Evaluation of Autoimmune Disease as a Risk Factor for Lymphoma

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A Thesis

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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JULY 2013

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EVALUATION OF AUTOIMMUNE DISEASE AS A RISK FACTOR FOR LYMPHOMA

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A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

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ACKNOWLEDGEMENTS

As a parent, the thing I long most to hear my own child say is, “Mom, I hope I’m like you one day.” As a mentee looking up to her mentors I can honestly tell you that I hope I can conduct myself with the same grace, softness, and intelligence that the three of my mentors carry themselves with.

To Dr. Jeffrey Bryan: Your friendship has been priceless to me. You have been an excellent mentor and friend to me. You are intelligent, motivated, and an excellent scientist. In so many ways, you gave me the tools to succeed. Your sense of humor will be missed, but your lessons will always be with me.

To Carolyn Henry: You gave me the shot at all of this. You believed in a naggy little wanna-be 4 years ago. It is only because of your desire to give me a shot at this residency thing, that I am here at all. You are a powerful woman and a figure that any aspiring leader can look up to. Thank you for your help with this master’s project and so much more. I will miss you greatly.

To Carol Reinero: You are not an oncologist and therefore had no obligation to me as a mentor. However, you have been an inspiration to me for a long time now. Watching you assess a problem or a challenge is what it means to be in the presence of a great mind. You have a good sense of life balance and you have been a really great friend and mentor. Thank you for your time with this project. Your insight is invaluable.

I would like to acknowledge my resident-mates, Chamisa Herrera,Lindsay Donnelly, Brian Flesner, and Katie Fitzpatrick for their support and friendship.

And I would like to acknowledge my faculty mentors in the VMTH Oncology Service for their continued leadership and guidance over the past 4 years.

I can not say enough thank you to my husband and daughter. Jason, thank you for taking care of me, and everything else, so that I could get this done. Marley, thank you for giving me up for this project you are always the light at the end of any dark tunnel.

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CHAPTER 1:
INTRODUCTION

The role of the immune system in the prevention and instigation of cancer is one of the most complex and oldest relationships in medical history. Thucydides first made reference to the immune system in fifth century BC in his mention of immunity of a “plague.” (Abbas 2003). Advancements in immunology after that time eventually led to an understanding of immunology via the mechanism of vaccination through Jenner’s small pox vaccine. Then in 1911, Peyton Rous discovered that a virus could cause sarcoma formation in the breast of chickens, noting one of the first associations between infectious disease and cancer formation.

The immune system is constantly sampling the environment and deciding whether or not to mount a response intended to protect the host from perceived danger. This concept is known as immune-surveillance. The notion that the immune system has a role in tumor prevention—via surveillance of antigens, both exogenous and endogenous—has grown (Vesely, Kershaw et al. 2011).

The immune system relies on a precarious balance between recognition of abnormal cells, protein signatures, and exogenous invaders (threats) and tolerance of cells belonging to the organism (non-threatening “self”) (Botti, Seregni et al. 1998). An immune system which interprets self as foreign may be primed for an inappropriate response resulting in autoimmunity. An immune

system which is overly tolerant of altered self or non-self antigens may allow tumor formation or be predisposed to infection.

The purpose of this communication is to discuss the role of the immune system in autoimmune disease as well as cancer. Evaluating the mechanisms that cause each of these events may give us clues to the link between immune dysfunction, cancer, and autoimmune diseases.

THE IMMUNE SYSTEM- WHAT IS THE NORMAL ROLE?

The immune system is made up of two general arms: the innate and the adaptive immune systems. These facets of the immune system are responsible for two complimentary and interposed aspects of reaction to antigen, both exogenous and endogenous. The innate immune system is responsible for recognition and initial response to foreign antigen. These defenses are present in the body long before the antigen ever appears to the immune system. Cells that do the work of the innate immune system are phagocytic cells like neutrophils and macrophages, natural killer cells, eosinophils, basophils and mast cells. Cytokines produced from these cells such as tumor necrosis factor (TNF); interleukin (IL)-1, IL-6, IL-10, IL-12; transforming growth factor (TGF) beta, and interferons are able to trigger the adaptive arm of the immune system. Innate antigen presenting cells consist of both macrophages and dendritic cells, and on occasion the adaptive immune system's B lymphocytes which, in addition to potentiating the innate inflammatory response, can process and present

antigen to activate T-lymphocytes. The macrophages and dendritic cells are able to signal the adaptive arm of the immune system. The innate immune system is primed for general immunity without being able to change or adapt to pathogens over the course of the lifespan of the organism; thus, adaptive immunity relies on recognition of evolutionarily conserved molecular markers on infectious organisms called pathogen-associated molecular patterns (PAMPs). Cells within the innate immune system are primed to recognize these molecular markers through toll-like receptors. These receptors recognize specific molecules of microbial origin or molecules that are induced by cell damage and will trigger cell lineage-specific responses. Macrophages play a large role both in the innate recognition of foreign invaders, and also direct effector cells towards the production of an adaptive immune response. Macrophages secrete cytokines such as TNFα, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and IFNγ, which contribute to the repair of damaged tissues, and phagocytize antigen. Macrophages play a role in immunosurveillance of cancer by processing and presenting tumor cell antigens to lymphocytes and converting the immune response into a long-lasting adaptive response (Guiducci, Vicari et al. 2005). Natural killer cells are lymphocytic cells that are activated immediately after antigen notification via IL-12,-15, and IL-18. Natural killer cells have a role in tumor antigen recognition and destruction (Whiteside and Herberman 1995). This mechanism is often by the recognition of a lack of major histocompatibility complex (MHC) class I. MHC class 1 is a receptor that is expressed on almost all nucleated cells in the body

to help the immune system identify self. MHCI is a receptor which promotes recognition of T cells so that perpetuation of immune responses can occur. MHC I is frequently not expressed by tumor cells (Tizard 2004). Another mechanism that triggers NK cell cytotoxicity is the recognition of MHC class chain-related A and B(MICA and MICB). This protein is expressed by cells undergoing stress. They are upregulated in tumor cells and virus infected cells (Tizard 2004). Natural killer cells then release interferon gamma which activate macrophages that process and present antigens to the adaptive immune system. Other cells that are part of the innate immune system, like neutrophils, contribute to immunosurveillance via generalized inflammation. In an effort to provide protection, the innate immune system may play a role in tumor formation by stimulating angiogenesis, inflammation, and free radicals(de Visser and Coussens 2006).

