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EVALUATION OF VALOSIN CONTAINING PROTEIN (P97) AS A CANCER BIOMARKER IN CANINE LYMPHOMAS

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Résumé

Le lymphome est l'une des tumeurs les plus communes tant chez le chien que l'humain. Chaque année, un nombre important de chiens développe ce cancer agressif. La majorité décédant un an suivant le diagnostic. Le lymphome canin est maintenant identifié comme un excellent modèle de recherche pour la tumeur chez l'homme, particulièrement en ce qui concerne la biologie moléculaire de la maladie. En conséquence, la recherche sur le lymphome canin sera bénéfique non seulement pour les chiens mais aussi pour l'oncologie humaine. Parmi les méthodes diagnostiques de choix pour dépister de façon hâtive le lymphome se trouve la mesure de marqueurs tumoraux. Ceci a l'avantage d'être peu invasive, simple et peu dispendieuse. Ainsi, dans le but d'évaluer la protéine VCP (valosin containing protein) comme biomarqueur tumoral dans les lymphomes canins à cellules B et T, nous avons évalué la protéine VCP par immunobuvardage sur sérums et tissus tumoraux de chiens atteints et par immunohistochimie sur des tumeurs de haut grade, grade intermédiaire et bas grade. Pour mieux définir l'expression de VCP dans les cellules cancéreuses, nous avons également examiné par immunobuvardage les niveaux de VCP dans 3 lignées cellulaires: CLBL-1, CL-1, et 17-71. Il s'avère que les lymphomes à cellules B de haut grade avaient une élévation significative du taux de VCP comparé aux tumeurs de bas grade ($P < 0,05$). De même, une accumulation importante de VCP a également été détectée dans les lignées tumorales comparées aux cellules mononucléaires du sang périphérique ($P < 0,05$). D'autre part, le taux sérique de VCP est resté similaire à ceux des chiens normaux. Ces résultats suggèrent une corrélation entre le taux de VCP et le degré de malignité des lymphomes à cellules B. En conclusion, la protéine VCP doit faire l'objet d'une évaluation approfondie pour déterminer son utilité comme marqueur pronostique.

Mots-clés : la protéine VCP, marqueurs tumoraux, lymphome canine, lymphome à cellules B, oncologie vétérinaire, chien, tumeur maligne

Abstract

Lymphoma is one of the common malignancies in both dogs and humans. Annually, an important number of canine patients develop this aggressive cancer and a majority succumbs to the disease within one year. In recent years, canine lymphoma has been increasingly recognized as an excellent model for the disease in humans, especially with regards to the molecular biology of the disease. Consequently, research targeted at canine lymphoma benefits not only dogs but the field of human oncology as well. Among the most desirable diagnostic and screening tests for lymphoma is the measurement of cancer biomarkers. They have the advantage of being minimally invasive, simple, and inexpensive. Thus, with the aim of evaluating valosin containing protein (VCP) as a cancer biomarker in canine B and T-cell lymphomas, we first performed western blots on sera and tumor tissue of dogs with lymphoma and then immunohistochemical analysis on low, intermediate and high-grade tumors. To further determine VCP expression in cancer cells, we also examined VCP levels by immunoblotting in 3 tumor cell lines: CLBL-1, CL-1, and 17-71. High-grade B-cell lymphomas had significantly increased levels of VCP compared to low-grade tumors ($P < 0.05$). Additionally, we detected a corresponding accumulation of VCP in tumor cells lines compared to peripheral blood mononuclear cells (PBMCs) ($P < 0.05$). In contrast, VCP levels were not elevated in sera of dogs with lymphoma compared to healthy controls. These results suggest that VCP positively correlates with malignancy in canine B-cell lymphomas. We conclude that VCP merits further investigation to determine its potential as a clinically useful prognosis biomarker for canine B-cell lymphoma.

Keywords : Valosin containing protein (p97), Cancer biomarker, Canine Lymphoma, B-cell lymphoma, Veterinary oncology, Dog, Malignancy

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List of acronyms and abbreviations

AAA+: ATPases-Associated with diverse cellular Activities

ACTB: β-Actin

ALS: amyotrphic lateral sclerosis

AUC: area under curve

CCOGC: Canine Comparative Oncology and Genomics Consortium

CGH: comparative genomic-hybridization

CHOP: cyclophosphamide (C), hydroxydaunorubicin or doxorubicin (H), oncovin or vincristine (O) and prednisone (P)

CID: inflammatory chronic disease

DNA: deoxyribonucleic acid

Eey 1 : Eeyarestatin 1

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmatic reticulum

ERAD: endoplasmatic reticulum associated degradation

FDA: US Food and Drug Administration

FFPE: formalin fixed paraffin embedded tissue

FISH: florescence *in situ* Hybridization

GCT: granulosa cell tumor

GEP: gene expression profiling

HDAC6: histone deacetylase 6

HER2: human epidermal growth factor receptor 2

IBMPFD: Inclusion Body Myopathy and Paget's disease of the bone and Frontotemporal Dementia

kDa : kilo Daltons

MALT: mucosa-associated lymphoid tissue

MDR: multiple drug resistance

NHL: Non-Hodgkin's Lymphoma

NPL4-UFD1: ubiquitin fusion degradation 1 – nuclear protein localization 4

NSCLC: non-small cell lung carcinoma

NSFL1C: N-ethylmaleimide-sensitive factor L 1 cofactor

PBMC: peripheral blood mononuclear cells

PCR: polymerase chain reaction

PICHROS: protein induced chromatin stress

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

ROC: receiver operating characteristic

RPMI: Roswell Park Memorial Institute

SEM: standard error of the mean

TK1: serum thymidine kinase 1

UBD: ubiquitin-fold domain

UBX: Ubiquitin regulatory X

UBXD1: ubiquitin regulatory X domain 1

UFD: ubiquitin-fusion domain

VCP: Valosin containing protein

VEGF: Vascular Endothelial Growth Factor

WB: western blot

WF: Working Formulation

WHO: World Health Organization

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Introduction

As presented in a plethora of studies, particularly in the last decade, cancer has become one of the major causes of death in humans and companion animals alike. The increasing incidence of diagnosed cases has pushed the research community to study and develop new screening and therapeutic methods for specific types of cancers. Among these, lymphoma, a common canine tumor, represents a continuing interest of research (1).

Lymphomas are a type of cancer characterized by the proliferation of malignant lymphoreticular cells, or cells pertaining to both lymphoid and reticuloendothelial systems, within solid organs (2). The primary lymphoid organs, represented by the bone marrow and thymus, along with the secondary lymphoid structures, such as lymph nodes and spleen, are the preferred sites of neoplastic growth (3). However, due to the facilitated access of lymphocytes to the bloodstream and lymph, the tumor can originate virtually in any organ (4).

In veterinary literature, lymphoma is presented as the most common hematopoietic canine neoplasm (4). Despite its ubiquity, the precise etiology of the disease has not yet been identified. Causes vary from genetic to environmental, with several breeds of dogs presenting a higher incidence for the disease (5). The tumor can manifest a wide spectrum of clinical and biological behaviours depending on its anatomical location and its stage of development (6). Furthermore, the symptomology of the disease can vary accordingly to the neoplastic transformation of either B or T-lymphocytes (7). To complicate matters even further, each individual tumor can present an innate heterogeneity, leaving early detection and complete curative therapies elusive for most veterinary patients.

During the last 25 years of continuous treatment development, late diagnosis has

proven to be the major limitation on the efficacy of available lymphoma treatments. Many patients seem to undergo intensive chemotherapy sessions with little apparent benefit in metastasis control or life expectancy (8). Along the years it was understood that early tumor detection has a strong correlation with net gains in quality of life and prolonged survival (4). As such, veterinary scientists are faced with the growing clinical demand to develop and validate new therapeutical targets that will assist oncologists in early cancer screening.

Among the most desirable tools for the diagnosis and prognosis of lymphomas are cancer biomarkers. They are defined as measurable molecules, in clinical or laboratory settings that appear in a modified state, quantitatively or qualitatively, in the presence of tumoral processes (9). Historically, the research for biomarkers was targeted at bodily fluids (blood, plasma, urine) and/or tumor tissue (10). Therefore, two distinctive categories of cancer biomarkers emerged: serum and tumor markers. Although, both operate on the same principle, their detection, measurement and utility are quite different.

Serum biomarkers are usually measured in bodily fluids, having considerable value in cancer screening, diagnosis, prognosis and follow-up of treatment efficacy (11, 12). As molecules, serum markers can be hormones, glycoproteins, or other proteins overexpressed by cancer cells. Although they are routinely used for screening and diagnosis of several human cancers, few serum markers have been identified in dogs, and none are currently used in clinical settings (13-15).

The ideal serum marker should have high sensitivity and/or high specificity. More specifically, the test needs to discriminate cancer patients form healthy subjects and/or patients with benign tumors or non-cancerous conditions. Furthermore, the markers need to show measurable plasma levels before the manifestation of symptoms (16). The measurement of

targeted proteins, done by enzyme-linked immunosorbent essay (ELISA) or western blot, has the advantage of being minimally invasive, simple, and inexpensive (10).

Tumor biomarkers, on the other hand, are measurable biochemical molecules associated with malignancy. They can be produced by tumors cells or by the body in response to the neoplastic process (9). Although measurement of tumor markers is only possible once the cancer has been identified and a biopsy has been obtained, making them ineffective for screening, they have an important role to play during the development of the disease (9). These markers are highly desirable in cancer staging, prognosis, monitoring treatment efficacy, and/or in evaluating the cancer's recurrence (9). The markers can be detected in tissue by several immunohistochemistry and immunofluorescence protocols, immunoblotting and mass spectrometry (10).

Despite their tremendous potential value, the use of serum and tumor biomarkers in veterinary oncology remains in its infancy. Recently, a new protein has been identified as a promising serum marker for human ovarian, breast, and colon cancer (17, 18). Based on our laboratory's preliminary results, valosin-containing protein, or VCP, also seems to have a higher sensibility for certain types of canine tumors, indicative of superior potential for early diagnosis (17). Lymphomas are found among the specific cancers with higher expression of VCP during their development.

As such, this thesis will present the evaluation of VCP expression as a cancer biomarker in canine lymphomas. First off, a review of the cancers in dogs and canine lymphoma will be presented, followed by current knowledge of the development of cancer biomarkers as a diagnostic tool in human and veterinary medicine, focusing on lymphoma. The emphasis is put on the use of serum and tissue markers as early detection and monitoring

tools. Next, the thesis will describe the cellular functions of VCP and will explain its potential usefulness as a diagnosis and prognosis biomarker for human and canine malignancies. Finally, the findings from this study will be presented and discussed.

