Summary Report — Emily Rout, DVM
AKC Canine Health Foundation Clinician-Scientist Fellowship — December 29, 2017

BACKGROUND

B-cell chronic lymphocytic leukemia (CLL) is a common tumor in dogs, but little is known about the pathophysiology or clinical progression of this disease. CLL accounts for approximately 10% of all lymphoid malignancies seen among canine samples submitted to the Colorado State University Clinical Immunology laboratory for immunophenotyping with suspicious of lymphoma or leukemia. Given the prevalence of this disease, there are relatively few studies in the primary literature assessing clinical presentation and outcome.

Canine CLL shares similarities with human CLL, which is the most common leukemia of adults in western countries. Both tumors are characterized by a clonal expansion of small B-cells in the blood and may involve secondary lymphoid tissues like lymph node and spleen. A study from our laboratory examined the clinical presentation of 491 canine patients with CLL and found similarities with human CLL. Both diseases affect older individuals, with a median age of diagnosis of 11 years in dogs. Human CLL is very rare in Asian countries, suggesting a genetic predisposition, which is also supported by an increased incidence of CLL in individuals with a familial history. Similarly, there is a strong breed predilection in canine CLL for small-sized breeds, with 11 breeds having increased odds of developing CLL, suggesting a genetic predisposition. Canine CLL frequently involves secondary lymphoid tissues like human CLL, with approximately 50% of cases having peripheral lymph node and splenic involvement. Also similar to human CLL, anemia affects 26% of canine CLL patients, thrombocytopenia is less frequent (6% of cases), and neutropenia is very rare.

Given the similarities in clinical presentation, we aimed to investigate other similarities between human and canine CLL. By studying these similarities, we can apply what is known about diagnosis, prognostication and treatment in human CLL, and improve our knowledge of canine CLL. Human CLL is known to have tremendous clinical heterogeneity, with marked variability in clinical outcome. A subset of human patients have indolent disease and often do not require therapy, with a median survival time >25 years and often a normal lifespan. Another subset have aggressive disease, with a shortened survival time of just 3-8 years even with aggressive therapy. Although many cases of canine CLL appear indolent, the two studies examining outcome report a wide range in survival times, suggesting there is a similar spectrum of disease in canine CLL. We are investigating three major aspects of canine CLL to better understand the clinical progression of this disease and identify prognostic markers to improve patient care: (1) immunoglobulin mutation status, (2) clinical outcome, (3) gene expression. All three components have been investigated during the time period supported by the Clinician-Scientist Fellowship, with the majority of funds contributing to the gene expression study.

AIM 1: Investigate immunoglobulin mutation status in canine CLL patients.

B-cell immunoglobulins play an important role in human CLL. One of the most important prognostic factors in human CLL is the mutation status of the immunoglobulin receptor on neoplastic B-cells. Mutation status refers to whether the immunoglobulin receptor has undergone somatic hypermutation, a process utilized by normal B-cells to increase diversity of immunoglobulins in the immune system. Mutated immunoglobulin receptors (that have undergone somatic hypermutation) in CLL patients are associated with an indolent disease course with patients often having a normal lifespan. Unmutated immunoglobulin receptors, having not undergone

somatic hypermutation, are associated with an aggressive clinical course with a median survival time of 3 years.

We investigated immunoglobulin mutation status in 55 canine CLL patients. CLL cases were defined by having lymphocytosis of >5,000 lymphocytes/µL in the peripheral blood and a homogeneous expansion (>60% of total lymphocytes) of small CD21+ lymphocytes by flow cytometry. Immunoglobulin mutation status is determined by sequencing the immunoglobulin heavy chain variable region gene in a patient's tumor cells and then comparing that sequence to the reference germline sequence. If there is a high degree of similarity between the tumor sequence and reference sequence, there has been little somatic hypermutation and the case is classified as unmutated. A low degree of similarity indicates somatic hypermutation has occurred and constitutes a mutated case. The majority (75%) of canine cases have mutated immunoglobulin receptors, predicted to cause an indolent clinical course. However, we have made the striking observation that Boxer dogs have a significant skewing toward unmutated cases (79% of cases), predicted to correlate with aggressive disease. This paper is currently under review at *PLOS ONE*.

AIM 2: Investigate clinical outcome in a large cohort of CLL patients, examining the clinical heterogeneity within canine CLL and identifying prognostic markers.

We aim to obtain 200 medical records for this study and are focusing on three major breed groups: Boxers, English Bulldogs and small breeds. Boxers are of interest because of their skewed use of unmutated immunoglobulin genes, predicted to cause aggressive disease. English Bulldogs are of interest because we previously found that they present at a younger age (6 yrs vs 11 yrs) and have a unique immunophenotype by flow cytometry (lower class II MHC and CD25 expression). Small breed dogs were previously identified as having increased odds of CLL. So far, we have examined records for 14 Boxers, 7 English Bulldogs, and 42 small breed dogs.

