THESIS

CHARACTERIZATION, QUANTIFICATION, AND BEHAVIOR OF NEOPLASTIC MONOCLONAL GAMMOPATHIES IN DOGS AND CATS

Submitted by

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ABSTRACT

CHARACTERIZATION, QUANTIFICATION, AND BEHAVIOR OF
NEOPLASTIC MONOCLONAL GAMMOPATHIES IN DOGS AND CATS

Monoclonal immunoglobulin (M-protein) production can occur in a number of myeloma related diseases (MRD) in domestic animals, including multiple myeloma (MM), extramedullary plasmacytomas, solitary osseous plasmacytoma, IgM Waldenstroms macroglobulinemia/lymphoplasmacytic lymphoma, immunoglobulin secreting lymphomas and leukemias, and plasma cell leukemia. This thesis seeks to improve upon the current knowledge of MRDs by examining diagnostic methods, possible biases (such as hyperproteinemia), and by describing a large population of dogs and cats with confirmed M-proteins.

Differentiation of polyclonal and monoclonal gammopathies can be achieved by serum protein electrophoresis (SPE) and immunofixation (IF). Agarose gel electrophoresis (AGE) is the most commonly used SPE method within veterinary medicine and a previous study validated a method for AGE SPE densitometric M-protein (dM-protein) quantification. Capillary zone electrophoresis (CZE) is another method of SPE that can be performed more rapidly than AGE SPE that may have increased sensitivity with similar specificity. We sought to compare these two methods of SPE to determine if CZE SPE is a comparable alternative method for dM-protein quantification in dogs and cats. We found that these methods performed similarly, and both appear to be acceptable methods for dM-protein quantification, but they should not be used interchangeably. This finding indicates that the previously published method for dM-protein quantification can also be used with CZE SPE in dogs and cats.

Hyperglobulinemia, hyperproteinemia, and hypoalbuminemia are frequently used criteria to prompt SPE in dogs and cats, but M-protein production can occur in humans and animals without these criteria being met. The assumption that these criteria need to be present to raise concern for M-protein production may lead to delayed diagnosis in patients that have early MRD or low concentration M-protein production. Retrospective evaluation of samples submitted to our lab for SPE and IF between January

2014 and December 2019 identified 18 cases of confirmed M-proteins in dogs with normal total protein concentrations. Most of these animals had confirmed, or suspected, myeloma related disease or lymphoproliferative disorders which prompted SPE. A subset of these cases were evaluated to highlight the diagnostic utility of IF in cases with low concentration M-proteins. In all 7 cases evaluated, IF was needed to make a definitive diagnosis of an M-protein. Based on these findings, we recommend running SPE and IF in tandem to increase diagnostic accuracy for M-protein detection.

Large studies characterizing dogs with monoclonal immunoglobulins are rare within the literature, with the largest study by Matus et al from 1984 describing 60 dogs with MM. We sought to retrospectively evaluate a large population of dogs with SPE and IF confirmed M-proteins to add to the available literature, evaluate previously published MM prognostic indicators, assess for novel prognostic indicators, and evaluate other clinicopathology and clinical variables. 113 canine cases were included in our analysis with a total of 75 cases having complete medical records available for analysis. MM was the most common diagnosis within our population, with fewer cases falling under the spectrum of MRD. The mean age of animals diagnosed with an M-protein was 9.9 years. Treatment of MM with prednisone and melphalan led to statistically longer MSTs in these cases when compared to single agent therapy with prednisone or melphalan. Clinical signs were frequently non-specific, but some cases presented with clinical signs that are potentially more specific for MRD such as collapse, evidence of bleeding diathesis, and musculoskeletal pain. Ancillary diagnostic testing such as PCR for antigen receptor rearrangement (PARR), flow cytometry, and/or immunohistochemistry/immunocytochemistry was required in some cases to fully categorize disease. Adoption of visceral organ involvement as a primary or alternative diagnostic criterion for MM may be more likely to appropriately categorize animals with MM, at least based on the clinical course of disease. Animals within our population also frequently had total hypercalcemia, proteinuria, and occasionally had renal dysfunction. Frequently used negative prognostic indicators failed to demonstrate statistical significance (except for renal disease), but occasionally appeared to have clinically significant impacts on survival time.

Similar to dogs, the available literature for cats with monoclonal immunoglobulins is sparse. The largest two studies in cats are Mellor et al and Patel et al, with 24 cats with MRD and 16 cats with MM, respectively. Again, we sought to add to the available data on cats with SPE/IF confirmed M-proteins by looking at the same criteria that were evaluated in dogs. Overall, cats with MRDs had a poorer prognosis when compared to dogs, with the exception of B cell chronic lymphocytic leukemia/lymphoma (BCLL) cases. Evidence of bleeding diathesis was not observed in our cat population and the presence of lytic bone lesions was uncommon. Hypocholesterolemia was a negative prognostic indicator for cats with MRDs (excluding BCLL, which was not included in these analyses). The validity of renal azotemia, hypercalcemia, proteinuria, and BJP as prognostic indicators could not be fully assessed in this study, due to a number of factors. Lastly, the addition of visceral organ involvement to the current veterinary MM diagnostic scheme may be warranted and can make categorization of MRDs and diagnosis of MM easier, while still correlating with the clinical behavior of these diseases in cats.

The work within this thesis adds to available knowledge of MRD in dogs and cats. We have shown that the lack of hyperproteinemia does not rule out the possibility of an M-protein, especially in cases with suspected MRD. Additionally, running SPE and IF in tandem can capture cases with M-protein production that may be missed with SPE alone. CZE SPE is an acceptable alternative method for dM-protein quantification and can be used when AGE SPE is not available. Finally, we present the largest retrospective analysis of dogs and cats with confirmed monoclonal immunoglobulins to date. Similar to previous works, we found that the course of disease is more aggressive in cats with MM when compared to MM in dogs. Renal disease in dogs and hypocholesterolemia in cats were found to be negative prognostic indicators in our study, but other negative prognostic factors either failed to reach statistical significance or could not be evaluated. We strongly recommend the consideration of visceral organ involvement as an additional or alternative criterion for the diagnosis of MM in dogs and cats, as animals diagnosed with MM with this scheme had disease courses similar to those diagnosed with the current scheme. Further work should examine the frequency of BJP, proteinuria, bone involvement in MM cases without apparent musculoskeletal clinical signs.

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Lastly, I want to sincerely thank my friends and family. I don't think I could've achieved all that I have without your support and love. I look forward to spending more time with you as I settle into post-academic life.

DEDICATION

I would like to dedicate this work to my big brother, Christopher Michael DeManicor. I love you and miss you terribly.

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CHAPTER 1: INTRODUCTION

BACKGROUND

Myeloma related disorders (MRD) are caused by malignant transformation within immunoglobulin secreting B lymphocytes or plasma cells. This disease group encompasses a number of diseases/syndromes, including multiple myeloma (MM), extramedullary plasmacytomas, solitary osseous plasmacytoma (SOP), IgM Waldenstroms macroglobulinemia/lymphoplasmacytic lymphoma, immunoglobulin secreting lymphomas and leukemias, and plasma cell leukemia.[1–3]

B cell development has two phases. The first phase is antigen independent and occurs within the bone marrow and ileal Peyer's patches.[3] The second phase, an antigen dependent phase, occurs in the peripheral lymphoid tissues.[3] B cell lymphocytes are responsible for the creation of antibodies. Once a satisfactory antibody is matured via the process of somatic mutation, the B cell produces terminally differentiated daughter cells known as plasma cells.[3] This process results in production of a polyclonal repertoire of immunoglobulins that are all slightly different from each other. When malignant transformation occurs, the clonal expansion of a single neoplastic cell results in production of a monoclonal immunoglobulin.[4,5]

Detection of monoclonal immunoglobulins

Serum protein electrophoresis (SPE) is utilized in human and veterinary medicine to evaluate protein fractions and aid in the diagnosis and detection of various disease conditions that can cause paraproteinemia and dysproteinemia.[1,3] Characterization of protein fractions in human and veterinary medicine is most frequently performed using agarose gel electrophoresis (AGE) and immunofixation (IF), but capillary zone electrophoresis (CZE) and immunosubtraction are also used.[6–9]

Separation of proteins in AGE occurs within a gel matrix and is based on size and electrical charge.[10] Conversely, CZE operates within an aqueous alkaline buffer filled capillary tube and is based on electroosmotic flow, resulting in separation of proteins based on their size and charge.[11,12] Due to the high

separation efficiency of CZE, the test can be carried out much quicker than AGE.[9,12] Previous work has reported that CZE has better resolution and increased sensitivity with comparable specificity for identification monoclonal immunoglobulins.[7,12] All of these factors make CZE an appealing SPE methodology.

Differentiating polyclonal and monoclonal immunoglobulins via serum protein electrophoresis is achieved by evaluating the electrophoretogram and associated gel. Polyclonal immunoglobulins are a heterogeneous protein population that produce a broad peak on the electrophoretogram and correspond to non-restricted smear of protein labeling on the associated gel.[6] As a single protein, a single immunoglobulin clone would be expected to resolve as a distinct tall narrow peak on the electrophoretogram that corresponds to a single restricted band on the associated gel. Based on this expected appearance, commonly used criteria to diagnose a monoclonal immunoglobin by electrophoresis alone is a tall peak that is as tall, or taller, than the albumin peak, as narrow, or narrower, than the albumin peak, or a peak that has a height:width ratio of 4:1.[13] With both polyclonal and monoclonal immunoglobulins, there can be an associated reduction in the albumin fraction concentration, likely secondary to the fact that albumin is a negative acute phase protein or as a compensatory mechanism to maintain plasma oncotic pressure in the face of hyperglobulinemia.[14,15]

Immunofixation is an adjunctive electrophoretic technique that can specifically label immunoglobulin classes and subclasses. It is recommended for isotype determination and can be essential in the identification of low amplitude monoclonal immunoglobulin peaks, including monoclonal free immunoglobulin light chains within the serum or urine.[6,14–17]

Many cases with an immunoglobulin secreting neoplasm will present with a single monoclonal immunoglobulin. Some neoplastic cases will present with 2 distinct monoclonal immunoglobulin isotypes as a result of either class switching within a subset of the tumor population or the concurrent presence of 2 different tumors.[18] Additionally, some monoclonal immunoglobulins will exist as monomers, dimers and/or multimers in the serum.[19] Both cases with 2 distinct monoclonal immunoglobulins and cases with a dimerized or multimerized monoclonal immunoglobulin will have an

electrophoretic pattern characterized by a pair of restricted peaks. Immunofixation can distinguish cases with 2 monoclonal appearing peaks that label with different immunoglobulin class but it cannot distinguish dimerized immunoglobulins from a pair of monoclonal immunoglobulins of the same class.

Several non-neoplastic conditions have been reported to produce an electrophoretic pattern suggestive of a monoclonal immunoglobulin. Some polyclonal immunoglobulin classes can migrate with a very restricted electrophoretic morphology that can be confused for a monoclonal immunoglobulin.[20] Inflammation can produce increases in non-immunoglobulin proteins that appear as an atypical, restricted pattern on electrophoresis. Additionally, some inflammatory responses can have a polyclonal immunoglobulin response which includes several restricted immunoglobulin bands which produce a monoclonal immunoglobulin pattern within a broader smearing of immunoglobulins.[15] Infectious and inflammatory etiologies (*Ehrlichia*, *Leishmania*, heartworm disease, pyoderma, and feline infectious peritonitis (FIP)) have been reported to produce monoclonal immunoglobulin patterns in animals.[4,6–8] Additionally, primary hyperparathyroidism in humans and animals has been reported to be associated with a monoclonal immunoglobulin pattern.[9,10] Whether these truly are a monoclonal expansion of immunoglobulin as part of a non-neoplastic process, a monoclonal immunoglobulin produced by a concurrent and unrelated lymphoid neoplasm, or a monoclonal appearing polyclonal immunoglobulin is not clear.[21]

The nomenclature used to describe monoclonal immunoglobulins has undergone change over time, resulting in a set of terms that can sometimes overlap and cause confusion. Recommendations have been made to standardize terminology in human medicine and these have been incorporated into the most recent human guidelines.[22,23] Much of the previous veterinary literature, including statements of diagnostic criteria for multiple myeloma and myeloma related disease originate from before these human recommendations: they use the term monoclonal gammopathy interchangeably to describe the electrophoretic pattern of a single or multiple restricted bands presumed to be immunoglobulin and the syndrome of having a monoclonal immunoglobulin. This work will conform with the human recommended terms. The terms ‘monoclonal protein’, ‘M-protein’, and ‘paraprotein’ will refer to a

monoclonal immunoglobulin, or immunoglobulin fragment. The electrophoretic pattern of a single atypical, restricted band that could be compatible with the presence of an M-protein will be referred to as a monoclonal gammopathy, independent of the confirmation of neoplasia or location within the electrophoretic tracing. Cases with a pair of monoclonal appearing restrictions in any location that are compatible with the presence of 1 or more M-proteins will be called a biclonal gammopathy and presumed to be a biclonal appearance of a single monoclonal immunoglobulin unless they can be confirmed to be the result of 2 different classes by immunofixation, which will be called a true biclonal gammopathy. When M-proteins composed of only the immunoglobulin light chain are found in the serum, they will be called serum monoclonal free light chains and when they are found in the urine, they will be called Bence-Jones Proteins (BJP), though admittedly they are biochemically the same protein being detected in 2 locations.

M-protein quantification methods

Work by Harris et al validated the AGE densitometric corrected perpendicular drop (area under the curve, AUC) method as the preferred method for the quantification of M-proteins (dM-proteins) in canine serum samples when compared to radial immunodiffusion (RID) and ELISA.[24] RID is the most commonly available method for M-protein quantification; however, RID has been shown to significantly overestimate M-protein concentrations and have difficulties correctly identifying IgM monoclonal proteins.[24] The current recommendation in human and veterinary medicine is that SPE and IF should be used for M-protein quantification and isotype determination, respectively.[16,24] To our knowledge, there had not been literature that assesses CZE as a method of M-protein quantification in veterinary medicine, although this is described in human medicine.[7–9,12] One of the aims of this project was to compare these two methods (AGE and CZE) of SPE to determine if CZE is a comparable method for densitometric quantification of M-proteins in small animals.

Myeloma related disease in humans

In human medicine, there has been significant effort to categorize myeloma related diseases, develop diagnostic criteria, identify prognostic factors, and optimize treatment regimens.[25–27] Human

multiple myeloma is almost always preceded by a premalignant asymptomatic stage known as monoclonal gammopathy of undetermined significance (MGUS).[28] MGUS is estimated to be present in approximately 3-4% of the population above 50 years of age, with a rate of progression to multiple myeloma of 0.5-1% per year.[28] In 2014, the International Myeloma Working Group (IMWG) recommended updates to the MM diagnostic criteria that better identify patients at risk of end organ damage (heart, kidney, eyes, and brain), promote earlier detection and treatment to minimize long term effects, and increase survival times in these patients. The IMWG also reached a consensus in 2011 which states that documentation of the presence of reliable biomarkers associated with approximately 80% probability of progression to MM within two years should result in a diagnosis of multiple myeloma and an offer of therapy.[28] The revised diagnostic criteria for multiple myeloma in people is ≥10% clonal bone marrow plasma cells or biopsy-proven bony or extramedullary plasmacytoma with any one or more of the following criteria being met: presence of myeloma defining events with evidence of end organ damage that can be attributable to the underlying plasma cell neoplasia (CRAB lesions; hypercalcemia, renal insufficiency, anemia, or bony lesions) and/or any number of biomarkers of malignancy (clonal bone marrow plasma cell percentage ≥60%, involved:uninvolved serum free light chain ratio ≥100, or >1 focal lesion on MRI studies).[28] Smoldering multiple myeloma was defined as serum monoclonal protein (IgG or IgA) ≥30g/L or urinary monoclonal protein ≥500mg/24 hours or 10-60% clonal plasma cells in bone marrow and absence of myeloma defining events or amyloidosis.[28] A recent human study concluded that people with the IgA M-protein isotype had a shorter mean survival time when compared to IgG isotypes.[10] With recent advancements in treatment options for people, treatments options now include chemotherapy and stem cell transplantations.[27,29]

Myeloma related diseases in dogs and cats

Multiple myeloma appears to be the most common veterinary MRD.[1] Most cases of MM are secretory in veterinary species and man, but there are rare case reports describing non-secretory MM in veterinary species.[30,31] No studies have been performed to determine the incidence of non-secretory MM in animals, likely due to the challenges faced in diagnosing these cases and confirming a lack of

immunoglobulin secretion. M-proteins have been found to occur in approximately 6% of canine lymphoma cases and in up to 68% of canine chronic lymphocytic leukemias (CLL) cases.[1,32] Cutaneous plasma cell tumors fall under the spectrum of MRDs, but are typically benign and surgical excision is curative. It is unclear how frequently these are secretory, but there are few case reports of cutaneous plasma cell tumors progressing to more aggressive diseases such as multiple myeloma or plasma cell leukemia.[2,33,34]

In veterinary medicine, there are diagnostic criteria for multiple myeloma, IgM Waldenstrom macroglobulinemia, and other B-cell lymphoproliferative diseases.[1,3] Much of this work will focus on MM and its diagnostic criteria since this is the most common cause of M-proteins within our study populations. Current veterinary diagnostic criteria for MM require that at least 2 criteria must be present (>20% bone marrow plasma cells, radiographic evidence of lytic bone lesions, serum monoclonal gammopathy, or BJP).[1,3] Using the definitions provided above, we interpret the diagnostic criteria for a serum monoclonal gammopathy to be fulfilled when a serum M-protein can be documented. While a monoclonal free light chain in the serum is biologically identical to documenting BJP, previous literature referring to BJP has only focused on urine: a serum monoclonal free light chain will be categorized as a serum M-protein and will be used to fulfill the diagnostic criteria of a serum monoclonal gammopathy. If both a serum monoclonal free light chain and BJP are documented in the same patient, they will be considered as meeting only 1 of the diagnostic criteria so as not to double count the monoclonal free light chain.

MRDs encompass the same spectrum of diseases in cats, and the criteria for diagnosis of MM is similar, with some literature suggesting that visceral (hepatic/splenic/lymph node) involvement can, or should, be used interchangeably with bone marrow plasma cell neoplasia in cats due to the lower incidence of bone marrow involvement in this species.[5,35] Common treatment regimens for MM in dogs consists of melphalan and prednisone, while feline MM is frequently treated with cyclophosphamide and prednisolone.[1]

The current recommendation for assessment of a patient's response to treatment is repeated monitoring of the M-protein concentration, as measured by SPE. If there is a lack of apparent M-protein on electrophoresis, IF is recommended to assess for residual low concentration M-protein; the lack of M-protein by IF suggests complete response.[36] Recent work has found that the human IMWG response criteria can be used in dogs with secretory MM and correlates with median survival time (MST) in this species.[36] More work needs to be done to see if this also translatable to other species with MM.

Monoclonal immunoglobulins in dogs and cats

Very few large studies of dogs with an electrophoretic pattern consistent with monoclonal immunoglobulins have been published. The two largest studies included a group of 60 dogs with MM and IgA or IgG paraproteins and a group of 18 cases with a monoclonal gammopathy pattern and both neoplastic and non-neoplastic causes.[4,37] There are other publications that discuss individual cases and small case series as well.[2,38–41] Matus et al did not find a significant correlation with M-protein isotype and prognosis in dogs.[37] Repeatedly documented negative prognostic indicators in canine MM include, hypercalcemia, proteinuria, BJP, extensive lytic bone lesions, and renal disease/azotemia.[1,4,37] Some publications suggest a male predisposition, while others have not supported this.[37,42] MST in dogs with MM treated with melphalan and prednisone is 540 days and 220 days for dogs treated with prednisone alone.[37]

The MST for cats with MM is less favorable than dogs, and is reported to be between 4-13 months, depending on the publication.[1] Classification of MM as aggressive and less aggressive in the cat has been proposed as a way of better predicting long-term outcome in cats.[43] Aggressive MM is characterized by hypercalcemia, bony lesions with pathologic fractures, anemia, presence of BJP, azotemia, persistence of high serum protein concentration after 8 weeks of treatment, and little or no clinical improvement.[43] Less aggressive MM is characterized by normal serum calcium/creatinine/blood urea nitrogen (BUN)/packed cell volume (PCV), presence of bony lesions without fractures, absence of BJP, and normalization of serum protein concentration after 8 weeks of treatment.[43] A single report

evaluated these criteria in 9 cats and found the MST for cats in the "aggressive" and "nonaggressive" groups was 5 days and 387 days, respectively.[43]

M-proteins in patients with normal total proteins

Hyperproteinemia and/or hyperglobulinemia are commonly used indications to perform SPE to assess for M-proteins, especially in cases of confirmed B cell lymphoid neoplasia or plasma cell tumors (mainly medullary, but occasionally extramedullary plasma cell tumors and SOP). SPE may also be employed in the initial work up for patients with hyperglobulinemia to differentiate polyclonal gammopathies due to infectious, inflammatory, or immune mediated processes from monoclonal gammopathies that warrant further evaluation of an immunoglobulin secreting neoplasm. Some literature suggests that a serum total protein concentration above 9.0 g/dl should raise suspicions for an underlying myeloma related disorder.[44] While a high total protein concentration could logically be used as a suggestion for a protein dyscrasia, several large studies in humans, one with 534 cases and another with 156 cases, revealed that a significant proportion of patients with M-proteins, 59% and 31% respectively, had normal total serum protein concentrations.[45,46]

There have been no large studies in veterinary medicine that assess whether similar findings are found in dogs and cats. However, upon searching the literature there are several small case series and individual case reports detailing the presence of an M-protein without concurrent elevation in the total protein or globulins.[36,47,48] It is unclear if these cases are truly rare, or simply underreported/diagnosed due to the lack of the classically observed hyperproteinemia.

Better characterizing the incidence of M-proteins in patients with normal total proteins and/or normal globulin concentrations would inform clinicians and provide potential guidelines and data to bolster the decision to pursue SPE in appropriate cases. In one of the aims of this project, we sought to characterize cases with normal serum total protein concentrations (≤ 7.5 g/dL for dogs) and M-proteins submitted to a veterinary diagnostic laboratory. We hypothesize the incidence of M-protein containing samples that have normal total proteins will be low and that additional diagnostics such as

immunofixation may be required in some cases to definitively identify low amplitude/concentration M-proteins.

PROJECT OVERVIEW

The overarching aims of this project include the characterization of M-proteins in dogs and cats. Assessment for additional or alternative methods for monoclonal protein quantification (CZE) may increase the availability of this diagnostic test and make diagnosis and monitoring more easily achieved. Additionally, we would like to characterize cases with confirmed M-proteins and normal total proteins to identify key features which may guide clinicians in the decision to pursue advanced testing, such as SPE and IF.

