400, Horiba). All samples were run in duplicate and the mean values are reported.

[3] http://www.matrixscience.com/

[4] ReactivLab, Glasgow, UK

2.5 Statistical Analyses

2.5.1 Definition of Normal One Dimensional Reference Ranges

To assess normality of data distribution from the dog's serum protein fractions, box and whisker plots of total protein serum concentrations alongside absolute protein fraction concentrations and relative protein fraction levels were made for the samples. The Anderson-Darling normality test was also applied to the data using computer software (Minitab 16, Minitab Ltd). Not all data sets were normally distributed therefore median values were calculated for the relative and absolute concentrations and subsequent statistical analyses therefore employed non-parametric tests.

2.5.2 Comparison of Control and Lymphoma Patient Serum Protein Fractions and Population Demographics

Absolute and relative protein values were compared between the lymphoid neoplasia group and healthy group using non-parametric Mann Whitney U-tests on commercially available software (Minitab 16, Minitab Ltd). Although subdivision of the globulin fractions beyond α1, α2, ß1, ß2 and γ was often possible the smaller sub-fractions were grouped into these 6 major globulin fractions for data presentation and statistical analysis. Mann Whitney U-tests were also used to compare the ages of the 2 groups and the number of peaks identified; a Chi square test was used to compare the sexes.

CHAPTER III

THE NORMAL CANINE SERUM PROTEOME CHARACTERISED USING A NOVEL, SECOND DIMENSION AGAROSE-POLYACRYLAMIDE GEL ELECTROPHORESIS TECHNIQUE FOLLOWED BY TANDEM MASS SPECTROMETRY

3.1 Introduction

The serum proteome has already been mapped using 2D PAGE and mass spectrometry in the human species (Pieper et al., 2003) and the domestic horse (*Equus caballus)* (Miller et al., 2004) but only one study with limited information has been obtained for the domestic dog (*Canis lupus familiaris)* (Presslmayer, 2002). Previously 1D agarose SPE has been performed on cat serum and combined with MS to analyse protein bands but this methodology did not give a high resolution in terms of protein separation (Gerou-Ferriani et al., 2011). The purpose of this part of the thesis was to characterise the normal canine serum proteome further by using a novel but simple methodology combining a 1D agarose SPE (performed on 17 healthy dogs' sera) with a second dimension PAGE system without isoelectric focusing (performed on the sera of two representative healthy dogs' sera). Following separation of serum proteins, mass spectrometry was performed on the second dimension PAGE gels from the sera of the two representative healthy adult dogs to identify the constituent serum proteins helping to characterise the serum proteome in the normal dog.

3.2 Results

3.2.1 Agarose Gel Electrophoresis

Electrophoretograms were generated for each of the 17 animals on the basis of densitometric scans. A minimum of 6 peaks could be identified for each animal (albumin, α1, α2 ß1, ß2 and γ) with further subdivision of some fractions (α1-a, α1-b, α2-a, α2-b, ß1-a and ß1-b) giving 9 peaks in 2 dogs. The median number of peaks for all traces was 8 (Fig. 5).

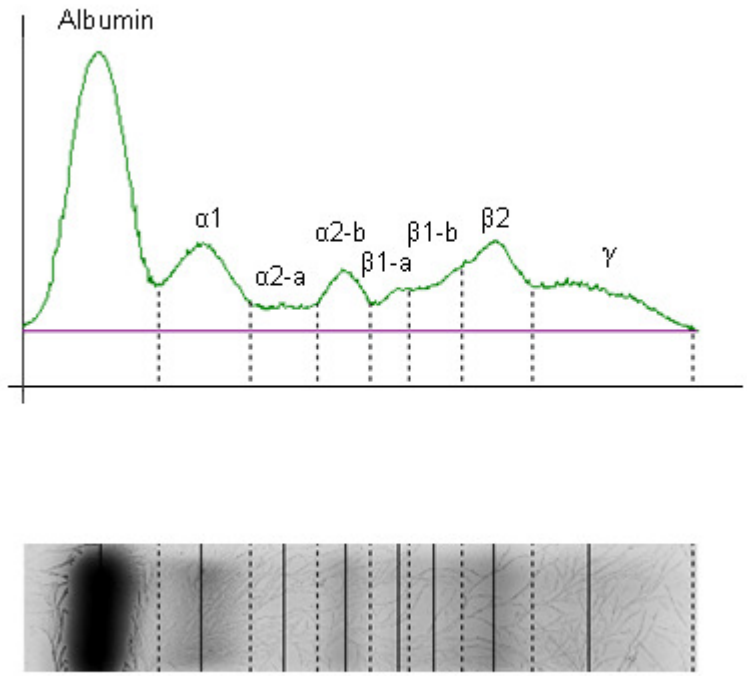


Figure 5 An electrophoretogram from a healthy one year old male entire Labrador blood donor showing subdivision of α2 and ß1 fractions to obtain eight bands. The top panel displays the electrophoretogram which is a graphical densitometric representation of the scanned gel lane seen in the corresponding bottom panel for both dogs displayed.

Median total protein values for all dogs (1-17) was 64 g/L (range 61-72). The absolute and relative values of the albumin and 5 major globulin fractions as well as the number of peaks identified for each dog were calculated (Table 4).

Table 4 Absolute and relative values of the different protein fractions from 17 healthy dogs. Median values and data ranges are displayed along the bottom of the table. The number of fractions into which each electrophoretogram can be divided is also displayed.

Dog number	Total protein (g/L)	Relative albumin (%)	Absolute albumin (g/L)	Relative α1 (%)	Absolute α1 (g/L)	Relative α2 (%)	Absolute α2 (g/L)	Relative β1 (%)	Absolute β1 (g/L)	Relative β2 (%)	Absolute β2 (g/L)	Relative γ (%)	Absolute γ (g/L)	Number of fractions	2D analysis
1	65	38.4	25.0	13.4	8.7	12.6	8.2	10.7	7.0	11.6	7.5	13.3	8.7	6	No
2	65	39.4	25.6	15.1	9.8	10.5	6.8	10.8	7.0	13.0	8.5	11.2	7.3	9	No
3	72	38.8	27.9	12.3	8.9	9.6	6.9	12.0	8.7	13.3	9.6	14.0	10.1	8	No
4	67	37.1	24.8	13.7	9.2	11.8	7.9	12.2	8.2	12.1	8.1	13.1	8.8	7	No
5	72	38.3	27.6	16.4	11.8	11.9	8.6	10.8	7.8	12.1	8.7	10.5	7.6	8	No
6	62	36.0	22.3	17.2	10.7	11.3	7.0	11.1	6.9	12.0	7.5	12.4	7.7	8	No
7	63	43.1	27.1	13.6	8.6	8.8	5.5	11.9	7.5	9.9	6.3	12.7	8.0	9	No
8	63	38.5	24.3	12.0	7.5	10.0	6.3	8.9	5.6	13.6	8.5	17.1	10.8	9	No
9	63	48.0	30.2	14.7	9.2	10.1	6.3	8.1	5.1	11.9	7.5	7.3	4.6	8	No
10	64	49.1	31.4	14.3	9.2	8.1	5.2	6.7	4.3	11.9	7.6	9.8	6.3	8	No
11	66	48.3	31.9	14.7	9.7	10.3	6.8	11.2	7.4	9.5	6.3	6.1	4.0	8	No
12	63	45.2	28.5	13.3	8.4	11.3	7.1	12.6	8.0	10.2	6.4	7.4	4.7	8	No
13	70	47.0	32.9	13.8	9.7	11.8	8.2	8.8	6.2	12.4	8.7	6.2	4.4	8	No

14	61	53.8	32.8	15.0	9.2	9.6	5.9	8.3	5.1	8.4	5.1	4.9	3.0	8	No
15	62	43.6	27.0	13.6	8.5	9.6	5.9	9.3	5.8	11.6	7.2	12.3	7.6	8	Yes
16	70	45.1	31.5	14.7	10.3	9.6	6.7	8.8	6.2	12.4	8.7	9.5	6.7	8	Yes
17	63	40.3	25.4	13.5	8.5	9.8	6.1	10.3	6.5	12.2	7.7	14.0	8.8	8	No
Median	64	43.1	27.6	13.8	9.2	10.1	6.8	10.6	6.9	12.0	7.6	11.2	7.6	8	
Range	61-72	36.0-53.8	22.3-32.9	12.0-17.1	7.5-11.8	8.1-12.6	5.2-8.6	6.7-12.6	4.3-8.7	8.43-13.6	5.1-9.6	4.9-17.1	3.0-10.8	6-9	

3.2.2 Second Dimension Electrophoresis

Following analysis of 1D gels 2 dogs (15 and 16) with 8 peaks in their electrophoretograms were selected for further separation of protein fractions in a second dimension on the basis of having the closest matching electrophoretograms to median values. One dimensional agarose gel electrophoresis was repeated using the Sebia system using serum from a 6 year old male neutered Flatcoated Retriever and a 3 year old female neutered Labrador Retriever. Visual inspection of the Sebia gel lanes revealed consistent migratory profiles with that of the previously run Beckman Coulter gels. This was followed by second dimension electrophoresis performed on polyacrylamide gel to separate each protein fraction further. A total of 26 protein bands were easily discernible on following 2D separation (Fig. 6). Other bands were present but not easily identified therefore precluding accurate excision from the gel. The globulin fractions separated into 9 bands in dog 15 and 7 bands in dog 16. Dog 15 had a single extra band in the α1 and β1 lanes (band 6 and band 8) but the remainder of bands were present across both PAGE gels in similar migratory positions. Within the albumin lanes 4 bands were excised from dog 15 and 6 bands from dog 16. Multiple other bands were present within the albumin lanes, however, it was not feasible to perform MS on all these bands and only a selection of bands across the albumin lanes were therefore submitted for proteomic analysis. The 26 bands identified from 2D PAGE were then excised and submitted for MS analysis.

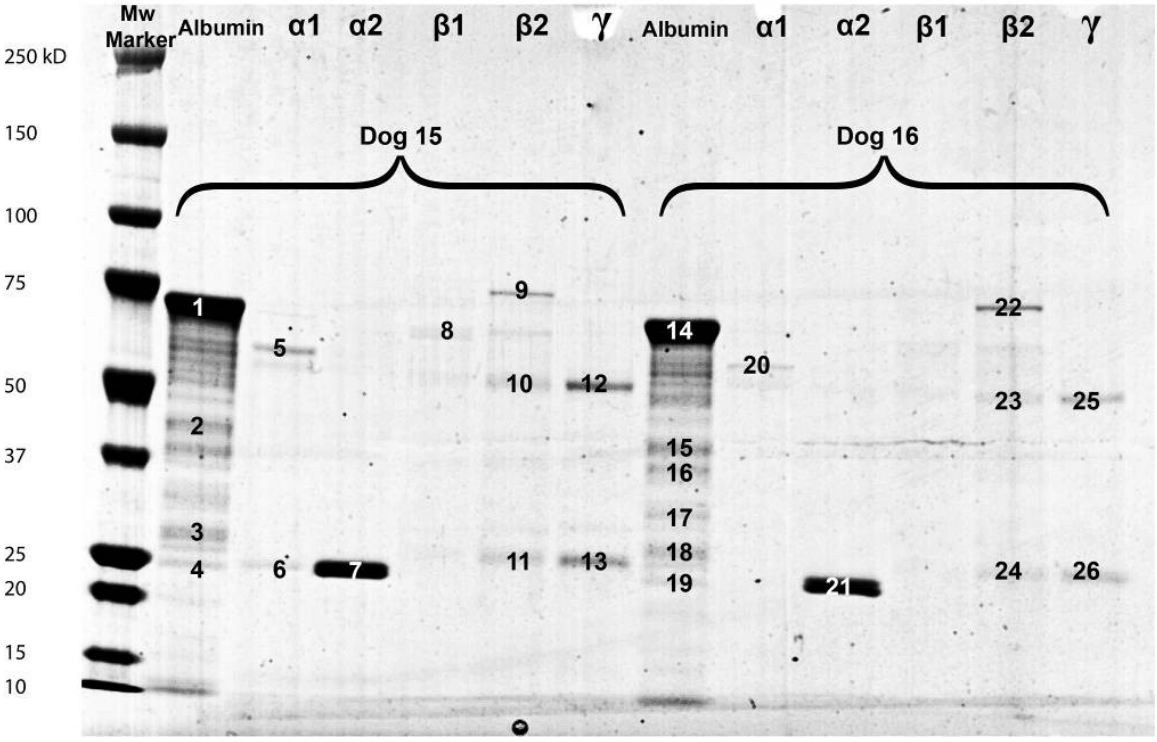


Figure 6 Further separation of sera from two healthy dogs (dogs 15 and 16) on second dimension (2D) polyacrylamide gel electrophoresis (PAGE). Each respective protein fraction is labelled at the top of the figure and the band numbers correspond to the proteins identified in Table 5. A molecular weight (Mw) control marker (Precision Plus Protein Standards All Blue, Bio-Rad Inc) was run in lane 1.

3.2.3 Mass Spectrometry

Submission of the MS data relating to the excised bands to the Mascot search engine generated a list of 32 individual proteins using a MOWSE score of ≥ 64 (which equates to $p<0.005$). These proteins comprised known sequenced canine proteins (for example canine albumin), proteins predicted from the canine genome (for example predicted canine Apolipoprotein A1) alongside proteins from other mammalian species (for example human gamma-actin). Table 5 lists the proteins present within each band with further information regarding the MS data generated for each protein matched including the MOWSE score, number of peptides matched, sequence coverage and nominal mass is available in supplementary table 1.

Table 5 Proteins identified by mass spectrometry in 2 healthy dogs within the bands labelled in Figure 7. Characters in bold represent proteins from the dog. Characters in italics represent proteins predicted from known genomic sequences. All proteins listed correspond to a MOWSE score ≥64 (*P* <0.005). Highest MOWSE scores are listed first in each band.

Band identification	Protein name, species of origin and accession number
1 (dog 15)	**Albumin [Canis lupus familiaris] gi|3319897**
2 (dog 15)	**Albumin [Canis lupus familiaris] gi|3319897** *Serum albumin-like isoform 1 [Ailuropoda melanoleuca] gi|301786252*
3 (dog 15)	**Albumin [Canis lupus familiaris] gi|3319897**
4 (dog 15)	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764** *Alpha 2 macroglobulin [Sus scrofa] gi|335288480* *T-cell receptor beta chain T17T-22-like [Sus scrofa] gi|350592642* Hemoglobin subunit alpha [Sus scrofa] gi|122465 Hemoglobin subunit beta [Ailurus fulgens] gi|122553 Inter-alpha (globulin) inhibitor H4 [Bos taurus] gi|59857769 *Serpin A3-6 [Sus scrofa] gi|194038353* *Alpha 1B glycoprotein-like [Sus scrofa] gi|311259609*
5 (dog 15)	**Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** ***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** ***Kininogen-1 isoform 2 [Canis lupus familiaris] gi|57109938***

6 (dog 15)	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** **Albumin [Canis lupus familiaris] gi|3319897** ***Vitamin D binding protein isoform 2 [Canis lupus familiaris] gi|73975215 Kininogen-1 isoform 2 [Canis lupus familiaris] gi|57109938***
7 (dog 15)	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106***
8 (dog 15)	***Complement C3 [Canis lupus familiaris] gi|359322249*** ***Hemopexin [Canis lupus familiaris] gi|73988725*** ***Complement C4-A [Canis lupus familiaris] gi|359320893***
9 (dog 15)	***Complement C4-A [Canis lupus familiaris] gi|359320893*** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009** ***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142***
10 (dog 15)	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528** **Immunoglobulin gamma heavy chain A [Canis lupus familiaris] gi|17066524** ***Complement C3 [Canis lupus familiaris] gi|359322249*** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526,**
11 (dog 15)	***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681*** ***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** ***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106***
12 (dog 15)	**Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528**

13 (dog 15)	***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** ***Immunoglobulin lambda light chain variable region [Canis lupus familiaris] gi|164430518***
14 (dog 16)	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764**
15 (dog 16)	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764** Mutant beta-actin (beta'-actin) [Homo sapiens] gi|28336 Histone H3 [Heterocephalus glaber] gi|351710220 Gamma-actin [Homo sapiens] gi|178045 Galectin-7 In Complex With Galactosamine [Homo sapiens] gi|3891470
16 (dog 16)	**Albumin [Canis lupus familiaris] gi|3319897**
17 (dog 16)	**Albumin [Canis lupus familiaris] gi|3319897**
18 (dog 16)	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764,**
19 (dog 16)	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** ***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106***
20 (dog 16)	**Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** **Albumin [Canis lupus familiaris] gi|3319897** ***Kininogen-1 isoform 2 [Canis lupus familiaris] gi|57109938*** ***Vitamin D binding protein isoform 2 [Canis lupus familiaris] gi|73975215***
21 (dog 16)	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106***

22 (dog 16)	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009** ***Complement C3 [Canis lupus familiaris] gi|359322249*** ***Complement C4-A [Canis lupus familiaris] gi|359320893***
23 (dog 16)	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528** **Immunoglobulin gamma heavy chain A [Canis lupus familiaris] gi|17066524** ***Complement C3 [Canis lupus familiaris] gi|359322249*** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526**
24 (dog 16)	***Serotransferrin isoform 1 [Canis lupus familiaris]*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** ***Immunoglobulin lambda-like polypeptide 5-like isoform 3 [Canis lupus familiaris] gi|345791535*** ***Complement C3 [Canis lupus familiaris] gi|359322249*** ***Uncharacterised protein LOC608320, partial [Canis lupus familiaris] gi|345806638***
25 (dog 16)	**Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528**
26 (dog 16)	***Immunoglobulin lambda-like polypeptide 5-like isoform 3 [Canis lupus familiaris] gi|345791535*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** ***Uncharacterised protein LOC608320, partial [Canis lupus familiaris] gi|345806638***

Twenty individual proteins were identified from Canine lupus familiaris within the bands from the polyacrylamide gels with some being found in multiple bands (Table 6). The canine proteins included apolipoprotein A1, serpin, kininogen, vitamin D binding protein, hemopexin, complement C4 and an uncharacterized protein.