Adaptive immunity is the second arm of the immune system. This response takes weeks to even months to form and provides specific immunity to different pathogens. The adaptive immune system effectors are comprised of B and T lymphocytes. Adaptive immune responses are triggered by signals from the innate immune system. It had long been thought that the adaptive immune system played a role in cancer protection only via the cytotoxic T cell component and cytokine-mediated lysis of tumor cells. However, the understanding of the role of the adaptive immune system in cancer has evolved, and we now know that a variety of tumor cell antigens may be recognized by B and T lymphocytes.

Studies have shown that mice deficient in B and T lymphocytes as well as NK cells are more likely to develop spontaneous neoplasia while aging(Shankaran, Ikeda et al. 2001). Lymphocyte-mediated cytotoxicity can protect against cancers like lymphoma(Smyth, Thia et al. 2000). Not only does the adaptive immune system protect against tumor formation, but this tumor suppressor function seems to be critical to the secretion of IFNγ(Shankaran, Ikeda et al. 2001). Lymphocytes can also shape the biologic behavior of a tumor, a process called "immunoediting." This process occurs when the immune system kills tumor cells that are susceptible to it, leaving the resistant, more aggressive cells behind to grow. This is evidenced by the fact that tumors that grow in the presence of an intact immune system tend to be less immunogenic than tumors grown in mice without competent immune systems (Shankaran, Ikeda et al. 2001). Tumors can down-regulate molecules like TAP1 and H2-K heavy chain to decrease immunogenicity to the immune system(Shankaran, Ikeda et al. 2001). Again, the adaptive immune response plays a role in tumor suppression. However, it can also paradoxically potentiate cancer via the differentiation of T regulatory cells, which suppress the immune response (de Visser and Coussens 2006; de Visser, Eichten et al. 2006).

The role of the immune system in cancer prevention "immunosurveillance" and the role of cancer in the alteration of cancer, "immunoediting," are well-studied entities. Decreased immune surveillance leads to an immune system which is likely to allow proliferation of neoplasia.

THE IMMUNE SYSTEM-WHAT IS ITS ROLE IN NEOPLASIA?

It is impossible to tease out the specific role that the immune system plays in cancer and it is likely to vary depending on the type of cancer, as well as other conditions (genetics, concurrent disease, etc). Does cancer arise because a dysfunction in the immune system is present, or does the immune system become dysfunctional as a result of the cancer? There is evidence for each of these scenarios. We do know that a normal immune system has a specific role in the fight against cancer. The immune system has the potential to fight against cancer in three specific ways. First, the immune system may be able to prevent viral infection which may transform cells through insertional mutagenesis, epigenetic modification, and upregulation of pathways and proteins needed for cell survival. Second, the immune system is responsible for prompt resolution of inflammation, without which a pro- inflammatory environments could favor tumorigenesis. There is a distinct association between chronic inflammatory conditions, such as inflammatory bowel disease or thyroiditis, and the development of cancer (Benjamin, Stephens et al. 1996). Chronic inflammation may cause cancer by induction of DNA damage through production of free radicals, signaling a cell not to proceed with apoptosis (through nuclear factor – KB), upregulation of COX-2, and suppression of anti-tumor adaptive immune responses (de Visser and Coussens 2006). Furthermore, chronic inflammation may cause neoplastic transformation through DNA methylation and histone

deacetylation (Niwa, Tsukamoto et al. 2010) . Chronic inflammation causes these cells to be bathed in cytokines that are produced during inflammation, allowing things like acetylation and cancer formation to occur (Rizzo, Pallone et al. 2011).

Lastly, the immune system may recognize specific tumor antigen signatures produced by many tumor types (Vesely, Kershaw et al. 2011). This last role of the immune system can be challenging because most of the proteins that arise from tumors are derived from self. However, the immune system is already prepared to search for one of four specific tumor signatures.

•Embryonic proteins: Embryonic proteins are normally only expressed and produced during fetal life. However, de-differentiated neoplastic cells produce these antigenic proteins during adult life. The immune system is designed to have a targeted response towards these cells. Notable examples include production of α-fetoprotein by hepatoma cells, carcinoembryonic antigen (CD66e) by gastrointestinal tumors (Lin, Chen et al. 2011; Thomas, Forse et al. 2011) and embryonic proteins by canine mammary carcinoma cells (Ferletta, Grawe et al. 2011; Weichselbaumer, Willmann et al. 2011).

•Excessive protein production: Excessive protein production is defined as over-expression of a particular protein that has the appropriate structure and conformation. The most notable example is increased expression of prostate specific antigen (PSA) by human prostatic carcinoma cells (Walsh 2012). This protein serves as an antigen that triggers an immune response. Canine

mammary cancer lines have similarly been known to produce excess progestin-induced growth hormones. This autocrine signaling serves to locally allow for disease progression in the microenvironment (Mol, Lantinga-van Leeuwen et al. 1999).

•Mutated proteins: Mutated proteins are similar to “normal” proteins produced by healthy cells, but have altered structure or charge. A notable example includes production of mutated signaling proteins from BRAF mutations in human melanomas (Pritchard and Hayward 2013). Canine acute lymphocytic leukemias express mutations in FLT3, a tyrosine kinase receptor, similarly to people (Suter, Small et al. 2011).

•Novel proteins: Novel proteins arise when neoplastic cells create new amino acid sequences that lead to the production of a novel protein. This is typically in response to a virally-initiated neoplastic change. Notable examples include production of oncornavirus cell membrane antigens on neoplastic lymphocytes in cats with feline leukemia virus, tumor specific antigens found in Marek’s disease in chickens, and novel fusion proteins seen in chronic myelogenous leukemia in both people and dogs (Burgess, Young et al. 2004; Beatty, Tasker et al. 2011; Avery 2012). These novel amino acid sequences can become an antigenic trigger for the immune system.

These protein signatures, along with the ability of natural killer cells to recognize cancer via decreased expression of MHC class I, make up the majority of the immune system’s natural protection against cancer. Altering the make-up

of the immune system means that it may not be able to recognize cancer and attack it before it can proliferate or metastasize.