Literature Review

Chapter 1: Cancer in dogs

Recent studies identify cancer as one of the most common causes of death among dogs, with values as high as 30% of registered cases (19). The total incidence of neoplasia in the United States was estimated at 1134 cases per 100,000 dogs per year (20). In 2000, a Canadian study conducted by Reid-Smith and colleagues reported 4817 cases per 100,000 dogs per year, almost four times more patients than the incidence rates reported in the States (20). Across the ocean, studies carried out in the United Kingdom present 2671 tumors per 100,000 dogs per year (20). Finally, an Italian study conducted in 2008 revealed an incidence of 1070 cases per 100,000 dogs per year (21). The discrepancy can be a direct consequence of the differences in the population of dogs found in the distinct geographical territories, since some breeds of dogs are reported to have higher genetic predispositions for lymphoma, and/or the method used in tumor classification and diagnosis (20). Whatever the variables influencing the data might be, the overall message remains the same: a relatively high incidence of canine neoplasia is encountered around the world.

The studies also presented estimated figures for the frequency of different types of canine tumors. Consequently, with small variations, certain types of cancers have been identified as having higher incidence and frequency. For female dogs, cancers of the mammary gland are the most significant, followed by Non-Hodgkin's Lymphoma (NHL) and other connective and soft tissue tumors (21). For males, lymphoma represents the most important neoplasm, before skin (excluding melanoma) and soft tissue cancers (21).

The major limitation in presenting specific canine cancers as more prevalent revolves around diagnosis and detection methods. Mammary, genital and skin tumors are easily recognizable during a general physical exam, whereas the detection of internal cancers is less obvious. The latter might require specific methods of diagnosis such as imagery, exploratory surgery and/or histopathological consult (21). Often, the client's decision to halt further clinical investigation represents a serious limitation in correctly estimating the incidence of different types of canine cancers such as lymphoma.

1.1. Lymphoma, a type of cancer most prevalent in dogs

Lymphomas, malignant or lymphosarcomas, are a diverse group of cancers (3). They present a high genetic variability with diverse, poorly understood causality (3). Clinical data combined with a comprehensive series of metanalyses, were able to thoroughly describe the biological behaviour of these cancers. In general, lymphomas have a high incidence with a rather aggressive development and a low life expectancy in the absence of treatment (3).

1.1.1. Incidence and prevalence

The annual incidence, or the number of new lymphoma cases per year, varies anywhere between 24 to 107 new cases per 100 00 dog, however there is a significant number of lymphoma case not recorded limiting our ability to detected the true incidence which seems to be much higher (3, 22, 23). In fact lymphoma occurs about 2 to 5 times in dogs as in people and it estimated that approximately 1 of every 15 dogs born today will get lymphoma at some point in his life. Incidence of lymphoma was found to increase with age, peaking at 10 years of age (24). As such, the incidence rates can be as low as 1.5 cases per 100,000 for dogs aged

less than one year, and as high as 84 cases per 100,000 for dogs older than 10 years (3). The maximal incidence can be found in the mid-age population, with dogs aged between 6 and 9 years old (25).

Although gender it is not an influential factor in lymphoma frequency, certain breeds appear to have a higher incidence for the disease (25). These include Golden Retrievers, Boxers, Bull Mastiffs, Basset Hounds, Saint Bernards, Scottish Terriers, Airedales and Bulldogs (26). Moreover, particular breeds present higher tendencies to develop specific types of lymphoma (25). Hence, Boxers and Shar-peis tend to develop alimentary lymphoma, Cocker Spaniels, Basset Hounds and Doberman Pinchers are more prone to develop B-cell lymphoma, and Boxers, Irish Wolfhounds, Siberian Huskies and Shih Tzus present more often than not T-cell lymphoma (27). Finally, Golden Retrievers can indiscriminately develop B or T-cell lymphomas (3).

Historically, lymphomas have a lower risk of appearance in intact females and in certain breeds, namely, Dachshunds and Pomeranians (3). Furthermore, because of closed gene pools and repeated inbreeding, purebred dogs present slightly higher tumor rates than crossbred dogs (28).

Lymphoma prevalence, also known as the cumulative incidence, is estimated at 7% to 24% of all canine neoplasia, with an average of 20% (3, 27). Among the hematopoietic malignancies, lymphoma occupies a staggering 83% of all registered cases (3). The most prevalent are the solid forms, such as lymphosarcomas and malign lymphomas (25). Within the solid forms, the most common type is diffuse large B-cell lymphoma, a similarity *canis familiaris* shares with humans (27).

1.1.2. Causes

The causes of lymphoma, despite extensive research, are still not completely understood and likely multifactorial. Among the etiological factors that trigger the cells' aberrant multiplication we can distinguish: genetics and molecular causes, infectious and environmental factors, and immunosuppression (3).

The recent advances in coding the canine genome have allowed detection of chromosomal aberrations in dogs affected by lymphoma. As such, studies have reported genetic material gain on canine chromosomes 13 and 31 and loss of it on chromosome 14 (3). Among other molecular events leading to tumorigenesis, deoxyribonucleic acid hypomethylation, and mutation of genes p53 and N-*ras* are common features of cancerous lymphocytes (3). The cellular pathways of the Bcl-2 family also seem to be involved in the process, however, more research is needed in order to fully understand their complete implication (3).

Besides the genetic causes, several studies have made a link between lymphomas and infectious and/or environmental factors. A weak association has been proposed between *Helicobacter pylori* infections and the development of gastric lymphomas (3). Viral infections appear to trigger the formation of mucosa-associated lymphoid tissue (MALT) lymphoma (3). Environmentally, the tumor appears to be related to the use of phenoxyacetic acid herbicides, particularly 2,4-dicholorophenoxyacetic acid (2,4-D) (3). In households were the substance was regularly used, dogs developed malignant lymphoma twice as frequently as in households where environmentally friendly or no herbicides were employed (3). In recent literature, there is a debate concerning the correlation between strong magnetic fields and the increased risk of canine lymphoma (25). Although the evidence is scarce, one study reported that dogs living in

households with very high current codes were 6.8 times more inclined to develop the disease (28).

Finally, immunosuppression is considered an important risk factor in the development of lymphomas. Immune system alterations, such as immune-mediated thrombocytopenia and immunosuppressive therapies (e.g., cyclosporine) are reported to help in the development of the neoplastic process (3).

1.1.3. Clinical findings

The prevalent ontogenic site of lymphomas is the lymphoid tissue (lymph nodes, spleen and bone marrow), however, almost any tissue can be affected (3). Due to this variability in anatomical locations, lymphoma presents with variously different clinical signs which are most often site dependent (29).

Multicentric lymphoma, the most common form encountered, presents a painless generalized lymphadenopathy, often accompanied by hepatosplenomegaly and bone marrow infiltration (3). The attack on the bone marrow provokes blood dyscrasias, a condition associated with anemia, thrombocytopenia and neutropenia (3). Due to the incapacity of the myeloid stem cells to replicate, the disorder can also lead to fever, sepsis and/or hemorrhage (3). Occasionally, the lesions will lead to nonspecific signs such as anorexia, weight loss, vomiting, diarrhea, emaciation, ascites, dyspnea, polydipsia, polyuria, and fever (3). T-cell lymphoma, a subtype of multicentric lymphoma, can add hypercalcemia to the long list of symptoms (25).

In the case of gastric or alimentary lymphoma, lesions to the mucosa and lamina propria will cause vomiting, diarrhea, weight loss, panhypoproteinemia, and malabsorption

(3). As secondary lesions, the tumor could metastasize to the mesenteric and pelvic lymph nodes, spleen and liver (3).

Canine lymphomas can also be encountered in the mediastinal cavity, where lesions create their own set of clinical symptoms. These types of tumors are characterized by the enlargement of the craniomediastinal structures and/or the thymus (3). Depending the extent of the disease, the animal might present with one or more of the following signs: respiratory distress, pleural effusion, exercise intolerance, regurgitation, polydipsia, polyuria, and possible hypercalcemia (3). If the mass compresses or invades the cranial vena cava, a pitting edema of the head, neck, and forelimbs might be observed (3).

The lesions for extranodal lymphomas are organ specific and, although important, their description exceeds the scope of this thesis. It is worth mentioning the involvement of tumors in the central nervous system (CNS) because of their serious consequences such as: paraparesis, ataxia, hyperesthesia, blindness, lethargy, and seizures (3).

1.1.4. Classification

Over the years, several classification systems have been used to differentiate between the various types of lymphomas. As such, the tumors can be classified on the basis of anatomical location, histopathological description and immunophenotypic analysis (3).

1.1.4.1. Anatomical classification

Multicentric lymphoma is encountered in approximately 80% of cases (29). It has the aspect of a ganglionary, rubbery, painless, mobile, smooth, and generally symmetrical tumor (25). The adenomegaly first affects the submandibular and retropharyngeal lymph nodes,

however, if left untreated, it will spread to other organs and parts of the lymphoreticular system (25).

The second most encountered form, representing 7% of cases, is gastrointestinal or alimentary lymphoma (29). The digestives lesions, mostly ulcers, can have different stages of infiltration throughout the submucosa and lamina propria (25). Mesenteric, gastric and retrocaecal adenopathies can be observed in the last stages of the disease (25). Historically, it was believed that alimentary lymphoma originates form B-cell lymphocytes, however, recent findings place the origin of the tumor as T-cell derived neoplasia (3).

Six percent of dogs affected by lymphoma develop the cutaneous or mucocutaneous form (29). The tumor is characterized by non-specific skin changes, progressing from scaly alopecia to thickened erythematous ulcerations to plaque-like lesions (29). Recent classifications have distinguished between two forms: epitheliotropic cutaneous and non-epitheliotropic cutaneous lymphomas (25). Epitheliotropic cutaneous lymphoma is encountered more often and is characterized by multicentricity and slow development, originating most often from CD8+ T-cells (3, 25). The latter type, although rarely encountered in clinical settings, develops in the deep dermis, being more invasive and difficult to treat (25).

Extranodal forms of lymphoma are rarely encountered, with a prevalence of only 3% of cases (29). The tumors appear randomly with predilections for eyes, central nervous system, bones, testis and nasal cavity (29). The form specific to the central nervous system is known as intravascular or angiotrophic lymphoma (3). It originates from either B or T-cell lymphocytes, and is characterized by diffuse infiltration of the wall and lumen of blood vessels (3).