Table 6 Summary of canine proteins identified in their respective protein fraction from the serum of two healthy dogs by polyacrylamide gel electrophoresis. Characters in italics represent proteins predicted from known genomic sequences. With the exception of proteins indicated by superscripts, proteins listed were found in both dogs 15 and 16.

Albumin	**Alpha 1**	**Alpha 2**	**Beta 1**	**Beta 2**	**Gamma**
Albumin	Albumin	*Apolipoprotein A1*	*Complement C3*[15]	*Apolipoprotein A1*[15]	Immunoglobulin Heavy Chains
Apolipoprotein A1	*Apolipoprotein A1*[15]		*Complement C4*[15]	*Complement C3*	*Immunoglobulin λ-like Polypeptide*
Serum Albumin Precursor[16]	*Kininogen-1 isoform 2*		*Hemopexin*[15]	*Complement C4*	*Uncharacterized Protein*[16]
	Serpin peptidase inhibitor			Immunoglobulin Heavy Chains	
	Vitamin D Binding Protein			*Immunoglobulin λ-like Polypeptide*	
				Serotransferrin isoform 1	
				Uncharacterized Protein[16]	

Several bands were apparent in the albumin lane and these were comprised of albumin and its precursor molecules. Albumin was also present within the α1 lane. Canine apolipoprotein A1 migrated across multiple bands (albumin, α1, α2 and ß2). Complement C3, C4 and various immunoglobulin classes were observed within both the ß and γ lanes. In nearly all of the distinct bands more than one protein could be identified suggesting complete separation into individual proteins was not fully achieved. All bands present in the globulin lanes, except bands 6 and 8 which were only present in dog 15, contained the same protein or groups of proteins between the two dogs.

3.3 Discussion

In this part of the study, two representative healthy animals were selected on the basis of their 1D agarose gel electrophoretograms and further separation of their serum proteins was performed using a novel but simple technique. After second dimension PAGE, MS enabled identification of proteins within healthy canine serum to a high level of confidence.

Both Keay (1982) and Tappin et al (2011) concluded that although normal reference intervals are useful, each laboratory performing SPE should devise its own set of reference intervals allowing for inter- lab variation in gel materials and methods of electrophoresis employed. We therefore performed 1 dimensional agarose SPE on 17 selected healthy animals in order to find representative normal samples and devise reference intervals. Our results are comparable to previous studies suggesting normal reference intervals for SPE (Keay, 1982; Tappin et al., 2011) with some minor expected variations from these results noted. To avoid biochemical disturbances such as sample haemolysis and hyperlipidaemia which can affect the results obtained by SPE (Martínez-Subiela et al., 2002b), samples were handled carefully and animals were fasted prior to blood collection. To achieve a uniform healthy population of animals all dogs had normal matching haematology and biochemistry profiles.

Full physical examination as well as recent medical history were deemed unremarkable in all cases. Variations of breed (Fayos et al., 2005) and nutritional status (Eckersall, 2008) are also known to influence serum protein values therefore a mixture of breeds were selected.

Although Tappin et al (2011) concluded that many diseases alter the serum protein patterns, 1D SPE on its own is unable to determine the exact nature of these changes as little is known about the constituents of the different protein fractions. A combination of high resolution electrophoresis, Sudan black staining, haemoglobin binding and electophoretic immunofixation was performed by Abate et al (2000) to identify the position of a few of the serum proteins within their respective electrophoretic fractions in a mixture of healthy and diseased dogs. This technique used antibodies specific to the various proteins expected within the serum and therefore proteins without appropriate anti-sera were not identified. Proteomics and more specifically, mass spectrometry, offer a huge improvement over such an immunologic technique and combined with genome sequencing data for the dog and other mammalian species enable identification of much large numbers of different proteins (Eckersall, 2008). Canine protein sequences are still quite incomplete however, sequencing of the canine genome (Lindblah-Toh et al., 2005) has enabled us to identify various other predicted proteins (i.e. canine proteins that have not yet been sequenced but whose structure and size can be predicted by the presence of DNA sequences in the canine genome that is homologous for the same gene encoding a known protein in a different species). Proteins matched in this study that were not of the species *Canine lupus familiaris* are likely homologues of dog proteins whose sequences are not contained in currently available genome datasets. Second dimension electrophoresis and mass spectrometry performed in this work has generated a list of proteins that one could expect to find within the normal serum proteome of the domestic dog by making use of large open access databases and the Mascot search engine.

Although traditional 2D PAGE requires IEF and IPGs to optimise the data acquired, both of which are technically difficult, the protein separation

achieved in this study does not require such techniques to be performed. The method applied here allows further separation within the globulin groups allowing us to define whether proteins identified were α, ß or γ globulins. This represents another possible advantage over traditional 2D PAGE.

By selecting proteins with a MOWSE score of at least 64 we were able to identify the various proteins listed with a high degree of confidence ($p<0.005$). As previously described (Abate et al., 2000) we were able to identify albumin definitively within the albumin band, complement C3 and transferrin within the ß band and various different immunoglobulin classes within the ß and γ bands. Unlike this previous work we failed to identify canine specific haptoglobin or canine specific α2 macroglobin within the α bands of these cases. Both of these proteins are known acute phase reactants and serum levels increase in inflammatory states (Murata et al., 2004). One explanation of their absence is that our animals were healthy individuals therefore serum concentrations of these proteins would be expected to be low as opposed to the work by Abate et al. (2000) in which diseased animal's sera were also analysed. Equally if a less stringent MOWSE score was applied further proteins may have been isolated from the dog's sera however, our confidence in these being genuine findings would also have diminished.

The migration patterns of albumin and apolipoprotein A1 were both of interest as they were found in multiple different band patterns. Two dimensional electrophoresis of equine serum also revealed multiple spots containing albumin (Miller et al., 2004). The reason for albumin being present in multiple spots, or in multiple bands as is the case here may well be due to fragmentation resulting in multiple peptide sequences of differing sizes. Alternatively the appearance of multiple spots may be due to alterations in the individual structure of proteins that could reflect post-translational modification. Overlap of protein spots, a phenomenon known as "smearing" in PAGE gels could also cause high abundance proteins to be present in multiple spots with prefractionation recommended to help prevent this (Herbert and Righetti, 2000). Similar explanations may also be applicable to the migration pattern of apolipoprotein A1. Apolipoprotein A1 was

clearly visible as a single band within the α2 lane of the second dimension gel (band numbers 7 and 21, figure 3) as well as in other bands in other fractions.

Albumin is the most abundant protein found in the sera of animals making up 35%-50% of total serum protein. This protein is manufactured and secreted by the liver and its chemical properties include a net negative charge and a molecular weight 66.4 kDa (David Eckersall, 2008). The latter 2 physical properties mean that albumin has the most anodal mobility of the serum proteins on an agarose gel electrophoresis (Stockham and Scott, 2008). Albumin has many vital homeostatic functions including maintaining colloid oncotic pressure, acting as a transport molecule for endogenous and exogenous products, moderating haemostasis and exerting anti-oxidative effects. Furthermore decreases in serum albumin concentrations are noted in many illnesses and in critical patients have been associated with a poor prognosis (Mazzaferro et al., 2002). In humans a precursor of albumin is present within hepatic tissue and similar molecules have also been documented in the livers of rats, cattle and also monkeys (Weigand and Alpert, 1981). The presence of serum albumin precursor in the dog's serum in this study may represent a similar precursor molecule and to the author's knowledge such a precursor has not previously been reported in the literature. Alternatively this protein may actually represent albumin rather than the precursor, as the two proteins share extensive structural homology within the rat (Ikehara et al., 1976) and a similar scenario may exist in the dog.

Apolipoprotein A1's main role within plasma is to act as a vehicle to transport macromolecular complexes of lipids throughout the body and of the high density lipoproteins apolipoprotein A1 is the most important molecule (Luo et al., 1989). The enzyme lecithin: cholesterol acetyltransferase is activated by apolipoprotein A1 and it is by this mechanism that apolipoprotein has a vital role in the transport and distribution of cholesterol throughout the body (Dergunov, 2012).

Serpin peptidase inhibitor (also know as α1 antitrypsin), one of the large serpin family of plasma proteins was clearly identified in both dog samples and can therefore be confidently identified as a canine α globulin for the first time. Abate et al (2000) were unable to confirm the presence of this protein in dog serum due to unavailability of specific antibodies, again highlighting the advantages of using the proteomic techniques reported in this paper. The serpins have multiple functions within the body and α1 antitrypsin which is regarded as the major plasma serpin in people accounts for 90% of the tryptic inhibitory capacity in humans (Patterson, 1991). In man serpins are primarily found in the plasma and represent 10% of its total content. Their role in inhibiting proteolytic enzymes is vitally important for normal homeostasis but they also have other minor roles in the transport of hormones and regulation of blood pressure (Potempa et al., 1994).

Canine kininogen was also found within the α1 globulin fraction of both dogs' sera. Kininogen, also known as high molecular weight kininogen (HMWK), is an α globulin found within circulating plasma in humans (Moreau et al., 2005). Our work has confirmed it as a canine α globulin for the first time. HMWK is a precursor (or cofactor) both within the inflammatory cascade leading to an end product of bradykinin and in the contact arm of the coagulation cascade. HMWK also influences cellular proliferation and apoptosis of endothelial cells (Schmaier and McCrae, 2007).

Another canine protein found and not previously reported within the α1 fraction was vitamin D binding protein. As its name suggests its major role is as a carrier protein for vitamin D but also has other functions including actin scavenging, fatty acid transport, macrophage activation and chemotaxis. Interestingly within human serum the concentrations of this protein exceed the concentrations of its ligand. This is in contrast to other hydrophobic hormone binding systems reported (Speeckaert et al., 2006).

Canine hemopexin and complement C4 A were also both identified definitively within the β globulin fractions for the first time. Hemopexin is a member of the acute phase reactants and has a key role in controlling oxidative stress in the body. Hemopexin helps reduce oxidative stress by binding heme which by producing hydroxyl free radicals has the potential to be highly toxic (Tolosano and Altruda, 2002). Complement C4 A and complement C3 were found in the sera of both dogs as was serotransferrin. Complement C4 A is a key early component in the classical and alternative complement pathways whereas C3 is the central component to these 2 pathways and it therefore follows that both molecules are vitally important for normal immune function (Kay et al., 1985). Iron is a vital mineral required by nearly every living cell therefore the transport of this metal needs to be under tight homeostatic control and this is mediated in the main part by serotransferrin; as with hemopexin serotransferrin is also involved in preventing oxidative damage being induced by circulating unbound ionic iron (Bou-Abdallah, 2012).

Multiple different immunoglobulin types were found within both the β and γ bands, consistent with the work performed by Abate et al (2000). An uncharacterised protein was also identified in both dogs; further analysis of this using the NCBI (National Center for Biotechnology Information) BLAST (Basic Logical Alignment Search Tool)[5] showed peptide sequence homology with the immunoglobulin superfamily. The presence of various types of immunoglobulin within the serum represents their varied antigenic binding ability and therefore is not an unexpected discovery and would appear representative of a normal immune system.

Although we have confirmed the presence of various proteins in the globulin fractions of healthy dogs, serum from only 2 animals was used for our 2D technique, which may limit the accuracy of our data. Another possible limitation is that proteins were not completely separated therefore meaning multiple proteins were identified within single bands on the 2D gels. A similar problem is

[5] http://blast.ncbi.nlm.nih.gov/Blast.cgi

encountered in traditional 2D PAGE, prefractionation of protein solutions prior to application to narrow range ("zoom") IPG-gels and immunoaffinity subtraction procedures to remove highly abundant serum proteins are 2 examples of proteomic techniques in 2D PAGE that have been applied to increase sample resolution (Görg et al.., 2004; Piper et al., 2003). Both of these methods are technically complex and were not applied in this study but may represent avenues for future investigation of the canine serum proteome. Interestingly when examining the data from 2 of the bands within the albumin sections of each dog (4 and 15) a variety of proteins that one would not expect to find within these groups were identified. Proteins such as porcine α2 macroglobin and human gamma-actin may have been contaminants but it is more likely they represent genuine homologous proteins within canine serum. Further 2nd dimension gels of more dog sera would help support the findings described here. Identification of more proteins would have been possible by decreasing the stringency of the MOWSE score, however this would have decreased the confidence in the results presented.

CHAPTER IV

DEMONSTRATION OF AN ALTERED SERUM PROTEOME IN ADVANCED STAGE CANINE LYMPHOMA USING TWO DIMENSIONAL AGAROSE-POLYACRYLAMIDE GEL ELECTROPHORESIS AND TANDEM MASS SPECTROMETRY

4.1 Introduction

Having developed a novel technique able to accurately define the serum proteome in healthy dogs the next part of the study was designed to analyse the proteome of dogs diagnosed with high grade multicentric lymphoma that had not been previously treated for this condition. Numerous studies have documented changes in the serum constituents of dogs suffering from lymphoma using a variety of different techniques including serum protein electrophoresis (Gavazza et al., 2009; Tappin et al., 2011), mass spectrometry (Gaines et al., 2007; Ratcliffe et al., 2009; Wilson et al., 2008) and measuring acute phase reactant concentrations (Merlo et al., 2007; Mischke et al., 2007). To date however, there has not been a single comprehensive study performed to clearly define any differences in the proteome of dogs suffering from lymphoma and to identify the proteins responsible for such changes. This part of the thesis describes differences between the healthy proteome defined above compared to lymphoma patients with the aims of uncovering potential biomarkers for canine lymphoma as well as helping to understand the pathogenesis of this commonly encountered neoplasm in the domestic dog. One dimensional SPE was performed on the sera from 21 lymphoma patients with further second dimension PAGE separation and mass spectrometry performed on 3 dogs' sera.

4.2 Results

4.2.1Animals

The lymphoma population comprised 21 dogs; all animals had high grade lymphoma confirmed cytologically (16 cases) or histologically (5 cases). Dogs presented with a variety of problems including peripheral lymphadenopathy (n=18), lethargy (n=6), vomiting (n=6), anorexia (n=5), polydipsia/ polyuria (n=5), diarrhoea (n=4), weight loss (n=4), blindness (n=3) and dyspnoea (n=1).

Eleven dogs presented with more than one clinical sign and all dogs had multicentric disease. Prior to presentation 5 of the cases had received fewer than 7 days of corticosteroids. One of the animals had concurrent endocardiosis, however, was not deemed to be in heart failure on the basis of echocardiography and thoracic radiography. All patients were fully staged using the WHO system (Owen, 1980) and there were 4 stage III patients, 7 stage IV patients and 10 stage V; most dogs were substage b (n=17). Immunophenotyping was performed on 9 animals resulting in 5 B-cell lymphomas, 3 T-cell and 1 null-cell lymphoma. Only 3 animals had normal haematologic profiles; the most common abnormalities included 13 dogs with lymphopenia (median 0.547 x 10^9/L (range 1-4.8)), 9 with anaemia (median haematocrit 34.9% (range 37-55)), 8 with thrombocytopenia (median 98 x 10^{12}/L (range 200-500)) and 7 with a neutrophilia (median 15.169 x 10^9/L (range 3-11.8)). Biochemically only 3 dogs had normal profiles and the most common biochemical changes included 11 dogs with increased aspartate transaminase (median 62 U/L (range <40)), 9 with increased alkaline phosphatase (median 558 U/L (range <230)), 6 with increased urea (median 13.8 mmol/L (range 2.5-8.5)), 6 with increased alanine transaminase (median 352.5 U/L (range <90)), 5 with increased creatinine (median 190 umol/L (range 45-155)), 4 with increased total bilirubin (median 24 umol/L (range <10)) and 3 with hypercalcaemia (median total calcium 3.58 mmol/L (range 2.34-3)).

The median age of the lymphoma population was significantly older than that of the healthy control population (p=0.0019). There was no difference between the genders of the 2 groups (p=0.796). A comparison of the total protein concentration revealed that the lymphoma group (median 58 g/L (range 42-77)) was significantly lower than that of the healthy group (median 64 g/L (range 61-72)). No animal in either group was found to be hyperproteinaemic (range 50-78 g/L).

Table 7 Summary of patient characteristics of the 21 dogs diagnosed with high grade multicentric lymphoma[6].