IMMUNE-MEDIATED DISEASE

While cancer can result from immunodysfunction, or even cause immunodysfunction, there are other diseases that result when the immune system is altered. Abbas defines disorders of the immune response as hypersensitivity disorders (Abbas 2003). An individual exposed to an antigen, in the context of other co-stimulatory signals such as CD28, CD80 or CD86, is then sensitized to further encounters with this antigen. This is an advantageous response when the antigen is a pathogen and the body must mount a rapid immune response; however, the sensitization process can occur to benign particles or even to self molecules. One of the common causes of this sensitivity is a lack of self tolerance, or an over-exuberant immune system (Abbas 2003). This will result in a number of autoimmune disorders. The main player in many autoimmune diseases is the T lymphocyte, due to the fact that T lymphocytes are critical for maintaining self tolerance (Steinman 2013). There are 4 types of hypersensitivity reactions that are defined by both the players in the pathologic process and the mechanism of disease.

•Type 1 hypersensitivity is mediated by a T helper 2 driven process eliciting allergen-specific IgE antibodies. T helper 2 cells produce a variety of cytokines such as TNF, IL-1, IL-4, IL-5, IL-6, IL-13, MIP-1a, MIP-1b, and GM-CSF

(granulocyte macrophage colony stimulation factor) which promote maturation and enhance survival of eosinophils (an important effector cell in this process) and stimulate B cells to undergo antibody class switching and ultimately terminally differentiate into plasma cells to produce IgE. In turn, IgE binds to Fc receptors on mast cells and triggers degranulation and elaboration of lipid mediators and other cytokines to perpetuate the inflammatory response. It is the most common type of hypersensitivity and results in diseases such as asthma, atopic dermatitis, and anaphylaxis.

•Type II hypersensitivity is mediated by IgM and IgG antibodies which are directed against cell surfaces or cell matrix antigens. Specifically, subclasses of IgG have been investigated and it is likely that IgG1 and IgG4 are the culprits capable of agglutinating RBCs from dogs with IMHA (Day 1999). The mechanism of reactivity is via autoreactive T lymphocytes. In clinically normal individuals, there exists a population of autoreactive T lymphocytes that is down regulated to prevent disease (Barker and Elson 1993). Patients who acquire autoimmune hemolytic anemia lose that tolerance. Opsonization and phagocytosis are the mechanisms for this disease process. This process leads to complement and Fc receptor-mediated recruitment with activation of leukocytes. Diseases like autoimmune hemolytic anemia, immune-mediated thrombocytopenia (thrombocytic purpura), and pemphigus diseases result from antibody activated mechanisms.

•Type III hypersensitivity is mediated by the complexing of IgM and IgG antibodies. These complexes may be composed of either self antigens or foreign antigens with bound antibodies. Complement and Fc receptor mediated recruitment of leukocytes is the mechanism for these diseases. The pathology of the disease is dependent on the site of immune complex deposition. However, the effects of this disease are systemic. As antigen-antibody complexes gather, complement becomes activated, which further recruits inflammatory cells. The process of antigen-antibody complexing is normal; however, this immune response occurs when the complexes are in excess. Diseases like autoimmune glomerulonephritis, systemic lupus erythematosis, and serum sickness are examples of diseases that result from this pathologic process.

•Type IV hypersensitivity is mediated by T lymphocytes; CD4+ T cells (T helper cells) of the Th1 subset, and CD8+ T cells (cytotoxic T cells). The mechanism of pathology for this disease is macrophage activation and induction of cytokine-mediated inflammation via the CD4+ cells or direct target killing via the CD8+ cells in a sustained fashion without a normal means to shut off the immune response. Autoantigens are frequent targets. Th1 lymphocytes secrete IFNγ in order to activate macrophages and induce inflammation. CD4+ cells directly mediate macrophage destruction of cells while CD8+ lymphocytes can directly cause tissue destruction. Diseases such as delayed contact sensitivities, inflammatory bowel disease, and insulin dependent diabetes mellitus result from this pathologic process.

Both immune- mediated hemolytic anemia and immune-mediated thrombocytopenia have two things in common: 1) self tolerance has failed and 2) self-reactive lymphocytes are activated. Lymphocytes develop in the thymus via variations in the variable, diversity and joining (V, D and J) chains so that they may develop a repertoire to recognize and respond to foreign antigen while recognizing and tolerating self antigen. The thymus is responsible for educating these cells and rejecting cells that recognize self MHC molecule too strongly or too weakly. The result of these cells is apoptotic death. Failure of self tolerance may occur at several points in this process. Failure to delete autoreactive lymphocytes or abnormal activation of autoreactive lymphocytes may result in autoimmune disease. The disease process may also take place after lymphocytes are targeted for destruction, but fail to, due to an inability to undergo apoptosis (Vossenkamper, Lutalo et al. 2012; Gatto, Iaccarino et al. 2013). Another place that immunodysfunction may occur is in the periphery. Somatic mutations in the periphery may result in an autoreactive population of cells. Such is the case with B cells in systemic lupus erythematosis (Dorner, Giesecke et al. 2011).

Immune-mediated hemolytic anemia is the most common autoimmune disease in the dog (Swann and Skelly 2013). Immune-mediated thrombocytopenia is another immune-mediated disease with severe morbidity and mortality (Grindem, Breitschwerdt et al. 1991). Both of these diseases occur via type II hypersensitivity reactions. In both of these diseases, antibodies are

directed against the antigens on red blood cells or platelets. Antibodies opsonize cells or activate the complement system, thereby destroying red blood cells or platelets. These cells are engulfed by phagocytes (neutrophils and monocytes) which express receptors for the Fc portions of the antibodies (Abbas 2003). The specific pathophysiology of these diseases encompasses a broad understanding of what is targeting these cells for destruction, as well as what is being targeted. Details for these mechanisms will be elucidated later in this communication. The connection between lymphoma and immune-mediated diseases has been documented before in people, as well as dogs (Keller 1992). Addressing these two diseases as possibly two sides to the same coin is logical because both diseases involve pathology of the immune system, more specifically the lymphocyte.