1.1.4.2. Histopathological classification

Due to numerous cellular analogies between human and canine malignancies, the histological classification systems have been adapted from human oncology (25). The current systems used in veterinary medicine include the Kiel classification, Working Formulation (WF) and World Health Organization (WHO) (3).

The Kiel classification is useful in distinguishing between B or T-cell tumors and in differentiating between high grade or low grade malignancies (25). We present the identifiable types of lymphoma using this system in table 1.

Table 1. The updated Kiel classification of canine lymphomas (25)

B-cell lymphomas	T-cell lymphomas
Low malignancy lymphomas	
Lymphocytic B-cell lymphoma Lymphoplasmacytic/limphoplasmacytoid Lymphoma Plasma cell lymphoma Centrocytic lymphoma Centroblastic – centrocytic lymphoma	Lymphocytic T-cell lymphoma Small cerebriforms cell lymphoma (Mycosis fungoides) Pleomorphic small cell lymphoma
High malignancy lymphoma	
Monomorphic centroblastic lymphoma Polymorphic centroblastic lymphoma Immunoblastic B-cell lymphoma Burkitt lymphoma Lymphoblastic B-cell lymphoma	Pleomorphic mixed cell lymphoma Pleomorphic large cell lymphoma Immunoblastic T-cell lymphoma Anablastic lymphoma Lymphoblastic T-cell lymphoma

The WF, developed by the National Cancer Institute, categorizes lymphomas as low, intermediate, and high grade, with no distinction between B or T-cell origin (3, 25). The tumoral classification mainly uses cytomorphological characteristics such as size, type (small cleaved cells, large cells, and immunoblastic cells), shape of the nucleus, and the architecture of the neoplastic tissue (follicular or diffuse) (25). The WF classification (table 2) is used to analyze the biology of the tumor and is better at predicting patient survival (3).

Table 2. The WF classification of Non Hodgkin's Canine Lymphomas (25)

Low malignancy lymphomas	Small cell lymphocytic lymphoma: - Diffuse small cell lymphocytic lymphoma - Plasmocytoid lymphoma Small cleaved cell follicular lymphoma Mixed small cleaved cell Large cell follicular lymphoma
Intermediate malignancy lymphomas	Large cell follicular lymphoma Diffuse small cleaved cell lymphoma Diffuse mixed small and large cell lymphoma Diffuse large cell lymphoma Diffuse large non-cleaved cell lymphoma
High malignancy lymphomas	Large cell immunoblastic lymphoma Convoluted cell lymphoblastic lymphoma Non-convoluted cell lymphoblastic lymphoma Burkitt lymphoma
Other types of lymphomas	Mixed lymphoma Mycosis fungoides Extramedullary plasmocytoma Histiocytic lymphoma Unclassifiable forms

The Veterinary Lymphoma Study has recently adapted the latest WHO classification of neoplastic diseases of lymphoid tissue in humans (30). The classification was developed to incorporate both morphological, genotypic, and immunohistologic criteria, thereby providing a system for categorizing lymphoid neoplasms according to the level of cellular maturation (3). The strength of this system resides in the capacity to categorize canine lymphomas within the B or T-cell subtypes as well as offering a clinical prognosis (31). The WHO classification combines with success the previous two systems, insuring a better diagnostical uniformity among veterinary pathologists.

1.1.4.3. Immunophenotypical classification

The development of monoclonal antibodies has made possible the detection of specific markers on the surface of lymphocytes, giving more insight into the classification of canine lymphomas. Based on this method, lymphomas are further differentiated into B, T or null-cell (neither B or T immunoreactive) tumors (29). The immunophenotypic classification has lead to the realization that most canine lymphomas have a B-cell immonophenotype (25). Thus, as presented by the veterinary literature, B-cell lymphoma accounts for 60% to 80% of cases, T-cell lymphomas for 10% to 38%, mixed B and T-cells for 22%, and null-cell for fewer than 5% (3). Furthermore, classifying lymphomas with the help of monoclonal antibodies led pathologists to the conclusion that T-cell lymphomas have a higher incidence than previously recorded (25). Clinically, making a clear distinction between a T-cell or a B-cell derived tumor has major importance since the latter can be more aggressive and present a lower response rate to treatment (25).

1.1.5. Diagnosis

When suspecting lymphoma, the veterinary clinical oncologist has to perform a thorough clinical evaluation which includes a complete physical examination, blood work, serum biochemistry, and urinalysis (3). Advanced imaging modalities like computer tomography, magnetic resonance imaging, and positron emission/computed tomography are becoming more accessible in veterinary medicine (3). The final diagnosis is largely given by the veterinary pathologist who will employ histologic and cytologic tissue evaluation, molecular analysis, immunophenotyping, and clonality assay as diagnostic tools (3).

Histological and cytological evaluations can be done on biopsied tissue using histochemical/immunohistochemical and cytochemical/immunocytochemical staining and/or flow cytometry (3). Although all protocols are clinically available, for the moment immunophenotyping seems to be the most reliable (3). The technique uses specific cellular membrane markers to improve the precision of diagnosis and provide the clinician with a certain prognosis (3). Antibodies against CD3+, CD4+ and CD8+ for T-cells and CD79a+ and CD21+ for B-cells are applied against tissue sections (immunohistochemistry), cytologic specimens (immunocytochemistry), or single cells (flow cytometry) (3). When the characterization of lymphomas proves to be challenging utilizing standard histology, PCR techniques are used to amplify the genes of the immunoglobulin receptor for either B or T-cell lymphocytes (3).

Once the precise diagnosis is reported by the pathologist, the WHO classification system (Table 3) is used to stage the tumor (3). Stages I and II are more favorable if index “b” is not present, however, the other higher stages carry with them a poor prognosis (25). Stage V

leads for the most part to the death of the animal in a matter of days to weeks (25). Because lower stages are asymptomatic and regular medical examination is not standard veterinary practice, most dogs present with advanced stages, thus reducing drastically the treatment's efficacy (3).

Table 3. WHO clinical staging for domestic animals with lymphoma

Stage	Criteria
I	Single lymph node a. Without clinical sign of disease b. With clinical signs of disease
II	Multiple lymph nodes in a regional area a. Without clinical signs of disease b. With clinical signs of disease
III	Generalized lymphadenopathy a. Without clinical signs of disease b. With clinical signs of disease
IV	Liver and/or spleen involvement (with or without stage III) a. Without clinical signs of disease b. With clinical signs of disease
V	Bone marrow or blood involvement and/or non-lymphoid organ (with or without stage I-IV) a. Without clinical signs of disease b. With clinical signs of disease

1.1.6. Treatment

In clinical practice treatment options and the order of their administration differ from patient to patient, depending on the type of lymphoma and the extent of the disease. The

current golden standard in veterinary oncology concerning lymphoma treatment includes: systemic chemotherapy and immunotherapy, radiation therapy, and surgery (29).

1.1.6.1. Chemotherapy

Veterinary oncologists have at their disposal a variety of chemotherapeutic protocols that can be adapted according to the stage and substance (the presence or absence of clinical signs) (12), the presence of paraneoplastic syndrome, and the dog's overall physical condition (3, 29). Moreover, the owners' financial situation and time constraints, along with possible apprehension vis-a-vis the side effects of treatment, may also play a deciding factor (3, 29).

Multidrug chemotherapy protocols were first introduced over 20 years ago and have developed considerably since (3, 29). The most used treatment is a modification of the CHOP protocol, used for the first time in human oncology (3). This first line treatment is composed of cyclophosphamide (C), hydroxydaunorubicin or doxorubicin (H), oncovin or vincristine (O) and prednisone (P) (32). For this protocol, remission rates can reach 80% to 90% and can present an average survival time of 8 to 12 months (29). In 20 to 25% of cases, survival times might be as high as two years, albeit complete healing can rarely be achieved (25).

When the CHOP-based protocol does not represent a desirable option, the oncologist can implement a single agent chemotherapy protocol. Several pharmaceutical agents are available: doxorubicin, L-asparaginase, polyethylene glycol-L-asparaginase, vincristine, cyclophosphamide, and prednisone (3). This type of protocol is often avoided as single agent chemotherapy induction does not lead to long-lasting remission, with the exception of doxorubicin (3). Compared to a complex chemotherapy treatment, a non-doxorubicin

treatment protocol has more often than not resulted in short-lived remission and survival times, and higher chances of developing multi drug resistance (3).

Despite the existence of several induction chemotherapy protocols, the majority of dogs will relapse (29). Several theories are trying to account for this regression, although the most accepted in the scientific literature is the emergence of multiple drug resistant cancer cells (25). Treating a patient with chemotherapeutic regimens exerts strong selective pressure on tumor cells: those that have activated genes that reduce their susceptibility to drugs will survive treatment and continue proliferating while those that have not will die. Chemoresistance comes in many forms, from molecular pumps that remove drugs from cells to impaired DNA damage response mechanisms that allow damaged cells to continue dividing In the eventuality the tumor will develop such cells, a new chemotherapy protocol has to be initiated: the reinduction protocol. The treatment is started with the previously used protocol and changed if remission delays appear (3). Favourable results are obtained in only 50% of cases with better outcomes if the patient fully completed the initial induction protocol (25).

The last resort for treating a lymphoma with the help of chemotherapy is the rescue protocol (29). The drugs used in a single agent or combination protocol may include, but not exclusive to, actinomycin D, mitoxantrone, lomustine etc. (33-35). The overall response rates rarely exceed 40% to 50% and the remission is short lived, with an average remission time of one and a half to 3 months (25).

Although chemotherapy is the treatment of choice in veterinary hospitals and clinics, the effectiveness of this method seems to have reached its limits. For the last couple of years, no important changes have been recorded in response and remission rates. New generations of chemotherapeutic drugs are needed along with the development of non-chemotherapeutic

agents such as immunoconjugate therapies (an antibody conjugated to a second molecule, usually a toxin or radioisotope) (36).

1.1.6.2. Immunotherapy

In recent years, several specific (i.e. bacterial vaccines) and non-specific (analogous lymphatic cells, monoclonal antibodies, anti-tumoral cell) immunotherapy trials have been developed (13). Unfortunately, these new treatment options have not yet passed phase two of clinical trials, leaving veterinary oncology without a viable chemotherapy replacement.