SPECIFIC PROJECT AIMS

Aim 1: Determine if CZE is a comparable method to AGE for M-protein densitometric concentration quantification in dog and cat sera.

Objectives:

1. Compare AGE and CZE densitometric M-protein concentrations using Pearson's correlation for the entire population, dogs only, and cats only.
2. Assess for, and compare, bias between both methods using Passing-Bablok regression analysis and Bland-Altman plot analysis.
3. Use method evaluation decision chart (MEDx chart) to compare performance between methods at a decision limit of 0.5g/dL for each group.

Aim 2: Characterize a population of dogs with neoplastic monoclonal immunoglobulins and normal total protein concentrations.

Objectives:

1. Estimate the incidence of normoproteinemia or nomoglobulinemia at the time of an SPE diagnosis of M-proteins in dogs evaluated in 2019.

2. Evaluate the strength of association between commonly accepted criteria used to prompt SPE (such as hyperproteinemia, hyperglobulinemia, and hypoalbuminemia) and the presence of an M-protein.

3. Describe population characteristics for patients with concurrent normoproteinemia and an M-protein and assess available clinical data to determine what prompted clinicians to perform SPE in these cases.

4. Illustrate the utility of IF to diagnose M-proteins using a blinded review of SPE data in several cases with low concentration/amplitude M-proteins.

Aim 3: Retrospective characterization of neoplastic monoclonal immunoglobulins in dogs.

Objectives:

1. Determine population characteristics of dogs diagnosed with neoplastic M-proteins between January 2014 through December 2020.

2. Assess and describe diagnoses, treatment regimens, median survival times, initial hematologic and biochemical abnormalities, frequency of presenting clinical signs, frequency of evidence of bleeding diathesis and coagulopathies, and cytologic and histopathologic abnormalities in dogs with myeloma related diseases with an emphasis on MM.

3. Evaluate the clinical utility of previously published prognostic indicators (such as hypercalcemia, proteinuria, renal azotemia, BJP, neutrophil to lymphocyte ratios (NLR)) in dogs with MM.

4. Assess for novel prognostic indicators in dogs with myeloma related diseases.

5. Evaluate various MM diagnostic schemes (current veterinary multiple myeloma diagnostic criteria, current human diagnostic criteria, and current veterinary criteria with inclusion of visceral organ involvement) to determine which diagnostic scheme best correlated with the clinical diagnoses in our sample population.

Aim 4: Retrospective characterization of neoplastic monoclonal immunoglobulins in cats.

Objectives:

1. Determine population characteristics of cats diagnosed with neoplastic M-proteins between January 2014 through December 2020.

2. Assess and describe diagnoses, treatment regimens, median survival times, initial hematologic and biochemical abnormalities, frequency of presenting clinical signs, frequency of evidence of bleeding diathesis and coagulopathies, and cytologic and histopathologic abnormalities in cats with myeloma related diseases

3. Evaluate previously published prognostic indicators (such as hypercalcemia, proteinuria, BJP, and hypocholesterolemia) in cats with MM/MRD.

4. Assess for novel prognostic indicators in cats with myeloma related diseases.

5. Evaluate various MM diagnostic schemes (current veterinary multiple myeloma diagnostic criteria, current human diagnostic criteria, and current veterinary criteria with inclusion of visceral organ involvement) to determine which diagnostic scheme best correlated with the clinical diagnoses in our sample population.

CHAPTER 2: M-PROTEIN QUANTIFICATION METHOD COMPARISON[1]

MATERIALS AND METHODS

Samples

The use of patient samples complied with institutional policies and owner consent. Laboratory identification numbers were used to ensure proper sample identification. A total of 51 serum samples from 22 dogs (33 samples) and 18 cats (18 samples) with adequate volume and previously diagnosed quantifiable (> 0.3 g/dL) monoclonal or biclonal gammopathies were used. Serial submissions for 3 dogs over the course of their disease were included. Samples had been submitted to the Colorado State University Veterinary Clinical Pathology Laboratory between January 2016 and December 2018. Samples were archived as part of normal sample processing within the clinical pathology laboratory, were free of significant hemolysis, icterus, and lipemia, and had been stored at -80°C until evaluation. Total protein concentration had been assessed at the time of initial submission using a biuret assay (Cobas c501: Roche Diagnostics. Indianapolis, IN, USA).

AGE SPE had been performed at the time of initial sample submission using amido black stained AGE (Sebia Hydrasys with Hydrogel Protein (E) with amido black kit, Sebia, France), a flat-bed scanner (Epson Perfection V700 Photo, Epson America, Inc, Long Beach, CA, USA), and Phoresis software (version 8.6.3, Sebia, France), as previously described.[24]

Involved immunoglobulin class was characterized using immunofixation if it had not been identified at the time of initial evaluation and there was sufficient sample volume. Routine immunofixation in dogs targeted IgG-FC, IgG4, IgA, and IgM heavy chain and light chain using canine-specific reagents and was performed as previously described.[24,49] Routine immunofixation in cats targeted IgG, IgA, and IgM heavy chain and light chain.[17] Free light chain (fLC) immunofixation had been

[1] Jeffries CM, Harris RA, Ashton L, Moore AR. Method comparison for serum protein electrophoretic M-protein quantification: Agarose gel electrophoresis and capillary zone electrophoresis in canine and feline sera. *Veterinary Clinical Pathology*. 2021;50(4):543-550. doi:10.1111/VCP.13029

performed using human-targeted fLC IF antibody set (Sebia Free light chains kit, Sebia, France) on a limited number of samples.[17] Serum fLC was found in samples at concentrations below the reportable limit of dM-protein (0.3 g/dL) and did not overlap with the complete M-protein bands and therefore the fLC bands were not further assessed for this study. The canine sample set included 4 IgG, 14 IgA, 3 IgM M-proteins, and 1 case with insufficient sample to perform immunofixation to identify the M-protein class. The feline sample set included 12 IgG, 3 IgA, 1 IgM, and 2 IgG and IgA true biclonal cases. Electrophoretic location of the M-proteins determined by AGE and IF, stratified by species and isotype is presented in Table 2.1. Cases with a biclonal appearance composed of bands of the same class were presumed to be the result of dimerization and treated as a single M-protein.[50] True biclonal cases were treated as 2 M-proteins. In one of the two IgG/IgA true biclonal cases, the amount of IgA M-protein was below the limits of detection; only the IgG M-protein was quantified in this case. The total number of M-proteins was 52. Distribution of involved immunoglobulin class and the number of samples for each class is listed in Table 2.2.

CZE was performed on archived samples in duplicate using Sebia Minicap Protein 6 (Sebia, France).[10] The manufacturer recommendations were followed for sample processing and samples were handled neat or diluted 1:2 with 0.9% saline if total protein was > 10 g/dL. Manufacturer supplied human-based quality control material (IT/IF control, Sebia Inc) that contained 3 restricted immunoglobulin bands in the β1 (IgM), β2 (IgA) and γ globulin (IgG) region was evaluated with each run and were within recommended limits.

The dM-protein concentration was determined in all 51 samples using both AGE and CZE on Phoresis software using the corrected perpendicular drop method, as previously described.[24]

Method Comparison

Intra-run variability for CZE dM-protein was determined from the repeat measurement of patient samples using the logarithmic method outlined by Bland and Altman.[51] Inter-run variability for CZE dM-Protein were measured using the control material. Comparison between the AGE determined dM-protein from the initial evaluation and the average of duplicate runs of CZE determined dM-protein was

performed as recommended.[7] Specifically, Shapiro-Wilks test was used to assess normality and distribution. Pearson's r correlation was evaluated followed by Passing-Bablok and Bland-Altman plot evaluation. Medical decision chart evaluation (Medx) was performed using the previously published AGE dM-protein inter-run CV of 3.5% and dM-protein total allowable error (TEa) of 20%, clinical decision limits of 0.5 g/dL and the median AGE based dM-protein concentration.[8,52]

Statistical analysis was performed using Excel (Microsoft Office 2016; Microsoft, Microsoft, Redmond, WA, USA) with the Real Statistics Resource Pack software (Release 5.4, www.real-statistics.com) and the ACOMED statistik Excel Tool Passing-Bablok Regression with CUSUM test (V03.0, www.acomed-statistik.de). Additional statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA, USA) and Medcalc (v 19.5.2, MedCalc Software Ltd, Ostend, Belgium). Alpha was set at 0.05.

RESULTS

Method Comparison

The M-proteins fell within a similar location on the electrophoretic tracings, for most cases, when AGE and CZE were compared, Figure 2.1. In 3 dogs with an IgG monoclonal gammopathy the M-protein band was in the β2 region by AGE and the mid-γ-globulin region by CZE, Table 2.1.

Shapiro-Wilk test revealed that the AGE and CZE dM-protein datasets were not normally distributed for any of the strata. Using commercially available human quality control material, the inter-run β1 region peak with a 7.3% mean AUC% and SD of 0.56% had an inter-run CV of 7.65% which was greater than the β2 region peak (mean AUC% = 8.7%, SD = 0.46%, inter-run CV = 5.28%) or the γ-region peak (mean AUC% = 12.6%, SD = 0.46%, inter-run CV = 3.71%). Using patient samples, the intra-run CV for the CZE was 4.18% for the entire population, 4.74% for dogs and 2.89% for cats.

The range ratio was large for all groups (all samples 39.7, canines only 31.4, and felines only 19.3) and simple linear regression correlation coefficient for comparison of AGE dM-protein to CZE dM-protein concentrations for the entire study population, canines only, and felines only was < 0.99 for some groups (Table 2.3, Figure 2.2). Pearson correlation coefficients indicated a stronger agreement between

AGE and CZE canine samples compared to feline samples (Table 2.3). Passing-Bablok regression analysis revealed a minimal amount of proportional bias (slope, Table 2.3) in the canine group and a slightly larger amount in feline samples. Systematic (y-intercept) bias was observed in the all samples group but was not apparent in the canine and feline only sample analysis; the 95% CI of the y-intercept failed to include 0 for the entire study population, but the canine only group and feline only group both included 1 in the 95% CI of the slope and zero in the 95% CI of the y-intercept.

Bland-Altman plots were prepared (Figure 2.3). Constant and proportional bias was not apparent for the entire population or when only canine cases were evaluated. The 95% CI of the y-intercept did not include 0 for the feline only dataset indicating potential proportional bias, Table 2.3. Proportional bias was visually apparent in the feline only dataset and the difference between methods was greater than could be explained by the combined imprecision of both assays in greater than the expected and allowable 5% of cases for both canine only samples (4/33, 87.8%) and feline only samples (2/18 88.9%), $p < 0.001$ Figure 2.3.

MEDx evaluation suggested poor performance at a decision limit of 0.5 g/dL for all strata. At median AGE dM-protein concentration, MEDx evaluation suggested marginal performance for all samples (3.74 g/dL decision limit), marginal performance for canine only samples (3.88 g/dL decision limit) and poor performance for feline only samples (5.26 g/dL decision limit).

DISCUSSION

Available data indicates that quantification of M-proteins using CZE in dogs and cats correlates well with the previously validated method for densitometric quantification of M-proteins in canine serum samples using AGE. However, the methods do not appear to be comparable under all conditions and so should not be used interchangeably without further support.

The intra-run CV for CZE-based methods was comparable but slightly higher than the reported AGE-based method. The inter-run CVs varied depending on the location of the control peaks. This phenomenon has been previously reported for CZE, and similar to our study, higher concentrations yielded smaller CVs.[8] This previous report also revealed that peaks within the albumin and γ globulin

fractions tended to have lower CVs, which was hypothesized to be related to the higher proportion of the total protein falling within these regions. Along those lines, peaks within the α and β regions tended to have higher CVs, which correlated with lower amplitude/concentration peaks.[8] A similar pattern was noted in our data with the lowest concentration peak, the β1 Ig peak, having the highest CV. Because CV is calculated as standard deviation/mean, when 2 processes have a similar standard deviation but one has a smaller mean, the process with the smaller mean will have a higher CV. Close evaluation of the data shows that not only did the β1 Ig peak have a lower mean it also had a higher SD than the other peaks, suggesting there truly may be a greater amount of variability in the measurement of M-protein bands in the β1 region compared to other regions. This may be due to effects of superposition of the M-protein over normal proteins in that electrophoretic region.

All strata had a simple linear regression correlation coefficient > 0.975. Passing-Bablok regression was used in conjunction because it does not require the gold standard method to be perfect and does not require error measurements to be normally distributed. Interestingly, the data was compatible with comparable performance in the canine only and feline only strata but this did not hold true when both groups were combined. Similar results were not found after Bland-Altman evaluation as there was the suggestion of proportional bias in the feline datasets and there was greater variation between the methods than could be explained by the effects of combined imprecision for both the canine and feline datasets. This data suggests that the methods were not comparable, but it should be noted that the deviation from the defined limits of acceptable performance was minimal. Previous reports have shown a positive bias of CZE dM-protein concentrations when compared to AGE, in human samples.[9] This positive bias may be due to the better resolution capable with CZE. It is unclear which method is more representative of the true M-protein concentration within the samples although they do yield similar results so establishing this may not be needed.

To evaluate if the methods were clinically comparable, the data was evaluated using TEa and MEDx evaluation. TEa was defined based on previous publication and the fact that current human recommendations distinguish clinical response based on changes of > 25%.[53] Interestingly, recent work

has characterized human M-protein CV_I at between 8.4 and 12.9%.[54,55] This could suggest that either the interpretive criteria need to be based on a greater degree of change or that TEa needs to be narrower. The CV_I for M-protein in the dog or cat is unknown but the human based response criteria appear to be clinically applicable in dogs with MM.[36] Nonetheless, using the current TEa estimate and available data, there appears to be a clinically significant difference between the methods, especially at lower concentrations and these methods should not be used interchangeably.

Densitometry based quantification is well justified in the dog and human, but this method is not fully validated in the cat. Full validation is beyond the scope of this work, but the available data suggests that the method performs relatively similar in the dog and cat. Additional feline samples would ideally be evaluated, but those samples were not available. Given the ability of the method to perform analytically and clinically in two other species and the available data, densitometric quantification of M-proteins in feline samples appears to be appropriate, however, additional data is needed.

There were several limitations to this study. AGE SPE had been performed on initial evaluation without duplication prior to storage. M-protein concentration has been shown to be stable for 5 years under the storage conditions used in this study, but lack of duplicate assessment and the minimal changes associated with storage would both be misattributed to variation in measurement between the 2 methods.[24] Per method comparison guidelines[56], a sample number of at least 40 samples is ideal for a method comparison study. Total sample number was 51 but the canine and feline subgroups were below this target. The range ratio was high, which likely made the data more robust, but a larger sample size would be appropriate.

This study concludes that AGE and CZE methods for M-protein quantification in canine and feline sera yield similar results and that there are inherent biases that may affect clinical utility. These methods should not be used interchangeably for quantification of M-protein.

Table 2.1. Electrophoretic location of the M-protein in 51 serum samples from dogs and cats, stratified by species and isotype. Isotype was not available (NA) for one canine patient. Electrophoretic location was determined using agarose gel electrophoresis (AGE) and capillary zone electrophoresis (CZE)

Species	Isotype	AGE					CZE				
		β1	β1-β2	β2	β2-γ	γ	β1	β1-β2	β2	β2-γ	γ
Feline	A					3					3
	G			1		11			1		11
	M					1					1
	A&G					2					2
Canine	A		3	3	7	1		3	3	7	1
	G			3		1					4
	M	2		1			2		1		
	NA				1					1	

Abbreviations: A, IgA; G, IgG; M, IgM; A&G, IgA and IgG true biclonal.

Table 2.2. Descriptive statistics for agarose gel electrophoresis (AGE) and capillary zone electrophoresis (CZE) based quantification of M-protein (dM-protein) concentration. Isotype was not available (NA) for 1 dog because there was insufficient sample volume for testing.

	Isotype	Number of Individuals	Number of M-proteins	AGE dM-protein concentration					CZE dM-protein concentration				
				Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max
Entire population	All	40	52	4.06	3	3.74	0.4	13.50	3.96	2.83	3.58	0.34	12.45
	IgG	18	18	5.11	3.24	4.15	1.24	13.50	4.79	3.07	3.96	0.71	12.45
	IgA	18	26	3.53	2.37	4.98	1.54	5.78	3.52	2.27	3.21	0.34	9.76
	IgM	4	7	2.42	2.28	0.66	0.42	5.90	2.54	2.41	0.58	0.43	5.94
	NA	1	1	10.67					10.34				
Canine only	All	22	33	3.38	2.7	3.88	0.4	10.7	3.42	2.62	2.70	0.34	10.34
	IgG	4	4	3.52	1.72	3.16	1.60	6.17	3.50	1.63	3.07	1.76	6.11
	IgA	14	22	3.39	2.45	2.93	0.41	9.75	3.47	2.39	2.94	0.34	9.76
	IgM	3	6	1.25	1.52	0.55	0.42	5.90	1.28	1.58	0.54	0.43	5.94
	NA	1	1	10.67					10.34				
Feline only	All	18	19	5.26	3.1	4.82	1.2	13.50	4.89	2.94	4.54	0.71	12.45
	IgG	14	14	5.56	3.42	4.59	1.24	13.50	5.15	3.28	4.37	0.71	12.45
	IgA	4	4	4.32	1.64	4.98	1.54	5.78	3.82	1.40	4.33	1.54	5.07
	IgM	1	1	4.82					5.48				

Table 2.3. Pearson's correlation, Passing-Bablok regression analysis and Bland-Altman difference plot analysis for comparison of dM-protein quantification using AGE and CZE based methods.

		Passing Bablok				Bland-Altman			
	Pearson r	Slope	95% CI	Y-intercept	95% CI	Slope	95% CI	Y-intercept	95% CI
Entire Population	0.99	0.94	0.88 - 1.00	0.28	0.04 - 0.45	0.01	-0.04 - 0.05	0.04	-0.17 - 0.25
Canine Only	0.994	0.98	0.92 - 1.03	0.12	-0.05 - 0.42	0.03	-0.01 - 0.07	-0.14	-0.31 - 0.04
Feline Only	0.985	0.93	0.82 - 1.10	0.16	-0.41 - 0.49	-0.05	-0.14 - 0.04	0.53	0.01 - 1.06

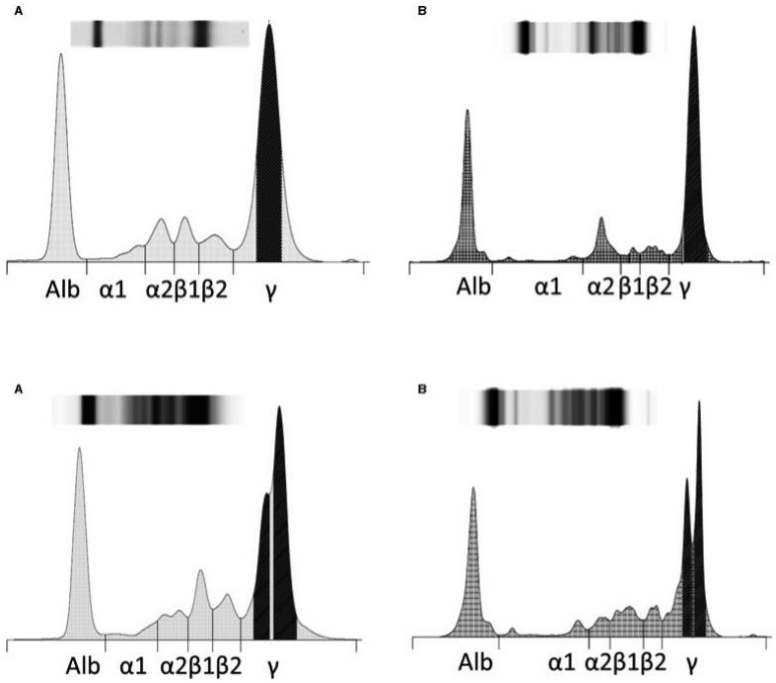

Figure 2.1. Comparison of canine electrophoretic tracings produced using agarose gel electrophoresis (A) or capillary zone electrophoresis (B). The M-protein was quantified using the gel or interpolated gel image using a perpendicular drop method.

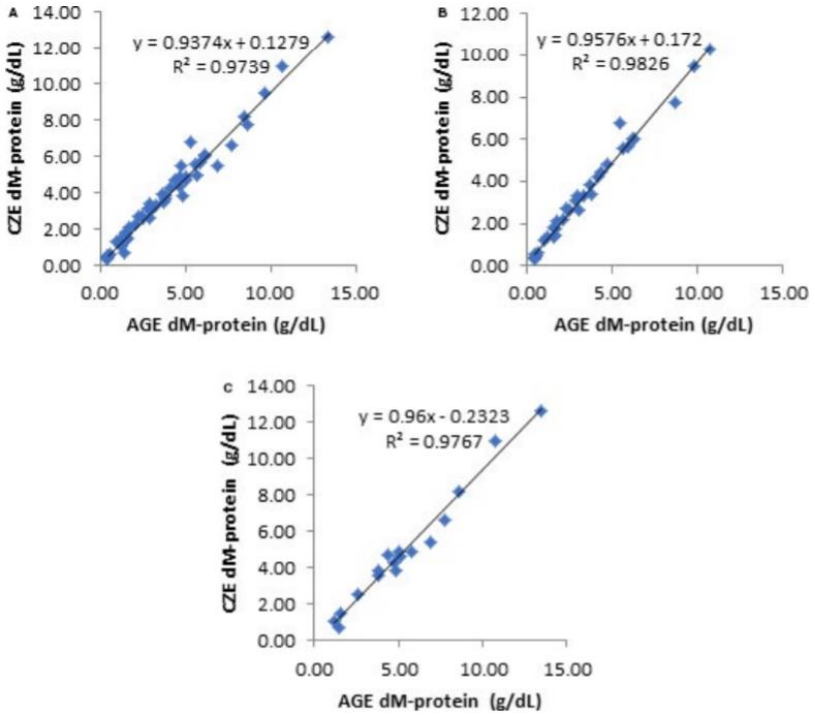

Figure 2.2. Simple linear regression comparison graphs for densitometric M-protein (dM-protein) measurements using agarose gel electrophoresis (AGE) or capillary zone electrophoresis (CZE). A, Entire population. B, Stratification for canine sample results. C, Stratification for feline sample results

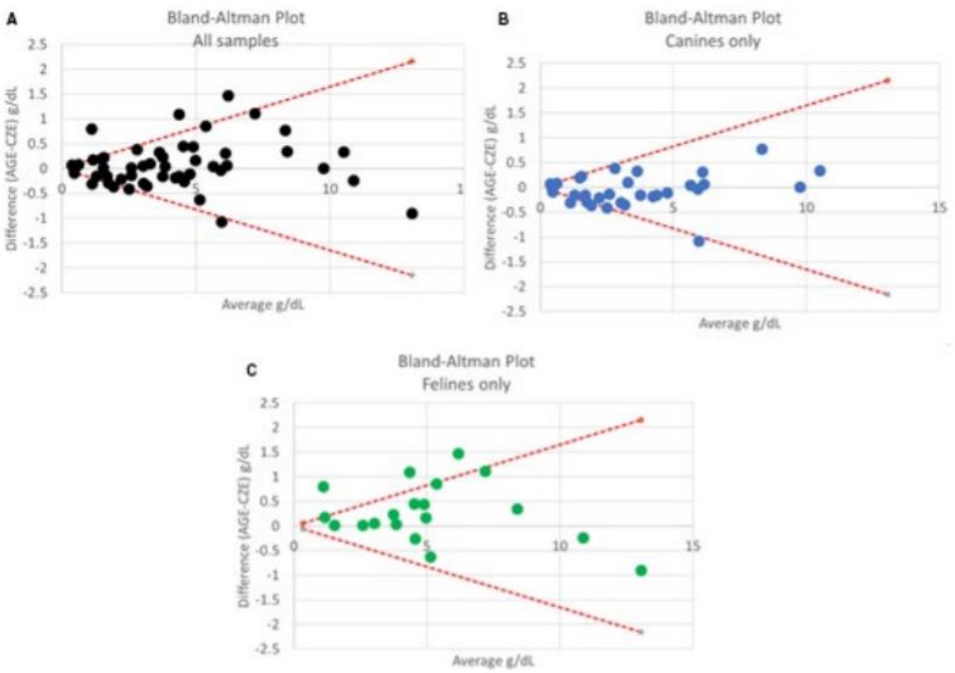

Figure 2.3. Bland-Altman difference plot for the comparison of densitometric M-protein (dM-protein) using agarose gel electrophoresis (AGE) or capillary zone electrophoresis (CZE) for all samples (A), and the canine-only (B), and feline-only (C) groups. Red dotted lines represent the 95% confidence interval derived by the combined imprecision of both methods

CHAPTER 3: NEOPLASTIC MONOCLONAL IMMUNOGLOBULINSIN DOGS WITH NORMAL TOTAL PROTEINS

MATERIALS AND METHODS

Retrospective study of medical records.