PATHOPHYSIOLOGY OF IMMUNE-MEDIATED HEMOLYTIC ANEMIA (IMHA)

Immune-mediated hemolytic anemia is the most common autoimmune disease of dogs, as well as one of the most commonly fatal diseases in veterinary medicine (McCullough 2003). Destruction of red blood cells is mediated by immunoglobulin, either IgG or IgM, presumptively coating self-antigen on red blood cells or a secondary effect from an immune response mounted against an infectious or neoplastic agent. The majority of canine cases involve the binding of IgG to red blood cells (Slappendel 1979). This attachment of immunoglobulin then activates the complement cascade or induces

phagocytosis by macrophages with Fc receptors for the immunoglobulin (McCullough 2003). Immune-mediated hemolytic anemia is a type II hypersensitivity reaction. IgG is a monomeric antibody and, therefore, contains only two binding sites. This feature renders it almost useless as a direct agglutinator unless a large amount of antibody is present (McCullough 2003). Instead, IgG-coated antibodies are phagocytized by macrophages via the Fc portion of the antibody binding Fc receptors on macrophages. This process of phagocytosis happens in large part in the spleen and only later in the course of the disease in the liver (Feldman, Handagama et al. 1985; Warren and Collins 1988). The pathogenesis of this disease is often multifactorial; however, one proposed mechanism is that the erythrocyte life cycle normally involves production of antibodies against RBC cytoskeletal components such as glycoprorins, band 3, and spectrin. This normal function is performed to remove senescent red blood cells from the body (Kay, Lake et al. 1995; Day 1999). Erythrocyte autoimmunity is closely monitored in normal animals (Day 1999). Normal individuals have autoreactive T lymphocytes that do react to self MHC molecules. These cells are controlled in the periphery by many mechanisms. One mechanism involves which RBC antigens the macrophages select to present to the T lymphoctyes and the phenotype of those macrophages (i.e., they are required to be activated). Loss of tolerance may occur as macrophages present a more antigenic peptide to the T lymphocyte, thereby triggering T lymphocyte proliferation and antibody production (Shen, Youssef et al. 2003).

Other causes of loss of tolerance include a relatively low number of self-reactive T cells that escape the thymus, as well as the fact that not all self antigens are presented in the thymus. Once the red cell is bound to antibody it has one of two fates: either activate the complement system and become lysed by the last step in the complement cascade(the membrane attack complex), or become phagocytized by macrophages that contain the appropriate Fc receptor. The complement system is activated by C1, a serine protease released by the liver (McCullough 2003).

As mentioned earlier, IgM may also initiate this process. IgM is a pentameric immunoglobulin. Its size keeps it from leaving the intravascular space (Tizard 2004). IgM, due to its multiple exposed Fc regions, has a strong ability to initiate the complement cascade. After initiation, IgM can leave the affected red cell and attach onto another one (McCullough 2003). The results of this pathology lead to high morbidity and often high mortality (Klag, Giger et al. 1993).

PATHOPHYSIOLOGY OF IMMUNE-MEDIATED THROMBOCYTOPENIA

Immune-mediated thrombocytopenia is an important disease in the canine patient (Botsch, Kuchenhoff et al. 2009). The pathophysiology is similar to IMHA in that antibodies are directed against antigens on the outside of the platelet membrane. This type II sensitivity is mostly directed against platelet glycoproteins. The major membrane proteins on platelets that result in antibody targets belong to the integrin and the glycoprotein families (Kunicki and Newman

1992). Evidence exists that these are common targets for autoimmune antibody production in dogs (Lewis and Meyers 1996). These proteins are normally responsible for platelet aggregation by binding to adhesion molecules on the endothelial surface. GPIIbIIIa binds von Willebrand's factor and is thought to be the primary mediator of platelet-vessel wall interactions. It is also possible for these antibodies to bind to platelet surface molecules and cause platelet dysfunction, rather than a decrease in absolute numbers (Thomas, Metcalfe et al. 2000).

Immune system dysfunction (loss of tolerance to self-antigen) seems to be at the root of both IMHA as well as ITP. Similarly, neoplastic conditions arise directly or indirectly from immune system dysfunction (poor ability to destroy non-self or altered self). It may be possible that immune system dysfunction at the genetic, epigenetic, or even transcriptional level may have a systemic effect not only on autoimmune diseases, but also on neoplastic processes..

ATOPIC DERMATITIS

Another disease which results from an over-exuberant immune system is canine atopic dermatitis. Atopic dermatitis results when dogs launch an antigen-specific immune response after being sensitized via inhalation or percutaneous exposure to allergens (Marsella, Sousa et al. 2012). This triggers the response of allergen-specific IgE antibodies. Subsequent exposure to these allergens causes degranulation of mast cells and basal cells and the clinical signs result from the granules and cytokines contained within these cells (Halliwell 1973).

While this disease results from an over-zealous immune system similar to IMHA and ITP, this disease process is a type I sensitivity reaction. Like the other disease processes, T lymphocytes play a critical role in this disease. Th2 cells predominate in the acute phases of atopic dermatitis, while Th1 cells predominate in the later stages. The mix of Th1 cells is likely due to a combination of secondary infection and self trauma (Marsella, Sousa et al. 2012). Cytokine profiles in atopic dogs are altered such that interleukin 4 is overexpressed in atopic dogs. This interleukin modulates IgE production as well as Th 2 cell differentiation. These patients also exhibit a lack of TGFβ, which is an immunosuppressant (Marsella, Sousa et al. 2012). This disease process is yet another example of dysfunction in the immune system, but it results from pathology in a different part of the immune system and results in disease that is not primarily hematopoietic. Potentially, the role of chronic inflammation can be linked to cancer in some ways. Chronic thyroid inflammation has been linked with thyroid cancer (Benjamin, Stephens et al. 1996). Chronic inflammation has also been linked with shorter disease free survival times in dogs with lymphoma (Baskin, Couto et al. 2000) as well as with cat with injection site sarcomas (Hershey, Sorenmo et al. 2000; Allenspach 2011). However, the link between atopic dermatitis and cancer in dogs has never been noted.

THE LINK BETWEEN CANCER AND AUTOIMMUNE DISEASE

Several autoimmune diseases have been linked to development of cancer in people(Martin, Mikhail et al. 2009). Genetic mutations in cell regulation checkpoints that result in cancer have also been noted in many autoimmune diseases (Goldin and Landgren 2009). These genetic defects may help promote the replication of cells with damaged DNA. There is a direct relationship between autoimmune diseases and lymphoma in people (Hyams, Fitzgerald et al. 1993; Hansen, Lipsky et al. 2007; Goldin and Landgren 2009; Martin, Mikhail et al. 2009). The incidence of chronic myelogenous leukemia, Hodgkin lymphoma, and lymphoplasmacytic lymphoma is greater in people who have been diagnosed with rheumatoid arthritis, Sjogren syndrome, or ulcerative colitis (Baecklund, Iliadou et al. 2006; Landgren, Engels et al. 2006; Goldin and Landgren 2009). Lymphoma has been linked with immune-mediated thrombocytopenia in dogs (Keller 1992). It is possible that the causes of these autoimmune diseases may somehow be linked to cancer. However, it is also possible that the treatments for autoimmune disease result in cancer (Goldin and Landgren 2009). People who have received organ transplants are at an increased risk of developing lymphoma and have an overall three to five time increased risk of developing cancer (Landgren, Engels et al. 2006). Cats receiving renal transplants who receive anti-rejection immunosuppressants are six times more likely to get cancer then immunocompetent cats (Schmiedt, Grimes et al. 2009). Some explanations for the links between these two diseases are the role of chronic

inflammation, infectious causes, epigenetic alterations, alterations in cell responsive signals to apoptosis.