Patient	Signalment	WHO stage	Substage	Immunophenotype	Method of diagnosis	Pretreatment	Hypercalcaemic	Concurrent disease
1	12YO ME GSD	III	a	Not performed	Cytology	None	No	None
2	4YO ME Newfoundland	IV	b	Not performed	Cytology	None	Yes	None
3	4YO FE Boxer	V	b	Not performed	Cytology	<7d steroids	No	None
4	8YO ME Springer Spaniel	IV	b	Not performed	Cytology	None	No	None
5	11YO ME Labrador	V	b	B cell	Cytology	None	No	None
6	8YO MN Golden Retriever	V	b	Not performed	Cytology	None	Yes	None
7	8YO FN Boxer	V	b	Not performed	Cytology	None	No	None
8	5YO FE Labrador	III	b	T-cell	Cytology	None	No	None
9	3YO ME Cocker Spaniel	IV	b	Not performed	Histology	None	Yes	None

[6] YO- years old, ME- male entire, MN- male neutered, FE- female entire, FN- female neutered, GSD- German Shepherd Dog, CKCS- Cavalier King Charles Spaniel.

10	8YO FN Labrador	V	b	Null cell	Cytology	None	No	None
11	5YO ME Doberman	V	b	Not performed	Histology	<7d steroids	No	None
12	6YO FE Bull mastiff	V	b	B-cell	Cytology	None	No	None
13	6YO FE Weimeraner	IV	a	B-cell	Cytology	None	No	None
14	6YO MN Labrador	V	b	Not performed	Cytology	None	No	None
15	4YO ME Labrador	IV	b	T-cell	Histology	<7d steroids	No	None
16	6YO ME Labrador	IV	b	Not performed	Cytology	None	No	None
17	5YO MN Great Dane	IV	a	B-cell	Cytology	<7d steroids	No	None
18	10YO MN Weimeraner	V	b	Not performed	Cytology	None	No	None
19	2YO MN Labrador	III	b	T-cell	Histology	None	No	None
20	6YO MN Labrador	V	b	B-cell	Cytology	<7d steroids	No	None
21	7YO FE CKCS	III	a	Not performed	Histology	None	No	Endocardiosis

4.2.2Agarose Gel Electrophoresis

Electrophoretograms were generated for each animal on the basis of densitometric scans. A minimum of 7 peaks could be identified for each animal (albumin, α1-a, α1-b, α2, ß1, ß2 and γ) with further subdivision of some fractions (α-1a, α-1b, α-2a α-2b, ß-1a and ß-1b) giving 9 peaks in 14 dogs. The median number of peaks for all traces was 9. Relative and absolute values for each of the protein fractions were calculated (Table 8).

Table 8 Absolute and relative values of the different protein fractions from 21 patients diagnosed with high grade multicentric lymphoma. Median values and data ranges are displayed along the bottom of the table. The number of fractions into which each electrophoretogram can be divided is also displayed.

Patient number	Total protein (g/L)	Relative albumin (%)	Absolute albumin (g/L)	Relative α1 (%)	Absolute α1 (g/L)	Relative α2 (%)	Absolute α2 (g/L)	Relative β1 (%)	Absolute β1 (g/L)	Relative β2 (%)	Absolute β2 (g/L)	Relative γ (%)	Absolute γ (g/L)	Number of fractions	2D analysis
1	70	46.7	32.7	10.1	7.1	15.8	11.1	7.6	5.3	9.8	6.9	10.1	7.1	7	Yes- dog 1
2	77	37.6	28.9	9.7	7.4	13.3	10.3	8.4	6.4	16.1	12.4	15	11.5	7	No
3	58	36.9	21.4	9.5	5.5	17	9.9	8.7	5.1	16.8	9.7	11.1	6.4	8	No
4	50	43.9	22	8.4	4.2	138	6.9	10.9	5.5	12.9	6.4	10.1	5.1	8	No
5	55	39.6	21.8	8.7	4.8	21.1	11.6	9.5	5.2	12.2	6.7	9	4.9	8	No- insufficient serum
6	61	39.1	23.8	11.6	7.1	12.9	7.9	15	9.2	11.3	6.9	10.1	6.1	8	No
7	50	28.4	14.2	13.4	6.7	23.2	11.6	15.6	7.8	11.5	5.8	8	4	9	Yes- dog 2
8	65	41.8	27.2	11.2	7.3	6.7	4.4	12.5	8.1	12.3	8	15.5	10	9	No
9	44	37.4	16.5	11.5	5.1	14.2	6.3	18.3	8.1	8.9	3.9	9.7	4.3	8	No
10	50	43.1	21.5	13.8	6.9	10.2	5.1	14.6	7.3	10.4	5.2	7.8	3.9	9	No
11	42	42.4	17.8	6.3	2.6	24.9	10.4	13	5.4	9.3	3.9	4.2	1.8	9	No
12	57	41.3	23.6	16.5	9.4	12.1	6.9	13	7.4	9.6	5.5	7.7	4.4	9	No
13	62	45.5	28.2	12.2	7.5	12.2	7.5	11.1	6.9	10.3	6.4	8.9	5.5	9	No

14	50	44.7	22.4	10.5	5.3	12.1	6.1	11.5	5.7	12.4	6.2	8.9	4.4	9	No
15	66	39.6	26.1	10	6.6	22.7	15	7.3	4.8	11.4	7.5	9	5.9	8	Yes- dog 3
16	55	42.2	23.2	9	5	19.3	10.6	9	5	12.1	6.7	8.5	4.7	9	No
16	58	39.3	22.8	12.2	7.1	18.8	10.9	8.4	4.8	11.5	6.7	9.9	5.8	8	No
18	50	45.3	22.6	10.5	5.2	14.5	7.3	13.8	6.9	9.1	4.6	6.9	3.5	9	No
19	64	48.2	30.8	13.1	8.4	10.7	6.9	11.4	7.3	9	5.7	7.6	4.9	9	No
20	58	46.1	26.7	13.9	8	13	7.5	9.6	5.6	10.1	5.8	7.4	4.3	9	No
21	61	46.5	28.4	12.3	7.5	10.3	6.3	11.7	7.1	12.2	7.4	7	4.3	9	No
Median	58	42.2	23.2	11.2	6.9	13.8	7.5	11.4	6.4	11.4	6.4	8.9	4.9	9	
Range	42-77	28.4-48.2	14.2-32.7	6.3-16.5	2.6-9.4	6.7-24.9	4.4-15	7.3-18.3	4.8-9.2	8.9-16.8	3.9-12.4	4.2-15.5	1.8-11.5	7-9	

There were significantly more peaks in the lymphoma group (median of 9) compared to healthy group (median of 8) (p=0.0317). All absolute values of the lymphoma fractions were significantly lower than that of the controls with the exception of the α-2 fraction that was significantly higher for the lymphoma group (Table 9, Fig. 7). The relative α-2 fraction of the lymphoma group (13.76%) was significantly higher than that of the controls (10.05%) which was in contrast to the relative α-1 value which was significantly lower for the lymphoma (11.22% vs. 13.78%) set of patients (p=0.0001 for both) (Fig. 8). There were no other significant differences in the relative values of the fractions.

Table 9 Comparison of absolute serum protein fractions derived from one dimensional serum protein electrophoresis in healthy dogs and those with multicentric lymphoma.

Fraction	**Control (17 dogs)** **Median** **(standard deviation)**	**Lymphoma (21 dogs)** **Median** **(standard deviation)**	**P value**
Total Protein	64 g/L (3.59)	58 g/L (8.57)	0.0008
Albumin	27.56 g/L (3.27)	23.18 g/L (4.61)	0.0058
α1	9.17 g/L (1)	6.92 g/L (1.59)	<0.001
α2	6.77 g/L (0.98)	7.53 g/L (2.67)	0.0371
β2	7.64 g/L (1.13)	6.44 g/L (1.89)	0.0082
γ	7.59 g/L (2.22)	4.86 g/L (2.15)	0.0188

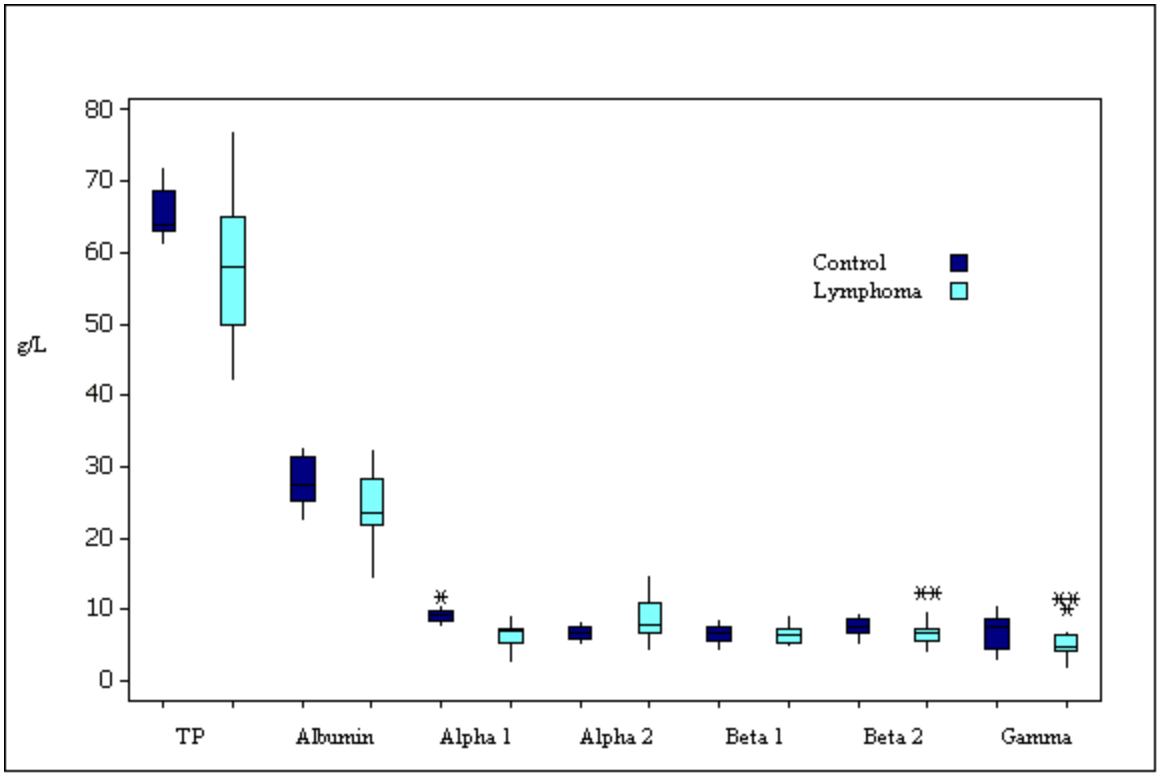


Figure 7 Box and whiskers plot displaying the absolute protein concentrations of the control group and canine lymphoma patients.

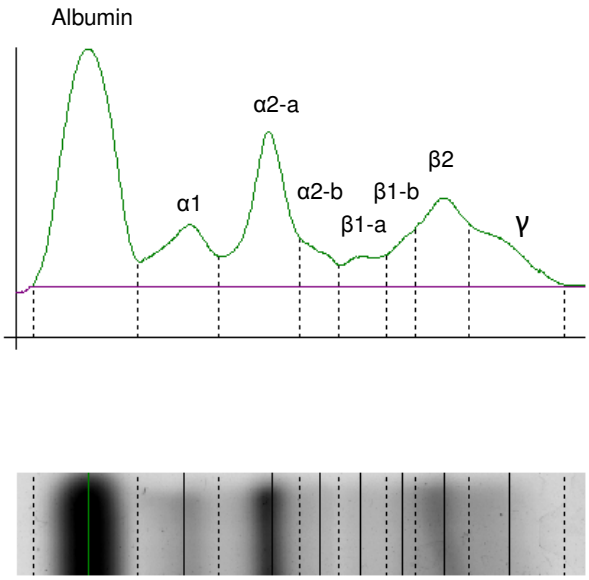


Figure 8 Electrophoretogram derived from a one dimensional (1D) agarose serum protein electrophoresis (SPE) gel showing an elevated spike in α2-a globulins in a four year old male entire Labrador diagnosed with stage IVb T-cell lymphoma. The top panel displays the electrophoretogram which is a graphical densitometric representation of the scanned gel lane seen in the bottom panel. The reader is referred back to Fig. 5 to compare the above trace with that of a representative control.

4.2.3 Second Dimension Electrophoresis

To further characterise the differences between the two populations a subset of animals was selected for additional separation of protein fractions by PAGE. Three lymphoma cases were selected on the basis having the highest absolute α2 concentration. The α2 globulin fraction was the only fraction in which both relative and absolute concentrations were greater in the lymphoma patients than the controls. The sera from these three patients were subjected to further analysis in order to determine the cause of the increased α2 concentration. One dimensional SPE was initially repeated using the Sebia system for the three dogs. Visual inspection of the Sebia gel lanes revealed consistent migratory profiles with that of the previously run Beckman Coulter gels. The three lymphoma patients comprised a 12 year old male entire German Shepherd Dog with stage IIIa lymphoma of unknown phenotype (dog 1), an 8 year old female spayed Boxer with stage Vb lymphoma of unknown phenotype (dog 2) and a 4 year old male entire Labrador Retriever with a stage IVb T-cell lymphoma (dog 3). Although patient number 5 also had one of the highest absolute α2 concentration further analysis of this dogs' proteome was not possible due insufficient serum volume.

On the second dimension PAGE gels all identifiable protein bands were excised from the α1, α2, ß1, ß2 and γ lanes and submitted for mass spectrometry; data was only available from the albumin, α1, α2 and ß2 lanes for dog 3. Multiple peaks were submitted from the albumin lanes however, since the manner of migration resulted in less easily discernible peaks in this lane, not every part of the gel containing protein was submitted. Some extra weak staining bands were identified in the globulin lanes however, they were not present in sufficient quantity to allow accurate excision from the gel. A total of 38 areas of gel were excised and submitted for analysis using mass spectrometry (19 from dog 1, 8 from dog 2 and 11 from dog 3) (Fig. 9). Multiple bands were present in the albumin lanes and a selection were dissected out for further analysis as it was unfeasible to submit all these bands for MS. Twenty six bands dissected from globulin lanes were submitted for proteomic analysis. Extra bands were consistently identified in the α-2 lanes of all 3 lymphoma samples in comparison

to the two previously described control samples. Of the 26 bands identified in the globulins, 12 were present in matching positions across all 3 gels (bands 7, 9, 11, 15, 21, 22, 23, 25, 33, 34, 36 and 37), 10 were present in corresponding positions in 2 gels (bands 10, 12, 16, 18, 19, 24, 26, 27, 35 and 38) and 4 were present in only 1 gel (bands 8, 13, 14 and 17, all from dog 1).

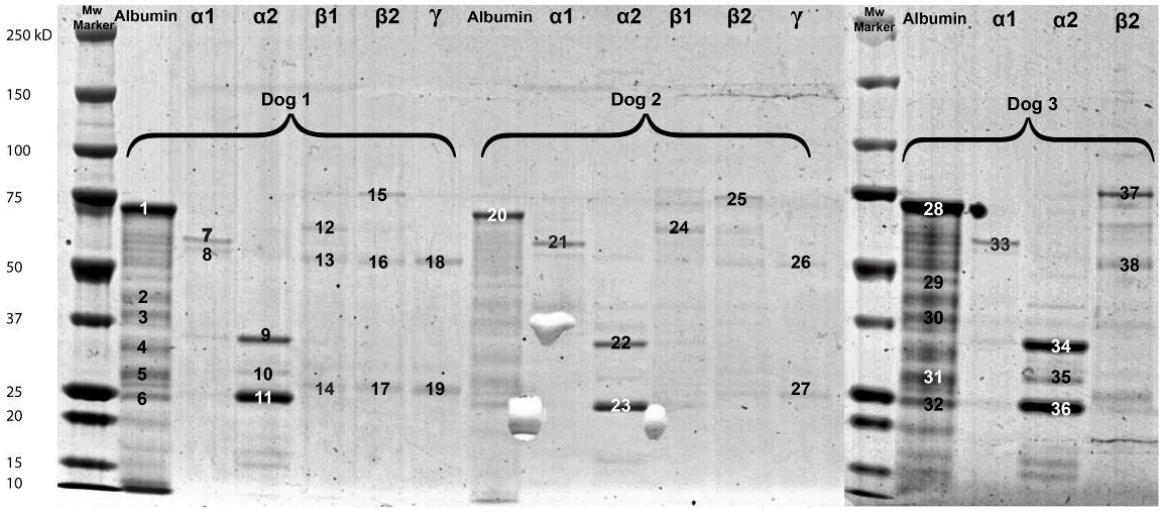


Figure 9 Second dimension polyacrylamide gel electrophoresis (PAGE) performed on three dogs with lymphoma: a 12 year-old male German shepherd diagnosed with stage IIIa lymphoma (dog 1), an eight year-old Boxer diagnosed with stage Vb lymphoma (dog 2) and a four year-old male Labrador retriever with stage IVb T-cell lymphoma (dog 3). Bands are labelled numerically corresponding to the proteins listed in Table 10. A molecular weight (Mw) control marker (Precision Plus Protein Standards All Blue, Bio-Rad) was run in first lanes of each of the 2 gels displayed here.