1.1.6.3. Radiation therapy

Used frequently in human oncology, radiation therapy has been used in veterinary medicine only for selected cases (3). Trained oncologists have the option of performing focal, whole body or half-body radiation. Focal radiotherapy is usually reserved only for localized stage I and II tumors (3). Whole body irradiation becomes a viable option when bone marrow transplant can be performed, and half-body irradiation only when the animal is in remission (3). Present investigations are in place to determine if radiation therapy could be used between chemotherapy sessions to reduce treatment length and induce faster remission (3).

1.1.6.4. Surgery

Curative surgical resection can only be used for ganglionary stage I that is localized and solitary extranodal lymphomas (25). Alimentary and cutaneous forms are the best candidates for this form of treatment (25). Dogs that present with massive splenomegaly can only benefit from splenectomy only when the cancer is not responsive to chemotherapy (37).

1.1.7. Prognosis

Historically, survival times for canine lymphoma have been associated with a number of factors, namely: the location of the disease, clinical stage, presence of clinical signs, histological grade, immonophenotype, previous treatment, development of multi-drug resistance, tumour's apoptosis and proliferation rate, presence of previous medical conditions, and paraneoplastic syndrome (3). According to the WHO, stage I and II carry a more favourable prognosis especially when associated with high and medium histopathological grades (3). Although high-grade tumors are normally associated with reduced survival times, in low stages they respond better to treatment. This is explained by their high mitotic rate which is directly targeted by the chemotherapeutic agents (3). For the same stage and grade a comparison between B-cell and T-cell lymphomas has shown for the latter shorter remission and survival times, especially when associated with hypercalcemia and reduced renal function (38, 39).

The multifactorial aspect of the disease makes it difficult to provide the client with a precise prognosis. Once the diagnosis is established, untreated dogs are expected to live 4 to 6 weeks, with some exceptions in the case of low-grade tumors (29). The prognosis improves to 8 to 12 months when the appropriate treatment is provided, however, the disease remains aggressive by nature and full recovery is rarely attained.

Chapter 2: Development of cancer biomarkers as tools for improving disease detection and treatment

2.1. Cancer biomarkers

Clinically, most cancers could be curable by conventional therapies if detected prematurely. Early, non-invasive cancer screening has been a long time endeavour for the scientific community. The realization that cancers could overexpress distinct biomolecules, in addition to the development of the field of proteomics, made scientists' efforts easier in identifying molecules overproduced in malignant processes. In turn, this has led to the possibility of early cancer detection and the development of individualized target therapies. As such, in the last decade, much attention was given to cancer biomarkers as a non-invasive and economically affordable method of diagnosis and treatment.

Cancer biomarkers can be defined as measurable molecules found in biological tissues or fluids that act as a surrogate indication for the presence of a tumor (40). They may be produced by the tumor itself or by the host in response to the presence of the tumor (40). The molecules may be measured qualitatively or quantitatively by several methods such as proteomic, genomic or chemical to determine their presence (40). The ideal tumor marker should be both specific and sensitive for the detection of a certain type of tumor early in its progression. Currently, most tumor markers are not sensitive or specific enough to be used in routine population screening and are used in monitoring patients after therapy (40).

Cancer biomarkers can take many different forms such as: DNA, RNA, proteins, gene-expression and proteomic signature, methylation patterns, metabolites, carbohydrates and lipids (40). The nucleic acid analysis has revealed that cancer cells can be detected due to

DNA based mutations, single nucleotide polymorphisms, altered expression of certain genes and epigenetic modifications that alter gene expression (41). Moreover, scientists realized that changes in peptide and protein levels could also represent a reliable way to detect malignant tumors (41). The small molecule metabolites resulting from the metabolic pathways of cancer cells, together with the concentration of certain micronutrients by some cancers represent yet another opportunity to identify tumors in their early stage of growth (41).

The opportunity to identify candidate cancer biomarkers has materialized in the past decade with the completion of the human genome sequence and the introduction of high throughput sequencing technologies, microarrays and mass spectrometric approaches for the identification of proteins. As a result, there have been large efforts in developing methods to identify novel cancer biomarkers that show clinical utility for diagnosis, prognosis or therapeutic monitoring. Two main approaches, genomic and proteomics are applied in today laboratories to the search for cancer biomarkers.

Historically, cancer biomarkers have been divided into serum and tumor markers, each with its own specific clinical application (16). Besides their biological classification, in human oncology three major utilitarian definitions have emerged for biomarkers: diagnostic biomarker, prognostic biomarker, and stratification or predictive biomarker (42). Diagnostic biomarkers are defined as molecules used to detect a specific type of cancer (42). On the other hand, prognostic biomarkers are used once the patient has a definite diagnosis and the clinician needs to predict the probable course of the disease including its recurrence (42). Finally, the measurement of a stratification biomarker is made when there is a need to predicting the response to a drug before treatment is started (42). In this case, the marker is

used to look for specific characteristics of the tumor that will inform the oncologist if the patient is likely to be responsive to treatment (42).

Besides the well-established clinical applications, cancer biomarkers have extended in the last decade into all the major segments of the treatment continuum. Table 4 depicts the full extent of the possible clinical utility of biomarkers according to their type.

Table 4. Use of cancer biomarkers in patient care (40)

Clinical Biomarker Use	Clinical objective	Type of marker
Risk stratification	Assess the likelihood that cancer will develop or recur	Serum
Chemoprevention	Identify and target molecular mechanisms of carcinogenesis in precancerous tissues	Tumor
Screening	Detect and early-treat cancers in the asymptomatic population	Serum
Diagnosis	Definitely establishes the presence of cancer	Serum
Classification	Classify patients by disease subset	Tumor
Prognosis	Predict the probable outcome of cancer regardless of the therapy, to determine the aggressiveness of treatment	Tumor
Prediction/Treatment stratification	Predict response to particular therapies and choose the drug that is most likely to yield a favourable response in a given patient	Tumor
Risk management	Identify patients with a high probability of adverse effects of a treatment	Tumor/Serum
Therapy monitoring	Determine whether a therapy is having the intended effect on a disease and whether adverse effects arise	Tumor

Post-treatment surveillance	Early detection and treatment of recurrent disease	Serum
Target validation	Demonstrate that a potential drug target plays a key role in the disease process	Tumor
Early compound screening	Identify compounds with the most promise for efficacy and safety	Serum
Pharmacodynamic assays	Determine drug activity; select dose and schedule	Tumor
Patient selection	In clinical trials, patient selection by disease subset or probability or response//adverse events	Serum
Surrogate end-point	Use of a short-term outcome measure in place of the long-term primary endpoint to determine more quickly whether the treatment is efficacious and safe in drug regulatory approval	Tumor

Clinical efficacy and analytical robustness are two of the main criteria used in selecting cancer biomarkers for use (16). A biomarker should be specific for its application, be it screening, prediction or therapeutic response monitoring. Overall it should exhibit high sensitivity and specificity with a high area under curve (AUC) in a receiver operating characteristic curve (ROC) (16). It should also be well-defined biochemically with the possibility of detection by reagents such as antibodies (16). The analytical method for measuring the marker should be accurate and precise with no cross-reactivity and should be automated for efficient execution (16, 40). In the following sections, this thesis will summarize the clinical efficacy of cancer biomarkers both in human and veterinary medicine.

2.2. Cancer biomarkers in human medicine

The emergence of evidence-based medicine in the late 19th century compelled human oncology to develop early ideas about premature cancer detection and personalized therapies. Consequently, human medicine already benefits from several FDA (Food and Drug Administration) approved cancer biomarker tests. The tests use quantifiable molecules to diagnose, prognose, monitor cancerous processes and evaluate treatment response (16). It is worth mentioning that, despite obvious progress, biomedical research has not yet been able to develop any reliable biomarker-based tests for early cancer screening.

The published literature reveals that in human medicine biomarkers of interest can be measured in serum or plasma, tumor, in vivo, urine, proximal fluids (pancreatic juices, ascites), exhaled breath, etc. (16). Due to the minor importance of the other sampling sources, this thesis will focus mainly on the utility of serum and tumor markers.

2.2.1. Serum markers in human medicine

Several serum biomarkers, mostly tumor derived soluble proteins, have been identified and are presently used in clinical settings (16). As aforementioned, their main clinical benefit is the ability to diagnose cancer and monitor its recurrence (16). The molecular biomarkers summarized in the table 5 are approved for clinical practice, thus used in oncological settings. Despite their large-scale usage none is clinically used in veterinary medicine.

Table 5. FDA approved serum biomarkers (16, 43)

Biomarker	*Type*	*Condition*	*Indicated use*	*Sample*
AFP (immunoassay)	Tumor-derived plasma protein	Nonseminomatous testicular cancer, foetal neural tube defects	Patient management, monitoring of disease progression	Serum
CA125 (immunoassay)	Tumor-derived plasma protein	Ovarian cancer	Patient management, monitoring of disease progression	Serum
CA15-3 (immunoassay)	Tumor-derived plasma protein	Breast cancer	Patient management, monitoring of disease progression	Serum
CA19-9 (immunoassay)	Tumor-derived plasma protein	Pancreatic cancer	Aid in patient management	Serum/plasma
CEA (immunoassay)	Tumor-derived plasma protein	Multiple cancers	Patient management, monitoring of disease progression	Serum/plasma Urine
HE4 (immunoassay)	Tumor-derived plasma protein	Ovarian cancer	Patient management, monitoring of disease progression	Serum
OVA1 (5 markers panel: APOA1, B2M, CA125, TF, TTR, immunoassay)	Tumor-derived plasma protein	Ovarian cancer	Aid in diagnosis	Serum

PSA (immunoassay)	Tumor-derived plasma protein	Prostate cancer	Diagnosis, patient management, monitoring of disease progression	Serum
ROMA (2-Marker panel: CA125, HE4, immunoassay)	Tumor-derived plasma protein	Ovarian cancer	Diagnosis	Serum
Alpha-Fetoprotein (AFP)	Glycoprotein	Nonseminomatous testicular cancer	Staging	Serum
Human chorionic gonadotropin-beta	Glycoprotein	Testicular cancer	Staging	Serum
Thyroglobulin (Tg)	Protein	Thyroid cancer	Monitoring	Serum
Progesterone receptor HER2/NEU	Protein	Breast cancer	Monitoring, prognosis, therapy selection	Serum/Tumor

Increasing research in the field of proteomic cancer profiling will most likely allow the discovery of new serum biomarkers (41). All the same, special attention has to be given to the improvement of present tests. Today's cancer biomarkers tend to have high sensitivity but low specificity since they do not detect cancer per se but rather a general cellular process intensified during pathological dysplasia (44). In this sense, early cancer detection proves to be a difficult task as numerous pathologies can have as a side effect an increased level of the molecule (41). Furthermore, not all cancer patients express high serum marker levels and

when they do, the cancer is too advanced, making the tests obsolete (41). To complicate matters even more, the majority of the biochemical and biocellular pathways involved in cancer growth are permanently active as part of the normal homeostatic process. Therefore, almost everyone has a trace amount of the interest molecule in their blood, thereby increasing the number of false positive tests (41). As such, it is imperative to remember that although an aggressive investigative approach may be warranted on the basis of raised serum marker values, treatment cannot be initiated without a confirmatory pathological report (44). For the moment, the development of a marker that would prove to be such a precise diagnostic tool is still in its infancy.