Other potential explanations exist that may support a hypothesis that autoimmune diseases and neoplasia are linked in prevalence. Viruses replicate by invading host cells and then commandeering DNA machinery to make progeny. In the process of commandeering a cell, virus particles may inadvertently transform an oncogene within a cell, giving it limitless potential to divide and resulting in cancer. Notable examples in medicine include the Epstein-Barr virus in Burkitts lymphoma, Hodgkins lymphoma, and nasopharyngeal malignancies; the Rous sarcoma virus in chickens; and the Feline Leukemia virus in cats. It is also possible that viruses may rearrange DNA such that a cell is imparted with other qualities including an ability to evade apoptosis or even to activate itself, in the case of a lymphocyte. It is hypothesized that herpes virus may be the cause of autoimmune diseases like pemphigus vulgaris (Senger and Sinha 2012). Epstein-Barr virus is a known tumor initiator and it has been implicated in rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosis (Lossius, Johansen et al. 2012). It is possible that viruses that cause autoimmune disease can recrudesce at later stages to cause cancer.

Epigenetics is a burgeoning new area of cancer research. Epigenetic changes are alterations in gene expression that occur without directly changing the genome. Genes are often expressed, or not expressed, depending on the level of methylation at certain points on the DNA. Epigenetic data are able to

provide us an abundance of information including diagnosis and prognosis of diseases, and help predict various drug outcomes (Gruver, Hudson et al. 2007). There are many known epigenetic alterations that result in cancer, for example; prostate tumors arise from alterations at the glutathione S transferase-1 gene (GST1) (Lee, Morton et al. 1994) and prostate, breast and colon cancer result from epigenetic alterations at RARB gene. Autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosis also have known gene loci where epigenetic alterations lead to autoimmune disease development. It is logical to consider that an epigenetic change may alter similar pathways to affect the same animal—yielding both cancer and autoimmune disease.

We have discussed alterations at the genome and at the effector cell. However, between DNA and the effector cell are innumerable pathways that must speak to one another. Another possible theory in the convergence of autoimmune disease and cancer may be that a dysregulated post-translational pathway is responsible for both diseases. The peptidylarginine deaminases (PADs) are a family of post-translational modification proteins that convert the positively charged arginine and methylarginine to citrulline. This process is called citrullination, and it is extremely important in the structure and function of many proteins and, therefore, in many cellular processes. Abnormalities in this pathway have been implicated in both autoimmune diseases and cancer (Mohanan, Cherrington et al. 2012). Citrullination has been implicated in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and psoriasis in people

(Chang, Yamada et al. 2005; Anzilotti, Pratesi et al. 2010). Citrullination and protein modification have been implicated in cancer in both people and animals (Chang and Han 2006). This is one of many protein alteration pathways that exist between the DNA and the protein product that performs cellular tasks.

Lastly, one must consider genetic inheritance as a risk for both of these diseases. Certain breeds such as the cocker spaniel are predisposed to immune-mediated disease, particularly immune-mediated thrombocytopenia (Jackson and Kruth 1985).Genes coding for MHC II or DLA (dog leukocyte antigen) and autoimmune disease have been evaluated for correlation. Nova Scotia Duck Tolling retrievers with homozygous risk haplotypes are at an increased risk for developing certain autoimmune diseases (Wilbe and Andersson 2012). Genetic mutations exist in certain breeds that confer increased risk for neoplastic conditions. Osteosarcoma is one cancer that results from genetic derangements (Selvarajah, Kirpensteijn et al. 2009). Genetic alterations may code for defects that can result in one or both diseases.

There is considerable overlap in disease process between cancer and autoimmune disease. Understanding the role that these diseases play in the lives of canine patients is the next step to understanding how these two processes affect one another.

CHAPTER 2:

AUTOIMMUNE DISEASE AS A RISK FACTOR FOR LYMPHOMA

The immune system is constantly sampling the environment and deciding whether or not to mount a response intended to protect the host from perceived danger. This concept is known as immune-surveillance (Burnet 1970). The immune system is responsible for protecting against inappropriate antigen, both endogenous and exogenous, making the immune system the first line of defense against neoplasia (Vesely, Kershaw et al. 2011). The immune system relies on a precarious balance between recognition of abnormal cells, protein signatures, exogenous invaders (threats) and tolerance of cells belonging to the organism (non-threatening "self")(Botti, Seregni et al. 1998). An immune system which interprets self as foreign may be primed for an inappropriate response resulting in autoimmunity. An immune system which is overly tolerant may allow for cancer formation.

A strong link between autoimmune disease and hematopoietic neoplasia has been identified in people and in dogs ((DeBoer and Madewell 1983; Madewell, Gieger et al. 2004; Martin, Mikhail et al. 2009). This relationship is discussed at length in chapter 1 of this thesis. The relationship between these

two diseases has been investigated briefly by Keller et al, 1992. In that paper, an association between dogs who presented with immune-mediated thrombocytopenia (ITP) and lymphoma was exposed. It is known that cancer can be the cause of immune-mediated disease (McCullough 2003); however, the link between autoimmune disease and neoplasia has not been fully elucidated. There is evidence that acquisition of these two diseases in the course of one individual's life span reflects an inherent flaw in the immune system. SCID mice are excellent models for tumor growth because a host with a defective immune system is more likely to contract and grow cancer. However, it is also possible that the treatments for autoimmune disease result in cancer(Goldin and Landgren 2009). Cats receiving renal transplants who receive anti-rejection immunosuppressants are six times more likely to get lymphoma than immunocompetent cats (Schmiedt, Grimes et al. 2009).