4.2.4 Mass Spectrometry

Submission of the data acquired from mass spectrometry performed on the 38 bands to the Mascot search engine generated a list of 36 different proteins using the criteria described within the materials and methods (Table 10). These proteins comprised known sequenced canine proteins, proteins predicted from the canine genome along with other mammalian proteins. Accession number, MOWSE score, number of peptides matched by mass spectrometry, sequence coverage and nominal mass were also recorded for each protein (Supplementary Table 2).

Table 10 Proteins identified by mass spectrometry within the bands labelled in Figure 9. Characters in bold represent proteins from the species *Canine lupus familiaris*. Characters in italics represent proteins predicted from known genomic sequences. All proteins listed correspond to a MOWSE score ≥64 (*P* <0.005). Highest MOWSE scores are listed first in each band.

Band identification	Protein name, species of origin and accession number
1	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** **Albumin [Canis lupus familiaris] gi|3319897**
2	**Albumin [Canis lupus familiaris] gi|3319897**
3	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764**
4	**Albumin [Canis lupus familiaris] gi|3319897**
5	**Albumin [Canis lupus familiaris] gi|3319897**
6	**Albumin [Canis lupus familiaris] gi|3319897** **Serum albumin precursor [Canis lupus familiaris] gi|55742764**
7	**Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** **Albumin [Canis lupus familiaris] gi|3319897** ***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106***
8	***Alpha 2 HS glycoprotein isoform 1 [Canis lupus familiaris] gi|359323766*** **Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** ***Vitamin D binding protein isoform 2 [Canis lupus familiaris] gi|73975215*** **Albumin [Canis lupus familiaris] gi|3319897**

9	**Haptoglobin [Canis lupus familiaris] gi|123511** Mutant beta-actin [Homo sapiens] gi|28336
10	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** **Haptoglobin [Canis lupus familiaris] gi|123511**
11	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** **Albumin [Canis lupus familiaris] gi|3319897** **Haptoglobin [Canis lupus familiaris] gi|123511** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681***
12	***Complement C3 [Canis lupus familiaris] gi|359322249*** ***Hemopexin [Canis lupus familiaris] gi|73988725*** ***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** ***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009**
13	*Histone cluster 1 [Oryctolagus cuniculus] gi|291410763* Selenium-binding protein 1 [Bos taurus] gi|114051361
14	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** **Immunoglobulin gamma heavy chain D [Canis lupus familiaris] gi|17066530** **Immunoglobulin heavy chain variable region [Canis lupus familiaris] gi|208342218** ***Uncharacterised protein LOC608320, partial [Canis lupus familiaris] gi|345806638***
15	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009**

16	**Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528** ***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142***
17	***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681*** ***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** ***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Apolipoprotein A1 Precursor [Canis lupus familiaris] gi|3915607**
18	**Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** **Immunoglobulin heavy chain V region [Canis lupus familiaris] gi|124389909** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528**
19	***Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687*** ***Immunoglobulin lambda-like polypeptide 5-like isoform 3 [Canis lupus familiaris] gi|345791535*** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526**
20	**Serum albumin precursor [Canis lupus familiaris] gi|55742764**
21	**Albumin [Canis lupus familiaris] gi|3319897** **Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** Alpha 2 macroglobulin [Sus scrofa] gi|41176597
22	**Haptoglobin [Canis lupus familiaris] gi|123511** **Clusterin precursor [Canis lupus familiaris] gi|50979240** **Apolipoprotein E [Canis lupus familiaris] gi|3915605** ***Alpha 2 macroglobulin [Canis lupus familiaris] gi|345792424***

23	*Apolipoprotein A-I [Canis lupus familiaris] gi|73955106* *Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681* **Haptoglobin [Canis lupus familiaris] gi|123511**
24	*Complement C3 [Canis lupus familiaris] gi|359322249* *Hemopexin [Canis lupus familiaris] gi|73988725* *Alpha 1 antichymotrypsin [Canis lupus familiaris] gi|73964432* *Lipopolysaccharide-binding protein [Canis lupus familiaris] gi|345789637* *Complement C4-A [Canis lupus familiaris] gi|359320893* *Antithrombin III isoform 1 [Canis lupus familiaris] gi|359320010* *Inter-alpha-trypsin inhibitor heavy chain H4 [Canis lupus familiaris] gi|345806499*
25	*Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142* *Complement C4-A [Canis lupus familiaris] gi|359320893*
26	**Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528** *Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*
27	*Apolipoprotein A-I [Canis lupus familiaris] gi|73955106* *Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995681* *Immunoglobulin lambda-like polypeptide 5-like [Canis lupus familiaris] gi|73995687* *Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142* *Uncharacterised protein LOC608320, partial [Canis lupus familiaris] gi|345806638*
28	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** **Albumin [Canis lupus familiaris] gi|3319897**

29	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** **Albumin [Canis lupus familiaris] gi|3319897**
30	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** **Albumin [Canis lupus familiaris] gi|3319897**
31	**Serum albumin precursor [Canis lupus familiaris] gi|55742764** **Albumin [Canis lupus familiaris] gi|3319897**
32	**Serum albumin precursor [Canis lupus familiaris] gi|55742764**
33	**Serpin peptidase inhibitor, clade A, member 1 precursor [Canis lupus familiaris] gi|121583756** **Albumin [Canis lupus familiaris] gi|3319897** ***Alpha 2 HS glycoprotein isoform 1 [Canis lupus familiaris] gi|359323766*** Alpha-amylase [Homo sapiens] gi|178585
34	**Haptoglobin [Canis lupus familiaris] gi|123511**
35	**Haptoglobin [Canis lupus familiaris] gi|123511** **Apolipoprotein A1 Precursor [Canis lupus familiaris] gi|3915607**
36	***Apolipoprotein A-I [Canis lupus familiaris] gi|73955106*** **Haptoglobin [Canis lupus familiaris] gi|123511**
37	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009** **Immunoglobulin heavy chain constant region CH4 [Canis lupus familiaris] gi|124390013** ***Complement C3 [Canis lupus familiaris] gi|359322249***

38	***Serotransferrin isoform 1 [Canis lupus familiaris] gi|73990142*** ***Complement C3 [Canis lupus familiaris] gi|359322249*** **Immunoglobulin gamma heavy chain C [Canis lupus familiaris] gi|17066528** **Immunoglobulin gamma heavy chain D [Canis lupus familiaris] gi|17066530** **Immunoglobulin gamma heavy chain B [Canis lupus familiaris] gi|17066526** **Immunoglobulin heavy chain constant region CH2 [Canis lupus familiaris] gi|124390009** Alpha-fibrinogen precursor [Homo sapiens] gi|182424

Thirty one individual proteins from the species *Canine lupus familiaris* were identified from polyacrylamide gels (Table 11). Ten canine proteins were identified in the lymphoma patients that had not previously been found in the proteome of the healthy dogs, including α2 HS glycoprotein, haptoglobin, clusterin precursor, α2 macroglobulin, apolipoprotein A-1 precursor, apolipoprotein E, α1 antichymotrypsin, lipopolysaccharide binding protein, antithrombin III and inter-α-trypsin inhibitor.

Table 11 *Canine lupus familiaris* proteins identified in their respective protein fraction from the serum of three dogs diagnosed with multicentric lymphoma. Characters in italics represent proteins predicted from known genomic sequences. * Indicates the proteins found in dogs with lymphoma but not seen in healthy control dogs.

Albumin	**Alpha 1**	**Alpha 2**	**Beta 1**	**Beta 2**	**Gamma**
Albumin	Albumin	*a2 Macroglobulin**	*a1 antichymotrypsin**	Apolipoprotein A-1 Precursor*	*Apolipoprotein A-1*
Serum albumin precursor	*a2 HS glycoprotein**	*Apolipoprotein A-1*	*Antithrombin III**	*Complement C3*	Immunoglobulin heavy chains
	Apolipoprotein A-1	Apolipoprotein A-1 Precursor*	*Apolipoprotein A-1*	*Complement C4*	*Immunoglobulin λ-like Polypeptide*
	Serpin peptidase inhibitor	Apolipoprotein E*	*Complement C3*	Immunoglobulin heavy chains	*Serotransferrin isoform 1*
	Vitamin D binding protein	Clusterin Precursor*	*Complement C4*	*Immunoglobulin λ- like Polypeptide*	*Uncharacterized Protein*
		Haptoglobin*	*Hemopexin*	*Serotransferrin isoform 1*	
		Immunoglobulin λ-like Polypeptide	Immunoglobulin heavy chains		
			Immunoglobulin λ-like Polypeptide		
			*Inter-a-trypsin inhibitor**		
			Lipopolysaccharide binding protein*		
			Serotransferrin isoform 1		
			Uncharacterized Protein		

The migration pattern seen within the albumin fraction was similar to that observed within the normal patients previously reported. Likewise apolipoprotein A1 was also found in multiple gel fractions. All three gels of the lymphoma patients had an extra band within the α2 fraction (bands 9, 22 and 34), which was identified as haptoglobin. Kininogen was not found in any of the α-1 bands from the lymphoma patients. The same protein or groups of proteins were identified in the 12 bands identified across all 3 gels or the 10 bands identified in 2 gels.

4.2.5 Haptoglobin and C-Reactive Protein Concentrations

Haptoglobin and CRP assays were performed in all 21 patients. Both the median CRP and haptoglobin concentrations were elevated above our reference intervals. The median haptoglobin concentration was 3.85 g/L with range from 0.03- 14.29 g/L (reference range 0- 3 g/L). All 3 of the patients selected for further analysis of sera using 2D PAGE had significantly elevated haptoglobin concentrations (10.71 g/L, 6.37 g/L and 14.29 g/L). The median CRP concentration was 32.98 mg/L and patient values ranged from 2.9- 198.1 mg/L (reference range 0-10 mg/L). CRP concentrations for the 3 dogs that underwent further 2D analysis were 5.89 mg/L (dog 1 suffering from stage IIIa lymphoma), 174.68 mg/L (dog 2 suffering from stage Vb lymphoma) and 32.98 mg/L (dog 3 suffering from stage IVb lymphoma).

4.3 Discussion

The work performed here has demonstrated significant differences in the electrophoretograms of dogs diagnosed with untreated canine lymphoma compared to healthy control dogs. Although only a small number of patients were selected for 2nd dimension PAGE and more uniform results might have been obtained using a single phenotype and stage of lymphoma in order to

minimise the variation exerted on the proteome by this naturally occurring tumour, numerous differences were determined between lymphoma and healthy dogs. The technique did not allow concentrations of proteins to be assessed accurately but documented the presence or absence of a protein. Using a high MOWSE score the presence of proteins in canine lymphoma was determined with high levels of confidence but may risk excluding some proteins that are expressed at lower levels within the serum.

Analysis of the data generated from the 1D SPE allowed us to compare the different protein fractions between healthy and control dogs. The total protein values and albumin levels were both reduced for patients suffering from lymphoma. Albumin, which is the most abundant protein within serum is a negative acute phase protein and its concentration will fall gradually in infectious and inflammatory disease therefore this change is not unexpected. ß globulin levels were not increased in our patients with lymphoma so this is discordant to previously documented data by Gavazza et al. (2009) however, little information was given with regards to the methodology of the SPE performed in this work and it is known that results generated from SPE can vary between different laboratories owing to alterations in equipment used and interpretation of the data generated (Tappin et al., 2011). α2 globulins were significantly higher in our lymphoma patients with respect to the control group. Work performed by Malpas and Fairley (1964) documented an increase in α-2 globulins in humans suffering from Hodgkin's disease and concluded that this was due to alterations in not just a single protein but rather a group of proteins.

Mass spectrometry performed on the bands observed within the second dimension PAGE gels allowed us to identify a large number of proteins with a high level of confidence ($P<0.005$). Many proteins previously identified within our control population were also present within the lymphoma subset and their respective distributions within the different serum globulin fractions was similar allowing us to conclude that this technique is reproducible in a diseased state. In addition 10 proteins were identified exclusively in lymphoma patients.

At least 1 extra band was identified for all three dogs with lymphoma in the α-2 lane (bands 9, 22 and 34). Mass spectrometry performed on this band revealed it to be haptoglobin . Haptoglobin is an acute phase protein which is increased in a variety of inflammatory diseases in dogs (Murata et al., 2004). Haptoglobin production can be induced by the administration of exogenous corticosteroids and increased levels are also observed in naturally occurring hyperadrenocorticism in dogs (Eckersall, 2008). Mischke et al (2007) documented increased levels of haptoglobin as well as C-reactive protein (CRP) in dogs with lymphatic neoplasia. Dogs with acute lymphoid leukaemia had the highest levels of these proteins but increased concentrations were also observed in dogs with malignant lymphoma and multiple myeloma when compared to healthy dogs. A commercially available test measuring serum concentrations of haptoglobin and CRP and applying these results in a Classification and Regression Tree (CART) analysis to diagnose canine lymphoma is now available[7]. Haptoglobin was increased in both Hodgkin's and non Hodgkin's lymphoma and partially accounted for elevations of the α2 globulin group in people with lymphoid neoplasia (Malpas and Fairley, 1964). The values obtained from our haptoglobin and CRP assays would support this finding as well as corroborating the inflammatory nature of canine multicentric lymphoma. CRP was not identified in our study using 2D-PAGE but was elevated in serum on immunoturbimetric assay. This is probably due to the fact that serum concentrations of CRP are too low to be identified using this method (CRP concentrations are mg/L whereas haptoglobin is g/L). Increased levels of IL-6 have been recorded in some human patients suffering from non Hodgkin's lymphoma. Although it was not identified in our canine lymphoma patients IL-6 is known to play an important role in B-cell maturation as well as proliferation in some B-cell malignancies (Legouffe et al., 1998). Increases in haptoglobin may therefore be due to elevations of IL-6 which is one of the major cytokines behind the acute phase response (Murata et al., 2004). As was the case for albumin and apolipoprotein A-1, haptoglobin was present in more than one band in individual dogs. Possible explanations for this include the presence of different isoforms of haptoglobin, fragmentation during electrophoresis of the protein into smaller peptides, post translational

[7] PetScreen Ltd, Biocity, Nottingham, UK

modification and “smearing”, as was postulated for the other proteins observed across multiple bands.

A number of other acute phase reactants including α2 macroglobulin, inter-α-trypsin inhibitor and lipopolysaccharide binding protein were identified in dogs with lymphoma again suggesting an inflammatory serum component to this pathologic process. It is possible that changes in these acute phase reactants may serve as future biomarkers in canine lymphoma. Many studies have sought to discover biomarkers in canine oncology. These have been recently reviewed (Henry, 2010) and are of particular current interest as they can have ramifications for diagnosis, stage, prognosis and monitoring of remission status in a wide variety of tumours.

Most dogs in our lymphoma population were stage V and substage b. Although many lymphoma dogs do not exhibit systemic signs (Vail and Young, 2007) the increased number of substage b animals in our study may be related to the high number of patients presenting with advanced stage V disease. This in turn may also reflect the inflammatory nature of the serum changes that were detected. Although one of the dogs (dog 3) that had further characterisation of its serum proteome received a short course of prednisolone prior to serum collection, which may have induced the production of haptoglobin, this dog also had an elevated CRP concentration. CRP concentrations are not significantly induced in dogs receiving corticosteroids (Martinez- Subiela et al., 2004) suggesting that the elevated CRP in dog 3 reflects a genuine acute inflammatory process and that the haptoglobin changes in the serum analysis in this patient could not be entirely explained by treatment with exogenous prednisolone. The other 2 patients did not receive any exogenous steroids therefore it can be concluded that this is not the sole reason for the elevations of serum haptoglobin concentrations observed.

α2 HS glycoprotein also known as fetuin-A was found in the sera of 2 lymphoma patients but was not present in either of the healthy controls. In people α-2 HS

glycoprotein is known to be a negative acute phase protein (Lebreton et al., 1979) but there is no direct evidence of this being the case in the dog and to the best of the authors' knowledge this protein has not previously been identified in dog serum. Fetuin-A has been recognised in the serum of lymphoma bearing mice and was proposed by the authors of this study to have a role in tumour progression (Caballero-Hernández et al., 2009). Contrary to this another study demonstrated decreases in the serum concentrations of α2 HS glycoprotein in people diagnosed with haematological malignancies including lymphoma. Further work is needed to find out what role, if any, α2 HS glycoprotein has in the pathogenesis of canine lymphoma.

Clusterin precursor was also identified in the α2 band of one of the lymphoma patients that was not receiving steroids (dog 2). Clusterin (CLU), also known as apoliprotein J, is a glycoprotein that exists in 2 different isoforms in human cells. To the best of the authors' knowledge this is the first time that clusterin has been identified in the serum of a dog with lymphoma. The nuclear form of the protein nCLU is thought to have pro-apoptotic roles in the cell which is in contrast to the secretory form sCLU which displays pro-survival properties (Shannan et al., 2006). Immunohistological evaluation of clusterin in human malignant lymphoma revealed high expression levels in systemic anaplastic large cell lymphoma as well as lower levels in a variety of other malignant lymphoid neoplasms (Saffer et al., 2002). Clusterin may well be an important anti-apoptotic protein in canine lymphoma alongside other known anti-apoptotic proteins such as survivin (Rebhun et al., 2008).