2.2.1.1. Specific serum markers for Lymphoma

Generally, the diagnosis of haematological cancers represents a difficult task. The malignant cells have the capacity of arising from different stages of hematopoietic differentiation giving birth to heterogeneous tumors (40). In recent years, several scientific advances have led to a better understanding of the biochemical processes underlying haematological malignancies. As such, potential biomolecular targets, with possible applications in detection, prognosis, and treatment monitoring, have been identified (40). Lymphoma was not deprived of the scientific advancement; several serum biomarkers have been acknowledged in human oncology. Table 6 presents the biomarkers that are of most interest, with the mention that only a small number are presently used in clinical settings mainly due to their low specificity or sensitivity (40).

Table 6. Lymphoma serum biomarkers identified in current literature (45-50)

Biomarker	*Type*	*Condition*	*Indicated use*	*Sample*
Serum Deoxythymidine (immunoassay)	Protein (enzyme)	Non-Hodgkin's Lymphoma	Prognostic and monitor of patients	Serum
Serum Ferritin	Protein	Lymphomas	Patient follow-up	Serum
Interleukin-2 Receptors	Protein	Non-Hodgkin's Lymphoma	Prognostic significance for survival	Serum
Beta-2 Microglobulin	Protein	Non-Hodgkin's Lymphoma	Prognostic significance for survival	Serum
CA125	Tumor-derived plasma protein	Non-Hodgkin's Lymphoma	Staging, monitoring and follow-up	Serum
TNF-R1 and CD27 (theoretical stage)	Proteins	Non-Hodgkin's Lymphoma	Cancer screening (increased future risk of NHL)	Serum
C-reactive Protein	Protein	Non-Hodgkin's Lymphoma	Prognosis	Serum

2.2.2. Tumor markers in human medicine

Cancer is a heterogeneous disease. During the tumorigenic process, different genetic or epigenetic lesions may occur that can result in distinct transcriptome, which is associated with a distinct tumor phenotype (51). Gene expression profiling (GEP) using microarray platforms represent a powerful tool to explore the expression of thousands of gene simultaneously (51,

52). In the context of cancer, GEP has been used to accurately classify tumors or define tumor subtypes. The molecular signatures derived from gene expression profiling might have an impact on diagnosis, prognosis, and therapy selection (52).

Recently, in human cancer studies, the application of a range of different microarray-based technologies such as, array-based comparative genomic-hybridization (CGH), cDNA microarray, and methylation array, has increased the understanding of cancer development, and, more importantly, has generated a large number of candidate molecular markers with potential clinical value (40). While several tumor biomarkers have made the object of clinical trials in the recent decade, due to inconclusive results, side effects of personalized treatments, or low efficacy compared to the golden standard, only a small number are present in clinical settings (40). Table 7 presents the FDA approved markers used in human medicine. Similar to serum markers, none of these molecules have applications in veterinary medicine.

Table 7. FDA approved tumor biomarkers (16)

Biomarker	***Type***	***Condition***	***Indicated use***	***Sample***
BRAF mutation (real time PCR)	Tumor DNA mutation	Melanoma	Treatment response	FFPE Tissue (Formalin Fixed Paraffin Embedded Tissue)
C-KIT (IHC)	Tumor-derived plasma protein	Gastrointestinal caners	Diagnosis, Treatment response	FFPE Tissue
EGFR (IHC)	Tumor-derived plasma protein	Colorectal cancer	Treatment response	FFPE Tissue

Mammaprint DNA microarray	Gene expression signature	Breast cancer	Prognosis	Fresh tissue
TOP2A (FISH)	Chromosomal aberration	Breast cancer	Prognosis	FFPE Tissue

2.2.2.1. Specific tumor markers for lymphoma

Non-Hodgkin lymphoma (NHL), or more commonly known as the solid tumor of lymphocytic origin, has the highest incidence among hematopoietic cancers in humans (53, 54). It is the fifth most common cancer in North America and its incidence has been increasing over the last three decades (54). NHL comprises a group of clinically and biological diverse diseases, which range from indolent to aggressive clinical course. Although, immunochemotherapy has significantly increased complete remission rates, leading to improved survival, cure rates reach only around 60% (55). Patients that develop resistance to the primary drug regiments have a poor survival, even with subsequent high-dose chemotherapy (55). Moreover, primary refractory patients have a dismal outcome (55). Many new treatment strategies are being explored, some involving targeted molecules (55).

A myriad of tumor markers have been described to help significantly increase survival rates. However, the field is constantly becoming more complex, and few markers have been validated in independent studies and have moved to controlled clinical trials. A PubMed search was performed using the terms “tumor biomarkers”, “lymphoma”, “prognosis”, “outcome”, and “survival”. Priority was given to studies analyzing the biomarkers already in clinical use. Table 8 summarizes the tumor biomarkers identified in the current literature.

Table 8. Tumor biomarkers for lymphoma identified in the current literature (55-59)

Biomarker	*Type*	*Condition*	*Indicated use*	*Sample*
MYC/BCL2 (double-hit)	Genetic abnormality	Diffuse Large B-Cell Lymphoma	Treatment response, Prognosis	FFPE Tissue
CD20	Transmembrane Protein	Lymphoma (better results for Diffuse Large B-Cell Lymphoma)	Treatment (Immunochemotherapy)	FFPE Tissue
LMO2, BCL6, FN1	Gene overexpression	Diffuse Large B-Cell Lymphoma	Prognosis	FFPE Tissue
CCND2, SCYA3, BCL2	Gene overexpression	Diffuse Large B-Cell Lymphoma	Prognosis	FFPE Tissue
CD30	Transmembrane Protein	Anaplastic Large Cell Lymphoma	Treatment (Immunochemotherapy)	FFPE Tissue
ALK-NPM1	Chromosomal translocation	Anaplastic Large Cell Lymphoma	Diagnosis	FFPE Tissue
C-MCY, IGH-BCL2, BCL6	Chromosomal translocation	Diffuse Large B-Cell Lymphoma/ Burkitt Lymphoma	Diagnosis	FFPE Tissue
IGH-BCL2	Chromosomal translocation	Follicular Lymphoma	Diagnosis	FFPE Tissue
CCND1-IGH	Chromosomal translocation	Mantle-Cell Lymphoma	Diagnosis	FFPE Tissue
API2-MALT1	Chromosomal translocation	Marginal Zone Lymphoma	Diagnosis	FFPE Tissue

Chapter 3: Cancers biomarkers in veterinary medicine

Although routinely used for screening, diagnosis and prognosis of several human cancers, few cancer biomarkers have been identified in dogs, and none are currently used in veterinary clinical settings (60). Moreover, the use of cancer biomarkers in veterinary medicine has been recently challenged on the basis that the identified biological molecules do not reach the specificity, sensitivity, and accuracy required for routine clinical practice (61). According to Mukaratirwa (61), despite the advances in human oncology, human markers cannot be simply used as multiple species, or "bridging biomarkers" since the molecular etiology of most types of cancers varies from humans to dogs.

Despite this criticism, other authors consider the pet dog population is an ideal model group for many diseases in humans, especially cancers (1, 62). Their large body size and similar metabolic rate make for easy translation of drugs and surgical techniques to the human population (62). Furthermore, they share a living environment with humans and are more closely genetically related to people than are mice (62). Compared to humans, dogs are a relatively inbred population; this may lend itself to successful identification of disease-causing genetic factors that are otherwise obscured in the human population (62). Finally, unlike mouse models, diseases in dogs are spontaneous and more likely to reflect similar human disease (1).

Just like in human medicine, there are several possible uses for cancer biomarkers in helping veterinary clinical management of patients with cancer. The properties of the biomarker and the eventual test used to measure it will dictate which application the marker is suitable for. These include cancer screening, diagnosis, prognosis, prediction of therapeutic response and monitoring effectiveness of therapy, determining tumor recurrence or remission,

or even targeted therapies.

In order to be considered as screening tests in human medicine, biomarkers need to meet two essential requirements: (1) they must be able to detect cancers at a sub-clinical state, and (2) early detection must contribute to the improvement of clinical outcome (63). By these standards, cancer screening in veterinary medicine is far from being a clinical reality. However, the obvious criticism could be opposed by the development of promising biomarker discovery tools such as transcriptomics (the study of RNA transcript expression), metabolomics (the study of metabolite[s]), and proteomics (the study of protein expression) (63). With the help of these large-scale comprehensive studies, veterinary scientists were able to develop new candidate molecules that are presently being evaluated (17). According to preliminary results, the future development of cancer screening tests in veterinary oncology is likely to enhance the prevention of cancer-related deaths and to reduce the morbidity associated with advanced tumors.

A tumour marker used in diagnosis of cancer involves the same considerations as a biomarker for screening. Most markers identified to date in veterinary research, not surprisingly, have either low diagnostic sensitivity or specificity (61). Notwithstanding these limitations, for patients in high-risk groups where prevalence is relatively high, such as dog with lymphoma (27), using a diagnostic marker would aid in deciding whether more invasive tests are required.

Determining the prognosis of a tumour is essential in deciding the course of therapy to be taken. Most tumour markers used in human medicine are correlated with cancer prognosis and other prognostic indications such as tumour grade and staging (16). However, due to the well-known limitations of biomarkers, the clinical decision to determine a therapeutic course

of action cannot be taken based on the levels of the marker alone. However, the availability of such a biomarker in veterinary clinical practice would obviously help the oncologists to predict and monitor therapeutic response. This determines if the therapy was effective and also determines if there is tumour recurrence, as the biomarker level would be seen to increase again.