Our hypothesis was that canine patients recorded in the veterinary medical database (VMDB) as having autoimmune diseases (specifically IMHA and ITP) would be more likely to appear a second time in the VMDB with a diagnosis of lymphoma than dogs recorded as having atopic dermatitis. We further hypothesized that there would be no relationship between patients who were initially diagnosed with either atopic dermatitis— a common disease that can affect healthy dogs-- or autoimmune disease and the incidence of soft tissue sarcoma, a non-hematopoietic, non-immune system based, neoplasia, as a control for disease relationship in the VMDB.

M ATERIALS AND METHODS

Records were retrieved from the Veterinary Medical Database. This is a database that sources outputs from 26 US veterinary referral institutions from 1964-2013. Dogs were identified as being recorded in the VMDB with either allergic dermatitis (AD) or autoimmune disease (AID). The autoimmune disease category was comprised of dogs who either had a diagnosis of immune mediated hemolytic anemia (IMHA) or immune mediated thrombocytopenia (ITP). We queried the system for dogs who were subsequently recorded at least six months later for soft tissue sarcoma or lymphoma. Using a Perl script, case numbers from the AD/AID case list were compared to case numbers in the lymphoma and soft-tissue sarcoma case lists and matches identified. Dogs were considered affected if their diagnosis of lymphoma followed a diagnosis of AD/AID by at least six months.

Statistical analysis- Proportion of dogs presenting with AD or AID subsequently entering the VMDB with a diagnosis of lymphoma was compared using a Chi Square test. A similar comparison was made for dogs that presented for AD or AID and subsequently entered the VMDB with a diagnosis of any soft tissue sarcoma. $P<0.05$ was considered significant. Sex and breed were evaluated to elucidate any predilections using a proc univariate analysis and a logistic

regression model. Relative risk was calculated where appropriate using the online software module http://www.medcalc.org/calc/relative_risk.php.

RESULTS

The VMDB query resulted in 3,092 dogs who presented for lymphoma, 32,340 dogs who presented for allergic dermatitis, 4,783 dogs who presented for autoimmune diseases, and 2,739 dogs who presented for soft tissue sarcomas. Among the affected dogs there were 14,091 spayed female dogs, 8,111 intact female dogs, 7,916 male neutered dogs, and 12,829 male intact dogs. The top 18 breeds presenting with any one of the four conditions are represented in table 1.

A total of 30 dogs who presented for either allergic dermatitis or autoimmune disease re-presented at least 6 months later with a diagnosis of lymphoma. Only 4 out of the 30 presented for a diagnosis of autoimmune disease after their diagnosis of lymphoma. Dogs presenting for autoimmune disease were evaluated for their likelihood of presenting again with lymphoma—when compared to dogs presenting with atopic dermatitis—via a chi square analysis. When we separated the two diagnoses out by more than 6 months, as initially stated in our study design, we found that dogs who presented for autoimmune disease were not any more likely to present, 6 months later, with lymphoma when compared to dogs who presented with atopic dermatitis ($p=0.748$).

However, when we took the time lapse out of the equation and allowed for diagnoses to be made where dogs could concurrently be diagnosed with lymphoma and autoimmune disease, we found that dogs with autoimmune disease were significantly more likely to re-present with lymphoma than dogs with atopic dermatitis ($p=0.0112$).

A total of 65 dogs presenting to a VMDB-participating institution with atopic dermatitis or autoimmune disease subsequently developed soft tissue sarcoma. Seven dogs out of the 60 total presented initially for autoimmune disease. The rest of the dogs presented for atopic dermatitis. Dogs presenting for autoimmune disease were evaluated for their likelihood of presenting again with soft tissue sarcoma—when compared to dogs presenting with allergic dermatitis—via a chi square analysis. Dogs who presented for autoimmune disease were not any more likely to present later with a soft tissue sarcoma ($p=0.607$).

Breed predilections were evaluated using Proc Univariate in SAS to pick out outliers in the 90% quintile. Variables were created by dividing the number of patients of a certain breed that presented for the 4 different disease categories by the total number of dogs of that breed that presented to the VMDB. Observational outliers represent dogs of a certain breed who make up a larger proportion than what their total numbers should indicate. For lymphoma, the Boxer and the Scottish Terrier were overrepresented breeds with a 0.596% chance and 0.815% chance of having lymphoma, compared to other breeds, respectively. For allergic dermatitis, the Bulldog and the Scottish Terrier were

overrepresented with a 2.73% and a 4.02% chance of having allergic dermatitis respectively. For autoimmune disease the Rottweiler and the cocker spaniel were over-represented with a 0.432% chance and a 0.898% chance respectively.

A logistical regression model was applied to the data set to detect likelihoods of various breeds presenting for soft tissue sarcoma versus lymphoma. Beagles(b=-0.50; p=0.027), collies (b=-0.59; p=0.027), Dobermans (b=-1.64; p=0.000), German Shepherd dogs (b=-1.20; p=0.000), toy poodles b=-1.25; p=0.000), and Schnauzers (b=-1.15; p=0.000), Rottweilers (b= -0.75; p=0.000), Labrador retrievers (b=-1.62; p=0.000), Golden Retrievers (b= -1.19; p=0.000), English Springer Spaniels (b=-.892; 0.000), Shetland Sheepdogs (-1.08; p=0.000), and Dachshunds (b=-1.17; p=0.000) were more likely to get soft tissue sarcomas than lymphoma. The logistical regression model showed that Dobermans (b=0.87; p=0.000), miniature poodles (b=1.84; p=0.000), Schnauzers (b=0.68; p=0.000), Cocker Spaniels (b=1.31; p=0.000), and Rottweilers (b=1.83; p=0.000) were more likely to present with autoimmune disease, rather than allergic dermatitis.

Using a logistic regression model, it was found that spayed female dogs were relatively protected from lymphoma and had a higher likelihood of presenting for soft tissue sarcomas (b=-.139; p=0.050).

Dogs who presented for autoimmune disease were statistically more likely to die or be euthanized at their initial diagnosis (p=0.0001) (table 2).