One of the proteins identified in multiple bands in both the lymphoma and control animals was apolipoprotein A-1. Apolipoprotein A-1 precursor was seen in multiple areas in the polyacrylamide gels of the lymphoma patients but was not identified in the 2 normal healthy controls. Apoplipoprotein E was found in one of the lymphoma patients and is known to have many diverse processes including reverse cholesterol transport, neuronal degeneration and immunomodulation (Luo et al., 1989). Possible reasons for this protein being present in a lymphoma patient and not in our healthy control patients are not clear at this time.

Interestingly kininogen (HMWK) was absent in all of 3 of the lymphoma patients but was present in both the α1 lanes of the control dogs previously reported. Kininogen acts as a precursor to the inflammatory protein bradykinin as well as being a cofactor for the coagulation factor XII involved in the intrinsic coagulation pathway (Schmaier and McCrae, 2007). It is possible that levels of kininogen may be reduced in the dogs with lymphoma due to consumption of this protein as part of a pro-inflammatory response in lymphoid neoplasia. Mischke et al. (1998) reported prolonged clotting times as demonstrated by increases in PT and aPPT in dogs with acute lymphoblastic leukaemia. This may in part be a consequence of reductions of HMWK concentrations in dogs with lymphoid neoplasia. Alongside its roles in inflammation and coagulation HMWK is also thought to have an anti-apoptotic and positive proliferative effect on endothelial and other cells (Schmaier and McCrae, 2007). Antithrombin III, a serine proteinase inhibitor, was found in one of the patients with lymphoma but was absent in both control patients. Antithrombin III acts as an anticoagulant and when deficient can lead to hypercoagulable states in the dog (Hibbetts et al., 1999). It is therefore conceivable that increases in antithrombin III concentrations could also be responsible for the increased clotting times reported by Mischke et al. (1998).

One limitation identified is that the lymphoma patients were older than the control dogs. This is not unexpected as the controls were blood donor dogs for which younger animals are recruited in comparison to selecting for a population of dogs with lymphoma; a disease which is more prevalent in middle age to elderly dogs (Vail and Young, 2007). The extent to which aging would alter the proteome in dogs is unknown. In a cross sectional study of the serum proteome in people of varying age groups some proteins were differentially expressed with age, however when analysing changes in these proteins in an attempt to classify a second set of individual's sera into age categories these proteins were not found to be an accurate predictor of age (Byerley et al., 2010). Another study focusing on immunoglobulin concentrations found these to decrease with age (Buckley and Dorsey, 1970). Ideally in this thesis the control and lymphoma groups would have been age matched but further work is required in dogs and humans to fully identify the effect of aging on the serum proteome. Only 9 of

the dogs with lymphoma had their immunophenotype recorded therefore statistical analysis to interrogate differences in the respective proteomes was not adequately powered. Future studies comparing possible differences between T-cell and B-cell lymphomas would help shed light on the effect of phenotype on the composition of the serum proteome.

CHAPTER V

CONCLUSIONS

5 Conclusions

This thesis has reported a novel technique allowing the identification of many proteins present within the serum proteome of healthy dogs, as well as allowing the stratification of the respective proteins within their serum globulin subclass. The method proposed alleviates the technical difficulties associated with using IEF and setting up IPGs. Widespread application of this type of 2D electrophoresis could give further information about the nature of changes in serum proteins in many different diseases of the domestic dog and other species and will help with the interpretation of traditional 1D agarose gel SPE.

The second part of the results presented in this thesis has documented many changes present in the dog's serum proteome with untreated advanced stage lymphoma despite having no increased protein concentrations on routine biochemistry. Application of this technique to a disease state has demonstrated its value in mapping changes of the canine serum proteome however, only three dogs with lymphoma and two healthy animals had full analysis performed therefore confirmation of these changes with increased population sizes will be necessary in the future. In addition some of the proteins unique to the lymphoma population were found in only one of the respective patients' sera therefore their significance is unclear. It would therefore be prudent to focus future studies on possible biomarkers for lymphoma on the proteins identified in either all 3 patients with lymphoma (e.g. haptoglobin) or at least 2 of the patients (e.g. α2 HS glycoprotein). Conversely as this technique may not be sensitive in detection of proteins present at lower circulating concentrations ignoring proteins found only in single patients (e.g. clusterin) may risk disregarding potential future biomarkers.

The proteins found would support a significant inflammatory component to this particular type of tumour as has been found in multiple studies in human lymphoid neoplasia. Although the majority of IL-6 is produced from endothelial

cells as well as fibroblasts and monocytes, IL-6 production from human neoplastic cell lines including a leukaemia and T cell lymphoma line has been documented (Heinrich et al., 1990). Another study found that a human histiocytic lymphoma cell line was capable of producing the pro-inflammatory cytokine IL-1 (Palacios et al., 2005) and work by Macia et al. (1996) documented increased serum concentrations of TNF-α in people diagnosed with non-Hodgkin's lymphoma. A "host response" subset of diffuse large B-cell lymphoma was identified in a study looking to further characterise this disease and was based on increased numbers of infiltrating non-neoplastic lymphocytes, natural killer cells and professional antigen presenting cells within neoplastic tissue (Monti et al., 2005). Further work is needed to shed light on the possible role of IL-6 and other cytokines in aetiopathogenesis of canine lymphoma as well as the acute phase response. Serial measurements of some of the acute phase proteins, such as haptoglobin, may be informative with regards to prognosis and response to therapy. Concentrations of IL-6 alongside acute phase reactants have been measured previously in dogs following noxious stimuli in an experimental setting (Yamashita et al., 1994) however, cytokine concentrations have not been measured serially alongside these proteins in canine lymphoma cases seen in the clinic. Serial measurements of IL-6 and haptoglobin during and after treatment may help give further information on the pathogenesis of this disease and could also be useful as markers of disease remission or progression.

Although changes in coagulation times and clotting factor concentrations have been reported in dogs with acute lymphoblastic leukaemia (Mischke et al., 1998) there are no specific studies assessing coagulation function in multicentric canine lymphoma. The alterations in the proteome documented here could also support alterations to clotting ability but future studies measuring coagulation times as well as serum concentrations of ATIII and kininogen are needed to further characterise this.

Two other potential candidate biomarkers identified in this thesis and worthy of future study are clusterin and α2 HS glycoprotein. An ELISA test has been validated to measure urinary clusterin in the dog (García-Martínez et al., 2012).

Applying this assay to the serum of larger numbers of dogs with lymphoma is warranted to assess its possible role as a biomarker as well as measuring concentrations in different stages and classes of the disease. To the best of the authors' knowledge no such assay exists for canine α2 HS glycoprotein however, in a study looking at people diagnosed with glioblastoma decreased concentrations of this protein demonstrated using turbidimetry were correlated with decreased survival times (Petrik et al., 2008). Any ongoing research assessing the potential suitability of α2 HS glycoprotein would first require either validation of this method in the canine species or alternatively development of a novel assay. Individual biomarkers may in certain scenarios be of limited use but by combining different circulating proteins into panels it is possible to develop minimally invasive clinical screening tests and one such test exists for people with gastric adenocarcinoma (Ahn et al., 2012).

The ultimate future goal would be to combine serum biomarkers with information gained from cytology, histology, immunophenotyping and molecular diagnostics of canine lymphoma patients to give an accurate prognosis for individuals with the next step being tailoring of therapy to individual patients. Such an approach is already being applied to certain human malignancies, for example the presence of mutations in BCR-ABL in chronic myeloid leukaemia not only helps predict prognosis but also informs the oncologist as to the most appropriate treatment regime for this malignancy (Redaelli et al., 2009).

This two dimensional electrophoresis technique could be applied to many other tumour types, amongst other conditions, in order to further knowledge on the pathogenesis of the respective disease and may help uncover potential candidate biomarkers. As this method is relatively easy to carry out in a clinical diagnostic pathology lab other tumour serum proteomes could be interrogated however, one disadvantage is the requirement for mass spectrometry which is costly and requires specialist laboratory equipment. Mast cell tumours could be considered for proteomic analysis as they are one of the most commonly encountered cutaneous tumours in dogs (Villamil et al., 2011). Canine patients diagnosed with mast cell tumours are predisposed to gastrointestinal ulceration,

alterations in serum concentrations of histamine and gastrin have also been documented (Fox et al., 1990). Application of this method to characterise the proteome of dogs with mast cell neoplasia could help validate the usefulness of this thesis and add valuable insight to another significant neoplasm diagnosed commonly in dogs.

APPENDIX

Supplementary Table 1 List of all proteins found in their respective bands labelled in Figure 7 including species of origin, accession number, MOWSE score, number of peptides matched by mass spectrometry, sequence coverage, nominal mass and the fraction in which each protein was found

ID	Protein name	Species of origin	Accession number	MOWSE score	Number of peptides matched	Sequence coverage (%)	Nominal mass (Da)	Fraction in which found
1	Albumin	[Canis lupus familiaris]	gi|3319897	3395	206	71	67857	Albumin
2	Albumin	[Canis lupus familiaris]	gi|3319897	1902	110	68	67857	Albumin
2	*Serum albumin-like isoform 1*	*[Ailuropoda melanoleuca]*	*gi|301786252*	*897*	*15*	*26*	*70619*	*Albumin*
3	Albumin	[Canis lupus familiaris]	gi|3319897	1467	86	69	67857	Albumin
4	Albumin	[Canis lupus familiaris]	gi|3319897	788	47	54	67857	Albumin
4	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	762	46	50	70556	Albumin
4	*Alpha 2 macroglobulin*	*[Sus scrofa]*	*gi|335288480*	*318*	*24*	*15*	*165223*	*Albumin*
4	*T-cell receptor beta chain T17T-22-like*	*[Sus scrofa]*	*gi|350592642*	*188*	*4*	*19*	*27584*	*Albumin*
4	Hemoglobin subunit alpha	[Sus scrofa]	gi|122465	98	5	30	15087	Albumin

4	Hemoglobin subunit beta	[Ailurus fulgens]	gi|122553	85	3	23	16054	Albumin
4	Inter-alpha (globulin)-inhibitor H4	[Bos taurus]	gi|59857769	77	1	1	101617	Albumin
4	*Serpin A3-6 [Sus scrofa]*	*[Sus scrofa]*	*gi|194038353*	*75*	*3*	*4*	*47363*	*Albumin*
4	*Alpha 1B glycoprotein-like*	*[Sus scrofa]*	*gi|311259609*	*65*	*3*	*6*	*54933*	*Albumin*
5	Serpin peptidase inhibitor, clade A member 1 precursor	[Canis lupus familiaris]	gi|121583756	256	29	30	46505	Alpha 1
5	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*128*	*8*	*31*	*30163*	*Alpha 1*
5	*Kininogen-1 isoform 2*	*[Canis lupus familiaris]*	*gi|57109938*	*114*	*5*	*16*	*49400*	*Alpha 1*
6	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*526*	*39*	*77*	*30163*	*Alpha 1*
6	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*155*	*12*	*21*	*67857*	*Alpha 1*
6	*Vitamin D binding protein isoform 2*	*[Canis lupus familiaris]*	*gi|73975215*	*76*	*3*	*6*	*54536*	*Alpha 1*
6	*Kininogen-1 isoform 2*	*[Canis lupus familiaris]*	*gi|57109938*	*66*	*2*	*5*	*49400*	*Alpha 1*
7	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*2392*	*126*	*77*	*30163*	*Alpha 2*

8	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*818*	*49*	*26*	*182611*	*Beta 1*
8	*Hemopexin*	*[Canis lupus familiaris]*	*gi|73988725*	*154*	*15*	*29*	*52047*	*Beta 1*
8	*Complement C4-A*	*[Canis lupus familiaris]*	*gi|359320893*	*105*	*4*	*2*	*200183*	*Beta 1*
9	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*805*	*54*	*50*	*80222*	*Beta 2*
9	Immunoglobulin heavy chain constant region CH2	[Canis lupus familiaris]	gi|124390009	113	4	28	12398	Beta 2
9	*Complement C4-A*	*[Canis lupus familiaris]*	*gi|359320893*	*75*	*1*	*0*	*200183*	*Beta 2*
10	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*353*	*28*	*25*	*25078*	*Beta 2*
10	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	246	18	22	52779	Beta 2
10	Immunoglobulin gamma heavy chain A	[Canis lupus familiaris]	gi|17066524	125	9	12	52383	Beta 2
10	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*152*	*9*	*6*	*182611*	*Beta 2*
10	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	126	10	16	52553	Beta 2

11	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*285*	*16*	*59*	*13434*	*Beta 2*
11	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*276*	*16*	*21*	*80222*	*Beta 2*
11	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*250*	*12*	*53*	*15118*	*Beta 2*
11	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*106*	*9*	*28*	*30163*	*Beta 2*
12	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	337	25	27	52553	Gamma
12	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	182	10	20	52779	Gamma
13	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*499*	*29*	*70*	*13434*	*Gamma*
13	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*466*	*27*	*63*	*15118*	*Gamma*
13	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	71	4	8	52553	Gamma
13	Immunoglobulin lambda light chain variable region	[Canis lupus familiaris]	gi|164430518	65	1	10	13807	Gamma
14	Albumin	[Canis lupus familiaris]	gi|3319897	3584	227	71	67857	Albumin

14	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	3492	223	70	70556	Albumin
15	Albumin	[Canis lupus familiaris]	gi|3319897	846	47	58	67857	Albumin
15	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	817	46	55	70556	Albumin
15	Mutant beta-actin	[Homo sapiens]	gi|28336	179	11	28	42128	Albumin
15	Histone H3	[Heterocephalus glaber]	gi|351710220	154	9	29	25151	Albumin
15	Gamma-actin	[Homo sapiens]	gi|178045	134	5	18	26147	Albumin
15	Chain A, Crystal Structure Of Human Galectin-7 In Complex With Galactosamine	[Homo sapiens]	gi|3891470	94	5	34	14992	Albumin
16	Albumin	[Canis lupus familiaris]	gi|3319897	1348	83	69	67857	Albumin
17	Albumin	[Canis lupus familiaris]	gi|3319897	1089	68	61	67857	Albumin
18	Albumin	[Canis lupus familiaris]	gi|3319897	1255	75	64	67857	Albumin
18	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	1169	74	61	70556	Albumin
19	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	863	52	58	70556	Albumin

19	Apolipoprotein A-I	[Canis lupus familiaris]	gi|73955106	82	5	19	30163	Albumin
20	Serpin peptidase inhibitor, clade A, member 1 precursor	[Canis lupus familiaris]	gi|121583756	288	19	22	46505	Alpha 1
20	Albumin	[Canis lupus familiaris]	gi|3319897	76	5	8	67857	Alpha 1
20	*Kininogen-1 isoform 2*	*[Canis lupus familiaris]*	*gi|57109938*	*76*	*2*	*6*	*49400*	*Alpha 1*
20	*Vitamin D binding protein isoform 2*	*[Canis lupus familiaris]*	*gi|73975215*	*66*	*2*	*4*	*54536*	*Alpha 1*
21	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*1453*	*78*	*77*	*30163*	*Alpha 2*
22	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*1237*	*72*	*61*	*80222*	*Beta 2*
22	Immunoglobulin heavy chain constant region CH2	[Canis lupus familiaris]	gi|124390009	153	5	28	12398	Beta 2
22	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*99*	*12*	*7*	*182611*	*Beta 2*
22	*Complement C4-A*	*[Canis lupus familiaris]*	*gi|359320893*	*70*	*4*	*2*	*200183*	*Beta 2*
23	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*391*	*27*	*30*	*80222*	*Beta 2*

23	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	179	12	17	52779	Beta 2
23	Immunoglobulin gamma heavy chain A	[Canis lupus familiaris]	gi|17066524	109	8	12	52383	Beta 2
23	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*106*	*6*	*4*	*182611*	*Beta 2*
23	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	65	11	22	52553	Beta 2
24	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*190*	*17*	*21*	*80222*	*Beta 2*
24	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*182*	*13*	*49*	*15118*	*Beta 2*
24	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|345791535*	*172*	*15*	*30*	*24916*	*Beta 2*
24	*Complement C3*	[Canis lupus familiaris]	gi|359322249	103	8	4	182611	Beta 2
24	*Uncharacterized protein LOC608320, partial*	*[Canis lupus familiaris]*	*gi|345806638*	*74*	*2*	*6*	*40739*	*Beta 2*
25	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	216	19	23	52553	Gamma
25	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	215	11	17	52779	Gamma

26	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|345791535*	*378*	*22*	*33*	*24916*	*Gamma*
26	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*331*	*17*	*49*	*15118*	*Gamma*
26	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	80	5	9	52553	Gamma
26	*Uncharacterized protein LOC608320, partial*	*[Canis lupus familiaris]*	*gi|345806638*	*75*	*2*	*6*	*40739*	*Gamma*

Supplementary Table 2 List of all proteins found in their respective bands labelled in Figure 9 including species of origin, accession number, MOWSE score, number of peptides matched by mass spectrometry, sequence coverage, nominal mass and the fraction in which each protein was found.