The archives at Colorado State University's Clinical Pathology Laboratory were retrospectively searched for samples from dogs that had SPE performed in 2019 (cohort 1). The final SPE interpretation and clinical data were recorded and evaluated. Additionally, cases with an M-protein that was confirmed by both SPE and IF performed between January 2014 and December 2019 and had a TP ≤ 7.5 g/dL were recorded and evaluated (cohort 2).

The albumin and globulin concentrations, albumin globulin ratio (A:G), age, and M-protein isotype and concentration were evaluated. Hypoalbuminemia was defined as an electrophoretically measured albumin fraction of < 3 g/dL. Hyperglobulinemia was defined as an electrophoretically measured globulin fraction of >3.4 g/dL. To demonstrate that IF is an integral part of the initial diagnosis, 7 electrophoretograms from 2019, with an IF confirmed M-protein, normal total protein, and normal globulin concentration were reviewed by 2 pathologists (PA/RAD) blinded to the IF results and classified as definitively containing an M-protein, suspicious for an M-protein, or definitively negative. Records and/or submission paperwork were obtained for 17/18 cases from cohort 2 and were evaluated to determine what criteria were used to prompt serum protein electrophoresis.

Serum Protein Electrophoresis and Immunofixation protocols

SPE was prospectively performed using amido black stained AGE (Sebia Hydrasys with Hydrogel Protein (E) with amido black kit, Sebia, France), a flat-bed scanner (Epson Perfection V700 Photo, Epson America, Inc, Long Beach, CA, USA), and Phoresis software (version 8.6.3, Sebia, France). The manufacturer recommendations were followed for the application of serum, gel processing, and staining procedures. Samples were loaded neat (unaltered/undiluted), except for cases with a total protein

> 10 g/dL which were diluted 1:2 with 0.9% saline, per standard laboratory protocol. Densitometric measurement of M-proteins was performed using a previously validated method.[24]

Routine IF in dogs targeted IgG-FC, IgG4, IgA, and IgM heavy chain and light chain using canine-specific reagents and was performed as previously described.[24,49] Free light chain (fLC) immunofixation was performed using human-targeted fLC IF antibody set (Sebia Free light chains kit, Sebia, France) on a limited number of samples.

Total protein concentration was assessed using a biuret total protein assay (Cobas c501: Roche Diagnostics. Indianapolis, IN, USA). Serum albumin concentrations were also obtained on the Cobas c501 using the bromocresol green method, with subsequent globulin determination using the difference between colorimetric total protein and albumin values.

Statistical Analysis

Simple logistic regression was performed on cohort 1 data to assess the association between the presence of an M-protein and hyperproteinemia, hyperglobulinemia, and hypoalbuminemia. Results are presented as odd ratios (OR), p values, and corresponding 95% CIs. Descriptive statistics (age at diagnosis, total protein concentration, globulin concentration, albumin concentration, albumin globulin ratio, and M-protein concentration) were also obtained for cohort 2 data with the mean, minimum, and maximum values being reported. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS

Cohort 1 – Evaluating odds ratios to determine strength of association between commonly accepted indications for SPE

In 2019, 127 canine samples were submitted to our laboratory for SPE. 68/127 also had IF performed. A total of 56/127 samples had a diagnosed M-protein and 31 of those cases were confirmed by IF. IF evaluation was available at M-protein diagnosis for 11/13 (84.6%) samples with a normal total protein, and 7/8 (87.5%) samples with a normal globulin (<3.4 g/dL). Simple logistic regression was performed using all 127 canine samples from 2019, comparing the presence of an M-protein with the

presence of hyperproteinemia (TP > 7.5 g/dL), hyperglobulinemia (globulin concentration >3.4 g/dL), and hypoalbuminemia (albumin concentration < 3 g/dL). This analysis revealed M-protein containing samples were not more likely to have hyperproteinemia (p = 0.11, OR 1.79, 95% CI 0.97 - 3.7), hyperglobulinemia (p = 0.83, OR 0.89, 95%CI 0.30 - 2.7), or hypoalbuminemia (p = 0.37, OR 1.57, 95% CI 0.60 - 4.5).

To demonstrate that IF is an integral part of the initial diagnosis in these types of cases, 7 electrophoretograms with an IF confirmed M-protein, normal total protein and normal globulin were reviewed by 2 pathologists (PA/ RAD) blinded to the IF results and patient history; the M-protein could not be definitively identified in any of the cases, though an atypical restricted band was noted in 5 samples. Due to lack of IF on all cohort 1 samples, the overall incidence of M-proteins could not be determined. Figure 3.1 provides an example of an electrophoretogram from an M-protein containing sample that cannot be positively diagnosed without IF.

Cohort 2 – Descriptive statistics of cases with a confirmed M-protein and normoproteinemia

Twenty-seven cases with TP ≤ 7.5 g/dl and an SPE and IF confirmed diagnosis of M-protein were identified between 2014 and 2019. 8/27 cases were excluded due to these samples being mid-treatment samples and a single case was excluded because it had a diagnosis of polyclonal B cell lymphocytosis (PBLEB). Records and/or submission paperwork (with sufficient history) were available for the 17/18 cases, which indicated SPE and IF were performed due to concern for an underlying paraproteinemia secondary to multiple myeloma, secretory plasma cell tumor, or B-CLL (B cell chronic lymphocytic leukemia/lymphoma). The mean age at diagnosis was 9.9 years (min 3 years, max 15 years). M-protein was identified in samples with a total protein as low as 5.5 g/dL. The mean albumin concentration was slightly decreased at 2.5 g/dL (min 1 g/dL, max 3.3 g/dL). The mean globulin concentration was slightly elevated at 4.2 g/dL (min 3.1 g/dL, max 6.3 g/dL) yet was within normal limits for 4/18 (22.2%) of cases. The mean A:G was slightly decreased at 0.63 (min 0.2, max 0.94). The M-protein concentration mean was 1.62 g/dL (min 0.5 g/dL, max 3.6 g/dL) and could not be quantified in one case due to being below our established lower limit of detection (<0.3 g/dL). The incidence of M-protein isotypes in this

population was 72.2% IgA (13/18), 11.1% IgG (2/18), 11.1% (2/18) light chain only, and 5.6% IgM (1/18). Complete results can be found in Table 3.1.

DISCUSSION

In cohort 1, we assessed whether the typical factors viewed as indications for SPE were truly associated with a higher likelihood of the presence on an M-protein. Interestingly, we found no association between hyperproteinemia, hyperglobulinemia, or hypoalbuminemia and the presence of a monoclonal immunoglobulin. This lack of association highlights that many etiologies, other than neoplasia, can be associated with hyperproteinemia, hyperglobulinemia, or hypoalbuminemia. These findings are supported by previous work that showed 56.4% of dogs with SPE performed were diagnosed with infectious or inflammatory diseases.[57] In that study by Tappin, only 5.7% (8/140) cases were diagnosed as monoclonal, with a variety of underlying neoplastic processes (4 MM, 1 splenic PCT, 1 hepatic PCT, and 1 lymphoma). Within that population of 140 dogs, the most common abnormalities were decreased albumin fraction concentrations in 59.3% of cases, increased gamma globulin fraction concentrations in 38.6% of cases, and decreased beta 1/2 globulin fraction concentrations in 36.4% and 30% of cases, respectively. This may suggest that the presence of other clinical or diagnostic findings associated with neoplasia (confirmed or suspicion for a lymphoproliferative disease, hypercalcemia, evidence of bleeding diathesis, lytic bones lesions, etc) should also be factored into the decision to pursue SPE and/or IF. Confirmed lymphoproliferative disease, or suspicions for, were present in the majority (13/18) of our cases.

Another important finding was the usefulness of immunofixation for the identification of low amplitude/concentration M-proteins. This has previously been reported within the veterinary literature.[22,23] The subset of cases we examined that had normal total proteins and normal globulin concentrations with concurrent immunofixation revealed that in all cases immunofixation was needed to reach a definitive diagnosis of an M-protein. In five out of seven of these cases there was an atypical band that prompted the recommendation for immunofixation; however, in two cases there was no suspicion for a monoclonal protein based on SPE alone. This raises the concern that M-proteins may be missed without evaluation of

SPE and IF together. We recommend running these in tandem in cases with confirmed B cell neoplasia or a strong concern for a myeloma related disorder, especially when the total protein and/or globulin concentrations are within normal limits. As an aside, light chain only disease is another disorder to consider and was identified in 11.1% (2/18) of our normal total protein population. Production of monoclonal light chain immunoglobulins by neoplastic cells often does not lead to significant accumulations of M-proteins in the blood, as light chains are freely filtered in the urine and thus are only present in low or undetectable concentrations in the blood.[17,40] For this reason, pairing SPE with routine immunofixation +/- free light chain immunofixation may be needed in cases suspected of light chain only disease.

In conclusion, a normal total protein or normal globulin concentration in dogs does not rule out the possibility of an M-protein, especially if there is confirmed (or suspected) lymphoproliferative disease, unexplained hypercalcemia, or lytic bone lesions. IF may be helpful in these circumstances to identify low amplitude M-proteins that may be missed with SPE alone. The addition of free light chain immunofixation may also be helpful in cases with suspected free light chain production. Additional studies are needed to determine if these findings translate to cats and if there are particular lymphoproliferative diseases (besides light chain only disease) that more frequently lead to low amplitude M-proteins.

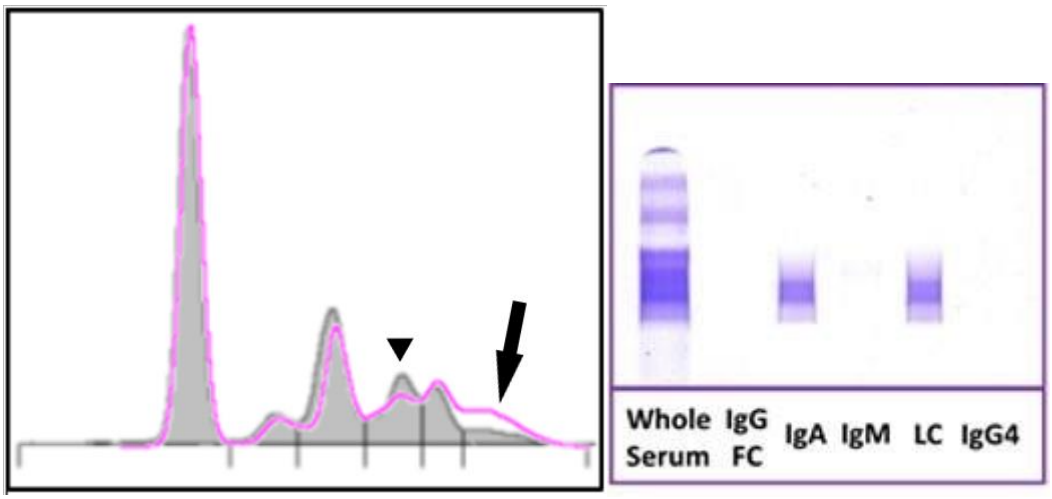

Fig 3.1 SPE and IF from a dog with normal total protein (5.2 g/dL) and globulin (2.41 g/dL) concentration. The M-protein is not readily apparent by SPE alone but can be diagnosed as an IgA biclonal gammopathy by addition of routine IF. The pink tracing is a canine control tracing that highlights a slightly increased beta 1 (arrowhead) and decreased gamma globulin fraction (arrow).

Table 3.1 Summary of case information including age, total protein/albumin/globulin concentrations, A:G, M-protein concentration, M-protein isotype, and historical/clinical information that prompted SPE.

SUMMARY DATA FOR INCLUDED CASES								
Case no.	Age (y)	TP (g/dL)	Alb (g/dL)	Glob (g/dL)	A:G	M-protein conc. (g/dL)	Isotype	Reason for SPE
1	10	6.9	3.3	3.6	0.9	1	A	Susp. MM, hepatosplenic PCT, T4 lytic bone lesion
2	10	6.1	2.4	3.7	0.65	2.02	A	PCT in pleural fluid
3	12	7.5	2.1	5.4	0.39	3.18	A	Limited clinical history provided
4	9	6.4	3.1	3.3	0.94	1.7	A	Suspected MM, T1 PCT
5	8	7.1	2.8	4.3	0.6	1	G	Hx of hypercalcemia, granulomatous lesions. Splenic lymphoid hyperplasia. Necropsy consistent with lymphoma
6	14	7.5	1.2	6.3	0.19	3.6	A	Spinal pain and paresis. Splenic mass showed histiocytic, plasma cell, and lymphoid population
7	12	7.23	2.8	4.43	0.63	1.7	A	Hypercalcemia, splenic and BM PCT
8	10	7.42	3.2	4.22	0.76	1.6	A	Splenic PCT
9	9	6.3	1.9	4.4	0.43	0.8	M	Limited clinical history provided
10	11	6.6	3.2	3.4	0.94	0.7	A	Round cell tumor of liver and spleen
11	9	7.4	2.4	5	0.48	2.3	A	BM PCT with pancytopenia
12	11	7.3	2.8	4.5	0.61	0.73	G	Oral PCT
13	10	6.6	2.7	3.9	0.69	1.87	A	Multifocal lytic bone lesions
14	10	6.2	1	5.2	0.2	3.1	A	Splenic PCT, lytic bone lesions
15	8	6	2.9	3.1	0.94	ND	L	Liver PCT
16	3	5.5	2.4	3.1	0.79	0.78	A	Hypercalcemia, liver mass
17	8	6	2.5	3.5	0.72	0.9	LC	Liver/spleen/bone PCT
18	15	6.3	1.9	4.4	0.42	0.5	A	Multiple PCT - spleen/RPLN/palatal mass

Abbreviations: TP; total protein, Alb; albumin, Glob; globulin, A:G; albumin globulin ratio, SPE; serum protein electrophoresis, MM; multiple myeloma, PCT; plasma cell tumor, BM; bone marrow, RPLN; retroperitoneal lymph node.

CHAPTER 4: RETROSPECTIVE STUDY ON MONOCLONAL IMMUNOGLOBULINS IN DOGS

MATERIALS AND METHODS

Case Selection

Cases submitted to the Colorado State University's Clinical Pathology Laboratory between January 2014 through December 2020 for performance of serum protein electrophoresis (SPE), with or without immunofixation (IF), were searched for patients that were diagnosed with a monoclonal immunoglobulin. Cases that had SPE performed and were interpreted as suspicious for a monoclonal immunoglobulin, but did not have immunofixation performed, were also selected if subsequent immunofixation confirmed a M-protein, yielding 122 cases. Records were collected from patients that met these inclusion criteria and reviewed for excluding factors such as certain infectious diseases (tick borne disease, heartworm disease, etc), PBLEB (Polyclonal B cell Lymphocytosis of English Bulldogs), and cases with insufficient evidence of myeloma related disease. A total of 10 additional cases were excluded based on this review, resulting in a study set of 112 cases. Complete records were obtained for 75 cases. Records from included cases were then reviewed and relevant variables were captured, which included diagnosis date, M-protein isotype, M-protein concentration, treatment protocol, response to treatment in patients with monitoring SPE performed, rescue treatment protocol (when available), initial clinical signs that prompted veterinary visit, whether there was evidence of bleeding diathesis, lytic bone lesions, selected initial biochemistry results (alkaline phosphatase [ALKP], aspartate aminotransferase [AST], gamma-glutamyl transferase [GGT], total bilirubin [Tbili], blood urea nitrogen [BUN], creatinine [Creat], phosphorus [Phos], total calcium [TCa], albumin [Alb], globulin [Glob]), selected initial complete blood count results (total nucleated cell count [TNCC], packed cell volume [PCV], leukocyte concentrations, platelet counts), coagulation testing results, urine specific gravity [USG], urine protein concentration, UPC, presence of BJP, infectious disease testing, previous/concurrent diagnoses, adverse reactions from treatment, and date of death or loss to follow up. Age in years, at time of diagnosis, was rounded to the nearest whole number. Since hematologic and biochemical analyses were run on various

instruments, only the associated reference intervals were used to determine if results were abnormal. Clinical signs at the time of diagnosis were categorized as none reported, gastrointestinal, pain, neurologic, constitutional (lethargy, anorexia, weight loss, etc), skin masses, renal disease, respiratory, or other. Evidence of bleeding diathesis was categorized as having one or more of the following, epistaxis, bruising (including petechiation, ecchymoses, or bruising), retinal hemorrhage, or other. Previous or concurrent diagnoses were categorized as endocrine, renal, hepatic, neoplastic, infectious, and other. Adverse reactions to treatment were categorized as none reported, neutropenia, thrombocytopenia, hepatic, renal, or local reactions to chemotherapeutics.

Serum protein electrophoresis and Immunofixation protocols

Samples were archived as part of normal sample processing within the clinical pathology laboratory, were free of significant hemolysis, icterus, and lipemia, and had been stored at -80°C until evaluation.

AGE SPE had been performed at the time of initial sample submission using amido black stained AGE (Sebia Hydrasys with Hydrogel Protein (E) with amido black kit, Sebia, France), a flat-bed scanner (Epson Perfection V700 Photo, Epson America, Inc, Long Beach, CA, USA), and Phoresis software (version 8.6.3, Sebia, France), as previously described.[24]

Involved immunoglobulin class was characterized using IF if it had not been identified at the time of initial evaluation and there was sufficient sample volume. Routine immunofixation in dogs targeted IgG-FC, IgG4, IgA, and IgM heavy chain and light chain using canine-specific reagents and was performed as previously described.[24,49] Free light chain (fLC) immunofixation had been performed using human-targeted fLC IF antibody set (Sebia Free light chains kit, Sebia, France) on a limited number of samples.[17] The manufacturer recommendations were followed for the application of serum, gel processing, and staining procedures. Samples were loaded neat (unaltered/undiluted), except for cases with a total protein concentration > 10 g/dL which were diluted 1:2 with 0.9% saline, per standard laboratory protocol. Densitometric measurement of M-proteins was performed using a previously validated method.[24]

Total protein concentration was assessed using a biuret total protein assay (Cobas c501: Roche Diagnostics. Indianapolis, IN, USA). Serum albumin concentrations were also obtained on the Cobas c501 using the bromocresol green method, with subsequent globulin concentration determined using the difference between colorimetric total protein and albumin.

RESULTS

Population characteristics, isotype distribution, and M-protein concentration

A total of 112 dogs were included for population characteristics. 58% (65/112) of the population were male, while 42% (47/112) were female (Figure 4.1). The MST of all patients, regardless of diagnosis or treatment protocol, was 502 days with 32.5% of patients surviving beyond 1000 days from diagnosis (Figure 4.2). The MST for females and males was 454 days and 502 days, respectively. The mean age at diagnosis was 9.9 years, with a range of 1-15 years (Figure 4.3). The breed was not recorded in 4 cases. Of the remaining 108 dogs, mixed breed dogs were most common, accounting for 21.3% (23/108) of the population. Other breeds with four or more animals included, Golden Retrievers 11.1% (12/108), Labrador Retrievers 10.2% (11/108), Shih Tzu 5.6% (6/108), German Shepherd Dogs 4.6% (5/108), and American Staffordshire Terriers 3.7% (4/108). Complete breed list is shown in Table 4.1.

The most common clinical signs that prompted veterinary examination were constitutional (60%, 45/75), pain (26.7%, 20/75), and gastrointestinal (21.3%, 16/75). Weight loss was noted by owners in 14.7% (11/75) of cases. Collapse prompted presentation for examination in 9.3% (7/75) of cases. Initial clinical signs are summarized in Table 4.2.