Finally, the advantage of actively researching for biomarkers in veterinary oncology, besides the obvious possible improvement in the remission rates and survival of the man's best friend, resides in the idea that the dog might be a possible animal model for human NHL. In dogs, the tumors seem to progress more rapidly, thus permitting clinical trials to be completed quickly, thereby acting as a bridge to human clinical applications (1). Furthermore, the short remission interval followed by resistance to treatment offers the possibility to test novel antitumoral drugs (1). Many interesting markers currently under investigation have proved efficacious in canine lymphoma (64-66).

The following sections of this thesis will focus on presenting the cancer biomarkers currently identified in the veterinary literature. Although, several publications address the need of advancing the study of cancer biomarkers, for now no ideal maker has been discovered for any canine tumor. As the progress in molecular techniques in oncology advances, so does the understanding of tumor biology, and more cancer biomarkers with better sensitivity and specificity will be found and used in clinical assay.

3.1. Serum markers in veterinary medicine

As in human medicine, measuring cancer biomarkers in bodily fluids has several advantaged such as easier sampling and handling, pain reduction in patients, and non-invasive

detection (17). These serum biomarkers have the potential to be beneficial not only for diagnostic of cancer, but also for monitoring the recurrence of tumors after surgical resection and the effect of anticancer drug therapies (67). Progressively, and with increasing rapidity, these prominent advantages have pushed the scientific community to the search for novel serum markers that could reliable be used in clinical settings. However, due to overlapping results, the data in not compelling enough to warrant routine use of these markers. The potential molecules are still not standardized and expensive for veterinary practice, thereby limited to preclinical exploratory research.

The following table (Table 9) summaries the serum markers mentioned in the veterinary literature. Most of the molecules present elevated serum levels in several cancers, however, their low specificity remains the dispute subject of several articles.

Table 9. Serum markers in veterinary medicine (68-72)

Biomarker	***Type***	***Condition***	***Potential use***	***Sample***
Serum Ferritin	Protein	Histiocytic Sarcoma	Diagnosis	Serum
Alpha1-acid glycoprotein	Protein	Carcinoma, Sarcoma and Round cell tumors	Diagnosis	Serum
CK19, ERBB2, CLDN7, ELF3	Gene overexpression	Mammary tumors	Diagnosis, Prognosis, Treatment response	Serum
VEGF	Protein	Several Aggressive canine tumors	Diagnosis	Serum
Thymidine Kinase 1	Protein	Hemangiosarcoma	Differential diagnosis	Serum

3.1.1. Detection and monitoring of lymphoma using serum makers

Research regarding the use of serum markers for the detection and monitoring of lymphoma in dogs reveals few markers of interest. To date, the measurements of serum thymidine kinase 1 (TK1) in canine lymphoma is the most studied (23, 72). Published results suggest that TK1 could potentially be used in prognosis since its elevation may precede clinical recurrence in some cases (23). Besides TK1, a variety of other markers have been the object of preclinical studies, however, no conclusion can be drawn as of yet on their potential clinical use. The table below (Table 10) summarizes the molecular targets published to date in veterinary articles.

Table 10. Lymphoma serum biomarkers detectable in dogs (23, 73-82)

Biomarker	*Type*	*Condition*	*Potential use*	*Sample*
Thymidine Kinase 1	Protein	Lymphoma	Prognosis, Disease Monitoring	Serum
Serum C-reactive Protein	Protein	Multicentric Lymphoma, High Grade Lymphoma	Prognosis	Serum
Lactate dehydrogenase	Protein	Lymphoma	Prognosis	Serum
High-mobility group B1 proteins	Protein	Lymphoma	Prognosis, Treatment outcome	Serum
Alpha-fetoprotein	Protein	Multicentric Lymphoma	Prognosis, Staging	Serum
VEGF	Protein	Lymphoma	Prognosis	Serum
Cobalamin	Vitamin	Multicentric Lymphoma	Prognosis	Serum

3.2. Tumor markers in veterinary medicine

Many proteins that could reflect the existence of the an early neoplastic process are not released or do not cross the cytoplasmic membrane of cancerous cells, making serum or plasma detection impossible (70). Moreover, the challenges in biomarker discovery using blood, is that plasma proteome is extensive, consisting not only of the indigenous proteins but also transient proteins which are secreted and shed into the bloodstream (83). The highly abundant proteins (i.e. albumin and IgG) make the low-abundant proteins that may serve as serum biomarkers difficult to identify (84). Consequently, the importance of using tumor markers in staging, prognosis, and treatment efficacy becomes evident.

In the past decade, the application of gene expression profiling (GEP) in tumors has contributed to the identification of unique gene expression signatures for major human cancers. Using GEP studies researchers have defined distinct molecular subtypes of different tumors (51, 52) that turn out to represent a significant difference in patient outcome following chemotherapy regiments.

In veterinary studies, GEP analysis in common canine cancers is currently underway, which, once complete, will allow the identification of informative expression signatures for several subtypes (85-87). To date, veterinary medicine lags years behind its human homologue, with only a handful of studies analyzing the specificity and sensitivity of tumor markers (Table 11). Although a few potential molecules have been identified, mainly for immunohistochemical study, there is yet no sufficient evidence to warrant their routine use in clinical practice (88).

Table 11. Tumor biomarkers in veterinary medicine (61, 88-92)

Biomarker	*Type*	*Condition*	*Potential use*	*Sample*
BMP2, LTBP4, DERL 1	Genes	Mammary Tumors	Malignancy diagnosis	Tissue
HBME-1	Epitope	Ovarian Tumors	Differential diagnosis	Tissue
c-KIT	Gene	Mast Cell Tumors	Prognostic	Tissue
Chondroitin sulfate proteoglycan - 4	Proteoglycan	Melanoma	Immunotherapeutical target	Tissue
Ki-67	Protein	Melanomas, Mast Cell Tumors	Prognostic	Tissue
Alpha-3 integrin	Protein	Melanoma	Treatment (delivery of cytotoxic agents)	Tissue

3.2.1. Detection and monitoring of lymphoma using tumor markers

The research for lymphoma tumor markers in veterinary medicine proves to be challenging. A review of the literature reveals only one potential biomarker: P-glycoprotein. The expression of the protein on malignant lymphocytes has a negative association with remission and survival times (4). The mechanism surrounding this predictive characteristic of the molecule is closely related to the acquisition by the malignant cells of multiple drug resistance (MDR). Belonging to a family of transmembrane transporter proteins, P-glycoprotein removes foreign molecules from the cell, reducing the effects of chemotherapy (4). Unfortunately, due to the fact that the development of MDR is not strictly related to the

expression of the protein, the research on possible therapeutic applications of the marker was discontinued.

In conclusion, despite tremendous potential value, the use of cancer biomarkers in veterinary oncology remains controversial at best. Potential canine biomarkers suffer from low sensitivity and/or specificity, limited applications and possible biases. Nevertheless, newly discovered biomarkers await validation and VCP is certainly one of them.

Chapter 4: Valosin containing protein (VCP) as a cancer biomarker

4.1. Structure of VCP

Valosin-containing protein (VCP), also known as p97/Cdc48 (*S. cerevisae*)/ter94 (*Drosophila*), is an ubiquitous type II AAA+ (ATPase associated with various cellular activities) protein which is highly evolutionarily conserved among multiple species (93). This diverse family of enzymes is associated with a wide range of cellular processes including proteolysis, DNA repair and membrane fusion that it carries out in an ATP-dependent manner (94). By electron microscopy, VCP forms a hexameric stricture composed of two internal AAA domains (also referred to as the D1 and D2 domains), a C-terminal regulatory and binding domain, and an N-terminal cofactor and substrate-binding domain (Figure 1) (95). The D1 domain is the region primarily responsible for oligomerization of VCP monomers into homohexamers (Figure 1) (96). On the other hand, the ATPase activity conferred by the D2 domain is essential for VCP's function as a molecular chaperone in a plethora of distinct cellular processes (97). The predominant view of VCP's structure-function relationship is that the large amount of energy released upon ATP hydrolysis by the ATPase activity-containing

D2 ring is transduced through the relatively fixed D1 ring to the substrate/cofactor-binding N-domain (96). This motion has been described as a "ratchet-like" mechanism (96).

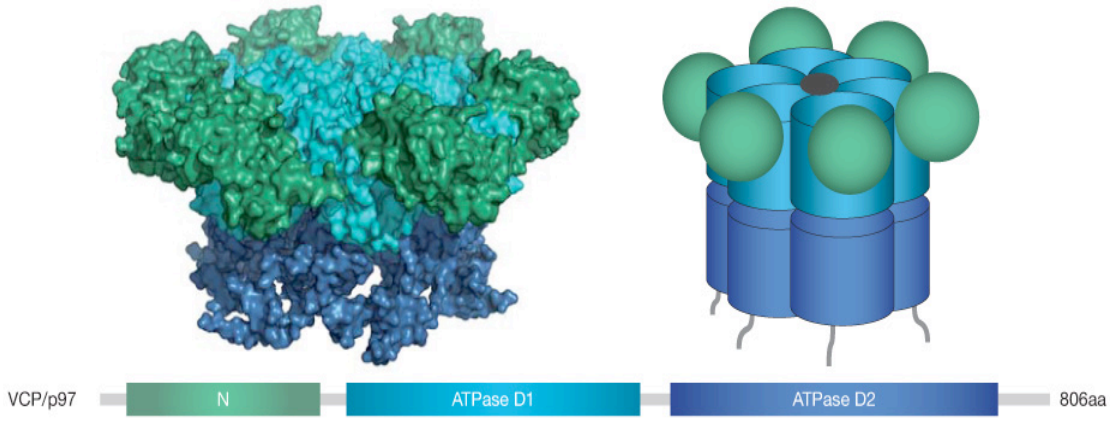

Figure 1. Structure of the hexameric AAA+ protein VCP/p97. Each subunit consists of a globular N-terminal domain (green), the two AAA ATPase domains D1 (cyan) and D2 (blue), and a C-terminal tail (grey) D1 and D2 form two stacked hexameric rings. The N-terminal globular domain is positioned at the periphery of the D1 ring.

Adapted from Mayer, Bug, & Bremer 2012.