Preliminary results indicate that Boxers and small breed dogs present at an older age (median age 9.4 years and 10.5 years, respectively), while English Bulldogs are significantly younger (median 6.2 years). There is no sex predilection among small breed dogs, but 6/7 English Bulldogs are male and 10/14 Boxers are female. Across the cohort, anemia is not uncommon (present in 47% of cases), thrombocytopenia is less common (26% of cases) and neutropenia is rare (1.4% of cases). There is a wide range in presenting lymphocyte count, with Boxers having significantly higher lymphocyte counts compared to both small breed dogs and English Bulldogs. Peripheral lymphadenopathy is common, particularly in Boxers (affecting 85% of cases) compared to small breed dogs (62% of cases) and English Bulldogs (33% of cases). English Bulldogs are significantly more likely to have splenic involvement (affecting all cases examined) as indicated by splenomegaly, ultrasonographic changes, cytology and/or histology compared to the other breed groups. In addition to lymph node and splenic involvement, rare cases also had rectal, pulmonary, central nervous system or cutaneous involvement. CLL involvement in these more unusual sites has been underappreciated with canine CLL, though all four systems are affected in human CLL.

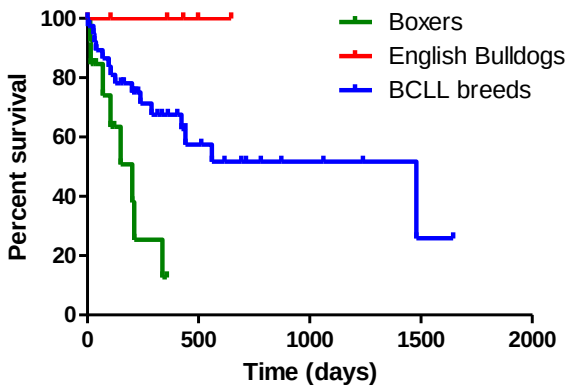

Figure 1. Survival (in days) from time of diagnosis. Boxers (green) have significantly shorter survival (MST 202 days), compared to small breed dogs (blue; MST 1478 days) and English Bulldogs (red; MST not reached).

We have found the majority of small breed CLL patients have a prolonged survival (median survival time (MST)=1478 days;

n=42 dogs), often with a normal lifespan, and frequently do not require chemotherapy, as seen in mutated human CLL (Figure 1). English Bulldogs appear to have prolonged survival (MST not reached), although only 7 records have been examined so far. The Boxers have much more aggressive disease, with a significantly shorter median survival time of 202 days (n=14 dogs). Given that Boxers almost exclusively have unmutated immunoglobulin receptors, this data suggests that like in human CLL, dogs with unmutated immunoglobulin receptors have aggressive disease. A future step is to correlate the mutation status of additional dogs with outcome data, to investigate the relationship of mutation status and survival outside of the Boxer breed. Additionally, we will examine the correlation between other clinical and immunophenotypic features with outcome, including age, sex, anemia, splenic and lymph node involvement, presenting lymphocyte count, class II MHC expression and CD25 expression.

AIM 3: Investigate the gene expression profile of canine CLL and examine differences in gene expression between Boxers, English Bulldogs and small breed dogs.

We performed a gene expression study to determine if canine and human CLL share expression profiles, and to compare gene expression between breeds. We measured expression of 261 genes that define CLL in the human literature using Nanostring technology, across three major disease groups: CLL, diffuse large B-cell lymphoma (DLBCL), and normal B-cells. RNA was isolated from CD21+ B-cells from 36 CLL peripheral blood samples, 20 DLBCL lymph node aspirate samples, and 8 normal lymph nodes. The CLL subset included three breed groups: Boxers (n=11), English Bulldogs (n=10), and small breed dogs (n=15). CLL samples clustered together by principal component analysis, separate from DLBCL samples and normal B-cells (Figure 2). Within the CLL group, English Bulldog and Boxer samples tended to group separate from one another. CLL small breed samples were distributed amongst the English Bulldog and Boxers samples, suggesting a spectrum of gene expression within this group.

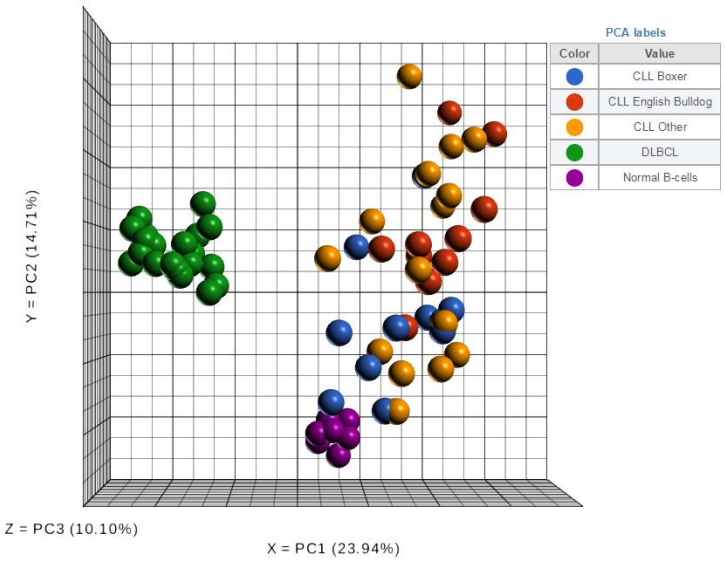

Figure 2. Principal component analysis plot of gene expression data of CLL samples, DLBCL samples and normal B-cells. Each dot is a sample. The axes show the first three principal components, with the fraction of explained variance (%). CLL samples form a cluster separate from DLBCL and normal B-cells. Within CLL, Boxers and English Bulldogs tend to cluster apart from one another. (Partek flow software)