DISCUSSION

Our study used a longer time frame, more retrospective cases and additional control populations to contradict what is known in veterinary medicine; that autoimmune disease is linked to lymphoma. To date, the veterinary literature has echoed the human literature by saying that autoimmune dysfunction is a risk factor for hematopoietic neoplasia. Keller et al. showed that dogs diagnosed with ITP were more likely to be also diagnosed with lymphoma. Similarly, cats receiving renal transplants who receive anti-rejection immunosuppressants are reported to be six times more likely to get lymphoma than immunocompetent cats (Schmiedt et al., 2009). Keller used the VMDB by selecting cases up to June of 1992. Our case selection included the same data but an additional 1,711 cases of lymphoma were added by including data up until 2004. Initial evaluation of our data from the VMDB yielded the same result. We found that dogs diagnosed with autoimmune disease were, indeed, more likely to be diagnosed with lymphoma. However, when we separated the diagnoses of autoimmune disease and lymphoma by 6 months, that significance disappeared. Why, then, were these findings so much different? We believe that by not allowing a time period to lapse between diagnoses that the Keller paper, and our initial findings, merely show that lymphoma may be a direct cause of immune-mediated disease. This may also potentially reflect that not enough dogs lived past their diagnosis to be later diagnosed with lymphoma. This does not necessarily show that these two separate disease entities are part of the same inherent dysfunction of the immune system. The study design only allowed dogs who first presented for an

autoimmune disease or an atopic dermatitis and then re-presented for lymphoma 6 months or more later to be included in the study. This setup allowed us to discern between two separate disease occurrences instead of accidentally including dogs who had autoimmune diseases imminently triggered by their lymphoma. Neoplastic disease processes are often direct triggers for IMHA and ITP (Helfand, 1988). Separating the diagnoses by 6 months allowed us to tease out potential occult dysfunction in the immune system versus direct opsonization and antigenic triggering of autoimmune disease by the cancer itself (Helfand 1988). The data also shows that dogs with autoimmune disease were no more likely to develop a soft tissue sarcoma than dogs with allergic dermatitis, as we predicted, given a lack of association between this cancer and immune dysfunction. The true measure of these disease links can only be evaluated after a set period of time has elapsed between the two diseases. This calls into question any of the body of literature which links immunodysfunction and lymphoma in dogs. We re-examined the relationship between the immune system and lymphoma in feline renal transplant patients. A Fischer's exact test was performed to assess statistical significance of receiving a renal transplant and anti-rejection medications and subsequently being diagnosed with lymphoma. The relative risk using the numbers presented in the paper was 15.6522 with a 95% confidence interval of 0.8619 to 284.2372, a non-significant relationship (P = 0.0629). It is unclear how the authors arrived at this association.

The study design includes several disease comparisons to enable accurate assessment of lymphoma as a sequel to autoimmune disease. Dogs presenting with autoimmune disease were compared to dogs presenting with atopic dermatitis. Atopic dermatitis is a type 1 hypersensitivity and does not involve autoreactive T cells. It is not known to be associated with autoimmune disease and presents commonly in an otherwise healthy population of dogs. This control population serves as an anchor with which to compare the background population of dogs entered into the VMDB. The VMDB was queried again to see if the dogs developed either soft tissue sarcomas or lymphoma. The soft tissue sarcoma population serves as another control to discern if these patients are more likely to be affected by lymphoma, a specific malignancy that arises from the immune system itself, or any cancer that is not necessarily arising from the immune system. Our findings show that dogs were no more likely to be diagnosed with soft tissue sarcomas after receiving a diagnosis of allergic dermatitis or autoimmune disease. This validates the null hypothesis and shows that there is no relationship between allergic dermatitis, autoimmune disease and soft tissue sarcomas.

Breed predilections in this paper are similar to those reported in other papers. Previously reported breed predilections include Doberman Pinschers, Cocker Spaniels (Klag, Giger et al. 1993) Miniature Poodles (Dodds 1983) Irish Setters, Collies, English Springer Spaniels and Old English Sheepdogs. Analysis of breed disease prevalence was conducted by sorting the most commonly represented

breeds out and performing statistical analysis on only these breeds. Other breeds that were not included may actually have a predisposition for one or more of the four different disease categories that were evaluated. Breed numbers that presented for these diagnoses may have been too limited to be included in the top 18 breeds. However, the relative incidence of these diseases may be large compared to the prevalence of this breed in the VMDB. We may not have included these breeds in our analysis and a significant difference may exist when we said that one did not. The finding that female sex hormones were protective against lymphoma was also validated in previous papers (Villamil, Henry et al. 2009).

Study limitations include several things inherent to the fact that it is both a retrospective study and that the data relies on reported evidence from the VMDB. This database is a recording from referral institutions. The data set that was provided is merely a representation of who actually re-presented to one of the 26 recording institutions. We have no way of measuring the actual re-occurrence incidence of lymphoma if some of the dogs did not make it to one of these referral institutions for treatment. The VMDB does allow for a plethora of retrospective information; however the data, compiled together, may not reflect current practices in veterinary medicine. The earliest recording from this study was from 1964. It is likely that practices such as blood transfusions, medications, and generalized standard of care for autoimmune disease have changed. Perhaps more owners were inclined to euthanize dogs for a bleaker prognosis of

autoimmune disease in 1964. This aspect of this study likely underrepresents the number of dogs who may have been referred back to the teaching hospital with a later diagnosis of lymphoma. It may also increase survival times in patients who may then live long enough to develop lymphoma.

The logistical regression model concluded that breeds such as beagles, collies, Dobermans, German shepherd dogs, miniature poodles, Golden retrievers, Labrador retrievers, mixed breed dogs, English springer spaniels, Schnauzers, Rottweilers, sheepdogs, and Dachshunds were all more likely to get soft tissue sarcomas than lymphoma. This finding is particularly interesting since genomic analysis of 87 dog breeds revealed that Labrador retrievers, Golden Retrievers, German shepherd dogs, and Dobermans have a higher risk for developing lymphoma than the background canine population (Modiano, Breen et al. 2005). Limitations in interpreting the logistical regression model may be at fault for this apparent discrepancy. The logistical regression model is designed to evaluate a dichotomous scenario. The patient can have disease A or disease B. It does not discern likelihood of disease risk compared to background population. These breeds may be more likely to get soft tissue sarcomas because soft tissue sarcomas are far more prevalent than lymphoma, as evidenced by the number of samples in each population. It would be erroneous to assume that this means that these breeds are protected from lymphoma as the regression model might seem to imply.

Another bias limitation in this study was the proportion of dogs who were euthanized during their visit for either allergic dermatitis or autoimmune disease. Dogs who presented for autoimmune disease were statistically more likely to die or be euthanized at their initial diagnosis. This provides a bias since death precludes the ability to follow those dogs out over time to see if they would later develop lymphoma or soft tissue sarcoma.