Isotype distribution was as follows; 63.4% (71/112, 95% CI 54.2-71.7%) IgA, 16.1% (18/112, 95% CI 10.4-24%) IgG, 12.5% (14/112, 95% CI 7.6-19.9%) IgM, 3.6% (4/112, 95% CI 1.4-8.8%) IgG4, 1.8% (2/112, 95% CI 0.3-6.3%) IgLC, and 2.7% (3/112, 95% CI 0.7-7.6%) true biclonal (1 – IgA and IgG, 1 – IgA and IgM, 1 – IgG and IgM) (Figure 4.4). The MST for IgA and IgG monoclonal immunoglobulins was 495 day and 475 days, respectively (Figure 4.5). All 3 of our cases with true biclonal gammopathies were diagnosed with MM. One patient (case no. 49) was a 6-year-old neutered

male Golden Retriever with IgA and IgG production, hepatosplenic and left popliteal lymph node plasma cell tumors, no evidence of lytic bone lesions, no BJP, and did not have bone marrow examination performed. This patient was treated with Tanovea and prednisone and survived 1183 days until progressive disease was detected, and he was euthanized. Another patient (case no. 70), was a 7-year-old neutered male Golden Retriever that presented with epistaxis, had IgA and IgM production, splenic plasma cell tumor, bone marrow plasma cell neoplasia, and the presence of BJP. This patient was treated with prednisone and melphalan until he was switched to cyclophosphamide, 1 month prior to euthanasia at 502 days after diagnosis. The last true biclonal gammopathy case (case no. 88) was an 11-year-old spayed female Australian Cattle Dog with IgG and IgM production, BJP, and suspected lymphoproliferative disease (plasmacytoid lymphoma versus PCT). No treatment or survival data was available on this patient. Two patients had production of monoclonal light chain immunoglobulins. One of these patients (case no. 38) was an 8-year-old spayed female Mixed Breed Dog that was diagnosed with MM and had hepatic PCT, BJP, mild plasma cell hyperplasia in the bone marrow, and renal azotemia (with previous diagnosis of chronic kidney disease). The other patient (case no. 42) was an 8-year-old spayed female Golden Retriever diagnosed with MM and had hepatosplenic and osseous PCT, multifocal lytic bone lesions (right proximal humerus, rib, thoracic vertebrae 13), BJP, 8.6% plasma cells on bone marrow evaluation, and lack of azotemia. The total protein and M-protein concentrations in these cases was 6.04 g/dL (M-protein below quantification lower limit) and 5.94 (M-protein concentration 0.9 g/dL), respectively.

The mean total protein concentration was 9.54 g/dL (min 5.53, max, 17.63, median 9.65). The mean albumin concentration was 2.18 g/dL (min 0.84, max 3.5, median 2.16). The mean globulin concentration was 7.44 g/dL (min 3.12, max 15.53, median 7.46). The mean M-protein concentration was 4.35 g/dL (min <0.3, max 11.55, median 4.12) (Figure 4.6). There was no statistically significant difference between the M-protein concentrations of different isotypes (Figure 4.7). A total of 23.4% (15/64) of patients were normoproteinemic on clinic run bloodwork at the time of diagnosis. 54% (34/63) of patients were normoalbuminemic, with the remaining 46% of patients being hypoalbuminemic. 12.5%

(8/64) of patients had a normoglobulinemia at the time of diagnosis, with the remaining 87.5% of patients having hyperglobulinemia.

Diagnosis

Complete medical records were evaluated for 75 patients to determine the diagnosis made by clinicians overseeing the cases. Table 4.3. Multiple myeloma was the most common diagnosis (66.7%, 50/75). One patient was diagnosed as probable multiple myeloma, two cases were diagnosed as MM versus BCLL, and a single case was diagnosed as MM versus MGUS. A total of 8% (6/75) of cases were diagnosed with BCLL. One case was diagnosed as a secretory plasma cell tumor after identification of neoplastic plasma cells in pleural fluid. This patient was euthanized before any additional work up could be performed. One patient was diagnosed with an extramedullary plasma cell tumor of the spleen without BJP and equivocal lytic bone lesions. Another patient was diagnosed as having multiple extramedullary plasma cell tumors (palatal, splenic, retropharyngeal lymph node). One patient was diagnosed with immune mediated hemolytic anemia and treated with immunosuppressive drugs without any additional clinical outcome data beyond the initial work up. Examination of the urine in this patient did not identify BJP; however, there were lytic bones lesions associated with unspecified vertebrae. One patient was diagnosed as having a monoclonal gammopathy and BJP, likely associated with a liver mass that was not interrogated further. Given the presence of a serum monoclonal gammopathy and BJP this patient would meet the current MM diagnostic criteria. A total of 13.3% (10/75) cases did not have a diagnosis due to insufficient diagnostic testing being performed. The MST for patients diagnosed, by their treating veterinarian, with MM or BCLL was 502 days and 716 days, respectively ($p = 0.69$).

Current veterinary diagnostic guidelines for MM, that stipulate 2 criteria (>20% bone marrow plasma cells, radiographic evidence of lytic bone lesions, serum M-protein, or BJP), must be present for a diagnosis of MM, 35 (46.7%) cases met diagnostic criteria for MM. When documentation of neoplastic plasma cells was broadened from > 20% bone marrow plasma cells to also include biopsy confirmed visceral organ plasma cell tumors (spleen, liver, GI), 51 (68%) cases met the diagnostic criteria. These cases behaved similar in terms of MST to those that met the current veterinary diagnostic criteria, MST

416 days versus 573 days, respectively. Using the new human criteria for the diagnosis of MM (clonal bone marrow plasma cells >10% or confirmed bony or extramedullary plasma cell tumor with a myeloma defining event or presence of a biomarker of malignancy) a total of 37 (49.3%) cases met inclusion criteria. Since the assessment of FLC ratios is not yet available in veterinary medicine, we were unable to use this as a criterion when categorizing our cases. All six BCLL cases were appropriately classified as not meeting MM diagnostic criteria, in all three diagnostic schemes evaluated. A summary of diagnostic schemes and number of cases diagnosed with MM with each, are found in Table 4.4. Comparison of MST between the current veterinary MM diagnostic criteria and the proposed criteria are found in Figure 4.9.

Treatment protocols were varied, but combination therapy with prednisone and melphalan was most common and was administered to 48.7% (36/74) of patients. Diagnoses in these cases were composed of 33/36 MM cases, one presumed MM case, one MM versus MGUS case, and one case without a definitive diagnosis. The MM versus MGUS case was presented for evaluation of a skin mass (soft tissue sarcoma) and did not have a substantial additional work up. The presumed MM case only had one MM diagnostic criteria met using the current veterinary scheme, but did have splenic plasmacytosis with atypia on cytology evaluated by the general practice veterinarian (but not evaluated by a pathologist). This patient also had gingival bleeding and petechiations noted in the mouth. The case without a definitive diagnosis was a 14-year-old neutered male Australian Shepherd who was presented with epistaxis and had mild elevations in ALT and AST on diagnostic bloodwork. No additional diagnostics were performed in this case and the patient was euthanized 98 days post diagnosis with an IgA monoclonal gammopathy (with biclonal morphology). Prednisone was the sole agent used in 12.2% (9/74) of patients; 5 MM cases, 2 cases without a definitive diagnosis, and a single MM versus BCLL case. Melphalan was the sole agent used in 4.1% (3/74) of patients with MM. No chemotherapeutics were administered in 14.9% (11/76) of patients; 5 cases without a definitive diagnosis, 2 extramedullary PCTs, and one patient each for MM, PCT, paraneoplastic IgG, and BCLL. A complete summary of treatment protocols and associated diagnoses are listed in Table 4.3. Combination therapy, prednisone and melphalan, was associated with a longer MST (MST 502 days, HR 0.0171e-13, 95% CI 1.57e-20-1.871e-

10, $p < 0.0001$) than melphalan only (MST 48 days, HR 5.84e+14, 95% CI 5.35e+9-6.37e+19) or patients that did not receive treatment (MST 93, HR 30.36, 95% CI 5.53-166.50, $p \leq 0.0001$). No statistically significant difference ($p = 0.05$) was noted when comparing the prednisone and melphalan treatment group against the prednisone only group (MST 311, HR 1.54, 95% CI 0.38-6.31). Figure 4.8.

Hematologic and Biochemical data

The number of patients with available data was different for each variable. For this reason, all abnormalities are followed by the number of patients with data available for that particular variable and how many of those were abnormal.

Anemia (55%, 33/60), thrombocytopenia (51.6%, 31/60), and lymphopenia (46.7%, 28/60) were the most common hematologic abnormalities at the time of diagnosis. Leukopenia (25.4%, 15/59), monocytopenia (16.9%, 10/59), and eosinopenia (32.1%, 18/56) were also noted. All hematologic abnormalities are shown in Figure 4.10. Bicytopenia or pancytopenia were present at the time of diagnosis in 39% of cases (bicytopenia 22%, 13/59; pancytopenia 16.9%, 10/59). The MST for dogs without cytopenias compared to those with pancytopenia was 502 days and 1183 days, respectively ($p = 0.23$). In this analysis there were 14 cases in the non-cytopenic group and 9 cases in the pancytopenic group (after exclusion of BCLL cases) with clinical data on date of death and date lost to follow-up. Additionally, there was a statistically significant prolonged survival time in patients with pancytopenia compared to animals with bicytopenia. The MST for dogs with bicytopenia and pancytopenia was statistically significantly different at 311 days and 1183 days, respectively ($p=0.004$) (Figure 4.11). 4/13 of the bicytopenia cases had evidence of neoplasia on bone marrow examination (two BCLL cases and two PCT cases). 6/10 of the pancytopenic cases had evidence of bone marrow plasma cell neoplasia. When comparing the neutrophil to lymphocyte ratios (NLR) in all animals except the BCLL cases, the MST for low NLR and high NLRs was 502 days and 475 days, respectively ($p = 0.95$) (Figure 4.12).

Several hepatic enzymes were evaluated and included, ALT, ALKP, AST, GGT, Total bilirubin, and Cholesterol (Figure 4.13). Hypocholesterolemia (33.9%, 20/59), increased AST (29.6%, 8/27), and increased ALKP (22.4%, 13/58) were the most common abnormalities. The MST in dogs (excluding

BCLL cases) with hypocholesterolemia and normocholesterolemia were 627 days and 475 days, respectively (p = 0.59) (Figure 4.14). Of the cases with confirmed hepatic plasma cell or BCLL neoplasia (excluding cholesterol), 4/9 had normal hepatic enzyme concentrations, 4/9 had elevations in a single enzyme (case no. 22 AST, case nos. 42 and 60 ALKP, and case no. 49 total bilirubin), and the single hepatic BCLL case had elevations in several enzymes (case no. 25 ALT, ALKP, AST) with normal GGT and total bilirubin. Abnormalities were typically mild to moderate and measured 1.69-2.5 times the upper reference interval limits. The hepatic BCLL case has slightly more profound derangements that measured 1.7-6.63 times the upper reference interval limits.

Renal parameters that were evaluated included BUN, creatinine, phosphorus, and total calcium (Figure 4.13). Azotemia was defined as an increase in the BUN and/or creatinine and was present in 44.8% (26/58) of patients at the time of diagnosis. Of the patients classified as azotemic, 26.9% (7/26) had elevations in only BUN, 42.3% (11/26) had elevations in creatinine only, and 30.8% (8/26) had elevations in both BUN and creatinine. The azotemia was classified as pre-renal azotemia (USG >1.030), renal azotemia (USG ≤ 1.030), and unclassified (when USG was not performed). Azotemia was classified as renal azotemia in 53.9% (14/26) of the patients (USG range 1.010-1.030). Pre-renal azotemia was present in 26.9% (7/26) of patients (USG range 1.034-1.050, with 3 patients having hyposthenuric urine with a USG of 1.007). Classification was not possible in 5 cases due to lack of a USG being performed at the time of diagnosis. The MST in dogs with renal azotemia and without renal azotemia (excluding BCLL cases) was 294 days and 573 days, respectively (p = 0.02) (Figure 4.15).

Calcium and phosphorous derangements can have both renal and non-renal causes. Total hypercalcemia was the most common abnormality noted (31%, 18/58). The MST for dogs with hypercalcemia and normocalcemia at the time of diagnosis was 383 days and 495 days, respectively (p = 0.41) (Figure 4.16). The adjusted calcium ((total calcium – albumin) + 3.5) was also assessed to see if this was a better prognostic indicator. The reference interval for total calcium, from the instrument used, was used to categorize the adjusted calcium as normal or increased. The MST for dogs with increased and normal adjusted calcium was 393 days and 573 days, respectively (p = 0.98). Hyperphosphatemia was

present in a smaller subset of the population (11.1%, 6/54). The calcium phosphorus product was calculated for 53 patients with both total calcium and phosphorus concentrations recorded. The mean calcium phosphorus product was 51.1 (min 15.1, max 134.3, median 45.9). A total of 6 cases had a calcium phosphorus product equal to or above 70. Of these 6 cases, 5 had azotemia (3 with elevated BUN and creatinine, 1 with only elevated BUN, and 1 with only elevated creatinine) and one had BUN and creatinine within the reference intervals.

85.4% (41/48) of patients had proteinuria at the time of diagnosis. The MST for patients with proteinuria was 502 days, but the MST for patients without proteinuria could not be determined due to a low number of non-proteinuric patients with survival data and high censoring of patients due to loss to follow up. A total of 17 patients were assessed for the presence of BJP and 52.9% had identifiable BJP. The MST for dogs with detectible BJP and those without detectible BJP was 688 days and 165 days, respectively ($p = 0.51$) (Figure 4.17).

A total of 19/69 patients had prothrombin (PT) and activated partial thromboplastin (aPTT) coagulation panels performed. 57.9% (11/19) of these patients had abnormal results. 54.5% (6/11) had prolongation of both PT and aPTT, 18.2% (2/11) had prolongation of PT only, 18.2% had prolongation of aPTT only, and 9% (1/11) had a reduced aPTT time.

Evidence of bleeding diathesis (EBD) was documented in 30.6% (23/75) of cases. 26.1% (6/23) of these cases had 2 criteria and 73.9% (17/23) had a single criterion. When present, epistaxis was present as the only sign of bleeding diathesis. Epistaxis was found in 39.1% (9/23) of cases in the group with EBD and 13% (9/69) of the entire population. 56.5% (13/23) of cases within the EBD group had coagulation panels run and of those 61.5% (8/13) were abnormal/prolonged. 17 cases with EBD also had platelet counts available at the time of diagnosis and 52.9% (9/17) were thrombocytopenic.

Various infectious disease tests were performed within the study population. A total of 55.3% (42/78) patients had 4Dx testing performed (*Ehrlichia canis*, *Anaplasma phagocytophilum*, *Dirofilaria immitis*, and *Borrelia burgdorferi* antibodies). A total of 10.5% (8/76) patients had extensive tick disease panels, 4% (3/76) had urine *Blastomyces/Histoplasma* urine antigen tests, and 2.6% (2/76) had rapid

leptospirosis tests performed. Other infectious disease tests were performed only in individual patients and included a single plague PCR, *aspergillus* EIA, and coccidioides titer. These tests were all negative. A single patient was positive for *Ehrlichia canis* antibodies. This case was included as the patient had been previously treated for E*hrlichia* and had splenic and bone marrow plasma cell neoplasia. A single patient had been previously treated for Lyme disease (*B. burgdorferi*) and was included because it tested negative for Lyme disease via a tick/vector PCR at the time of MM diagnosis. This case also had sufficient evidence of MM, which included multifocal lytic bone lesions, left/right mandibular lymph node PCTs, splenic PCT, and osseous PCT.

The presence of bony lesions was noted in 23.3% of cases (17/73). When stratifying for M-protein isotype in these cases, 14/17 were IgA, 2/17 were IgG, and 1/18 was light chain only disease. Animals without apparent musculoskeletal pain or lameness were infrequently assessed for bony lesions. Locations of these bony lesions varied, but vertebral lesions were most common. Summarized in Table 4.5.

Cytologic and Histopathologic data

Cytology was performed in 62.7% (47/75) of cases. Spleen was the most commonly sampled organ, with 72.3% (34/47) of cytologic cases having splenic aspirates performed. Splenic plasma cell tumors were diagnosed in 55.9% (19/34) of these cases. 32.4% (11/34) of splenic aspirates were equivocal for a diagnosis of plasma cell tumor versus markedly reactive lymphoid tissue. Small cell lymphoma was diagnosed in a single case. Cytology of one spleen was concerning for lymphoma (this patient had confirmed lymphoma in a lymph node). Liver was aspirated in 11 patients, of which hepatic plasma cell tumors were diagnosed in 6/11 cases, plasma cell tumor versus reactive lymphoid tissue in 2/11 cases, lymphoma 2/11 cases, suspicious for lymphoma in a single case (lymphoma was ultimately confirmed with flow cytometry), and suppurative inflammation in one case. Lymph nodes were aspirated in 9 cases, which were interpreted as plasma cell tumors in 5/9 cases, equivocal/suspicious for PCT in 2/9 cases, lymphoma in 2/9 cases, equivocal for lymphoma versus plasma cell tumor in one case, and normal in one case. Lytic bone lesion aspirates were taken in 3/47 cases and were consistent with

plasma cell tumors in all cases. A solitary cutaneous plasma cell tumor was also diagnosed in a patient. Plasma cell tumors were also found in a cranial mediastinal mass, pleural fluid, intrabdominal mass near urinary bladder, and a neoplastic abdominal effusion. Detailed results can be found in Table 4.6.

Histopathology was performed in 16 cases, with 4 of those cases having histopathologic specimens collected during necropsy. 11/16 cases had plasma cell tumors and 1/16 had lymphoma diagnosed on histopathologic evaluation of tissues. The remaining 4/16 cases were considered suspicious for neoplasia and in one case previously diagnosed with BCLL, histologic evaluation at the time of necropsy did not diagnose neoplasia. This patient was a 9-year-old spayed female Labrador Retriever diagnosed with BCLL on histopathology of the liver and flow cytometry (CD21+ lymphocytosis) and treated with prednisone and cyclophosphamide and died of suspected sepsis and/or disseminated intravascular coagulation post abdominal exploratory surgery for bile peritonitis.

Bone marrow was examined antemortem in 21 patients. Plasma cell neoplasia was diagnosed in 12/21 cases, with plasmacytosis (% plasma cells 8.6-20%) diagnosed in an additional 4/21 cases. Small cell lymphoma was diagnosed in 2/21 cases, with BCLL (CD21+ lymphocytosis) being confirmed by flow cytometry in both cases. Detailed results can be found in Table 4.7.

DISCUSSION

Myeloma-related diseases encompass several distinct clinical syndromes characterized by a clonal population of plasma cells or immunoglobulin-producing B-lymphocyte precursors. The animals in our study population demonstrated many of the classic clinical manifestations of this proliferation of neoplastic cells and production of monoclonal immunoglobulin.

Similar to some previous reports[37,42], male dogs were more commonly observed within our population of animals with monoclonal immunoglobulinss with a male to female ratio of 1.4:1. Most animals diagnosed with monoclonal immunoglobulin were older; however, disease still occurred in animals as young as one year of age. Mixed breed dogs were the breed group with the highest number of cases; however, Golden Retrievers and Labrador Retrievers were most commonly observed at 21.3% of the total population when combined. Another study that evaluated two melphalan protocols in dogs with

multiple myeloma had 21.5% (8/38) Golden Retrievers and 15.8% (6/38) Labrador Retrievers.[42] Previous reports have suggested that German Shepherd dogs are overrepresented[37], but they made up only 4.6% of our study population. Additionally, these two studies were looking specifically at dogs with MM and our population contained several different MRDs. It is challenging to determine if these represent true breed predispositions, or if this is simply a reflection of normal hospital presented dogs from our hospital and submitting clinics.

Non-specific constitutional clinical signs included lethargy, anorexia/inappetence, and weight loss and were present in most patients, similar to previous studies. These clinical signs are by no means specific, but persistence or severity of signs such as these should prompt further investigation for an underlying etiology. Interestingly, collapse was the initial presented concern in 9.3% of cases and increased panting was noted in 4% (3/75) of cases. These signs were potentially associated with anemia, dehydration, hyperviscosity syndrome, and/or musculoskeletal pain.

Most patients regardless of type of MRD (64.9%, 48/74) were treated with a combination of prednisone and melphalan, or single agent therapy with either prednisone or melphalan. There was a statistically significantly longer MST in patients treated with combination therapy as opposed to either agent alone. While the number of BCLL cases within our population was low, which isn't surprising given the lower frequency of monoclonal immunoglobulins in lymphoma/leukemias, these patients were treated with a variety of chemotherapy protocols that did not include melphalan. A single BCLL case was not treated because it did not meet the standard criteria used to prompt treatment in this disease.

Hyperglobulinemia, hypoalbuminemia, and hyperproteinemia were frequently present at the time of diagnosis, which isn't surprising; however, nearly one in four of patients were normoproteinemic at the time of diagnosis. This, along with the work from Chapter 3, highlights the fact that M-proteins do not have to have a measurable effect on total protein content and a normal total protein concentration should not be used as a sole contraindication for SPE/IF.

Monoclonal IgA production was present in a majority of cases, and most often had a biclonal morphology. As mentioned earlier, this is thought to be secondary to dimerization of IgA proteins

creating a biclonal appearance.[58] Biclonal peaks associated with a single immunoglobulin isotype were present in 70 cases (67 IgA, 2 IgG, and 1 IgM). This biclonal morphology is not to be confused with true biclonal gammopathies (two distinct immunoglobulin classes), which was only present in 2.7% of patients. This is thought to occur secondary to immunoglobulin production from two distinct plasma cell clones or from a single plasma cell clone that has undergone class switching.[59] Little information is available in the veterinary literature about true biclonal gammopathies[18,60], but human literature suggests that true biclonal gammopathies occur in 1.5-4% of all monoclonal immunoglobulin producing cases and these patients typically respond similarly to treatment as patients with single monoclonal immunoglobulin.[59] This suggests that the human and canine incidence of true biclonal gammopathies in similar.

Two patients had production of monoclonal light chains only. Previous literature has suggested that light chain only disease can result in low concentrations of M-protein in the serum, as much of the M-protein is freely filtered by the glomeruli and lost in the urine.[36] Without performing fLC IF on all patients, we are unable to determine the incidence of light chain only disease; however, this is an area that warrants additional research. Based on this data, a reasonable recommendation is if there is a suspicion for a MRD and no M-protein is identified on routine SPE or IF, fLC IF should be performed on the urine and/or serum to assess for the presence of low amplitude M-proteins.

Multiple myeloma was the most common MRD diagnosis, similar to previous reports.[1,57] There has been concern about the current MM diagnostic guidelines, in both human and veterinary medicine, that they may fail to capture all animals/people with the disease.[28,31] Based on clinical records and treatment, it would appear that clinicians were not following a strict interpretation of the veterinary guidelines that discuss diagnosis by bone marrow involvement (35 cases met criteria) but were also including the use of documented visceral organ involvement without documentation of bone marrow involvement as sufficient for a working diagnosis and treatment protocol consistent with MM (51 cases met criteria). This practice appears to be clinically justified as there was not an apparent difference in MST between these 2 groups. The addition of visceral organ involvement may minimize the diagnostic

workup currently needed to meet diagnostic criteria and could increase the certainty of diagnosis in cases where a bone marrow sampling is not feasible, but less invasive and less costly organ aspirates can be obtained.

Applying the current human diagnostic guidelines yielded a total number of MM cases similar to the current veterinary guidelines but application of the IMWG recommendations was technically challenging. For one, MRI evaluation for bony lesions is not routine practice in veterinary medicine. Additionally, assessing kappa and lambda FLC ratios is not yet performed within veterinary medicine. With that said, often the presence of extramedullary plasma cell tumors and presence of CRAB lesions were met in the cases diagnosed with MM in this diagnostic scheme. While this does not appear to be a scheme that should be adopted within veterinary medicine based on current diagnostic capabilities and limitations, it may be appropriate to revisit these criteria as additional diagnostic modalities become available. Notably, in humans, the κ:λ ratio is expected to be abnormal in all FLC MM, 68% of non-secretory MM, and 95% of intact immunoglobulin secretory MM. If this test becomes available and performs similarly in the dog, application of the IMWG scheme may be appropriate.