ID	Protein name	Species of origin	Accession number	MOWSE score	Number of peptides matched	Sequence coverage (%)	Nominal mass (Da)	Fraction in which found
1	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	2388	45	72	70556	Albumin
1	Albumin	[Canis lupus familiaris]	gi|3319897	2371	147	67	67857	Albumin
2	Albumin	[Canis lupus familiaris]	gi|3319897	1441	92	58	67857	Albumin
3	Albumin	[Canis lupus familiaris]	gi|3319897	1881	104	62	67857	Albumin
3	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	1805	102	59	70556	Albumin
4	Albumin	[Canis lupus familiaris]	gi|3319897	1598	100	65	67857	Albumin
5	Albumin	[Canis lupus familiaris]	gi|3319897	1748	108	61	67857	Albumin
6	Albumin	[Canis lupus familiaris]	gi|3319897	1681	100	64	67857	Albumin
6	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	1648	100	61	70556	Albumin
7	Serpin peptidase inhibitor, clade A, member 1 precursor	[Canis lupus familiaris]	gi|121583756	260	16	22	46505	Alpha 1
7	Albumin	[Canis lupus familiaris]	gi|3319897	128	9	15	67857	Alpha 1
7	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*74*	*3*	*10*	*30163*	*Alpha 1*

8	*Alpha 2 HS glycoprotein isoform 1*	*[Canis lupus familiaris]*	*gi|359323766*	*145*	*8*	*18*	*40021*	*Alpha 1*
8	Serpin peptidase inhibitor, clade A, member 1 precursor	[Canis lupus familiaris]	gi|121583756	136	10	16	46505	Alpha 1
8	*Vitamin D binding protein isoform 2*	*[Canis lupus familiaris]*	*gi|73975215*	*80*	*10*	*15*	*54536*	*Alpha 1*
8	Albumin	[Canis lupus familiaris]	gi|3319897	77	11	17	67857	Alpha 1
9	Haptoglobin	[Canis lupus familiaris]	gi|123511	382	43	45	36890	Alpha 2
9	Mutant beta-actin (beta'-actin)	[Homo sapiens]	gi|28336	96	4	10	42128	Alpha 2
10	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*275*	*14*	*42*	*30163*	*Alpha 2*
10	Haptoglobin	[Canis lupus familiaris]	gi|123511	186	29	39	36890	Alpha 2
11	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*2013*	*100*	*72*	*30163*	*Alpha 2*
11	Albumin	[Canis lupus familiaris]	gi|3319897	500	27	35	67857	Alpha 2
11	Haptoglobin	[Canis lupus familiaris]	gi|123511	98	13	24	36890	Alpha 2
11	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*87*	*3*	*23*	*13434*	*Alpha 2*
12	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*742*	*52*	*28*	*182611*	*Beta 1*
12	*Hemopexin*	*[Canis lupus familiaris]*	*gi|73988725*	*143*	*21*	*36*	*52047*	*Beta 1*
12	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*108*	*7*	*9*	*80222*	*Beta 1*
12	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*79*	*6*	*19*	*30163*	*Beta 1*
12	Immunoglobulin heavy chain constant region CH2	[Canis lupus familiaris]	gi|124390009	77	2	20	12398	Beta 1

13	*Histone cluster 1, H2ag-like*	*[Oryctolagus cuniculus]*	*gi|291410763*	*99*	*5*	*16*	*27347*	*Beta 1*
13	Selenium-binding protein 1	[Bos taurus]	gi|114051361	90	3	7	53092	Beta 1
14	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*311*	*25*	*69*	*30163*	*Beta 1*
14	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*217*	*12*	*55*	*13434*	*Beta 1*
14	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*193*	*8*	*49*	*15118*	*Beta 1*
14	Immunoglobulin gamma heavy chain D	[Canis lupus familiaris]	gi|17066530	97	6	12	52169	Beta 1
14	Immunoglobulin heavy chain variable region	[Canis lupus familiaris]	gi|208342218	76	2	24	13143	Beta 1
14	*Uncharacterized protein LOC608320, partial*	*[Canis lupus familiaris]*	*gi|345806638*	*75*	*2*	*6*	*40739*	*Beta 1*
15	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*499*	*36*	*43*	*80222*	*Beta 2*
15	Immunoglobulin heavy chain constant region CH2	[Canis lupus familiaris]	gi|124390009	83	3	21	12398	Beta 2
16	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	129	8	17	52779	Beta 2
16	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*106*	*10*	*13*	*80222*	*Beta 2*
17	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*293*	*17*	*44*	*13434*	*Beta 2*
17	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*277*	*13*	*36*	*15118*	*Beta 2*
17	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*163*	*13*	*16*	*80222*	*Beta 2*
17	Apolipoprotein A1 Precursor	[Canis lupus familiaris]	gi|3915607	74	13	40	30178	Beta 2
18	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	200	16	23	52553	Gamma

18	Immunoglobulin heavy chain V region	[Canis lupus familiaris]	gi|124389909	107	3	20	15280	Gamma
18	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	100	7	15	52779	Gamma
19	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*299*	*20*	*49*	*15118*	*Gamma*
19	*Immunoglobulin lambda-like polypeptide 5-like isoform 3*	*[Canis lupus familiaris]*	*gi|345791535*	*285*	*21*	*32*	*24916*	*Gamma*
19	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	149	10	16	52553	Gamma
20	Serum albumin precursor	[Canis lupus familiaris]	gi|55742764	1712	99	56	70556	Albumin
21	Albumin	[Canis lupus familiaris]	gi|3319897	193	14	23	67857	Alpha 1
21	Serpin peptidase inhibitor, clade A, member 1 precursor	[Canis lupus familiaris]	gi|121583756	173	16	19	46505	Alpha 1
21	Alpha-2-macroglobulin	[Sus scrofa]	gi|41176597	67	1	9	15357	Alpha 1
22	Haptoglobin	[Canis lupus familiaris]	gi|12351	463	60	45	36890	Alpha 2
22	Clusterin precursor	[Canis lupus familiaris]	gi|50979240	119	9	17	52327	Alpha 2
22	Apolipoprotein E	[Canis lupus familiaris]	gi|3915605	116	7	15	35332	Alpha 2
22	*Alpha 2 macroglobulin*	*[Canis lupus familiaris]*	*gi|345792424*	*81*	*4*	*2*	*166540*	*Alpha 2*
23	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*1427*	*83*	*74*	*30163*	*Alpha 2*
23	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*86*	*3*	*23*	*13434*	*Alpha 2*
23	Haptoglobin	[Canis lupus familiaris]	gi|123511	75	12	24	36890	Alpha 2

24	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*768*	*56*	*27*	*182611*	*Beta 1*
24	*Hemopexin*	*[Canis lupus familiaris]*	*gi|73988725*	*139*	*14*	*32*	*52047*	*Beta 1*
24	*Alpha 1 antichymotrypsin*	*[Canis lupus familiaris]*	*gi|73964432*	*130*	*7*	*20*	*47338*	*Beta 1*
24	*Lipopolysaccharide-binding protein*	*[Canis lupus familiaris]*	*gi|345789637*	*126*	*4*	*11*	*53444*	*Beta 1*
24	*Complement C4-A*	*[Canis lupus familiaris]*	*gi|359320893*	*104*	*8*	*5*	*200183*	*Beta 1*
24	*Antithrombin III isoform 1*	*[Canis lupus familiaris]*	*gi|359320010*	*100*	*6*	*13*	*53003*	*Beta 1*
24	*Inter-alpha-trypsin inhibitor heavy chain H4*	*[Canis lupus familiaris]*	*gi|345806499*	*94*	*5*	*5*	*101512*	*Beta 1*
25	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*622*	*35*	*45*	*80222*	*Beta 2*
25	*Complement C4-A*	*[Canis lupus familiaris]*	*gi|359320893*	*74*	*1*	*0*	*200183*	*Beta 2*
26	Immunoglobulin gamma heavy chain B	[Canis lupus familiaris]	gi|17066526	224	15	22	52553	Gamma
26	Immunoglobulin gamma heavy chain C	[Canis lupus familiaris]	gi|17066528	165	7	17	52779	Gamma
26	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*157*	*12*	*18*	*80222*	*Gamma*
27	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*253*	*16*	*56*	*30163*	*Gamma*
27	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995681*	*206*	*16*	*58*	*13434*	*Gamma*
27	*Immunoglobulin lambda-like polypeptide 5-like*	*[Canis lupus familiaris]*	*gi|73995687*	*201*	*12*	*48*	*15118*	*Gamma*
27	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*90*	*8*	*9*	*80222*	*Gamma*
27	*Uncharacterized protein LOC608320, partial*	*[Canis lupus familiaris]*	*gi|345806638*	*79*	*2*	*6*	*40739*	*Gamma*

28	*Serum albumin precursor*	*[Canis lupus familiaris]*	*gi|55742764*	*2781*	*176*	*68*	*70556*	*Albumin*
28	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*2730*	*168*	*67*	*67857*	*Albumin*
29	*Serum albumin precursor*	*[Canis lupus familiaris]*	*gi|55742764*	*1764*	*99*	*68*	*70556*	*Albumin*
29	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*1720*	*97*	*69*	*67857*	*Albumin*
30	*Serum albumin precursor*	*[Canis lupus familiaris]*	*gi|55742764*	*1909*	*107*	*61*	*70556*	*Albumin*
30	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*1864*	*103*	*61*	*67857*	*Albumin*
31	*Serum albumin precursor*	*[Canis lupus familiaris]*	*gi|55742764*	*1578*	*95*	*56*	*70556*	*Albumin*
31	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*1540*	*94*	*56*	*67857*	*Albumin*
31	*Serum albumin-like isoform 1*	*[Ailuropoda melanoleuca]*	*gi|301786252*	*619*	*39*	*26*	*70619*	*Albumin*
32	*Serum albumin precursor*	*[Canis lupus familiaris]*	*gi|55742764*	*1465*	*93*	*63*	*70556*	*Albumin*
33	*Serpin peptidase inhibitor, clade A, member 1 precursor*	*[Canis lupus familiaris]*	*gi|121583756*	*366*	*22*	*24*	*46505*	*Alpha 1*
33	*Albumin*	*[Canis lupus familiaris]*	*gi|3319897*	*245*	*16*	*26*	*67857*	*Alpha 1*
33	*Alpha 2 HS glycoprotein isoform 1*	*[Canis lupus familiaris]*	*gi|359323766*	*112*	*5*	*18*	*40021*	*Alpha 1*
33	*Alpha-amylase*	*[Homo sapiens]*	*gi|178585*	*84*	*2*	*5*	*58398*	*Alpha 1*
34	*Haptoglobin*	*[Canis lupus familiaris]*	*gi|123511*	*651*	*116*	*47*	*36890*	*Alpha 2*
35	*Haptoglobin*	*[Canis lupus familiaris]*	*gi|123511*	*382*	*57*	*48*	*36890*	*Alpha 2*

35	*Apolipoprotein A1 Precursor*	*[Canis lupus familiaris]*	*gi|3915607*	*160*	*8*	*33*	*30178*	*Alpha 2*
36	*Apolipoprotein A-I*	*[Canis lupus familiaris]*	*gi|73955106*	*2533*	*128*	*77*	*30163*	*Alpha 2*
36	*Haptoglobin*	*[Canis lupus familiaris]*	*gi|123511*	*189*	*24*	*45*	*36890*	*Alpha 2*
37	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*863*	*54*	*54*	*80222*	*Beta 2*
37	*Immunoglobulin heavy chain constant region CH2*	*[Canis lupus familiaris]*	*gi|124390009*	*161*	*5*	*28*	*12398*	*Beta 2*
37	*Immunoglobulin heavy chain constant region CH4*	*[Canis lupus familiaris]*	*gi|124390013*	*79*	*6*	*44*	*14396*	*Beta 2*
37	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*76*	*11*	*7*	*182611*	*Beta 2*
38	*Serotransferrin isoform 1*	*[Canis lupus familiaris]*	*gi|73990142*	*376*	*25*	*28*	*80222*	*Beta 2*
38	*Immunoglobulin gamma heavy chain C*	*[Canis lupus familiaris]*	*gi|17066528*	*238*	*14*	*22*	*52779*	*Beta 2*
38	*Complement C3*	*[Canis lupus familiaris]*	*gi|359322249*	*100*	*11*	*7*	*182611*	*Beta 2*
38	*Immunoglobulin gamma heavy chain D*	*[Canis lupus familiaris]*	*gi|17066530*	*100*	*6*	*12*	*52169*	*Beta 2*
38	*Immunoglobulin gamma heavy chain B*	*[Canis lupus familiaris]*	*gi|17066526*	*85*	*8*	*14*	*52553*	*Beta 2*
38	*Immunoglobulin heavy chain constant region CH2*	*[Canis lupus familiaris]*	*gi|124390009*	*71*	*2*	*21*	*12398*	*Beta 2*
38	*Alpha-fibrinogen precursor*	*[Homo sapiens]*	*gi|182424*	*66*	*1*	*2*	*70223*	*Beta 2*

LIST OF REFERENCES

Abate, O., Zanatta, R., Malisano, T. & Dotta, U., 2000. Canine serum protein patterns using high-resolution electrophoresis (HRE). The Veterinary Journal 159: 154–160.

Adida, C., Haioun, C., Gaulard, P., Lepage, E., Morel, P., Briere, J., Dombret, H., Reyes, F., Diebold, J., Gisselbrecht, C., Salles, G., Altieri, D.C. & Molina, T.J., 2000. Prognostic significance of survivin expression in diffuse large B-cell lymphomas. Blood 96: 1921–1925.

Aebersold, R. & Mann, M., 2003. Mass spectrometry-based proteomics. Nature 422: 198–207.

Ahn, H.S., Shin, Y.S., Park, P.J., Kang, K.N., Kim, Y., Lee, H.J., Yang, H.K. & Kim, C.W., 2012. Serum biomarker panels for the diagnosis of gastric adenocarcinoma. British Journal of Cancer 106: 733–739.

Anderson, N.L. & Anderson, N.G., 2002. The Human Plasma Proteome History, Character, and Diagnostic Prospects. Molecular and Cellular Proteomics 1: 845–867.

Baskin, C.R., Couto, C.G. & Wittum, T.E., 2000. Factors influencing first remission and survival in 145 dogs with lymphoma: a retrospective study. Journal of the American Animal Hospital Association 36: 404–409.

Batchelor, D., Bright, S., Ibarrola, P., Tzannes, S. & Blackwood, L., 2006. Long-term survival after combination chemotherapy for bilateral renal malignant lymphoma in a dog. New Zealand Veterinary Journal 54: 147–150.

Baumann, H. & Gauldie, J., 1994. The acute phase response. Immunology Today 15: 74–80.

Birgegard, G., Aapro, M.S., Bokemeyer, C., Dicato, M., Drings, P., Hornedo, J., Krzakowski, M., Ludwig, H., Pecorelli, S., Schmoll, H., Schneider, M., Schrijvers, D., Shasha, D. & Van Belle, S., 2005. Cancer-Related Anemia: Pathogenesis, Prevalence and Treatment. Oncology 68: 3–11.

Bou-Abdallah, F., 2012. Transferrins: Molecular mechanisms of iron transport and disorders. Biochimica et Biophysica Acta (BBA) - General Subjects 1820: 157–158.

Breen, M. & Modiano, J.F., 2008. Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans–man and his best friend share more than companionship. Chromosome Research 1: 145–154.

Buckley, C.E. &, Dorsey, F.E., 1970. The effect of aging on human serum immunoglobulin concentrations. Journal of Immunology 105: 964-972.

Burnett, R.C., Vernau, W., Modiano, J.F., Olver, C.S., Moore, P.F. & Avery, A.C., 2003. Diagnosis of Canine Lymphoid Neoplasia Using Clonal Rearrangements of Antigen Receptor Genes. Veterinary Pathology 40: 32–41.

Byerley, O.L., Leamy, L., Tam, S.W., Chou, C-W. & Ravussin, E., 2010. Development of a serum profile for healthy aging. Age. 32: 497-507.

Caballero-Hernández, D., Gomez-Flores, R., Tamez-Guerra, P., Tamez-Guerra, R. & Rodríguez-Padilla, C., 2009. Role of immunogenic fetuin A on L5178Y-R lymphoma tumorigenesis. Cancer Investigation 27: 257–263.

Caniatti, M., Roccabianca, P., Scanziani, E., Paltrinieri, S. & Moore, P.F., 1996. Canine Lymphoma: Immunocytochemical Analysis of Fine-needle Aspiration Biopsy. Veterinary Pathology 33: 204–212.

Carter, R.F., Valli, V.E. & Lumsden, J.H., 1986. The cytology, histology and prevalence of cell types in canine lymphoma classified according to the National Cancer Institute Working Formulation. Canadian Journal of Veterinary Research 50: 154–164.

Caspi, D., Snel, F.W., Batt, R.M., Bennett, D., Rutteman, G.R., Hartman, E.G., Baltz, M.L., Gruys, E. & Pepys, M.B., 1987. C-reactive protein in dogs. American Journal of Veterinary Research 48: 919-921.

Comazzi, S., Gelain, M.E., Riondato, F. & Paltrinieri, S., 2006. Flow cytometric expression of common antigens CD18/CD45 in blood from dogs with lymphoid malignancies: a semi-quantitative study. Veterinary Immunology and Immunopathology 112: 243–252.

Conner, J.G., Eckersall, P.D., Ferguson, J., Douglas, T.A., 1988. Acute phase response in the dog following surgical trauma. Research in Veterinary Science 45: 107–110.