In conclusion, this paper shows that dogs with autoimmune disease do not appear to be at greater risk for developing lymphoma than the control population in the VMDB, contrary to what has been reported previously. It does show that cancer is a significant risk for secondary immune-mediated disease in the immediate peri-neoplastic diagnosis

CHAPTER 3:
CONCLUSIONS AND FUTURE DIRECTIONS

This thesis questions the validity of some of the previous studies performed in veterinary medicine that underpin the archetypal notion of how the immune system acts in relation to lymphomagenesis. However, these new findings raise the question of what is the true relationship between autoimmune disease and lymphoma? The VMDB is not an ideal resource for identifying dogs who concurrently present with lymphoma and autoimmune disease. Many, perhaps most, practitioners would attribute the autoimmune disease to the lymphoma and simply record one diagnosis via the VMDB instead of the two simultaneous

diagnoses of lymphoma and immune-mediated disease. What percentage of dogs with lymphoma actually get concurrent autoimmune disease? We know that a relationship between neoplasia and autoimmune disease is well established in the literature (Helfand 1988; O'Keefe and Couto 1988; Hammer, Couto et al. 1991). The bulk of the work was done several decades ago (Gordon, Moroff et al. 1980; DeBoer and Madewell 1983); however, further investigation has not really been undertaken since. A prospective study that, at its most basic, will document dogs with thrombocytopenia below 100,000 platelets with no lymphoma bone marrow involvement would give a truer incidence of the disease linkage.

Currently underway is a Golden Retriever study evaluating 3,000 Golden Retrievers from birth to death. A study like this would be an excellent model to use prospectively to evaluate how frequently dysfunction in the immune system occurs prior to a disease like lymphoma. We know that Golden Retrievers are prone to lymphoproliferative diseases (Modiano, Breen et al. 2005; Suter, Small et al. 2011). Several studies have documented immunodysfunction during the course of neoplasia. An increase in T regulatory cells and a decrease in CD8+ T cells has been noted in dogs with neoplasia (Biller et al., 2007; Biller et al., 2010). The immune system seems to be tipped towards immunosuppression and not immunoreactivity. This coincides with our findings that autoimmune disease is not highly associated with lymphoma in the VMDB. The bigger question at hand is, do these dogs have immunodysfunction prior to getting lymphoma? Is there a

certain composition of immune regulatory cells that may predispose dogs to not only getting lymphoma, but also developing autoimmune disease previously or concurrently? Evaluating the number and phenotype of immune cells before, during, and after lymphoma and before, during and after immune disease may give us a clue as to where the dysfunction exists.

Beyond documentation of what types of cells are present, assays are needed to determine the function of these cells. It has been shown that the immune system in dogs with lymphoma retains adequate function to mount an antibody response to vaccination (Henry, McCaw et al. 2001). However, IMHA and ITP are examples of a type 2 hypersensitivity which is mediated by autoreactive T lymphocytes. The functional component to these cells in the immune system have been evaluated in dogs with lymphoma or autoimmune disease. T cell proliferation assays have been validated in dogs and have been used to study infectious diseases like leishmaniasis (Messaritakis, Mazeris et al. 2010). They have also been used to study response to alkylating agents such as cyclophosphamide (Kan, Hazama et al. 2012). Assays such as the CFSE T Cell Proliferation Assays exist to evaluate activated dendritic cell presentation of antigen to T cells. This assay is commonly used to test the antigenicity of drugs in early phase 1 study in people (Ha, Klemen et al. 2010). To the author's knowledge, the functional components of T lymphocytes have not been evaluated in dogs with lymphoma or dogs with autoimmune disease, much less dogs with both concurrent disease processes. One could use a known antigenic

stimulant identified by such assay to stimulate the T cells in dogs with lymphoma and then measure proliferation via flow cytometry. The quantity of cytokines expressed by these T cells could also be measured via colorimetric sandwich ELISA assays (Fowler, Axiak et al. 2011).

This paper serves as an initial report from which additional more focused prospective studies can be designed. Dogs with autoimmune disease do not present again later with lymphoma to the VMDB, either because this association is not present or because the high mortality rate associated with IMHA and ITP does not allow for future cancer development. The current study was not capable of determining the role of chronic immunosuppressive medications used for immune-mediated disease on the development of cancer, which is an additional suspected contributor if causation could be proven. However, lymphoma is currently an underinvestigated potential cause of secondary autoimmune disease in canine patients. The next steps need to lead us towards more fully understanding the relationship between the immune system and lymphoma. We need to use prospective studies to evaluate predictive models of who may get lymphoma, and we need to use functionality assays to assess how these immune cells function before, during, and after lymphoma and immune-mediated disease.

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APPENDIX

Table 1: Breed distribution and representation of disease categories reported in the VMDB

Breed	Lymphoma N=	Dermatitis N=	IMHA N=	ITP N=	Sarcoma N=	Total dogs N= (%)
Bassett Hound	82	316	4	0	42	444(2.5%)
Beagle	68	428	28	5	53	582 (1.6%)
Boxer	148	345	20	22	70	605 (2.4%)
Bulldog	29	406	8	1	16	460 (3.0%)
Collie	45	396	28	9	38	516 (1.3%)
Doberman	87	460	65	60	196	868 (1.5%)
GSD	155	2066	42	29	213	2505 (1.9%)
Min/toy Poodle	82	1087	110	106	83	1468 (1.2%)
Golden Retriever	329	1659	76	73	512	2649 (3.3%)
Labrador Retriever	167	2102	93	88	380	2830 (2.3%)
Mixed Breed dog	686	7570	617	404	1080	10357 (1.9%)
English Springer spaniel	44	541	71	28	51	735 (2.9%)
Schnauzer (min)	83	855	147	5	105	1195 (3.1%)
Cocker Spaniel (American and English)	135	1951	494	230	95	2905 (3.6%)
Rottweiler	103	167	51	65	99	485 (1.8%)
Scottish Terrier	87	430	9	9	40	575 (5.4%)
Shetland Sheepdog	71	500	18	18	95	702 (3.3%)
West Highland White Terrier	20	742	10	12	16	800 (2.8%)
Dachshund	21	542	91	3	29	686 (1.8%)

N= number of dogs of that breed total in the VMDB

Table 2: Number of Cases Alive or Dead at Discharge

Disease (total cases)	Dogs alive at discharge	Dogs dead at discharge
AD (32,339)	32,245	94
AID (4,791)	3640	1151

Deceased dogs include both dogs who died and dogs who were euthanized

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