The remaining 25/75 cases were composed of 6 BCLL cases, 9 MRD cases (MM versus BCLL or MGUS, a single suspected MM case, and multiple cases of extramedullary plasma cell tumors), and 10 cases with inadequate work up to obtain a definitive diagnosis.

Anemia (55%, 33/60), thrombocytopenia (51.6%, 31/60), and lymphopenia (46.7%, 28/60) were the most common hematologic abnormalities in cases with a M-protein and these results are similar to other studies.[4,37,42] Bicytopenia or pancytopenia were noted in nearly 40% of all cases at the time of diagnosis. This is suspected to be associated with bone marrow involvement and myelopthesis of the marrow cavity with neoplastic cells.[1] Another potential mechanism for this could include splenic involvement, decreasing extramedullary hematopoietic capacity. Interestingly, when comparing the survival times for patients without cytopenias to patients with pancytopenia at the time of diagnosis, there was a notably longer survival in dogs with pancytopenia. To our knowledge, this has not been reported before and previous case series have not examined bicytopenia or pancytopenia in relation to MST. It is

not clear why pancytopenia should be associated with a longer survival time. It is possible that clinicians treated patients with panyctyopenia more aggressively because of a perceived poorer clinical condition, that cases with pancytopenia had disease that was more sensitive to therapy or that there were other biological mechanisms for this longer survival. Alternatively, although unlikely, this could be an artifact of sampling that would not be repeated in analysis of a second cohort.

Hepatic enzyme elevations were observed frequently within our study population. Increased AST and ALKP and decreased cholesterol concentrations were most common. In people, hypocholesterolemia in MM is thought to occur secondary to increased clearance of LDLs and potential increased use by neoplastic cells.[61] In our study, a total of 5/9 cases with hepatic aspirates and cytology confirmed neoplasia (PCT and a single BCLL case) there was an increase in one or more of the hepatic enzymes (ALT, AST, ALKP, GGT, TBILI). Increased hepatic enzyme activity may be a helpful indicator of infiltrative hepatic neoplasia and could offer additional clinical support for pursuing hepatic aspirates. A prospective look at hepatic biochemical analytes paired with hepatic aspirates, in cases with MM and other MRDs, may be helpful in further characterization this possible association.

Hypercalcemia was present in approximately 31% of our population. Two previous studies reported that hypercalcemia occurred in 16.6%[33] and 50%[36] of patients with MM, respectively. The former paper reflecting only animals with hypercalcemia after correction for albumin concentration.[37] Hypercalcemia associated with MRDs is thought to occur secondary to production of osteoclast activating factor by neoplastic cells and cytokines such as IL-1, IL-6, and TNF-α.[1] Matus et al found statistically significant differences in MST between MM dogs with hypercalcemia (>11.5 g/dL, adjusted for albumin concentrations – although the formula was not provided) and dogs without hypercalcemia,[37] but other reports have failed to find statistical significance.[42] In this study, adjusted calcium did not appear to be associated with survival time, and thus is not recommended as a guide for assessing prognosis.

Azotemia was commonly present at the time of diagnosis and is likely related to renal parenchymal damage from a variety of disease related factors such as hyperviscosity, hypercalcemia, glomerular damage secondary to free immunoglobulin light chain filtration, coagulopathy, and others.[1,37]

Renal disease was associated with a statistically significant shorter MST of 294 days versus 573 days. A majority of cases (85.4%, 41/47) also had proteinuria at the time of diagnosis; however, only a small subset of cases were analyzed for the presence of BJP. 52.9% of these cases had identifiable BJP. This is a higher frequency than what has been previously reported. Previous studies used an alternative, heat precipitation method to document BJP that is no longer used in human medicine due to its limitations and reported that BJP occurs in 25-40% of canine MM.[37,62,63] Interestingly, the immunofixation based assay used to detect BJP is not as sensitive in the dog as it is documented to be in humans; it is likely that the observed incidence of BJP in this study is an under representation of the true incidence of monoclonal free light chain presence in dogs with MRD. Additional studies in patients diagnosed with monoclonal immunoglobulins to further explore the incidence of BJP could provide clinicians additional guidance on whether evaluation for BJP or free light chain serum immunoglobulin is a high yield test, or if it should be reserved for cases with a suspicion of production of monoclonal immunoglobulin without evidence of complete immunoglobulins (IgA, IgG, etc).

The presence of bony lytic or osteoporotic lesions was assessed in numerous patients; however, in patients without significant evidence of bony or musculoskeletal pain, thorough radiographic screening for these lesions was lacking. This makes it difficult to determine the frequency of bony lesions in these patients and thus our numbers should be considered a minimum. The patients within our study who had evidence of these lesions typically had lesions associated with marrow containing long bones and the vertebrae.

Patients were twice as likely to have cytology performed compared to bone marrow evaluation. There are several proposed reasons for this discrepancy. Bone marrow collection is a more invasive procedure that often requires heavy sedation or generalized anesthesia. While fine needle aspiration of tissue and organs is often less invasive, less expensive and only occasionally requires sedation. Clinician comfortability with the process of collecting bone marrow may have also contributed. Splenic, liver, and lymph node plasma cell tumors were present in over half of the cases in which these tissues were sampled for cytologic evaluation. These findings speak to the utility of cytology in the assessment of disease

distribution and diagnosis. With that said, there were a number of cases where a definitive diagnosis couldn't be made based on cytology alone. Additional diagnostic techniques, such as PCR for Antigen Receptor Rearrangement (PARR) to confirm clonality or flow cytometry, and immunocytochemistry to evaluate surface marker expression can be used as complementary tools for further clarification. Although bone marrow samples were less commonly obtained in our population, over half of these cases (12/21) were diagnosed with plasma cell neoplasia affecting the bone marrow and 8/12 of these cases had bi- or pancytopenia. The presence of cytopenias in a majority of these cases with confirmed bone marrow neoplasia is most likely associated with myelopthesis with neoplastic cells, leading to a reduced capacity of the hematopoietic cells to mount an appropriate regenerative response. 75% (12/16) of cases with histopathologic evaluation of tissue were diagnosed with plasma cell tumors.

Evidence of bleeding diathesis was noted in 30.6% (23/75) of patients. Interestingly, epistaxis was found in 13% of the population and was the only sign of bleeding diathesis in these patients. Prolongation of coagulation times (PT/aPTT) were also present in 57.9% of evaluated samples, which suggests assessment for coagulopathies in patients suspected of having a MRD is warranted and should be done prior to performing certain diagnostics that may increase the risk of hemorrhage. If this testing is unavailable, close monitoring of these patients for hemorrhage after fine needle aspirates and/or bone marrow collection is recommended.

Infectious disease testing is an important part of the diagnostic work up in patients diagnosed with a monoclonal gammopathy. This is because these diseases produce serum protein changes which have been interpreted as a monoclonal gammopathy pattern and because, many tick-borne diseases can also cause similar clinical signs to MRD, such as vomiting, epistaxis, lethargy, and thrombocytopenia.[64] Treatment with immunosuppressants and/or chemotherapeutics appropriate for neoplasia would be detrimental in infectious disease cases. Additionally, a diagnosis of cancer may prompt euthanasia in some cases where owners would have been willing to treat an infectious disease. The most common infectious disease diagnostic screening test performed in our population was the IDEXX 4Dx snap test. This was negative in 41/42 cases, with a single case positive for E. canis antibodies. A subsequent PCR

test for E. canis was negative in this case, ruling out active infection. Other infectious disease testing was also pursued in numerous cases for various fungal and bacterial agents. Test results were negative for all other patients except for a case with a *L. pomona* microscopic agglutination titer (MAT) of 1:200. This was suspected to be associated with previous vaccination, as vaccination can induce MAT titers ≥1: 800.[65] Cases with confirmed infectious disease and concurrent lack of significant evidence of neoplasia were excluded from our analyses and thus the frequency of infectious disease related monoclonal gammopathies is not assessed here.

Adjusted calcium, NLR, sex, and isotype did not appear to have clinically significant different MSTs. The lack of a significant difference in survival based on M-protein isotype is similar to previous veterinary reports[37], and contrary to what has been reported in human medicine.[66] Total hypercalcemia was associated with a shorter MST (383d versus 495d) in our study population. Due to the low number of patients without proteinuria within our study population, we were unable to compare the MST between animals with and without proteinuria. Unfortunately, only a small portion of the animals in our study were interrogated for BJP and a larger cohort of animals with screening for BJP would have been helpful in exploring this unexpected finding. Additional studies are needed to assess the frequency at which BJP occurs in dogs with MM. Previous studies in people and cats with MM have found a decreased MST in patients with hypocholesterolemia. That was not apparent in our dog population. In fact, animals with a lower cholesterol concentration at the time of diagnosis had a longer MST (627d versus 475d). Previous studies in dogs and humans have also found the NLR to be associated with survival time. Using cutoffs defined in previous literature[42], we assessed this in our population. There was no statistically, or clinically, significant difference in these two groups. Attempting to establish different NLR cutoffs is beyond the scope of this work, but may be worthwhile to determine the significance of the NLR in patients with MM.

Median survival time was assessed in regard to its association with numerous variables and these results did not always agree with previously published works. The findings of statistically and clinically significant shorter MSTs were associated with renal azotemia and melphalan only treatment, and match

expected results. For some previously identified prognostic indicators, close evaluation of the survival data suggested a trend toward shorter survival (hypercalcemia and BJP), however, the observed differences in this study did not reach statistical significance. While the observed trend agrees with previously published data that these measurands can be used for prognostic significance, the data could be equally compatible with a finding that these measurands do not have prognostic utility. Given the sum of the published data and our findings, we expect that future studies will find these measurands prognostically useful. For other previously identified prognostic indicators, there was very little support of its utility in these patients. The lack of statistical significance may be potentially due to differences in sampling, inclusion criteria, or high censorship for cases lost to follow up but at the least, the lack of repeatability to document a prognostic utility in this study should warrant a degree of caution in the continued use and further investigation before these prognostic criteria are abandoned.

Neoplastic monoclonal immunoglobulins in dogs were most commonly associated with MM, with fewer cases falling under the spectrum of MRD. Treatment of MM with prednisone and melphalan led to statistically longer MSTs in these cases when compared to single agent therapy with prednisone or melphalan. Clinical signs were frequently non-specific, but some cases presented with clinical signs that are potentially more specific for MRD such as collapse, evidence of bleeding diathesis, and musculoskeletal pain. Cases with suspected, or confirmed, lymphoproliferative diseases are good candidates for diagnostic SPE/IF even if there is not an apparent hyperproteinemia or hyperglobulinemia as the presence of a neoplastic M-protein could be useful in diagnosis and monitoring the response to treatment. Ancillary diagnostic testing such as PARR, flow cytometry, and/or immunohistochemistry/ immunocytochemistry may be required in some cases to fully categorize disease. Adoption of visceral organ involvement as a primary or alternative diagnostic criterion for MM may be more likely to appropriately categorize animals with MM, at least based on the clinical course of disease. Dogs within our population also frequently had total hypercalcemia, proteinuria, and occasionally renal dysfunction. Renal azotemia was associated with a shorter MST, but other frequently used negative prognostic

indicators failed to demonstrate statistical significance, but often appeared to have clinically significant impacts on survival time, with the exception of the NLR.

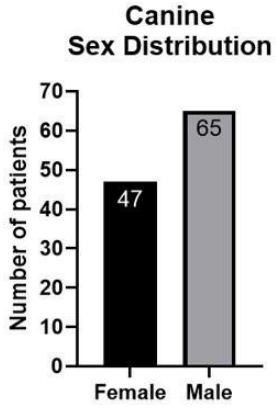

Figure 4.1. Bar chart showing the number of female and male dogs diagnosed with M-proteins.

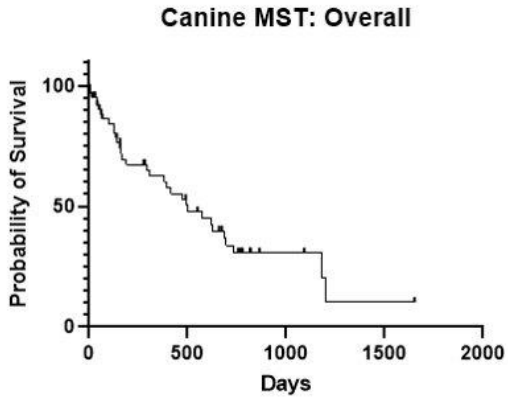

Figure 4.2. Kaplan-Meier survival curve for entire dog population regardless of diagnosis or treatment protocol. MST time was 502 days, with 32.5% of patients surviving beyond 1000 days after diagnosis with an M-protein.

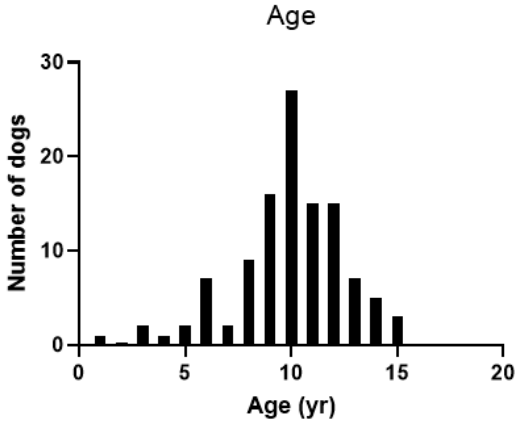

Figure 4.3. Histogram showing the age of 112 dogs diagnosed with M-proteins. Range 1-15 years, mean 9.9 years.

Table 4.1. Complete list of breeds represented within our study population and number of dogs from each respective breed.

CANINE BREEDS REPRESENTED IN STUDY POPULATION			
Breed	**#**	**Breed**	**#**
Mixed Breed Dog	23	Bouvier De Flanders	1
Golden Retriever	12	Spitz	1
Labrador Retriever	11	Great Dane	1
Shih Tzu	6	Terrier	1
German Shepherd	5	Great Pyrenees	1
American Staffordshire Terrier	4	Boxer	1
Beagle	3	Afghan hound	1
Poodle	3	Pomeranian	1
Chihuahua	2	Jack Russell Terrier	1
American Eskimo	2	Portuguese water dog	1
Havanese	2	Rottweiler	1
Australian Shepherd	2	Bulldog	1
Husky	2	Shetland Sheepdog	1
Cocker Spaniel	1	Catahoula	1
Pointer	1	Coonhound	1
English Mastiff	1	Maltese	1
Airedale	1	St. Bernard	1
Basset	1	Standard Schnauzer	1
Doberman Pinscher	1	Mini Australian Shepherd	1
Border Collie	1	Whippet	1
Australian Cattle Dog	2	Mini schnauzer	1
Schipperke	1		
	Total		**108**

Table 4.2. Summary of reported presenting clinical signs in 75 dogs with an M-protein.

REPORTED CLINICAL SIGNS		
Clinical Sign	**Percent**	**Number**
Constitutional	60	45/75
Weight loss	15	11/75
Collapse	9	7/75
Pain	27	20/75
Collapse	9	7/75
Gastrointestinal	21	16/75
Chronic diarrhea	8	6/75
Renal	8	6/75
Neurologic	7	5/75
Respiratory	5	4/75
Skin masses	4	3/75

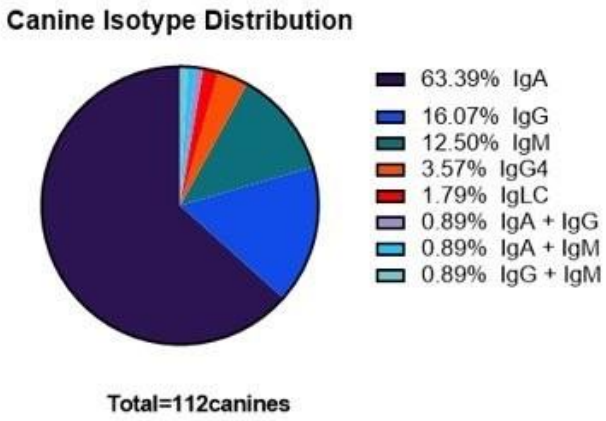

Figure 4.4. Pie chart demonstrating the distribution of isotypes in dogs with M-proteins. Color and isotype key to the right of the chart.

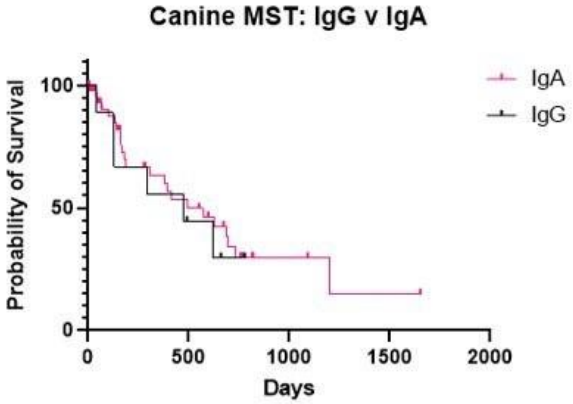

Figure 4.5. Kaplan-Meier survival curve comparison showing dogs with IgA M-proteins (pink) and IgG M-proteins (black). BCLL cases were excluded. The MST for patients with IgA M-proteins was 495 days (HR 1.04, 95% CI 0.42-2.57). The MST for patients with IgG M-proteins was 475 days (HR 0.96, 95% CI 0.39-2.37). p = 0.65.

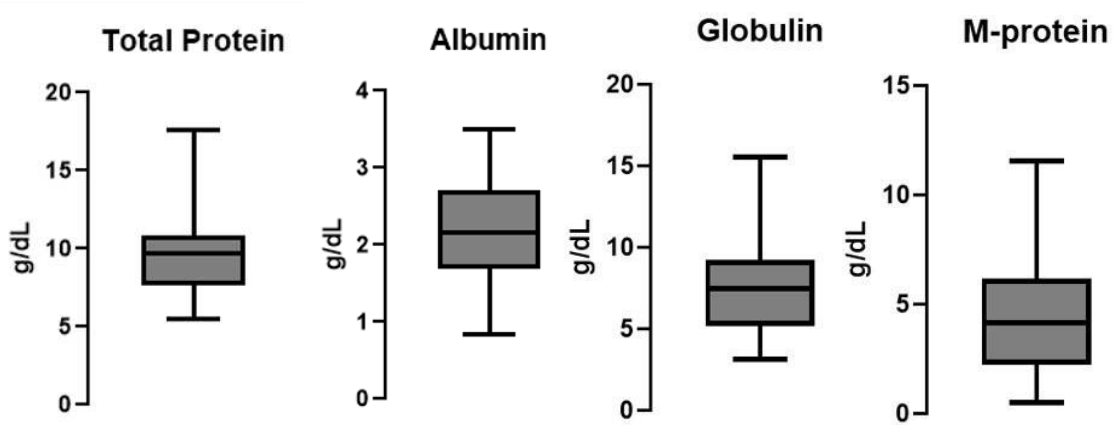

Figure 4.6. Box and whisker plots demonstrating the dog population total protein, albumin, globulin, and M-protein concentrations. Minimum and maximum are represented by the whiskers, the box extends from the 25th to 75th percentile, and the median is represented by the horizontal line within the box.

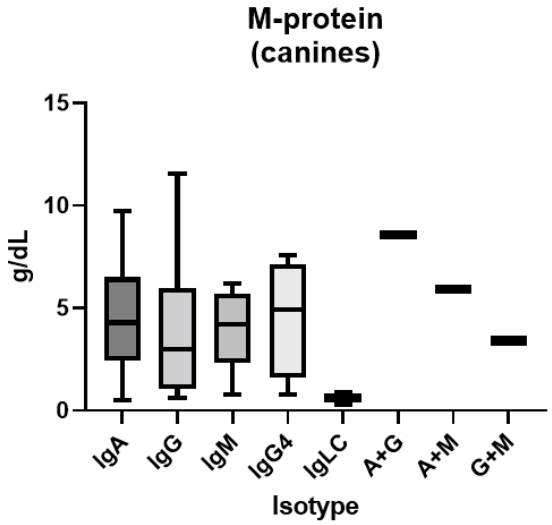

Figure 4.7. Box and whisker plots demonstrating dog M-protein concentrations stratified by isotype. There was no statistically significant difference between the groups. Minimum and maximum are represented by the whiskers, the box extends from the 25th to 75th percentile, and the median is represented by the horizontal line within the box.

Table 4.3. Complete summary of clinical diagnoses and treatment agents in dogs with M-proteins. Abbreviations: MM; multiple myeloma, PCT; plasma cell tumor, EPCT; extramedullary plasma cell tumor, PMM; presumed multiple myeloma, MG; monoclonal gammopathy, IMHA; immune mediate hemolytic anemia, MGUS; monoclonal gammopathy of undetermined significance, BCLL; B cell chronic lymphocytic leukemia, CHOP; cyclophosphamide/doxorubicin/vincristine/prednisone, RT; radiation therapy.

SUMMARY OF CANINE DIAGNOSES AND TREATMENT REGIMENS											
	MM	PCT	EPCT	PMM	MG	IMHA	MM/BCLL	MM/MGUS	BCLL	Unknown	
Treatment regimen	Number of patients										
Prednisone/Melphalan	33			1				1		1	36
Prednisone	5					1	1			2	9
Melphalan	3										3
CHOP	1								1		2
Prednisone/Cyclophosphamide	1								1		2
Tanovea/Prednisone/RT	3										3
Prednisone/Melphalan/RT	1										1
Chlorambucil									1		1
Prednisone/Cyclo/Chlorambucil									1		1
Prednisone/Chlorambucil							1		1		2
None	2	1	2		1				1	7	14
Unknown	1										1
Number of cases	50	1	2	1	1	1	2	1	6	10	75

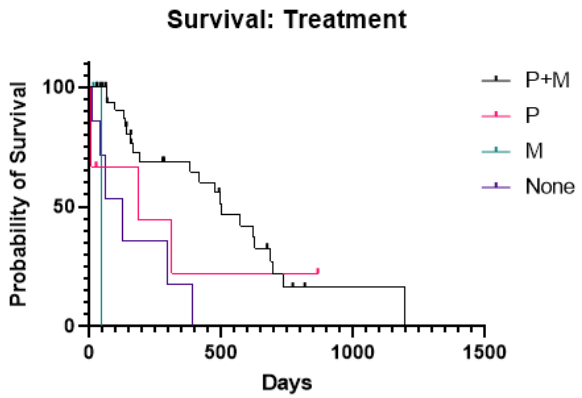

Figure 4.8. Kaplan-Meier survival curve comparison between dog treatment groups. P+M; prednisone and melphalan, P; prednisone only, M; melphalan only, and None; no chemotherapeutic treatment documented.