Couto, C.G., Rutgers, H.C., Sherding, R.G. & Rojko, J., 1989. Gastrointestinal Lymphoma in 20 Dogs. Journal of Veterinary Internal Medicine 3: 73–78.

Dank, G., Rassnick, K.M., Kristal, O., Rodriguez, C.O., Clifford, C.A., Ward, R., Mallett, C.L., Gieger, T. & Segev, G., 2011. Clinical characteristics, treatment, and outcome of dogs with presumed primary hepatic lymphoma: 18 cases (1992-2008). Journal of the American Veterinary. Medical Association 239: 966–971.

Dergunov, A.D., 2012. A mechanistic model of lecithin: cholesterol acyltransferase activity exploits discoidal HDL composition and structure. Archives of Biochemistry and Biophysics 520: 81–87.

Diamandis, E.P., 2004. Mass Spectrometry as a Diagnostic and a Cancer Biomarker Discovery Tool Opportunities and Potential Limitations. Molecular and Cellular Proteomics 3: 367–378.

Dobson, J.M., Blackwood, L.B., Mcinnes, E.F., Bostock, D.E., Nicholls, P., Hoather, T.M. & Tom, B.D.M., 2001. Prognostic variables in canine multicentric lymphosarcoma. Journal of Small Animal Practice 42: 377–384.

Dobson, J.M., Samuel, S., Milstein, H., Rogers, K. & Wood, J.L.N., 2002. Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. Journal of Small Animal Practice 43: 240–246.

Doumas, B.T., Ard Watson, W. & Biggs, H.G., 1971. Albumin standards and the measurement of serum albumin with bromcresol green. Clinica Chimica Acta 31: 87–96.

Dunn, M.J., Corbett, J.M. & Wheeler, C.H., 1997. HSC-2DPAGE and the two-dimensional gel electrophoresis database of dog heart proteins. Electrophoresis 18: 2795–2802.

Eckersall, P., Duthie, S., Safi, S., Moffatt, D., Horadagoda, N., Doyle, S., Parton, R., Bennett, D. & Fitzpatrick, J., 1999. An automated biochemical assay for haptoglobin: Prevention of interference from albumin. Comparative Haematology International 9: 117–124.

Eckersall, P.D., Conner, J.G., Harvie, J., 1991. An immunoturbidimetric assay for canine C-reactive protein. Veterinary Research Communications 15: 17–24.

Eckersall, P.D., 2008. Proteins, proteomics, and the dysproteinemias. In: Kaneko, J.J., Harvey, J.W., Bruss, M.L. (Eds). Clinical Biochemistry of Domestic Animals, 6th ed. Academic Press, San Diego. pp. 117-155.

Eckersall, P.D. & Bell, R., 2010. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. The Veterinary Journal 185: 23–27.

Edwards, D.S., Henley, W.E., Harding, E.F., Dobson, J.M. & Wood, J.L.N., 2003. Breed incidence of lymphoma in a UK population of insured dogs. Veterinary and Comparative Oncology 1: 200–206.

Evans, T.W., 2002. Albumin as a drug—biological effects of albumin unrelated to oncotic pressure. Alimentary Pharmacology and Therapeutics 16: 6–11.

Fayos, M., Couto, C.G., Iazbik, M.C. & Wellman, M.L., 2005. Serum protein electrophoresis in retired racing Greyhounds. Veterinary Clinical Pathology 34: 397–400.

Finnie, J.W. & Wilks, C.R., 1982. Two cases of multiple myeloma in the dog. Journal of Small Animal Practice 23: 19–27.

Flood-Knapik, K.E., Durham, A.C., Gregor, T.P., Sánchez, M.D., Durney, M.E. & Sorenmo, K.U., 2012. Clinical, histopathological and immunohistochemical characterization of canine indolent lymphoma. Veterinary and Comparative Oncology In Press.

Fournel-Fleury, C., Magnol, J.P., Bricaire, P., Marchal, T., Chabanne, L., Delverdier, A., Bryon, P.A. & Felman, P., 1997. Cytohistological and immunological classification of canine malignant lymphomas: Comparison with human non-Hodgkin's lymphomas. Journal of Comparative Pathology 117: 35–59.

Fournel-Fleury, C., Ponce, F., Felman, P., Blavier, A., Bonnefont, C., Chabanne, L., Marchal, T., Cadore, J.L., Goy-Thollot, I., Ledieu, D., Ghernati, I. & Magnol, J.P., 2002. Canine T-cell Lymphomas: A Morphological, Immunological, and Clinical Study of 46 New Cases. Veterinary Pathology 39: 92–109.

Fox, L.E., Rosenthal, R.C., Twedt, D.C., Dubielzig, R.R., MacEwen, E.G. & Grauer, G.F., 1990. Plasma Histamine and Gastrin Concentrations in 17 Dogs With Mast Cell Tumors. Journal of Veterinary Internal Medicine 4: 242–246.

Gaines, P.J., Powell, T.D., Walmsley, S.J., Estredge, K.L., Wisnewski, N., Stinchcomb, D.T., Withrow, S.J. & Lana, S.E., 2007. Identification of serum biomarkers for canine B-cell lymphoma by use of surface-enhanced laser desorption-ionization time-of-flight mass spectrometry. American Journal of Veterinary Research 68: 405–410.

García-Martínez, J.D., Tvarijonaviciute, A., Cerón, J.J., Caldin, M. & Martínez-Subiela, S., 2012. Urinary clusterin as a renal marker in dogs. Journal of Veterinary Diagnostic Investigation 24: 301–306.

Garrett, L.D., Thamm, D.H., Chun, R., Dudley, R. & Vail, D.M., 2002. Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. Journal of Veterinary Internal Medicine 16: 704–709.

Gavazza, A., Sacchini, F., Lubas, G., Gugliucci, B. & Valori, E., 2009. Clinical, laboratory, diagnostic and prognostic aspects of canine lymphoma: a retrospective study. Comparative Clinical Pathology 18: 291–299.

Gentilini, F., Calzolari, C., Buonacucina, A., Di Tommaso, M., Militerno, G. & Famigli Bergamini, P., 2005. Different biological behaviour of Waldenström macroglobulinemia in two dogs. Veterinary and Comparative Oncology 3: 61-121.

Gerou-Ferriani, M., McBrearty, A.R., Burchmore, R.J., Jayawardena, K.G.I., Eckersall, P.D. & Morris, J.S., 2011. Agarose gel serum protein electrophoresis in cats with and without lymphoma and preliminary results of tandem mass fingerprinting analysis. Veterinary Clinical Pathology 40: 159–173.

Giordano, A. & Paltrinieri, S., 2010. Interpretation of capillary zone electrophoresis compared with cellulose acetate and agarose gel electrophoresis: reference intervals and diagnostic efficiency in dogs and cats. Veterinary Clinical Pathology 39: 464–473.

Giraudel, J.M., Pagès, J.-P. & Guelfi, J.-F., 2002. Monoclonal gammopathies in the dog: a retrospective study of 18 cases (1986-1999) and literature review. Journal of the American Animal Hospital Association 38: 135–147.

Görg, A., Weiss, W. & Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4: 3665–3685.

Gornall, A.G., Bardawill, C.J. & David, M.M., 1949. Determination of Serum Proteins by Means of the Biuret Reaction. Journal of Biological Chemistry. 177: 751–766.

Greenlee, P.G., Filippa, D.A., Quimby, F.W., Patnaik, A.K., Calvano, S.E., Matus, R.E., Kimmel, M., Hurvitz, A.I. & Lieberman, P.H., 1990. Lymphomas in dogs a morphologic, immunologic, and clinical study. Cancer 66: 480–490.

Grindem, C.B., Breitschwerdt, E.B., Corbett, W.T., Page, R.L. & Jans, H.E., 1994. Thrombocytopenia associated with neoplasia in dogs. Journal of Veterinary Internal Medicine 8: 400–405.

Hallek, M., Wanders, L., Strohmeyer, S. & Emmerich, B., 1992. Thymidine kinase: a tumor marker with prognostic value for non-Hodgkin's lymphoma and a broad range of potential clinical applications. Annals of Hematology 65: 1–5.

Halleux, C.M., Takahashi, M., Delporte, M.L., Detry, R., Funahashi, T., Matsuzawa, Y. & Brichard, S.M., 2001. Secretion of Adiponectin and Regulation of apM1 Gene Expression in Human Visceral Adipose Tissue. Biochemical and Biophysical Research Communications 288: 1102–1107.

Han, X., Aslanian, A. & Yates, J.R., 2008. Mass spectrometry for proteomics. Current Opinion in Chemical Biology 12: 483–490.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A. & Bloomfield, C.D., 1999. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. Journal of Clinical Oncology 17: 3835–3849.

Harris, N.L., Jaffe, E.S., Stein, H., Banks, P.M., Chan, J.K., Cleary, M.L., Delsol, G., De Wolf-Peeters, C., Falini, B. & Gatter, K.C., 1994. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 84: 1361–1392.

Heinrich, P.C., Jose V. Castell, J.V. & Andust, T., 1990. IL-6 and the acute phase response. Biochemical Journal 265: 621-636.

Henry, C.J., 2010. Biomarkers in veterinary cancer screening: Applications, limitations and expectations. The Veterinary Journal. 185: 10–14.

Herbert, B. & Righetti, P.G., 2000. A turning point in proteome analysis: Sample prefractionation via multicompartment electrolyzers with isoelectric membranes. Electrophoresis 21: 3639–3648.

Hibbetts, K., Hines, B. & Williams, D., 1999. An overview of proteinase inhibitors. Journal of Veterinary Internal Medicine 13: 302–308.

Honda, K., Ishiko, O., Tatsuta, I., Deguchi, M., Hirai, K., Nakata, S., Sumi, T., Yasui, T. & Ogita, S., 1995. Anemia-inducing substance from plasma of patients with advanced malignant neoplasms. Cancer Research 55: 3623–3628.

Hutchings, M. & Barrington, S.F., 2009. PET/CT for therapy response assessment in lymphoma. Journal of Nuclear Medicine 50, Supplement 1: 21–30.

Ikehara, Y., Oda, K. & Kato, K., 1976. Conversion of proalbumin into serum albumin in the secretory vesicles of rat liver. Biochemical and Biophysical Research Communications 72: 319–326.

Issaq, H. & Veenstra, T., 2008. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. BioTechniques 44: 697-700.

Jagielski, D., Lechowski, R., Hoffmann-Jagielska, M. & Winiarczyk, S., 2002. A retrospective study of the incidence and prognostic factors of multicentric lymphoma in dogs (1998–2000). Journal of Veterinary Medicine 49: 419–424.

Kay, P.H., Dawkins, R.L. & Penhale, J.W., 1985. The molecular structure of different polymorphic forms of canine C3 and C4. Immunogenetics 21: 313–319.

Keay, G., 1982. Serum protein values from clinically normal cats and dogs determined by agarose gel electrophoresis. Research in Veterinary Science. 33: 343–346.

Keller, E.T., MacEwen, E.G., Rosenthal, R.C., Helfand, S.C. & Fox, L.E., 1993. Evaluation of prognostic factors and sequential combination chemotherapy with doxorubicin for canine lymphoma. Journal of Veterinary Internal Medicine 7: 289–295.

Lana, S., Plaza, S., Hampe, K., Burnett, R. & Avery, A.C., 2006. Diagnosis of mediastinal masses in dogs by flow cytometry. Journal of veterinary internal medicine 20: 1161–1165.

Leblanc, A.K., Jakoby, B.W., Townsend, D.W. & Daniel, G.B., 2009. 18FDG-PET imaging in canine lymphoma and cutaneous mast cell tumor. Veterinary Radiology and Ultrasound 50: 215–223.

Lebreton, J.P., Joisel, F., Raoult, J.P., Lannuzel, B., Rogez, J.P. & Humbert, G., 1979. Serum concentration of human alpha 2 HS glycoprotein during the inflammatory process: evidence that alpha 2 HS glycoprotein is a negative acute-phase reactant. Journal of Clinical Investigation 64: 1118–1129.

Legouffe, E., Rodriguez, C., Picot, M.C., Richard, B., Klein, B., Rossi, J.F. & Commes, T., 1998. C-reactive protein serum level is a valuable and simple prognostic marker in non Hodgkin's lymphoma. Leukaemia and Lymphoma 31: 351–357.

Lehninger, A.L., Nelson, D.L. & Cox, M., 1993. Principles of Biochemistry, 2nd ed. Worth Publishers, New York. 1013pp.

Lennert, K., Stein, H. & Kaiserling, E., 1975. Cytological and functional criteria for the classification of malignant lymphomata. British Journal of Cancer Supplement 2: 29–43.

Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., Karlsson, E.K., Jaffe, D.B., et al., 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438: 803–819.

Long, S.N., Johnston, P.E.J. & Anderson, T.J., 2001. Primary T-cell lymphoma of the central nervous system in a dog. Journal of the American Veterinary Medical Association 218: 719–722.

Lossos, I.S., Jones, C.D., Warnke, R., Natkunam, Y., Kaizer, H., Zehnder, J.L., Tibshirani, R. & Levy, R., 2001. Expression of a single gene, BCL-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. Blood 98: 945–951.

Lowrie, M., Penderis, J., McLaughlin, M., Eckersall, P. D. & Anderson, T. J., 2009. Steroid Responsive Meningitis-Arteritis: A Prospective Study of Potential Disease Markers, Prednisolone Treatment, and Long-Term Outcome in 20 Dogs (2006–2008). Journal of Veterinary Internal Medicine 23: 862–870.

Lukes, R.J. & Collins, R.D., 1974. Immunologic characterization of human malignant lymphomas. Cancer 34: 1488–1503.

Luo, C.C., Li, W.H. & Chan, L., 1989. Structure and expression of dog apolipoprotein A-I, E, and C-I mRNAs: implications for the evolution and functional constraints of apolipoprotein structure. Journal of Lipid Research 30: 1735–1746.

Macia, J., Gomez, X., Esquerda, A., Perez, B., Callao, V. & Marzo, C., 1996. Value of the determination of TNF-α in the plasma of patients with non-Hodgkin's lymphoma. Leukaemia and Lymphoma 20: 481-486.

Macpherson, R., Scherer, J., Ross, M.L. & Gentry, P.A., 1999. Factor VII deficiency in a mixed breed dog. Canadian Veterinary Journal 40: 503–505.

Maddison, J.E., 1992. Hepatic encephalopathy. Current concepts of the pathogenesis. Journal of Veterinary Internal Medicine 6: 341–353.

Malpas, J.S. & Fairley, G.H., 1964. Changes in serum alpha-2 globulins in reticuloses. Journal of Clinical Pathology 17: 651–654.

Marconato, L., 2011. The staging and treatment of multicentric high-grade lymphoma in dogs: A review of recent developments and future prospects. The Veterinary Journal 188: 34–38.

Martínez-Subiela, S., Tecles, F., Cerón, J.J. & Eckersall, P.D., 2002a. Serum concentrations of acute phase proteins in dogs with leishmaniasis. Veterinary Record 150: 241–244.

Martínez-Subiela, S., Tecles, F., Montes, A., Gutiérrez, C. & Cerón, J.J., 2002b. Effects of haemolysis, lipaemia, bilirubinaemia and fibrinogen on protein electropherogram of canine samples analysed by capillary zone electrophoresis. The Veterinary Journal 164: 261–268.

Martínez-Subiela, S., Ginel, P.J. & Cerón, J.J., 2004. Effects of different glucocorticoid treatments on serum acute phase proteins in dogs. Veterinary Record 154: 814–817.

Massa, K.L., Gilger, B.C., Miller, T.L. & Davidson, M.G., 2002. Causes of uveitis in dogs: 102 cases (1989–2000). Veterinary Ophthalmology 5: 93–98.

Mazzaferro, E.M., Rudloff, E. & Kirby, R., 2002. The role of albumin replacement in the critically ill veterinary patient. Journal of Veterinary Emergency and Critical Care 12: 113–124.

McCaw, D.L., Chan, A.S., Stegner, A.L., Mooney, B., Bryan, J.N., Turnquist, S.E., Henry, C.J., Alexander, H. & Alexander, S., 2007. Proteomics of canine lymphoma identifies potential cancer-specific protein markers. Clinical Cancer Research 13: 2496–2503.

McGrotty, Y.L., Arteaga, A., Knottenbelt, C.M., Ramsey, I.K. & Eckersall, P.D., 2005. Haptoglobin concentrations in dogs undergoing trilostane treatment for hyperadrenocorticism. Veterinary Clinical Pathology 34: 255–258.

McGrotty, Y.L. & Tennant, K., 2005. Disorders of plasma proteins. In: Villiers, E. & Blackwood, L. (Eds.). BSAVA Manual of Clinical Pathology, 2nd ed. British Small Animal Veterinary Association, Gloucester. pp. 99-112.

McLafferty, F.W., 1980. Tandem mass spectrometry (MS/MS): a promising new analytical technique for specific component determination in complex mixtures. Accounts of Chemical Research 13: 33–39.

Merlo, A., Rezende, B.C.G., Franchini, M.L., Simões, D.M.N. & Lucas, S.R.R., 2007. Serum C-reactive protein concentrations in dogs with multicentric lymphoma undergoing chemotherapy. Journal of the American Veterinary Medical Association 230: 522–526.