4.2. Role of VCP in the cell

The staggering variety of binding partners that VCP can interact with through both its N-domain and C-terminal domain allows for a wide and diverse array of cellular functions (95). The N-terminal domain binds both directly to poly-ubiquitin chains, as well as to adaptor proteins. Three core adaptors have been identified in the regulation of diverse cellular functions: NPL4-UFD1 (ubiquitin fusion degradation 1 – nuclear protein localization 4), UBXD1 (ubiquitin regulatory X domain 1) and p47 or NSFL1C (N-ethylmaleimide-sensitive

factor L 1 cofactor) (Figure 2) (95). Two of these proteins that bind the N-terminus of VCP are p47, which contains a UBX (Ubiquitin regulatory X) domain and is involved in membrane fusion, and NLP4-UFD1, which contains a UBD (ubiquitin-fold domain) and is involved in proteasome targeting of substrates (95, 96). The C-terminal domain of VCP can bind proteins with various functions through the UBXD1 cofactor (95).

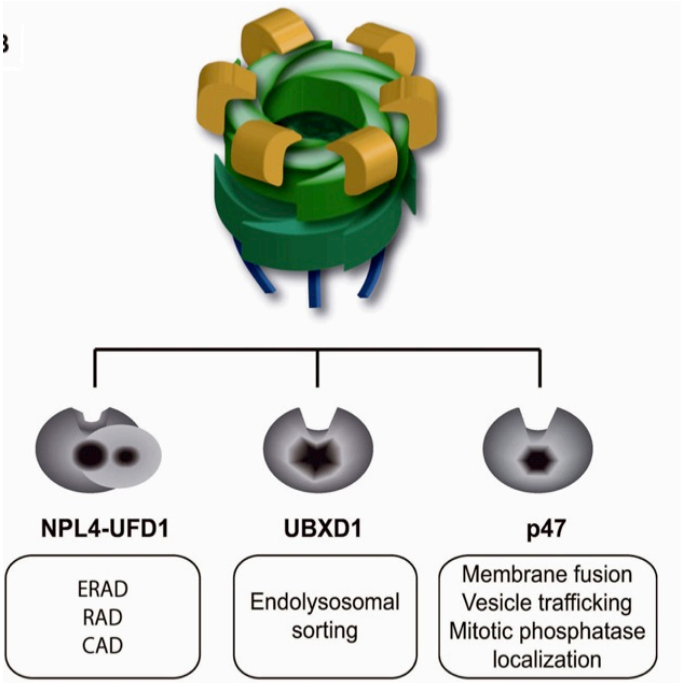

Figure 2. VCP and its three core adaptors regulate diverse cellular functions. VCP homohexamer forms a barrel-like structure that mutually binds one of three core adaptor complexes, Npl4-Ufd1 heterodimer, UBXD1 or p47, and regulates diverse cellular functions as indicated in white boxes. ERAD, endoplasmic reticulum-associated degradation; RAD, ribosomal-associated degradation; CAD, chromatin-associated degradation.

Adapted from Vaz, Halder, & Ramadan 2013

VCP coordinates a number of ubiquitin-regulated processes through its basic function as a molecular segregase that utilizes ATP-powered conformational changes in the assembly and disassembly of macromolecular machineries (Figure 3) (98). VCP is essential to some aspects of ubiquitin-dependent degradation including endoplasmic reticulum-associated degradation (ERAD), degradation of some cytosolic proteins by the ubiquitin-fusion domain

(UFD) pathway and delivery of substrates to the proteasome, and rapid maturation of autophagosomes. VCP also is integral to some non-proteolytic aspects of ubiquitin signalling, including chromatin decondensation following mitosis, nuclear envelope assembly, DNA replication and repair, and post-mitotic Golgi reassembly (95, 99, 100).

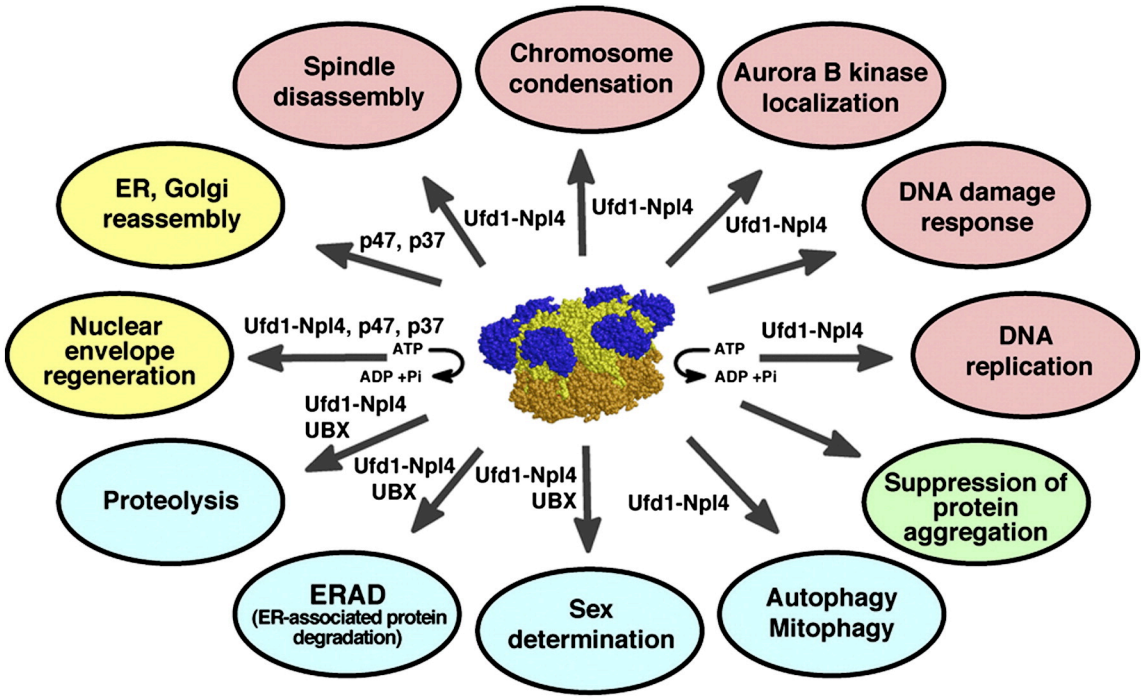

Figure 3. Multiple functions of VCP. N-terminal cofactors involved in each function are also indicated. Functions shown in yellow are related to membrane reformations. Functions shown in blue pertain to protein degradation. Functions shown in green pertain to protein aggregation. Functions shown in pink functions are key to cell cycle and DNA replication.

Adapted from Yamanaka, Sasagawa, & Ogura 2012.

VCP is essential for ERAD, a process in which defective or abnormally folded ER proteins are degraded (99). As with many other functions, VCP's participation in ERAD is

dependent on one specific cofactor, NPL4-UFD1 (95). Specifically, substrates destined for proteasomal degradation are translocated from the ER lumen to its cytosolic face where they are bound by the VCP/ NPL4-UFD1 complex (95). The ERAD substrates are subsequently ubiquitinated and transferred to the proteasome for degradation (99).

While a large amount of work done regarding VCP's role in protein turnover has focused on ERAD, a role for VCP in cytosolic protein degradation has also been outlined (98, 99, 101, 102). As support for this role, VCP has also been shown to be required for the ubiquitin-independent proteasomal degradation of some proteins (103). There is not yet a consensus mechanism for VCP's role in cytosolic protein turnover, being described interchangeably as a scaffold, shuttling factor, or segregase of ubiquitinated proteins (100). It is likely to play all of these roles depending on both the substrate protein as well as the cofactors bound to VCP (97).

VCP has more recently been implicated in the autophagic process as well, and seems to be required specifically for the maturation of the autophagosomes into autolysosomes (97). The protein also facilitates the proteasome-independent degradation in the lysosome (97). Through a gear box-like function, it controls protein sorting in the endocytic pathway and autophagy (96). Finally, VCP protects the cell from protein induced stress by degrading or recycling unwanted proteins and protein aggregates situated in the outer mitochondrial membrane and cytosol (96).

Direct evidence links VCP with genome stability (95). Intentional VCP inactivation slows the progression of the cellular cycle through anaphase and, in some cases, even the discontinuation of mitosis (96). This phenomenon is explained by the VCP's role in removing and/or degrading cellular cycle related proteins, regulating DNA replication, assuring proper

chromosome alignment, segregation, decondensation and nuclear envelope formation (95). The hypothesis linking VCP and genome stability is further supported by the direct control the protein has on the gap-filling DNA synthesis after UV damage (95). These activities prevent the accumulation of ubiquitinated substrates on the chromatin, and consequently the appearance of PICHROS (protein-induced chromatin stress) and genome instability, intracellular processes characteristic of cancer development (95).

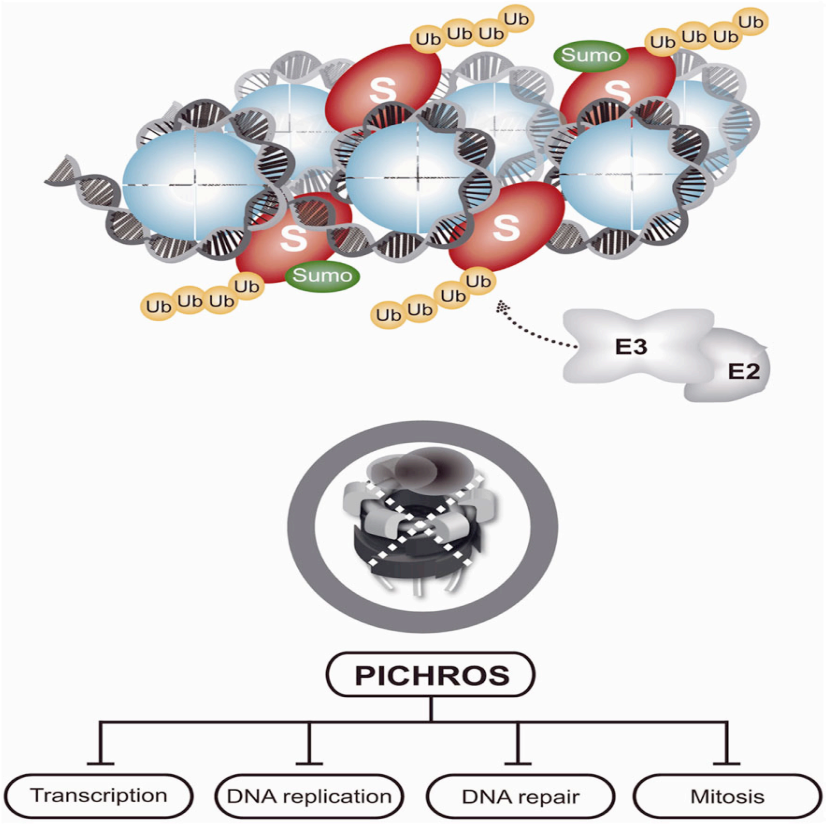

Figure 4. Protein-induced chromatin stress (PICHROS). Inactivation of VCP causes the accumulation of polyubiquitinated substrates on chromatin, causing PICHROS that inhibits essential DNA metabolic processes, such as DNA replication, transcription, or DNA repair, and leads to genome instability.