Table 4.4. Summary of MM diagnostic schemes and number of dogs that met the minimum diagnostic criteria for MM in each.

COMPARISON BETWEEN MM DIAGNOSTIC SCHEMES		
Current veterinary MM diagnostic criteria	Proposed veterinary criteria	Human criteria
Need 2 of more criteria		
Serum M-protein	Serum M-protein	≥10% clonal bone marrow plasma cells or biopsy proven bony or extramedullary plasmacytoma
Lytic bone lesion/s	Lytic bone lesion/s	AND
≥ 20% plasma cells in bone marrow	≥ 20% plasma cells in bone marrow	CRAB lesion/s (hypercalcemia, renal dysfunction, anemia, bony lesions)
FLC proteinuria (BJP)	FLC proteinuria (BJP)	AND/OR
	Visceral organ PCT	Biomarkers of malignancy (clonal bone marrow plasma cell percentage ≥60%, involved:uninvolved serum free light chain ratio ≥100, or >1 focal lesion on MRI studies)
Number of cases that were diagnosed with MM		
35	51	37

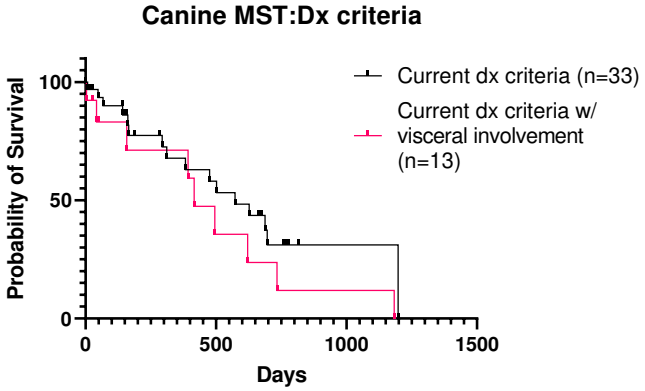

Figure 4.9. Kaplan-Meier survival curve comparison between dogs diagnosed with MM using the current veterinary diagnostic criteria (black) and the proposed diagnostic criteria (pink). Only cases that failed to meet the current diagnostic criteria but met the proposed new criteria were included in that group. The MST for the current diagnostic criteria group was 573 days and the MST for the proposed criteria group was 416. p = 0.19.

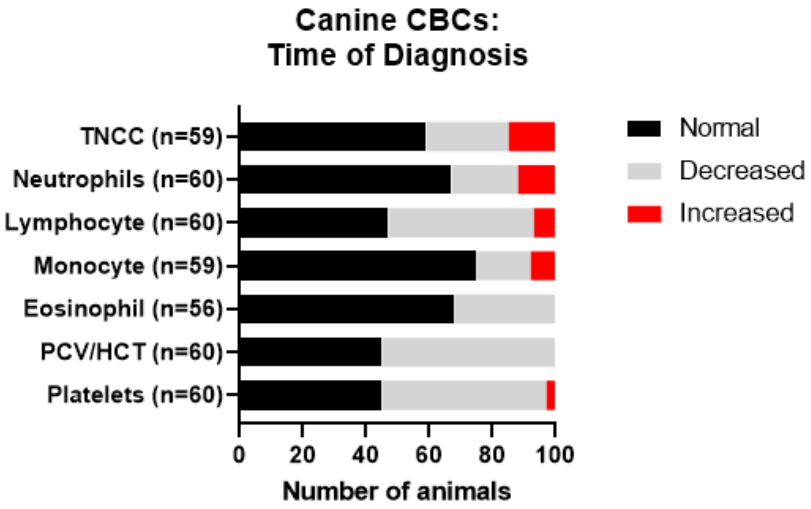

Figure 4.10: Complete blood count results of interest. Percentage of animals with results within the reference interval are represented in black. Percentage of animals with results below the lower reference interval limit are represented in light gray. Percentage of animals with results above the upper end of the reference interval are represented in red. The number of animals with data available for each variable is found in parentheses next to the variable title.

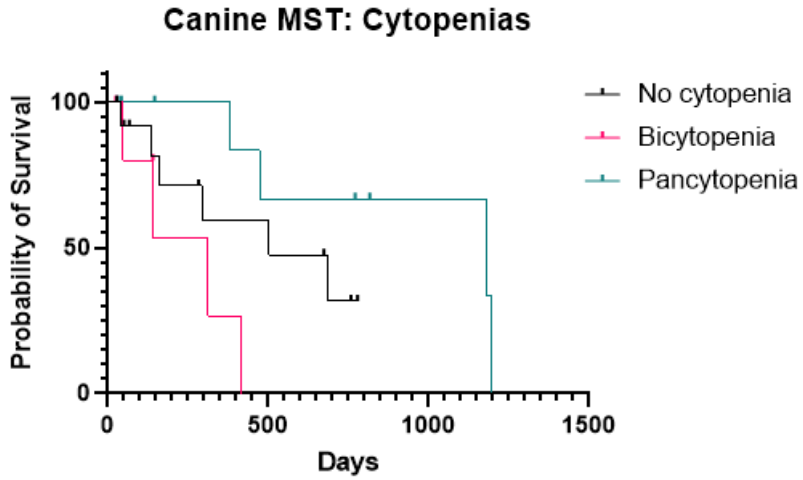

Figure 4.11. Kaplan-Meier survival curve comparison should dogs without cytopenias (black), dogs with bicytopenia (pink), and dogs with pancytopenia (green). The MST in animals without cytopenia was 502 days. The MST in animals with bicytopenia was 311 days and in pancytopenic dogs it was 1183 days. Statistically significant difference ($p = 0.004$) was present between the bicytopenia and pancytopenia groups.

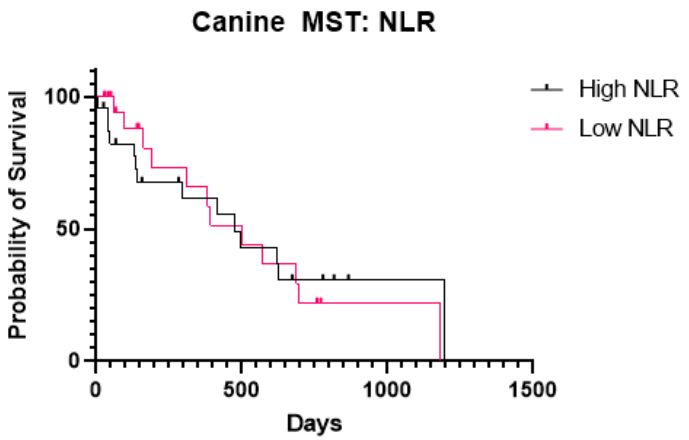

Figure 4.12. Kaplan-Meier survival curve comparison showing dogs in the high neutrophil to lymphocyte ratio (NLR) group (black) and those in the low NLR group (pink). The MST in the high NLR group was 475 days (HR 1.06, 95% CI 0.49-2.29). The MST in the low NLR group was 475 days (HR 0.95, 95% CI 0.44-2.05). p = 0.95.

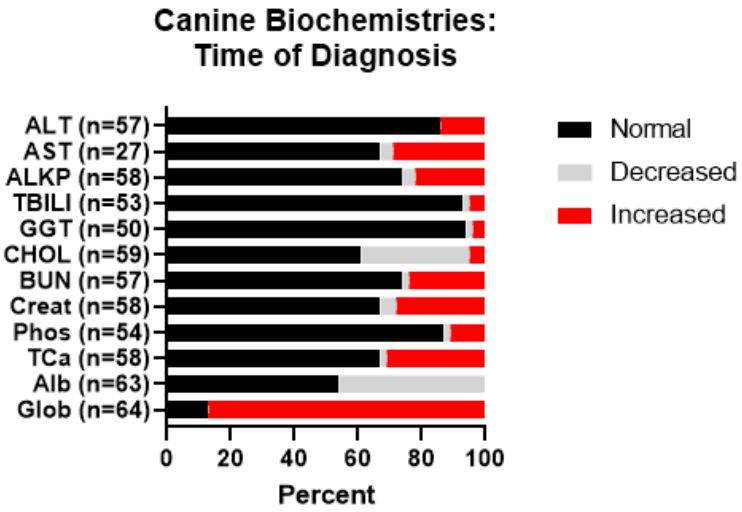

Figure 4.13. **S**erum/plasma biochemistry results. Percentage of animals with results within the reference interval are represented in black. Percentage of animals with results below the lower reference interval limit are represented in light gray. Percentage of animals with results above the upper end of the reference interval are represented in red. The number of animals with data available for each variable is found in parentheses next to the variable title.

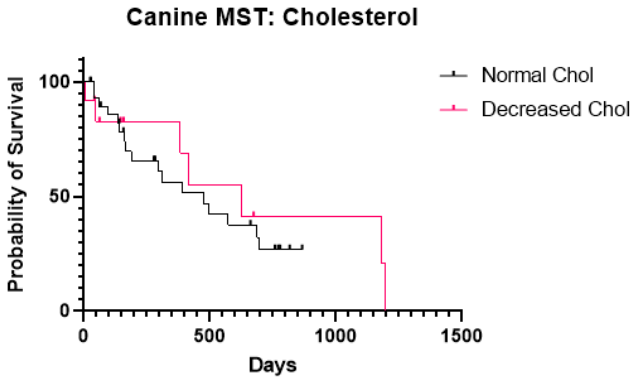

Figure 4.14. Kaplan-Meier survival curve comparison showing dogs with normal cholesterol concentrations (black) and those with hypocholesterolemia (pink). The MST for animals with normal cholesterol concentrations was 475 days (HR 1.30, 95% CI 0.51-3.30). The MST in animals with hypocholesterolemia was 627 days (HR 0.77, 95% CI 0.30-1.96). p = 0.59.

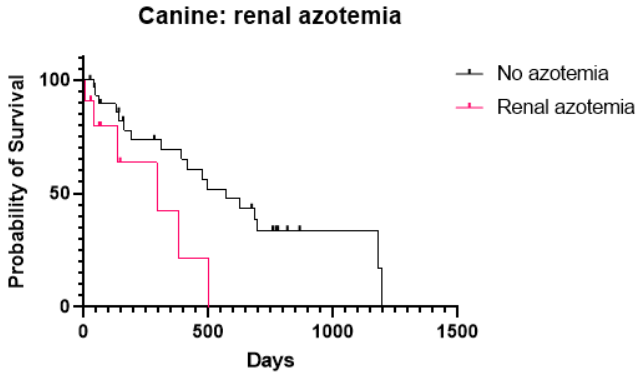

Figure 4.15. Kaplan-Meier survival curve comparison showing dogs without renal azotemia (black) and with renal azotemia (pink). BCLL cases were excluded. The MST for dogs without renal azotemia was 573 days (HR 0.20, 95% CI 0.05-0.77). The MST for dogs with renal azotemia was 294 days (HR 4.97, 95% CI 1.30-19.0). p = 0.02.

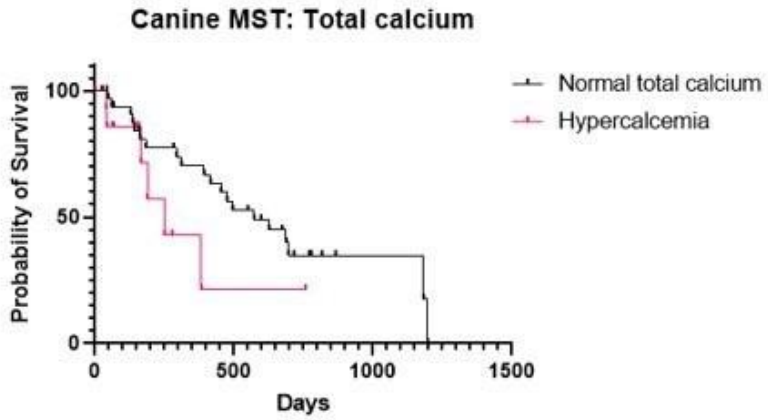

Figure 4.16. Kaplan-Meier survival curve comparison showing dogs with normal total calcium concentrations (black) and dogs with total hypercalcemia (pink). MST for patients with normal total calcium concentrations was 383 days (HR 1.63, 95% CI 0.51- 5.24). MST for patients with total hypercalcemia was 573 days (HR 0.43, 95% CI 0.14-1.37). p = 0.41.

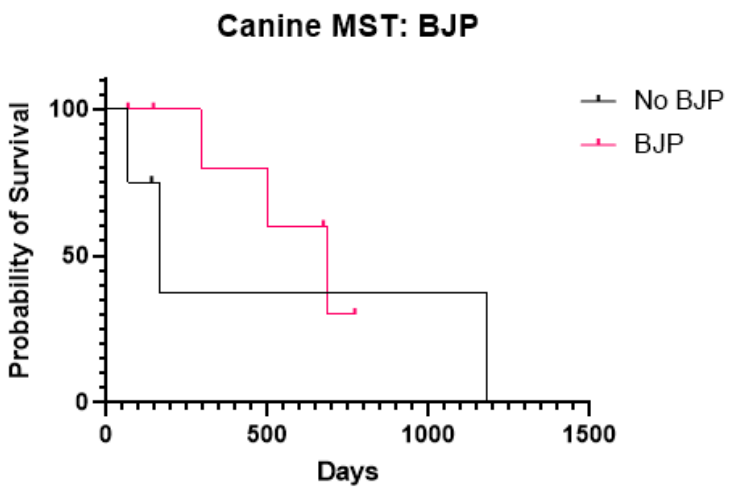

Figure 4.17. Kaplan-Meier survival curve comparison showing dogs without Bence-Jones Proteinuria (BJP) (black) and with BJP (pink). The MST for dogs without BJP was 165 days (HR 1.97, 95% CI 0.27-14.65). The MST for dogs with BJP was 688 days (HR 0.51, 95% CI 0.07-3.76). p = 0.51.

Table 4.5. Summary of confirmed lytic bone lesions and M-protein isotype in dogs with M-proteins.

SUMMARY OF CONFIRMED LYTIC BONE LESIONS		
CASE #	**Isotype**	**Location**
6	A	L4 vertebra, left ilium, humerus
7	A	L2 dorsal spinous process
17	A	Vertebrae (unspecified)
19	A	Proximal tibia
24	A	Left femur, both tibia, pelvis, os penis, and possibly the vertebrae
25	A	Scapula
27	G	T7, L3-L4 vertebrae
30	G	T1 vertebra
42	LC	T13 vertebrae, right proximal humerus, rib
43	A	Ribs (unspecified)
44	A	C2-C3 and C6-C7 vertebrae
48	A	C2 vertebra and dorsal spinous processes of lumbar vertebrae
51	A	Vertebrae (unspecified) and right femur
56	A	C2 and T1 vertebrae, rib
60	A	T4 vertebra
62	A	Sternebrae
64	A	All cervical/thoracic vertebrae, ribs, humerus

Table 4.6**:** Summary of cytologic findings in dogs with cytology performed.

CYTOLOGY RESULTS		
Case #	**Organ sampled**	**Interpretation**
3	Lymph node	Lymphoma (BCLL on flow cytometry)
5	Spleen	PCT
6	Lymph node (mandibular)	Normal
	Spleen	Plasmacytosis
7	Liver	Reactive/hyperplastic lymphoid tissue
	Spleen	Reactive/hyperplastic lymphoid tissue
8	Skin	PCT
9	Spleen	PCT
10	Spleen	PCT
11	Spleen	PCT
13	Cranial mediastinal mass	PCT
14	Spleen	Plasmacytosis with atypia
16	Spleen	Expanded intermediate sized lymphocytes
18	Spleen	PCT
19	Liver	Probable PCT
	Spleen	Probable PCT
20	Spleen	Marked plasmacytosis
21	Pleural fluid	PCT
22	Liver	PCT
23	Liver	BCLL (CD21+ lymphocytosis on flow cytometry)
24	Left femur	PCT
25	Spleen	PCT
26	Spleen	Probable atypical PCT
29	Spleen	Small cell lymphoma
30	T1 vertebral body	PCT
35	Spleen	PCT
37	Liver	Reactive lymphoid tissue
	Spleen	Suppurative inflammation
38	Liver	PCT
39	Spleen	EMH and reactive lymphoid tissue (BCLL by flow cytometry)
41	Iliac lymph node	Marked plasmacytosis
	Spleen	Mild lymphoid and plasma cell hyperplasia
42	Bone	PCT
	Liver	PCT
	Spleen	PCT
44	Gluteal mass	MCT
	Spleen	Marked plasmacytosis, concern for histiocytic sarcoma
45	Spleen	PCT
49	Left popliteal lymph node	PCT
	Liver	PCT
	Spleen	PCT
51	Intra-abdominal mass (near bladder)	PCT
	Spleen	EMH
54	Effusion	Plasma cell neoplastic effusion
56	Liver	Low numbers of plasma cells
	Spleen	Low numbers of plasma cells
60	Liver	PCT
	Spleen	PCT
62	CSF	Normal
	Liver	PCT
	Spleen	PCT
63	Retropharyngeal lymph node	PCT
	Spleen	PCT
64	Prescapular lymph node	PCT
65	Lymph node	Lymphoma
	Spleen	Concerning for lymphoma with EMH
68	Spleen	PCT
69	Spleen	PCT
70	Spleen	PCT
71	Spleen	PCT
72	Popliteal and iliac lymph nodes	PCT
75	Spleen	PCT
78	Spleen	PCT
88	Lymph node	Lymphoproliferative disease (PCT vs plasamacytoid lymphoma)

Abbreviations: BCLL, B cell Chronic Lymphocytic Leukemia; PCT, plasma cell tumor; EMH, extramedullary hematopoiesis; MCT, mast cell tumor; CSF, cerebrospinal fluid.

Table 4.7. Summary of bone marrow findings in dogs with bone marrow evaluations.

Bone marrow results	
Case #	**Interpretation**
3	BCLL (confirmed with flow cytometry)
4	PCT
5	Plasmacytosis (13-20% plasma cells)
11	PCT
20	Plasmacytosis (17% plasma cells)
22	PCT/MM
27	Normal
29	Small cell lymphoma
38	Mild plasma cell hyperplasia
42	Plasmacytosis (8.6% well-differentiated plasma cells)
45	PCT (80% plasma cells)
46	PCT
53	PCT (65% plasma cells)
57	Myeloma vs myeloid leukemia
59	PCT (39% plasma cells)
61	PCT
64	PCT
66	PCT/MM
70	PCT/MM
71	Plasma cell dyscrasia
73	PCT/MM (54.6% plasma cells)

CHAPTER 5: RETROSPECTIVE STUDY ON MONOCLONAL IMMUNOGLOBULINS IN CATS

MATERIALS AND METHODS

Case Selection

Cases submitted to the Colorado State University's Clinical Pathology Laboratory between January 2014 through December 2020 for performance of serum protein electrophoresis, with or without immunofixation, were searched for cats that were diagnosed with a monoclonal immunoglobulin. Cases that had serum protein electrophoresis performed and were interpreted as suspicious for a monoclonal immunoglobulin, but did not have immunofixation performed, were also selected if subsequent immunofixation confirmed a monoclonal immunoglobulin yielding 46 cases. Records were collected from patients that met these inclusion criteria and reviewed for excluding factors such as certain infectious diseases (*Blastomyces, Histoplasmosis, etc*) and cases with insufficient evidence of myeloma related disease. A total of 4 cases were excluded based on this review. Records from included cases were then reviewed and a number of variables were captured, which included diagnosis date, M-protein isotype, M-protein concentration, treatment protocol, response to treatment in patients with monitoring SPE performed, rescue treatment protocol (if applicable), initial clinical signs that prompted veterinary visit, whether there was evidence of bleeding diathesis, lytic bone lesions, selected initial biochemistry results (ALKP, AST, GGT, Tbili, BUN, Creat, Phos, TCa, Alb, Glob), selected initial complete blood count results (TNCC, leukocyte concentrations, platelet counts), coagulation panel results, urine specific gravity, urine protein concentration, UPC, presence of BJP, infectious disease testing, previous/concurrent diagnoses, adverse reactions from treatment, and date of death or loss to follow up. Age at time of diagnosis was rounded to the nearest whole number. Since hematologic and biochemical analyses were run on various instruments, only the associated reference intervals were used to determine if results were abnormal. Clinical signs at the time of diagnoses were categorized as none reported, gastrointestinal, pain, neurologic, constitutional (lethargy, anorexia, weight loss, etc), skin masses, renal disease, respiratory, or

other. Evidence of bleeding diathesis was categorized as having one or more of the following, epistaxis, bruising (including petechiation, ecchymoses, or bruising), retinal hemorrhage, or other. Previous or concurrent diagnoses were categorized as endocrine, renal, hepatic, neoplastic, infectious, and other. Adverse reactions to treatment were categorized as none reported, neutropenia, thrombocytopenia, hepatic, renal, or local reactions to chemotherapeutics.

Serum protein electrophoresis and Immunofixation protocols

Samples were archived as part of normal sample processing within the clinical pathology laboratory, were free of significant hemolysis, icterus, and lipemia, and had been stored at -80°C until evaluation.

AGE SPE had been performed at the time of initial sample submission using amido black stained AGE (Sebia Hydrasys with Hydrogel Protein (E) with amido black kit, Sebia, France), a flat-bed scanner (Epson Perfection V700 Photo, Epson America, Inc, Long Beach, CA, USA), and Phoresis software (version 8.6.3, Sebia, France), as previously described.[24]

Involved immunoglobulin class was characterized if it had not been identified at the time of initial evaluation and there was sufficient sample volume. Routine immunofixation in cats targeted IgG-FC, IgA, and IgM heavy chain and light chain using feline-specific reagents and was performed as previously described.[24,49] Free light chain (fLC) immunofixation had been performed using human-targeted fLC IF antibody set (Sebia Free light chains kit, Sebia, France) on a limited number of samples.[17] The manufacturer recommendations were followed for the application of serum, gel processing, and staining procedures. Samples were loaded neat (unaltered/undiluted), except for cases with a total protein > 10 g/dL which were diluted 1:2 with 0.9% saline per standard laboratory protocol. Densitometric measurement of M-proteins was performed using previously a validated method.[24]

Total protein was assessed using a biuret total protein assay (Cobas c501: Roche Diagnostics. Indianapolis, IN, USA). Serum albumin concentrations were also obtained on the Cobas c501 using the bromocresol green method, with subsequent globulin determination using the difference between colorimetric total protein and albumin.