Merlo, A., Rezende, B.C.G., Franchini, M.L., Monteiro, P.R.G. & Lucas, S.R.R., 2008. Serum amyloid A is not a marker for relapse of multicentric lymphoma in dogs. Veterinary Clinical Pathology 37: 79–85.

Miller, A. g., Morley, P. S., Rao, S., Avery, A. C., Lana, S. E. & Olver, C. S., 2009. Anemia is associated with decreased survival time in dogs with lymphoma. Journal of Veterinary Internal Medicine 23: 116–122.

Miller, I., Friedlein, A., Tsangaris, G., Maris, A., Fountoulakis, M. & Gemeiner, M., 2004. The serum proteome of Equus caballus. Proteomics 4: 3227–3234.

Milner, R.J., Pearson, J., Nesbit, J.W. & Close, P., 1996. Immunophenotypic classification of canine malignant lymphoma on formalin-mixed paraffin wax-embedded tissue by means of CD3 and CD79a cell markers. Journal of Veterinary Research 63: 309–313.

Mischke, R., Freund, M., Leinemann-Fink, T., Eisenberger, B., Casper, J. & Nolte, I., 1998. Changes in hemostasis of dogs with acute lymphoblastic leukemia. Berliner und Münchener Tierärztliche Wochenschrift 111: 53-59.

Mischke, R., Waterston, M. & Eckersall, P.D., 2007. Changes in C-reactive protein and haptoglobin in dogs with lymphatic neoplasia. The Veterinary Journal. 174: 188–192.

Modiano, J.F., Breen, M., Valli, V.E.O., Wojcieszyn, J.W. & Cutter, G.R., 2007. Predictive value of p16 or Rb inactivation in a model of naturally occurring canine non-Hodgkin's lymphoma. Leukemia 21: 184–187.

Monti, S., Savage, K.J., Kutok, J.L., Feuerhake, F., Kurtin, P., et al., 2005. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. Blood 105: 1851-1861.

Moreau, M.E., Garbacki, N., Molinaro, G., Brown, N.J., Marceau, F. & Adam, A., 2005. The kallikrein-kinin system: current and future pharmacological targets. Journal of Pharmaceutical Sciences 99: 6–38.

Moshage, H., 1997. Cytokines and the hepatic acute phase response. The Journal of Pathology 181: 257–266.

Moulton, J.E., 1990. Tumors in Domestic Animals, 3rd ed. University of California Press, Berkeley. 684pp.

Murata, H., Shimada, N. & Yoshioka, M., 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. The Veterinary Journal 168: 28–40.

Mustard, J.F., Secord, D., Hoeksema, T.D., Downie, H.G. & Rowsell, H.C., 1962. Canine factor-VII deficiency. British Journal of Haematology. 8: 43–47.

Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R., 2003. A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. Analytical Chemistry. 75: 4646–4658.

Neuhaus, O.W., Balegno, H.F. & Chandler, A.M., 1966. Induction of plasma protein synthesis in response to trauma. American Journal of Physiology. 211: 151–156.

Owen, L.N., 1980. TNM classification of tumours in domestic animals. World Health Organization (WHO), Geneva. 53pp.

Palacios, R., Ivhed, I., Sideras, P., Nilsson, K., Sugawara, I. & Fernandez, C., 2005. Accessory function of human tumor cell lines I Production of interleukin 1 by the human histiocytic lymphoma cell line U-937. European Journal of Immunology 12: 895-899.

Palus, V., Volk, H.A., Lamb, C.R., Targett, M.P. & Cherubini, G.B., 2012. MRI features of CNS lymphoma in dogs and cats. Veterinary Radiology and Ultrasound 53: 44–49.

Patterson, S.D., 1991. Mammalian alpha 1-antitrypsins: comparative biochemistry and genetics of the major plasma serpin. Comparative Biochemistry and Physiology - Part B: Biochemistry and Molecular Biology 100: 439–454.

Petrik, V., Saadoun, S., Loosemore, A., Hobbs, J., Opstad, K.S., Sheldon, J., Tarelli, E., Howe, F.A., Bell, B.A. & Papadopoulos, M.C., 2008. Serum alpha 2-HS glycoprotein predicts survival in patients with glioblastoma Clinical Chemistry 54: 713–722.

Pieper, R., Gatlin, C.L., Makusky, A.J., Russo, P.S., Schatz, C.R., Miller, S.S., Su, Q., McGrath, A.M., Estock, M.A., Parmar, P.P., Zhao, M., Huang, S.-T., Zhou, J., Wang, F., Esquer-Blasco, R., Anderson, N.L., Taylor, J. & Steiner, S., 2003. The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Proteomics 3: 1345–1364.

Ponce, F., Magnol, J.P., Marchal, T., Chabanne, L., Ledieu, D., Bonnefont, C., Felman, P. & Fournel-Fleury, C., 2003. High-grade canine T-cell lymphoma/leukemia with plasmacytoid morphology: a clinical pathological study of nine cases. Journal of Veterinary Diagnostic Investigation 15: 330–337.

Ponce, F., Magnol, J.-P., Ledieu, D., Marchal, T., Turinelli, V., Chalvet-Monfray, K. & Fournel-Fleury, C., 2004. Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. The Veterinary Journal 167: 158–166.

Ponce, F., Marchal, T., Magnol, J.P., Turinelli, V., Ledieu, D., Bonnefont, C., Pastor, M., Delignette, M.L. & Fournel-Fleury, C., 2010. A morphological study of 608 cases of canine malignant lymphoma in France with a focus on comparative similarities between canine and human lymphoma morphology. Veterinary Pathology 47: 414–433.

Potempa, J., Korzus, E. & Travis, J., 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. Journal of Biological Chemistry 269: 15957–15960.

Presslmayer, A., 2002. Identifizierung caniner serumproteine mittels elektrophoretischer verfahren. Master of Science Thesis, University of Natural Resources and Applied Life Science Sciences, Vienna, Austria. 156pp.

Presthus, J. & Teige, J., 1986. Peripheral neuropathy associated with lymphosarcoma in a dog. Journal of Small Animal Practice 27: 463–469.

Ramaiah, S.K., Seguin, M.A., Carwile, H.F. & Raskin, R.E., 2002. Biclonal gammopathy associated with immunoglobulin A in a dog with multiple myeloma. Veterinary Clinical Pathology 31: 83–89.

Rao, S., Lana, S., Eickhoff, J., Marcus, E., Avery, P. R., Morley, P. S. & Avery, A. C., 2011. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. Journal of Veterinary Internal Medicine 25: 1097–1105.

Rappaport H, 1966. Tumors of the Haematopoietic System. Armed Forces Institute of Pathology, Washington DC. 442pp.

Raskin, R.E. & Krehbiel, J.D., 1989. Prevalence of leukemic blood and bone marrow in dogs with multicentric lymphoma. Journal of the American Veterinary Medical Association 194: 1427–1429.

Rassnick, K.M., Moore, A.S., Collister, K.E., Northrup, N.C., Kristal, O., Chretin, J.D. & Bailey, D.B., 2009. Efficacy of combination chemotherapy for treatment of gastrointestinal lymphoma in dogs. Journal of Veterinary Internal Medicine 23: 317–322.

Ratcliffe, L., Mian, S., Slater, K., King, H., Napolitano, M., Aucoin, D. & Mobasheri, A., 2009. Proteomic identification and profiling of canine lymphoma patients. Veterinary and Comparative Oncology 7: 92–105.

Rebhun, R.B., Lana, S.E., Ehrhart, E.J., Charles, J.B. & Thamm, D.H., 2008. Comparative analysis of survivin expression in untreated and relapsed canine lymphoma. Journal of Veterinary Internal Medicine 22: 989–995.

Redaelli, S., Piazza, R., Rostagno, R., Magistroni, V., Perini, P., Marega, M., Gambacorti-Passerini, C. & Boschelli, F., 2009. Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants. Journal of Clinical Oncology 27: 469–471.

Ritchie, R.F., Palomaki, G.E., Neveux, L.M., Navolotskaia, O., Ledue, T.B. & Craig, W.Y., 1999. Reference distributions for the negative acute-phase serum proteins, albumin, transferrin and transthyretin: a practical, simple and clinically relevant approach in a large cohort. Journal of Clinical Laboratory Analysis 13: 273–279.

Rosenberg, M.P., Matus, R.E. & Patnaik, A.K., 1991. Prognostic factors in dogs with lymphoma and associated hypercalcemia. Journal of Veterinary Internal Medicine 5: 268–271.

Rosenburg, S.A., Berard, C.W., Brown, W.B., Burke, J., Dorfman, R.F., et al., 1982. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. The Non-Hodgkin's Lymphoma Pathologic Classification Project. Cancer 49: 2112–2135.

Rothe, G. & Schmitz, G., 1996. Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies. Working Group on Flow Cytometry and Image Analysis. Leukemia 10: 877-895.

Ruslander, D.A., Gebhard, D.H., Tompkins, M.B., Grindem, C.B. & Page, R.L., 1997. Immunophenotypic characterization of canine lymphoproliferative disorders. In Vivo 11: 169-172.

Rylander, H., 2010. Neurologic manifestations of systemic disease. In: Ettinger, S.J. & Feldman, E.C. (Eds). Textbook of Veterinary Internal Medicine, 7th ed. Saunders Elsevier, Missouri. pp. 212-216.

Saffer, H., Wahed, A., Rassidakis, G.Z. & Medeiros, L.J., 2002. Clusterin expression in malignant lymphomas: a survey of 266 cases. Modern Pathology 15: 1221–1226.

Sato, M., Kanemoto, H., Kagawa, Y., Kobayashi, T., Goto-Koshino, Y., Mochizuki, H., Takahashi, M., Fujino, Y., Ohno, K. & Tsujimoto, H., 2012. Evaluation of the prognostic significance of BCL6 gene expression in canine high-grade B-cell lymphoma. The Veterinary Journal 191: 108–114.

Schmaier, A.H. & McCrae, K.R., 2007. The plasma kallikrein-kinin system: its evolution from contact activation. Journal of Thrombosis and Haemostasis 5: 2323–2329.

Shannan, B., Seifert, M., Leskov, K., Willis, J., Boothman, D., Tilgen, W. & Reichrath, J., 2006. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. Cell Death and Differentiation. 13: 12–19.

Smithies, O., Poulik, M.D., 1956. Two-dimensional electrophoresis of serum proteins. Nature 177: 1033.

Sözmen, M., Tasca, S., Carli, E., Lorenzi, D.D., Furlanello, T. & Caldin, M., 2005. Use of fine needle aspirates and flow cytometry for the diagnosis, classification, and immunophenotyping of canine lymphomas. Journal of Veterinary Diagnostic Investigation 17: 323–329.

Speeckaert, M., Huang, G., Delanghe, J.R. & Taes, Y.E.C., 2006. Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. Clinica Chimica Acta 372: 33–42.

Srinivas, P.R., Kramer, B.S. & Srivastava, S., 2001. Trends in biomarker research for cancer detection. The Lancet Oncology 2: 698–704.

Stockham, S.L., Scott, M.A., 2008. Fundamentals of Veterinary Clinical Pathology, 2nd ed. Blackwell, Iowa. 936pp.

Tappin, S.W., Taylor, S.S., Tasker, S., Dodkin, S.J., Papasouliotis, K. & Murphy, K.F., 2011. Serum protein electrophoresis in 147 dogs. Veterinary Record 168: 456.

Tecles, F., Spiranelli, E., Bonfanti, U., Cerón, J. j. & Paltrinieri, S., 2005. Preliminary studies of serum acute–phase protein concentrations in hematologic and neoplastic diseases of the dog. Journal of Veterinary Internal Medicine 19: 865–870.

Teske, E. & Van Heerde, P., 1996. Diagnostic value and reproducibility of fine-needle aspiration cytology in canine malignant lymphoma. Veterinary Quarterly 18: 112–115.

Teske, E., Van Heerde, P., Rutteman, G.R., Kurzman, I.D., Moore, P.F. & MacEwen, E.G., 1994. Prognostic factors for treatment of malignant lymphoma in dogs. Journal of the American Veterinary Medical Association 205: 1722–1728.

Theilen, G.H. & Madewell, B.R., 1987. Veterinary cancer medicine, 2nd ed. Lea and Febiger, Philadelphia. 677pp.

Tizzard, I.R., 2004. Veterinary Immunology: An Introduction, 7th ed. Saunders, Philadelphia. 494pp.

Tolosano, E. & Altruda, F., 2002. Hemopexin: structure, function, and regulation. DNA and Cell Biology 21: 297–306.

Tvarijonaviciute, A., Gutiérrez, A.M., Miller, I., Razzazi-Fazeli, E., Tecles, F. & Ceron, J.J., 2012. A proteomic analysis of serum from dogs before and after a controlled weight-loss program. Domestic Animal Endocrinology 43: 271–277.

Ünlü, M., Morgan, M.E. & Minden, J.S., 1997. Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 18: 2071–2077.

Vail, D.M. & Macewen, E.G., 2000. Spontaneously occurring tumors of companion animals as models for human cancer. Cancer investigation 18: 781–792.

Vail, D.M., Ogilvie, G.K., Wheeler, S.L., Fettman, M.J., Johnston, S.D. & Hegstad, R.L., 1990. Alterations in carbohydrate metabolism in canine lymphoma. Journal of Veterinary Internal Medicine 4: 8–11.

Vail, D.M. & Young, K.M., 2007. Hematopoietic tumors. In: Withrow, S.J. & Vail, D.M. (Eds). Withrow and MacEwen's Small Animal Clinical Oncology. Elsevier Health Sciences, St Louis. pp. 699-732.

Valli, V.E., Myint, M.S., Barthel, A., Bienzle, D., Caswell, J., Colbatzky, F., Durham, A., Ehrhart, E.J., Johnson, Y., Jones, C., Kiupel, M., Labelle, P., Lester, S., Miller, M., Moore, P., Moroff, S., Roccabianca, P., Ramos-Vara, J., Ross, A., Scase, T., Tvedten, H. & Vernau, W., 2011. Classification of canine malignant lymphomas according to the world health organization criteria. Veterinary Pathology 48: 198–211.

Villamil, J.A., Henry, C.J., Bryan, J.N., Ellersieck, M., Schultz, L., Tyler, J.W. & Hahn, A.W., 2011. Identification of the most common cutaneous neoplasms in dogs and evaluation of breed and age distributions for selected neoplasms. Journal of the American Veterinary Medical Association 239: 960–965.

Von Euler, H., Einarsson, R., Olsson, U., Lagerstedt, A.-S. & Eriksson, S., 2004. Serum thymidine kinase activity in dogs with malignant lymphoma: a potent marker for prognosis and monitoring the disease. Journal of Veterinary Internal Medicine 18: 696–702.

Von Euler, H.P., Öhrvik, A.B. & Eriksson, S.K., 2006. A non-radiometric method for measuring serum thymidine kinase activity in malignant lymphoma in dogs. Research in Veterinary Science 80: 17–24.

Von Euler, H.P., Rivera, P., Aronsson, A.-C., Bengtsson, C., Hansson, L.-O. & Eriksson, S.K., 2009. Monitoring therapy in canine malignant lymphoma and leukemia with serum thymidine kinase 1 activity- evaluation of a new, fully automated non-radiometric assay. International Journal of Oncology 34: 505–510.

Wait, R., Miller, I., Eberini, I., Cairoli, F., Veronesi, C., Battocchio, M., Gemeiner, M. & Gianazza, E., 2002. Strategies for proteomics with incompletely characterized genomes: The proteome of Bos taurus serum. Electrophoresis 23: 3418–3427.

Weigand, K. & Alpert, E., 1981. Human albumin synthesis via an albumin precursor in liver tissue slices. Cellular and Molecular Life Sciences 37: 1145–1147.

Wilkerson, M.J., Dolce, K., Koopman, T., Shuman, W., Chun, R., Garrett, L., Barber, L. & Avery, A., 2005. Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules. Veterinary Immunology and Immunopathology 106: 179–196.

Williams, M.J., Avery, A.C., Lana, S.E., Hillers, K.R., Bachand, A.M. & Avery, P.R., 2008. Canine lymphoproliferative disease characterized by lymphocytosis: immunophenotypic markers of prognosis. Journal of Veterinary Internal Medicine 22: 596–601.

Wilson, C.R., Regnier, F.E., Knapp, D.W., Raskin, R.E., Andrews, D.A. & Hooser, S.B., 2008. Glycoproteomic profiling of serum peptides in canine lymphoma and transitional cell carcinoma. Veterinary and Comparative Oncololgy 6: 171–181.

Yamashita, K., Fujinaga, T., Miyamoto, T., Hagio, M., Izumisawa, Y. & Kotani, T.,1994. Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. The Journal of Veterinary Medical Science/The Japanese Society of Veterinary Science 56: 487–492.

Yamazaki, J., Baba, K., Goto-Koshino, Y., Setoguchi-Mukai, A., Fujino, Y., Ohno, K. & Tsujimoto, H., 2008. Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. Veterinary Immunology and Immunopathology 126: 321–331.

Yoon, J., Feeney, D.A., Cronk, D.E., Anderson, K.L. & Ziegler, L.E., 2004. Computed tomographic evaluation of canine and feline mediastinal masses in 14 patients. Veterinary Radiology and Ultrasound 45: 542–546.