Of the 261 genes examined, 137 genes were differentially expressed between canine CLL and normal B-cells, and 171 genes were differentially expressed between CLL and DLBCL. The canine CLL gene signature was significantly enriched for the human CLL genes using Gene Set Enrichment Analysis (GSEA). Of the genes differentially expressed between canine CLL and normal B-cells, 82% were differentially expressed in the direction predicted by the human literature. This gene probe set also identified differences among Boxers and English Bulldogs with CLL, with 70 genes differentially expressed between the two breed groups. Boxers had significant enrichment in pathways involved in mitosis and proliferation compared to English Bulldogs, with the following pathways most significantly enriched in Boxers using GO enrichment analysis (p-

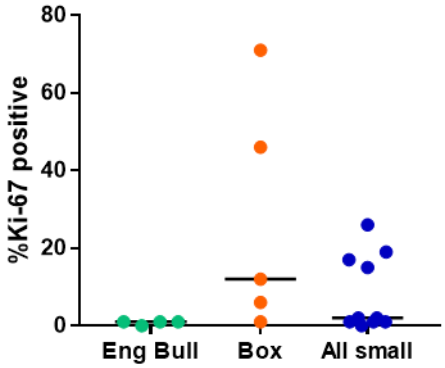

Figure 3. Ki-67 expression in CLL peripheral blood B-cells by flow cytometry. Boxers have increased Ki-67 expression compared to English Bulldogs. The majority of small breed cases have low Ki-67 expression, with a few cases having increased expression.

value < 0.01): (1) positive regulation of mitotic cell cycle, (2) regulation of nuclear division, (3) cytoplasm, (4) regulation of mitotic cell cycle, (5) regulation of chromosome segregation, (6) regulation of mitotic nuclear division. These findings suggested Boxer CLL cells are more proliferative compared to English Bulldogs, which may contribute to the poor outcome in Boxer CLL compared to English Bulldogs. To investigate this hypothesis functionally, we measured Ki-67 as a marker of cell proliferation in CLL cases by flow cytometry (Figure 3). In cases examined so far, Boxers had increased Ki-67 expression in B-cells in the peripheral blood compared to English Bulldogs. Small breed CLL cases predominantly expressed low levels of Ki-67, like the Bulldogs, but few cases had increased expression. These preliminary results suggest that Boxers and a subset of small breed dogs with CLL may have more proliferative tumor cells. Given the poor outcome in Boxers with CLL, these results suggest that Ki-67 may be a useful prognostic marker; however, more cases need to be examined for direct correlation between Ki-67 expression and survival.

FELLOWSHIP OUTCOMES

While supported by the AKC Canine Health Foundation Clinician-Scientist Fellowship, I have completed the aim investigating immunoglobulin mutation status in canine CLL and a manuscript is in the final stages of review at *PLOS ONE*. The CLL outcome study is underway and approximately half of the targeted medical records have been reviewed. This study will be the largest report on canine CLL outcome in the literature, and we hope it will help to improve the diagnosis and management of dogs with this tumor. The gene expression study has laid the foundation to further investigate differences amongst breeds with CLL. We will correlate these differences with outcome to improve prognostic information and evaluate these differences functionally to better understand the molecular mechanisms driving CLL and its clinical heterogeneity. The work described here has been presented at annual meetings for the American College of Veterinary Pathologists, the American College of Veterinary Internal Medicine, and the Veterinary Cancer Society.

I completed my clinical pathology residency and am a full-time PhD student under the mentorship of Dr. Anne Avery. Data collected during this Fellowship is contributing to my dissertation work and provided a significant amount of preliminary data for additional grant proposals. I received an NIH T32 Institution National Research Service Training Grant and a grant from the Morris Animal Foundation for research investigating canine CLL. We are currently writing a proposal for an NIH/NCI - Predoctoral to Postdoctoral Fellow Transition Award (RFA-CA-18-001). As a clinical pathology resident and PhD student, my interests are focused on hematopoietic neoplasms and I have published several manuscripts and a book chapter on canine hematopoietic tumors. After completing my dissertation, my goal is to attain an academic appointment improving diagnosis of hematopoietic tumors through my experience in clinical pathology and flow cytometric immunophenotyping, and studying canine lymphoid neoplasms as a primary investigator in cancer research.