RESULTS

Population characteristics, isotype distribution, and M-protein concentration

A total of 42 felines with monoclonal immunoglobulin were included for the descriptive population characteristics. 59.5% (25/42) of the population were males and 40.5% (17/42) were female (Figure 5.1). The MST for females and males, regardless of diagnosis or treatment protocol, was 217 days and 193 days, respectively ($p = 0.77$). The MST of all patients, regardless of treatment protocol or diagnosis, was 193 days with approximately 32.9% of patients alive at one-year post diagnosis (Figure 5.2). The mean age at diagnosis was 12.5 years, with a range of 6-18 years (Figure 5.3). Domestic short hair cats made up 61% (25/41) of the study population. Domestic long hair cats 12.2% (5/41), domestic medium hair cats 7.3% (3/41), Maine Coons 4.9% (2/41), and various pure breed cats (6 total, with one cat from each breed and a single mixed pure breed cat) were also represented. Complete breed list is shown in Table 5.1.

The most common clinical signs that prompted veterinary examination were constitutional (88.9%, 24/27), gastrointestinal (48.2%, 13/27), and pain (18.5%, 5/27). No initial clinical signs were reported in 7.4% (2/27) of cases. A total of 39.3% (11/28) cats had heart murmurs noted on examination, 35.7% (10/28) had underlying or previous endocrine disease (6/10 hyperthyroidism, 2/10 diabetes mellitus, and 2/10 pancreatitis), and 21.4% (6/28) had historical chronic kidney disease. Hepatic disease, previous neoplasia, infections/infectious disease, and other previous diagnoses were less common. Initial clinical signs are summarized in Table 5.2.

Isotype distribution was as follows; 57.1% (24/42, 95% CI 42.2-70.9%) IgG, 21.4% (9/42, 95% CI 11.7-35.9%) IgA, 2.4% (1/42, 95% CI 0.1-12.3%) IgM, and 19.1% (8/42, 95% CI 10-33.3%) true biclonal (5 – IgA and IgG, 2 – IgG and IgM, and 1 – IgG and LC) (Figure 5.4). The MST for cats with IgG and IgA monoclonal immunoglobulins, excluding BCLL cases, was 343 days and 109.5 days, respectively ($p = 0.33$) (Figure 5.5). The mean total protein was 10.85 g/dL (min 7, max, 18.7). The mean albumin concentration was 2.68 g/dL (min 1.6, max 4.7). The mean globulin concentration was 8.10 g/dL (min 3.7, max 15.8). Figure 5.6. The mean M protein concentration was 4.65 g/dL (min 0.5, max 13.49).

No statistically significant difference was apparent between M-protein isotypes and M-protein concentration (Figure 5.7). 16.7% (4/24) of patients had normal total protein concentrations (< 8.0g/dl) at the time of diagnosis. 66.7% (16/24) of patients had normal albumin concentrations at the time of diagnosis. 22.8% of patients had a normal globulin concentration at the time of diagnosis, while the remaining 79.2% had hyperglobulinemia.

Diagnosis

Complete medical records were obtained and evaluated for a total of 28 patients to determine the diagnosis made by clinicians overseeing the cases. Table 5.3. Multiple myeloma was the most common diagnosis (25%, 7/28) with two additional cases diagnosed as suspected MM, one with PCT versus MM, and one as MM or MM. A total of 17.9% (5/28) cases were diagnosed as MRD with a single additional case diagnosed as suspected MRD. BCLL was diagnosed in 7.1% (2/28) cases and BCLL versus MM in an additional 14.3% (4/28) cases. Hepatic or splenic PCT tumors were diagnosed in 7.1% (2/28) cases. One case was diagnosed with cutaneous T cell lymphoma. This patient was a 10-year-old spayed female DMH cat who was presented for cutaneous skin masses that had clonal TCR (T cell receptor) on PARR. Bone marrow examination and/or assessment for BJP was not performed in this case, and cytology of the left popliteal and inguinal lymph nodes revealed a reactive and hyperplastic lymphoid populations, respectively. This patient was treated with prednisolone and cyclophosphamide and lost to follow-up 112 days after diagnosis with a true biclonal IgA and IgG gammopathy. Another patient was suspected of having FIP or neoplasia. This patient was an 11-year-old neutered male DSH cat who was presented for gastrointestinal and constitutional clinical signs. Bone marrow examination and/or assessment for BJP was also not performed in this case. Liver cytology was consistent with lymphoid hyperplasia versus lymphoproliferative disease and the spleen had moderate plasma cell hyperplasia on cytologic evaluation. There was also a potential mass associated with the duodenal papilla in this case that was not interrogated further. This patient was treated with prednisolone and chlorambucil for hepatic/biliary inflammation and possible neoplasia and was lost to follow-up 107 days after diagnosis with a true biclonal IgA and IgG gammopathy. The last case was diagnosed as having a spinal compressive tumor of thoracic vertebrae 11-

12. This patient was an 8-year-old neutered male mixed breed cat that was presented for evaluation of pain, neurologic signs, constitutional clinical signs, and weakness. This patient was tested, and negative, for *Toxoplasmosis* antibodies IgG and IgM. No additional work up was performed in this case and the patient was sent home on prednisolone and lost to follow up 3 days after initial presentation and diagnosis with an IgG monoclonal gammopathy.

Using the current veterinary diagnostic guidelines for MM, a total of 17.9% (5/28) of cases met the diagnostic criteria. With the addition of visceral organ involvement, a total of 60.7% (17/28) case met inclusion criteria. The number of cases managed as MM/MRD was 64.3% (18/27). Table 5.4. A Kaplan-Meier survival curve comparison of the current veterinary diagnostic criteria and proposed criteria (addition of visceral organ involvement) can be reviewed in Figure 5.9.

Treatment protocols varied, but combination therapy with prednisolone/melphalan and prednisolone/cyclophosphamide was most common and was used in 6 cases each (21.4%, 6/28 each). Diagnoses for patients treated with prednisolone/melphalan were 4 MM cases, one MRD, and one MM versus MRD diagnosis. Diagnoses in patients treated with prednisolone/cyclophosphamide were two MRDs, one MM, one suspected MM, one hepatic and splenic PCT, and one case with cutaneous T cell LSA. Prednisolone monotherapy was given to 17.9% (5/28) of patients (2 MM, 2 MRD, 1 suspected MRD, and a single animal with a T11/12 compressive bone lesion). A complete summary of diagnoses and treatments can be found in Table 5.3. Comparison between several treatment protocols and MST are shown in Figure 5.8.

Hematologic and Biochemical data

Anemia (60%, 15/25), thrombocytopenia (60, 15/25), and lymphopenia (44%, 11/25) were the most common hematologic abnormalities at the time of diagnosis. Leukopenia (24%, 6/25), neutrophilia (16%, 4/25), neutropenia (12%, 3/25), and leukocytosis (12%, 3/25) were also noted. All hematologic abnormalities are shown in Figure 5.10. Bicytopenia or pancytopenia were present in 44% of cases at the time of diagnosis (bicytopenia 24%, 6/25; pancytopenia 20%, 5/25). Bicytopenia was always associated with decreased platelet concentrations and anemia (the leukocyte concentrations were normal, or slightly

elevated, in these animals). No statistically significant difference was noted between animals with no or monocytopenia (MST 260 days) and those with bicytopenia (MST 118 days, p = 0.29, HR 2.18, 95% CI 0.52-9.20) or pancytopenia (MST 141 days, p = 0.42, HR 1.91, 95% CI 0.39-9.32) (Figure 5.11).

Several hepatic enzymes were evaluated and included, ALT, ALKP, AST, GGT, Total bilirubin, and Cholesterol (Figure 5.12). Increased AST (30%, 3/10), hypocholesterolemia (22.7%, 5/22), and increased ALKP (20%, 5/25) were the most common abnormalities. A statistically significant (p = 0.0014) difference was noted in animals with hypocholesterolemia (MST 31 days, HR 78.51, 95% CI 5.41-1140)) compared to animals with normal cholesterol (MST 343 days, HR 0.013, 95% CI 0.0009-0.19, Figure 5.13). Of the cases with confirmed hepatic plasma cell or BCLL neoplasia (excluding cholesterol), 2/6 had normal hepatic enzyme concentrations, 2/6 had elevations in a single hepatic enzyme (case no. 122 ALKP and case no. 132 GGT), and 2/6 had elevations in multiple hepatic enzymes (case no. 120 ALKP and total bilirubin, case no. 121 ALT, ALKP, AST, GGT, total bilirubin). Abnormalities were typically mild to moderate and measured 1.05-4.0 times the upper reference interval limits. The single case (case no. 121) with elevations in all the hepatic enzymes was the only case diagnosed with hepatic BCLL. This case also had more profound derangements in these hepatic enzymes that measured 1.85-50 times the upper reference interval limits.

Renal parameters that were evaluated included BUN, creatinine, phosphorus, and total calcium (Figure 5.12). Azotemia was defined as an increase in the BUN and/or creatinine and was present in 36% (9/25) of patients at the time of diagnosis. 3 of the 25 patients had a decreased BUN at the time of diagnosis. Of the patients categorized as azotemic, 55.6% had elevations in only BUN, 33.3% (3/9) had elevations in creatinine only, and 11.1% (1/9) had elevations in both BUN and creatinine. The azotemia was classified as pre-renal (USG >1.035), renal (USG ≤ 1.035), and unclassified (when USG was not performed). Azotemia was classified as renal azotemia in 44.4% (4/9) of the patients (USG range 1.014-1.018). Pre-renal azotemia was present in 22.2% (2/9) of patients (USG range 1.054-1.060). Azotemia was unclassified in 3 patients. The MST in cats (excluding BCLL cases) with renal azotemia and without renal azotemia was 396 days and 141 days, respectively (p = 0.35) (Figure 5.14).

Two cases had total hypercalcemia. The MST for patients with total hypercalcemia could not be assessed due to only two patients being hypercalcemic. One was lost to follow up at 97 days after diagnosis and the other was euthanized 396 days after diagnosis (diagnosed with MRD via necropsy). The MST in patients without total hypercalcemia was 141 days, and approximately 27.9% of these patients were alive at one-year post diagnosis. The calcium phosphorus product was calculated for 24 patients with both total calcium and phosphorus concentrations recorded. A total of 3 cases had a calcium phosphorus product equal to or above 70. Two of these cases had azotemia (1 with elevated BUN and creatinine and 1 with only elevated BUN) and one had BUN and creatinine within reference intervals.

A total of 4/27 patients had prothrombin (PT) and activated partial thromboplastin (aPTT) coagulation panels performed. 2/4 of these patients had abnormal results. One patient had prolongation of both PT (15.6s, RI 10.9-13) and aPTT (19.4s, RI 10.5-13). The other patient had a normal PT (10.1s, RI 6-11) and prolongation of the aPTT (42.1s, RI 10-25). The remaining 2/4 patients had results within reference intervals. Clinical evidence of bleeding diathesis was not documented in any of the cases.

Testing for FIV/FeLV was performed in 40.7% (11/27) of cases and was negative in all cases. Expanded infectious disease testing was performed in 5 cases. An infectious disease panel was performed in a single cat that was positive for *M. Turicensis* and *B. clarridgalae*. One cat was PCR positive for *Mycoplasma hemominutum*. One cat was negative for *Toxoplasma* IgG and IgM antibodies and another cat was negative for *Mycoplasma sp* via PCR. Our case that had co-infection with *M. turicensis* and *B. clarridgalae* was an 18-year-old neutered male mixed breed cat who was presented for gastrointestinal and constitutional clinical signs and was diagnosed as probable MM. During the work up for this cat he was diagnosed with an IgG M-protein, had mild plasmacytosis on bone marrow examination, mild plasmacytosis of the liver and spleen, and reactive lymphoid tissue of the colonic lymph node. This case was treated symptomatically with aluminum hydroxide and bisphosphonates and died 396 days after diagnosis. On post-mortem necropsy a diagnosis of MRD was made based on examination of the spleen and bone marrow. The cat that was PCR positive for *Mycoplasma hemominutum* was a 13-year-old neutered male DLH cat that was presented for gastrointestinal and constitutional clinical signs. This

patient was diagnosed with an IgA M-protein (with biclonal morphology), hepatosplenic PCT, and had marked erythroid hypoplasia and myelofibrosis on bone marrow biopsy. This patient was treated with prednisolone and cyclophosphamide and was euthanized 18 days after initial presentation.

Proteinuria was present in 91% (20/22) of cats with urinalysis data available. Only 4 patients were assessed for the presence of BPJ and a single patient had detectable BJP. Due to the low number of patients screened for BJP we could not determine the MST for this group.

The presence of bony lesions was noted in 3/27 cases. One patient had lesions associated with thoracic vertebrae 11-12 that lead to weakness and pain. This patient was lost to follow-up after discharge on prednisolone. Another patient had a pathologic fracture of the right femur and this lesion was PARR positive for a clonal B Cell Receptor. This patient also had a splenic PCT on cytology and bone marrow evaluation that was diagnosed as a possible PCT. The last patient had a moth-eaten bony lesion on the right proximal humerus and PCT diagnosed on cytologic evaluation of an abdominal lymph node and dorsal skin mass. All three cases were associated with IgG M-proteins.

Cytologic and Histopathologic data

Cytology was performed in 77.8% (21/27) of cases. Spleen was the most commonly sampled organ, with 76.2% (16/21) of cytologic cases having splenic aspirates performed. Splenic plasma cell tumors were diagnosed in 62.5% (10/16) of these cases. 18.8% (3/16) of splenic aspirates were equivocal for a diagnosis of plasma cell tumor versus marked reactive lymphoid tissue. The remaining three cases included a low cellularity splenic aspirate sample, spleen with lymphoid hyperplasia and extramedullary hematopoiesis, and a spleen with no cytologic abnormalities. Liver was the second most commonly aspirated organ, with 57.1% (12/21) of cytologic cases having hepatic aspirates evaluated. Hepatic plasma cell tumors were diagnosed in 41.7% (5/12) of cases. 16.7% (2/12) were equivocal for plasma cell tumor versus plasmacytosis. A single case was diagnosed with hepatic lymphoma (T lymphocyte marker CD3 negative, B lymphocyte marker PAX5 positive). The remaining cases consisted of two cases diagnosed with lymphoplasmacytic inflammation, one case that was equivocal for lymphoid hyperplasia versus a lymphoproliferative disease, and a single hepatic aspirate from another patient was cytologically normal.

Lymph nodes aspirates were examined in 33.3% (7/21) of cases. Plasma cell neoplasia was diagnosed in a single case. Two cases were diagnosed as suspicious for plasma cell neoplasia. Four cases were diagnosed as lymphoid hyperplasia/reactivity with one of these cases also considered to potentially be emerging lymphoma. A single case had a skin aspirate that was evaluated as a plasma cell tumor. Detailed results can be found in Table 5.5.

Histopathology was performed in 4 of 27 patients. Results consisted of a single plasma cell tumor of the eye, renal B cell lymphoma, hepatic B cell lymphoma, and a jejunal round cell tumor (plasma cell tumor versus small cell lymphoma).

Bone marrow was examined in 4 of 27 patients (excluding patients who had bone marrow examinations associated with necropsy). Possible plasma cell neoplasia was diagnosed in a single sample. The remaining samples were composed of a case with mild plasmacytosis, one with marked erythroid hypoplasia with myelofibrosis, and a single case that had no cytologic abnormalities.

DISCUSSION

Within our study population males were slightly overrepresented with a male to female ratio of 1.47:1. The mean age of cats at the time of diagnosis was higher in the canine population studied (12.5 years and 9.9 years, respectively). Similar to previous reports[5,35,43], domestic short haired cats were the most common breed affected within our population. Assessment of the sex and breed demographics of our general sample submission population is needed to definitively state these findings are different than that population. A vast majority of patients were presented for evaluation due to constitutional clinical signs (lethargy, anorexia, behavioral changes). Interestingly, 39.3% of animals had heart murmurs noted on examination. While it is tempting to explain this incidence as the M-protein induced hyperviscosity syndrome leading to increased workload of the cardiovascular system, and ultimately cardiomyopathies, the prevalence of heart murmurs in cats is surprisingly high.[67,68] One study with 780 cats, and another with 103 cats, identified heart murmurs in 44% and 21% of apparently healthy cats, respectively.[67,68] When interrogated further via echocardiography, these murmurs were frequently associated with structural heart disease.[67,68] The incidence of heart murmurs in this population statistically fits with the

general population. Based on this information, the actual association of monoclonal gammopathies with cardiac pathology is uncertain.

The feline M-protein isotype distribution included 19.1% true biclonal cases, suggesting that true biclonal gammopathies occur more frequently in cats, compared to humans and dogs. The significance of this is unclear, but this higher incidence of true biclonal gammopathies in cats may make them an ideal model for further research into whether these behave differently than cases with a single monoclonal immunoglobulin. The predominant isotype was IgG, which correlates with a single previous case series that used RID to determine isotype in felines with MM and found IgG production in the 7 cases evaluated.[35] Unfortunately, of the 3 case series describing MM and MRD in cats, none have included IF interrogation to differentiate true biclonal immunoglobulins from dimerization of a single immunoglobulin type. No cases of light chain only disease were detected, but this may be in part due to the low number of cases submitted for this type of analysis (4/28 cases). While there were no statistically significant differences in the MST based on isotype, close evaluation of the data shows that most of the IgA cases had a shorter survival time with the MST for IgG M-proteins being 343 days, compared to 109.5 days in patients with IgA M-proteins. This may suggest a similar correlation with isotype and prognosis in cats, as was recently documented in the human literature.[66] This human report also documented the patients with IgA M-proteins were less likely to have bony lesions than IgG patients.[66] The 3/27 cats in our study with confirmed bony lesions had IgG M-proteins, which may warrant further investigation to determine if IgG secreting cases are truly more prone to radiographically apparent bony lesions.

Based on the clinical diagnoses in these cases and previous literature[35], there appears to be some ambiguity surrounding the categorization of MRDs in the cat. This is likely associated with the fact that the veterinary diagnostic criteria for MM includes bone marrow plasma cell neoplasia (>20% plasma cells in the bone marrow) as one of the criteria and bone marrow involvement appears to occur less commonly in the cat compared to humans and dogs. For this reason, many cases without confirmation of bone marrow involvement but the presence of visceral organ involvement plasma cell neoplasia, are simply

categorized as MRD or EPCT (extramedullary plasma cell tumor), even though these may behave aggressively like multiple myeloma cases. Inclusion of visceral organ involvement as a criterion for the diagnosis of MM may better classify these cases, based on their clinical course, as MM.[35]

Hematologic abnormalities (anemia, thrombocytopenia, and lymphopenia) and, to a lesser extent, biochemical abnormalities (azotemia and hepatic enzyme elevations) were observed in a number of patients and likely secondary to visceral organ involvement, end organ damage related to hyperviscosity and/or light chain damage accumulation/deposition within glomeruli and small capillaries, and potential bone marrow involvement in these animals. Proteinuria was very common in our population (91%) but was rarely further characterized by evaluation for the presence of BJP (only 4 patients were evaluated for BJP and one case had detectable BJP).

Interestingly, evidence of bleeding diathesis was not noted within our study population. Comparing to our canine monoclonal immunoglobulin study, evidence of bleeding diathesis was noted in 30.6% of canine cases. Previous feline studies have reported evidence of bleeding diathesis in around 13% of feline patients with myeloma related disease.[35] Coagulation testing was only performed in a small subset of patients (4/27) and two of the animals had prolongation of one or both PT and aPTT. Based on the low number of animals assessed for coagulopathies it is difficult to make any meaningful interpretations in regard to how frequently this was present in our study population.

Infectious disease testing was frequently performed and included assessment for FIV/FeLV primarily. Previous studies have shown no association between MRD and FIV/FeLV status.[35,43] Two cats tested positive for infectious disease. One of our cats was co-infected with M. turicensis and B. clarridgalae and the other patient was PCR positive for Mycoplasma hemominutum. One previous report detailed a cat diagnosed with multiple myeloma and found to be co-infected with *Anaplasma platys*, *Bartonella henselae*, *Bartonella koehlerae*, and *Candidatus Mycoplasma haemominutum*.[69] The authors of this paper suspected immune system dysregulation caused by MM allowed a niche for this co-infection to take place. Another postulated explanation was initial infection with one or more of these organisms and low-level chronic inflammation initiating malignant transformation and ultimate MM.

Lytic bone lesions were rarely identified (11.1%, 3/27 cases), which is similar to previous reports.[35,43] However, given the low number of animals assessed for bony lesions this should be considered a minimum number. Cats with lytic bone lesions in our study were all associated with IgG monoclonal immunoglobulin. Further studies to assess whether there is a correlation with IgG M-protein production and lytic bone lesions, particularly in felines, may be beneficial. Additionally, prospective studies performing more thorough radiographic evaluation for bone lesions in cats with suspected MM could shed light on whether cats without apparent lameness or musculoskeletal pain may have subclinical bone lesions.

Splenic and hepatic PCTs were present at initial diagnosis in 62.5% and 41.7% cases where these organs were sampled by cytology, respectively. Several additional cases from each site were also equivocal for PCTs. This suggests that splenic and/or hepatic aspirates evaluated cytologically are frequently helpful in diagnosing MRDs in feline patients. Similar to canines, additional diagnostic testing such as PARR and/or immunocytochemistry for lymphoid and plasma cell specific markers can be informative in cases with equivocal cytologic results.

The overall MST for cats diagnosed with monoclonal immunoglobulins in this study was 193 days, which is much shorter than the overall MST in our canine population (502 days). This is similar to the current literature that states MRDs tend to have a less favorable prognosis in cats.[1] Hypocholesterolemia was a negative prognostic indicator in our population (when excluding BCLL cases). This supports previous literature that found cats with MM that had hypocholesterolemia had a shorter MST.[43,70] There was the suggestion of trends towards shorter survival times in animals with bicytopenia (MST 118 versus 678 days in animals without cytopenias), pancytopenia (MST 141), and IgA M-proteins (109.5 days versus 343 days in IgG M-protein cases). We were unable to compare MSTs in cats in relation to calcium concentration, proteinuria, or BJP due to low numbers of animals with hypercalcemia, lack of proteinuria being rare, and only a few cases evaluated for the presence of BJP.

Based on the available records, it appears that clinicians include evidence of visceral organ involvement as a criterion of MM in the cat. The MST data suggests that there may be no outcome

difference between cats diagnosed MM using a strict interpretation of the diagnostic criteria and those who diagnoses relied on visceral organ involvement. Additionally, no BCLL cases were misclassified as MM with this proposed scheme. With the current scheme there are major challenges that clinicians face in categorizing MRDs in cats. Without the presence or confirmation of bone marrow plasma cell numbers equal to or above 20% and lack of lytic bone lesions, clinicians are dependent on using the remaining two criteria to diagnose MM. This ultimately leads to many cats receiving a generic diagnosis of MRD or MRD versus MM, which can cause delays in treatment due to lack of a clear understanding of the disease to be treated. Additionally, the available literature indicates that lytic bone lesions and bone marrow involvement are less common in cats, which arguably makes these MM diagnostic criteria antiquated in cats. Our survival curve comparisons between animals diagnosed with MM with the proposed scheme and those being treated as MM or MRD revealed these curves were not significantly different.

Overall, cats with MRDs had a poorer prognosis when compared to dogs, with the exception of BCLL cases. Evidence of bleeding diathesis was not observed in our population and the presence of lytic bone lesions was uncommon in our population. Hypocholesterolemia was a negative prognostic indicator for animals with MRDs (excluding BCLL, which was not included in these analyses). The validity of renal azotemia, hypercalcemia, proteinuria, and BJP as prognostic indicators could not be fully assessed in this study, due to a number of factors. Lastly, the addition of visceral organ involvement to the current veterinary MM diagnostic scheme may be warranted and can make categorization of MRDs and diagnosis of MM easier, while still correlating with the clinical behavior of these diseases in cats.

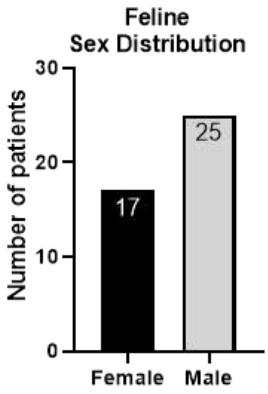

Figure 5.1. Bar chart showing the number of female and male cats diagnosed with M-proteins.

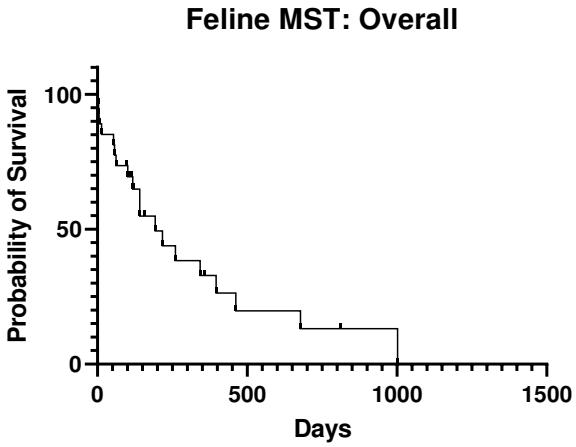

Figure 5.2. Kaplan-Meier survival curve for entire cat population regardless of diagnosis or treatment protocol. MST 193 days with approximately 32.9% of cats alive at one-year post diagnosis.

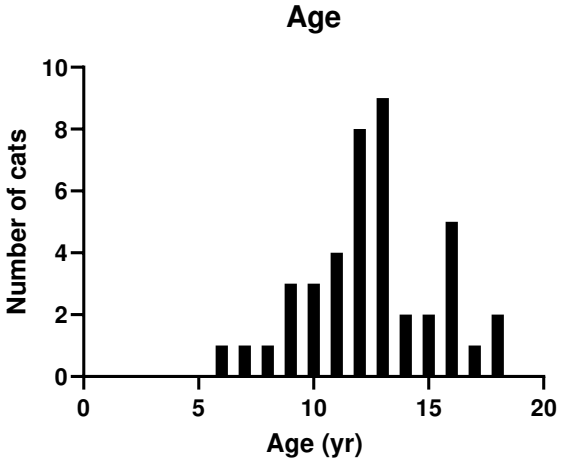

Figure 5.3. Histogram showing the age distribution for cats diagnosed with an M-protein. Range 6-18 years, mean 12.5 years.

Table 5.1. Complete list of cat breeds represented in our cat study population with M-proteins. Abbreviations: DSH; domestic short hair, DLH; domestic long hair, DMH; and domestic medium hair.

SUMMARY OF BREEDS	
Breed	**Number of animals**
DSH	25
DLH	5
DMH	3
Maine Coon	2
Cornish rex	1
Tabby	1
Snowshoe	1
Bengal	1
Siamese	1
Mixed	1
Total	**41**

Table 5.2. Summary of presenting clinical signs in 27 cats with an M-proteins.

REPORTED CLINICAL SIGNS		
Clinical sign	**Percent**	**Number**
None reported	7.4	2/27
Constitutional	88.9	24/27
Gastrointestinal	48.2	13/27
Pain	18.5	5/27
Neurologic	3.7	1/27
Skin masses	7.3	2/27
Respiratory	3.7	1/27

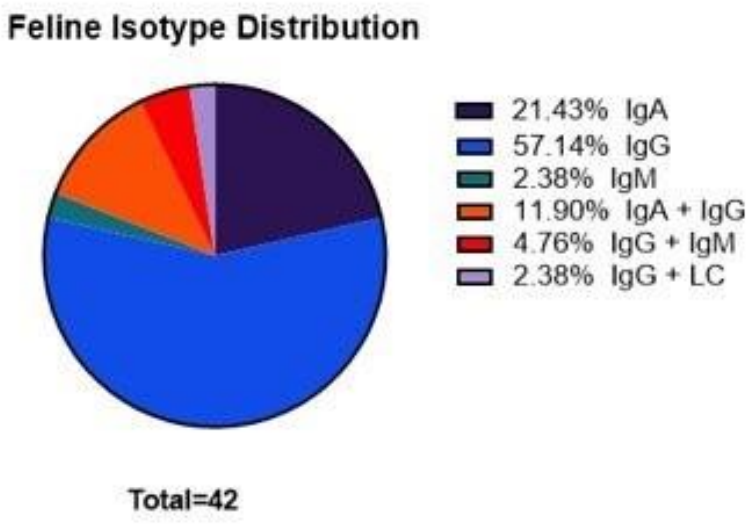

Figure 5.4. Pie chart demonstrating the distribution of isotypes in cats with M-proteins. Color and isotype key to the right of the chart.

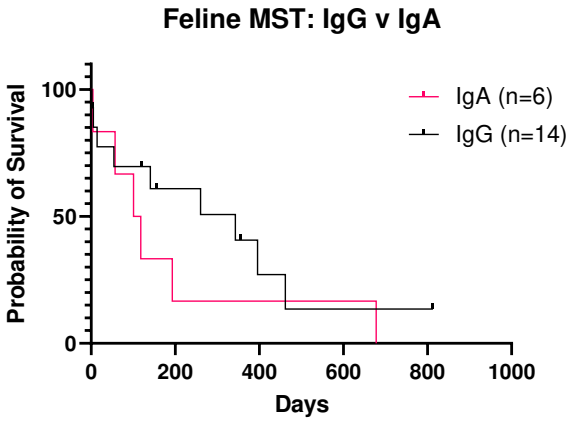

Figure 5.5. Kaplan-Meier survival curve comparison between cats with IgG (black) and IgA (pink) M-proteins. No statistically significant difference was noted between cats with IgG M-proteins (MST 343 days, HR 0.57, 95% CI 0.18-1.78) and cats with IgA M-proteins (MST 109.5 days, HR 1.75, 95% CI 0.56-5.46). p = 0.33.

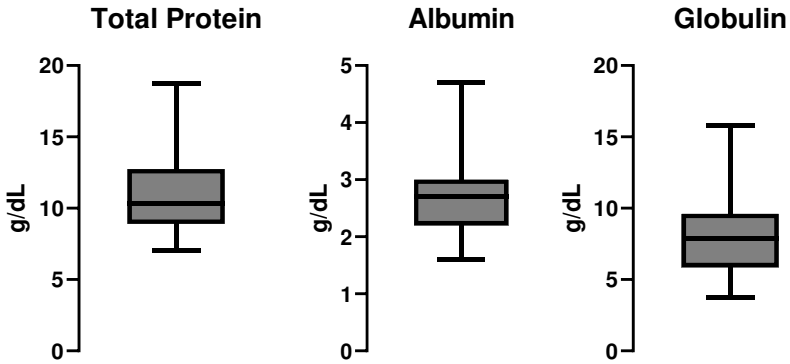

Figure 5.6. Box and whisker plots demonstrating the cat population total protein, albumin, and globulin concentrations. Minimum and maximum are represented by the whisker, the box extended from the 25th to 75th percentile, and the median is represented by the horizontal line within the box.

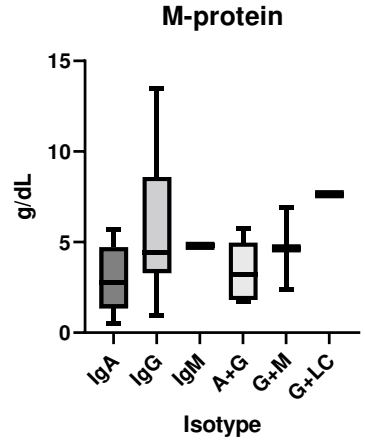

Figure 5.7. Box and whisker plots demonstrating cat M-protein concentrations stratified by isotype. There was no statically significant difference between the groups. Minimum and maximum are represented by the whiskers, the box extends from the 25th and 75th percentile, and the median is represented by the horizontal line within the box.

Table 5.3. Complete summary of clinical diagnoses and treatment agents for cats with M-proteins. Abbreviations: MM; multiple myeloma, MRD; myeloma related disease, BCLL; B cell chronic lymphocytic leukemia, PCT; plasma cell tumor, PMM; presumed multiple myeloma, PMRD; presumed myeloma related disease, CTLSA; cutaneous T cell lymphoma, HS PCT; hepatosplenic plasma cell tumor, FIP; feline infectious peritonitis.

SUMMARY OF FELINE DIAGNOSES AND TREATMENT REGIMENS													
	MM	MRD	BCLL	BCLL/MRD	MRD/MM	PCT/MM	PMM	PMRD	CTLSA	HS PCT	Spinal PCT	FIP/neoplasia	
Treatment regimen	Number of patients												
Prednisone/Melphalan	3	1			1								5
Prednisone	2	1						1			1		5
CHOP			1										1
Prednisone/Cyclophosphamide	1	2					1		1	1			6
Cyclophosphamide				1									1
Prednisone/Chlorambucil/Palladia		1											1
Prednisolone/Chlorambucil				1						1		1	3
Prednisolone/Lomustine			1	1									2
None	1			1			1						3
Unknown						1							1
Number of cases	7	5	2	4	1	1	2	1	1	2	1	1	28

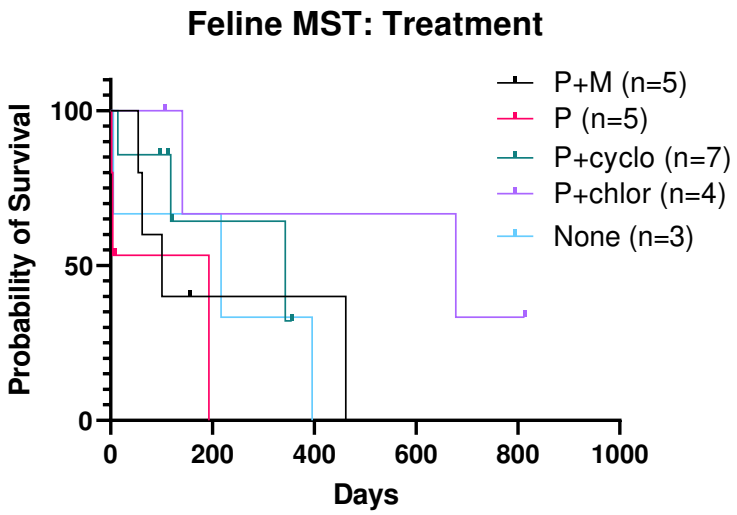

Figure 5.8. Kaplan-Meier survival curve comparison between cat treatment groups. P+M; prednisolone and melphalan, P; prednisolone, P+cyclo; prednisolone and cyclophosphamide, P+chlor; prednisolone and chlorambucil, None; no chemotherapeutic treatment documented.

Table 5.4. Comparison between MM diagnostic schemes and number of cats that met the diagnostic criteria for MM in each.

COMPARISON BETWEEN MM DIAGNOSTIC SCHEMES		
Current veterinary MM diagnostic criteria	Proposed veterinary criteria	Human criteria
Need 2 of more criteria		
Serum M-protein	Serum M-protein	≥10% clonal bone marrow plasma cells or biopsy proven bony or extramedullary plasmacytoma
Lytic bone lesion/s	Lytic bone lesion/s	AND
≥ 20% plasma cells in bone marrow	≥ 20% plasma cells in bone marrow	CRAB lesion/s (hypercalcemia, renal dysfunction, anemia, bony lesions)
FLC proteinuria (BJP)	FLC proteinuria (BJP)	AND/OR
	Visceral organ PCT	Biomarkers of malignancy (clonal bone marrow plasma cell percentage ≥60%, involved:uninvolved serum free light chain ratio ≥100, or >1 focal lesion on MRI studies)
Number of cases that were diagnosed with MM		
5	17	12

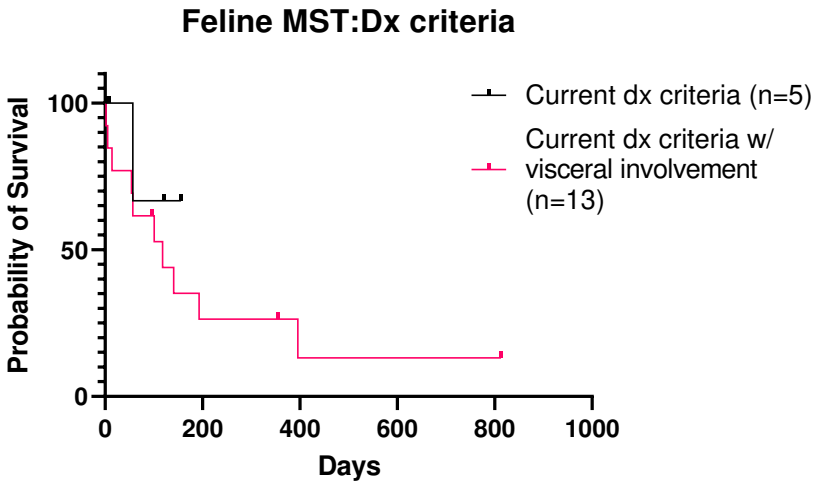

Figure 5.9. Kaplan-Meier survival curve comparison between cats diagnosed with MM using the current veterinary diagnostic criteria (black) and the proposed diagnostic criteria (pink). Only cases that failed to meet the current diagnostic criteria but met the proposed new criteria were included in that group. The MST for the current diagnostic criteria group could not be calculated and the MST for the proposed criteria group was 118. $p = 0.31$.

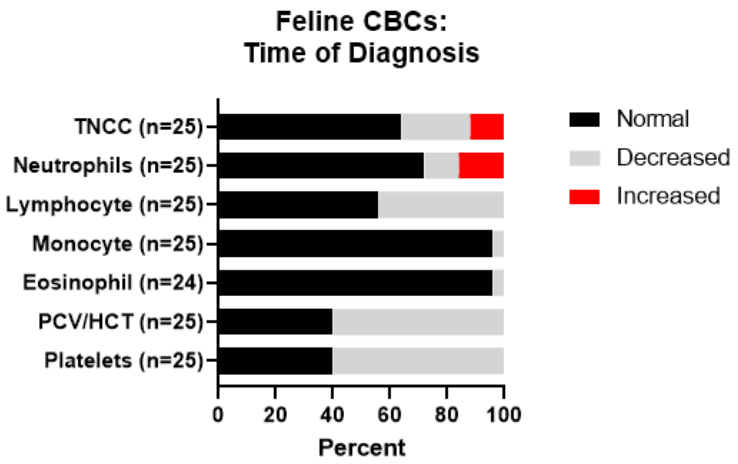

Figure 5.10. Complete blood count results of interest. Percentage of animals with results within the reference interval are represented in black. Percentage of animals with results below the lower reference interval limit are represented in light gray. Percentage of animals with results above the upper end of the reference interval are represented in red. The number of animals with data available for each variable is found in parentheses next to the variable title.

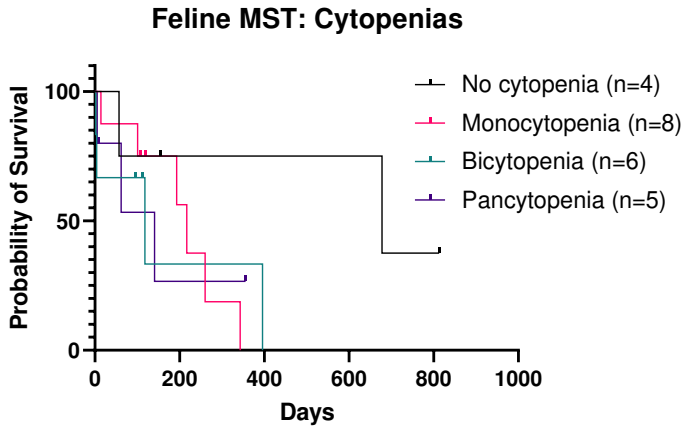

Figure 5.11. Kaplan-Meier survival curve comparison between animals with no cytopenia (black), monocytopenic cats (pink), bicytopenic cats (blue), and pancytopenic animals (purple). No statistically significant difference was noted between animals with no or monocytopenia (MST 260 days) and those with bicytopenia (MST 118 days, p = 0.29, HR 2.18, 95% CI 0.52-9.20) or pancytopenia (MST 141 days, p = 0.42, HR 1.91, 95% CI 0.39-9.32)

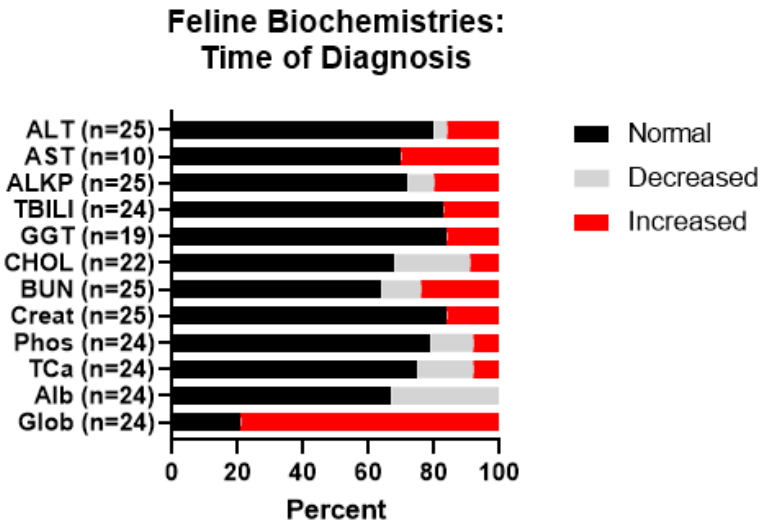

Figure 5.12. Serum/plasma biochemistry results. Percentage of animals with results within the reference interval are represented in black. Percentage of animals with results below the lower reference interval limit are represented in light gray. Percentage of animals with results above the upper end of the reference interval are represented in red. The number of animals with data available for each variable is found in parentheses next to the variable title.

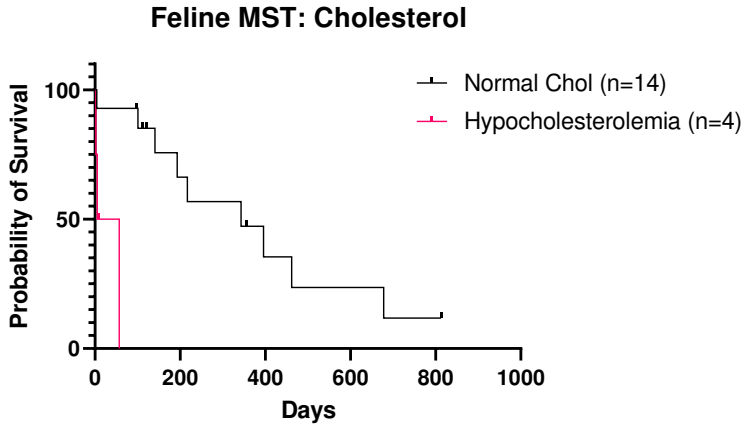

Figure 5.13. Kaplan-Meier survival curve comparison between cats with normal cholesterol concentrations (black) and those with hypocholesterolemia (pink). A statistically significant (p = 0.0014) difference was noted in animals with hypocholesterolemia (MST 31 days, HR 78.51, 95% CI 5.41-1140)) compared to animals with normal cholesterols (MST 343 days, HR 0.013, 95% CI 0.0009-0.19).

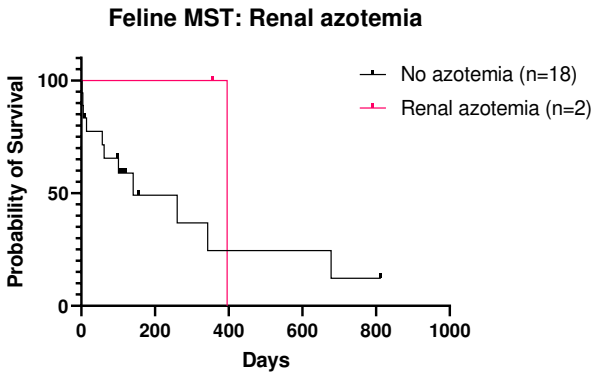

Figure 5.14. Kaplan-Meier survival curve comparison between cats without renal azotemia (black) and those with renal azotemia (pink). No statistically significant difference in MST was noted between animals without renal azotemia (141 days, HR 2.08, 95% CI 0.45-9.60) and those with renal azotemia 396 days (HR 0.48, 95% CI 0.10-2.22). p = 0.35.

Table 5.5. Summary of cytology results in cats with M-proteins.

	CYTOLOGY RESULTS	
Case #	**Organ samples**	**Interpretation**
118	Spleen	PCT
120	Liver	PCT
	Spleen	PCT
121	Liver	BCLL
122	Liver	PCT
	Spleen	PCT
124	Jejunal LN	Probable PCT
	Liver	NSF
	Spleen	NSF
125	Colonic LN	Reactive lymphoid hyperplasia
	Liver	Mild plasmacytosis
	Spleen	Mild plasmacytosis
126	Liver	Probable PCT
	Spleen	Probable PCT
127	Liver	Lymphoid hyperplasia vs lymphoproliferative disease
	Spleen	Moderate plasma cell hyperplasia
128	Jejunal LN	Marked lymphoid hyperplasia vs emerging LSA
129	Liver	PCT
	Spleen	PCT
131	Left popliteal LN	Reactive lymphoid tissue
	Iliac LN	Reactive lymphoid hyperplasia
	Liver	Lymphoplasmacytic inflammation
132	Liver	PCT
	Spleen	PCT
133	Mesenteric LN	Plasmacytosis, possible PCT
134	Spleen	PCT
135	RPSLN/abdominal mass	Reactive lymphoid tissue
	Liver	Marked cholestasis with lymphoplasmacytic inflammation
	Spleen	Lymphoid hyperplasia, EMH
138	Liver	PCT
	Spleen	Low cellularity sample
139	Spleen	PCT
140	Spleen	PCT
141	Spleen	PCT
143	Spleen	PCT
144	Abdominal LN	PCT
	Dorsum skin mass	PCT

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