Adapted from Vaz, Halder, & Ramadan 2013

The functions of VCP and its adaptor complexes described here are only a few of the better-characterized functions of VCP. It is clear though that the binding of the cofactors to VCP determines its diverse cellular functions in all compartments of the cell. Despite knowing the identity of over 40 adaptors, the association between VCP and such a wide range of proteins is still poorly understood.

4.3. VCP as cancer biomarker in human medicine

The diversity of VCP functions within the cell implicates the protein in a possible large number of diseases, especially related to protein quality control, offering a potentially interesting biomarker and/or therapeutic target. VCP has been associated with various neurodegenerative disorders (104). These include senile plaques in Alzheimer's disease, Lewy bodies in Parkinson's disease, neuronal intranuclear inclusions in polyglutamine diseases, and ubiquitin-positive inclusions in amyotrphic lateral sclerosis (ALS) (104, 105). In pathology studies, VCP has been shown to localize with nuclear inclusions in Huntington and Machado-Joseph disease (104). Most interestingly, the disorder Inclusion Body Myopathy and Paget's disease of the bone and Frontotemporal Dementia (IBMPFD) has been linked to various mutations of the VCP gene (104).

Prior to the recognition that mutations in VCP were causative of neurodegenerative disorders, VCP was implicated as playing a role in cancer. VCP has been found overexpressed in several human carcinomas (93, 100). Furthermore, RNA interference or inhibition of VCP in tumor cell lines has been shown to cause cell death (106-108). Although there is no evidence that mutations of VCP are related to cancer, the involvement of the molecule in

genome stability, protein degradation, and cell cycle has led scientists to speculate that cancer relies on intact VCP functions (95, 109-111), rendering the protein a potential highly sensitive cancer biomarker.

4.3.1. VCP as a tumor marker

There is a small but increasing number of studies focusing on the association between VCP overexpression in tumor tissue and the development of several human carcinomas. Most of the recent attention in the preclinical setting has been focused on the predictive nature of VCP and its significance as a tumor maker. One group, Yamamoto *et al.*, examined between 2003 and 2004 in a series of 9 studies the expression levels of VCP in different human carcinomas (112-120). The authors reported that VCP overexpression, as determined by immunohistochemistry, is a useful marker for predicting tumor extension, lymph node metastasis, and prognosis of esophageal carcinoma (112), non-small-cell lung carcinoma (113), pancreatic ductal adenocarcinoma (114), colorectal carcinoma (115), gastric carcinoma (116), gingival squamous cell carcinoma (117), pancreatic endocrine carcinomas (118), hepatocellular carcinoma (119), and follicular thyroid cancer (120). The authors further propose that besides predicting patient prognosis VCP might also provide a novel way to explore effective treatment modalities for the mentioned malignancies. In addition and as validation that VCP is associated with metastasis and prognosis, another group, Tsujimoto *et al.*, came to the same conclusions when studying VCP expression in prostate cancer (18).

Interestingly and of importance for this thesis, one recent study has investigated the expression levels of VCP in primary orbital MALT lymphoma, a type of NHL (121). Zhu *et al.* observed that VCP could be a useful tumor marker for predicting the prognosis of this sub-

type of lymphoma, since the 5-year disease-free and overall survival rate of patients with lower VCP expression levels was significantly better than that of higher expression levels (121). Significantly, VCP levels were correlated with recurrence and tumor size (121).

While there are no clinical tests that use VCP as a prognostic biomarker for human cancers, the emerging field of tumor markers is undoubtedly progressing and the vast body of knowledge from basic science and pre-clinical work is reassuring. Moreover, these results make a strong case for the effectiveness of VCP as a tumor marker for several human cancers.

4.3.2. VCP as a serum marker

Despite the great potential shown by VCP as a tumor marker, there has been only one study measuring the overexpression of the protein in sera of cancer patients. In an experiment designed to identify novel serum diagnostic markers for human ovarian granulosa cell tumor (GCT), Laguë *et al.*, detected using proteomic profiling by mass spectrometry elevated VCP levels in the preoperative serum of GCT patients (17). Motivated by these results, the authors decided to determine the specificity of VCP in sera of patients with ovarian, breast, colon, pancreatic, lung, and prostate cancer, and, again of interest to us, NHL (17). Interestingly, increased serum VCP levels were observed in all malignancies with the exception of lung and prostate cancer (17). The results obtained by Laguë *et al.* in 2012 argued for the potential use of VCP as a sensitive serum marker for NHL, forming the basis for the present study.

4.4. VCP as cancer biomarker in veterinary medicine

In veterinary studies, cancer biomarker analysis in common canine malignancies is currently underway, which, once complete, will allow the identification of

promising makers and development of clinically applicable tests. Although several different studies in human malignancies have identified VCP as a promising serum and tumor biomarker, being useful for the detection of a variety of neoplastic diseases including NHL, at the initiation of this research there was no study investigating VCP as a potential cancer biomarker in veterinary medicine. With knowledge generated by the previous studies, this thesis is the first to evaluate VCP as a cancer biomarker for canine lymphoma.

Hypothesis and objectives

Several molecular products metabolized and secreted by neoplastic tissue could be characterized biochemically in cells, tumor tissues and/or body fluids. They may act as cancer biomarkers, useful as indicators of tumor stage and grade as well as for monitoring responses to treatment and predicting recurrence. Few studies have focused on investigating the potential of such markers in veterinary medicine. These molecules could be used to aid in the diagnosis and determining the prognosis of canine lymphoma, which represents one of the most prevalent types of cancers in dogs. Among these proteins, VCP has been described as significantly increased both in sera and tumor tissue of several human carcinomas. Giving that the molecular and genetic processes that drive cancer development in humans and other mammals are highly related, we hypothesize that VCP will be significantly increased in sera and tumor tissue of dogs with lymphoma compared to healthy dogs. Moreover we hypothesize that VCP levels will vary according to the tumour's grade, being highest in high-grade lymphomas.

In order to test this hypothesis, this study's objectives were to:

1. Evaluate the expression of VCP in canine lymphoma compared to normal lymph nodes and also to see if this expression differs among different tumor types and grades
2. Determine if VCP could be useful serum and tumor biomarker in canine lymphoma
3. Determine any potential correlation between tumor and serum VCP expression in canine lymphoma.

Publication as first author

Valosin containing protein (p97) expression positively correlates with malignancy in canine B-cell lymphomas

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ABSTRACT

Although routinely used for diagnosis, prognosis, treatment monitoring, and recurrence of several human cancers, few cancer biomarkers have been identified in dogs, and none are currently used in the veterinary clinical setting. Valosin containing protein (VCP), an essential biochemical component of a wide-range of ubiquitin-linked cell biological reactions, was recently identified as a potential promising cancer biomarker. Given that the molecular and genetic processes driving lymphoma's development in humans and dogs share many similarities, we hypothesize that VCP will be significantly increased in sera and tumor tissue of dogs with lymphoma, more specifically in high-grade tumors, compared to healthy dogs. VCP expression was determined by immunohistochemistry in formalin-fixed tissues from healthy and cancer-bearing dogs. Canine lymphoma cell lines CLBL1, 17-71 and CL1, peripheral blood mononuclear cells (PBMCs), and sera VCP were determined by immunoblotting and signal was quantified by densitometry. Increased VCP levels were observed in tumor tissue of dogs with high-grade B-cell lymphomas compared to low-grade tumors ($P < 0.05$). Furthermore, we observed a corresponding accumulation of the protein in tumor cell line compared to PBMCs ($P < 0.05$). Conversely, no significant difference was found in the VCP expression in the sera of dogs with lymphoma compared to healthy controls. Together, these results suggest that VCP positively correlates with malignancy in canine B-cell lymphomas. This study represents an important step towards providing veterinary oncologists with a simple but effective prognosis tool for canine B-cell lymphoma.

INTRODUCTION

Recent development of veterinary care has led to a significant extension of dogs' lifespan and allowed the diagnosis and treatment of a growing number of different diseases in this species (1). Unfortunately, among all diseases in dogs, cancer is considered the main cause of mortality, with lymphoproliferative disorders accounting for up to 30% of all canine cancers (2). As in humans, the heterogeneous disease is one of the most common dog neoplasms with an incidence of more than 33 cases per 100 000 dogs per year (3-5). Unlike humans, multicentric lymphoma accounts for 80% to 85% of reported cases, and diffuse large B-cell lymphoma is the most common histomorphologic variant (6). Moreover, due to the rapid course of the disease and lack of both baseline and follow-up medical examinations (7), our canine patients are still diagnosed late in the course of their disease. This represents a major limitation to the success of our therapies, but more notably highlights the importance of identifying better tools to assist veterinarians in the early diagnosis of this insidious disease.

Classically, the diagnosis of canine lymphoma was dependent on morphologic characteristics established by cytology and/or histopathology (8). In the past decade, much research has been turned toward additional assays such as immunocytochemistry, immunohistochemistry, flow cytometry, and PRC to detect clonal antigen receptor gene rearrangement (8). However, the diagnostic tests are only truly useful when the tumor can be biopsied, in many cases when the disease is well advanced. It would be extremely valuable for clinicians to detect, stage, monitor and evaluate cancer’s recurrence for risk group stratification, effective treatment intervention and outcome prediction at an early stage of the disease (9, 10).

Although routinely used for diagnosis, prognosis, treatment monitoring, and recurrence of several human cancers, few cancer biomarkers have been identified in dogs, and none are currently used in the veterinary clinical setting (11). Historically, human oncological research has focused its attention on two distinct categories of cancer biomarkers: serum and tumor markers. Serum makers have considerable value for clinical screening, diagnosis, and follow-up (12). On the other hand, tumors markers give valuable insight in the development of the disease by helping clinicians, stage, prognosticate, monitor treatment efficacy, and/or evaluate cancer recurrence (9). For veterinary oncology, the identification of both new serum and tumor biomarkers would prove a valuable tool for the fight against lymphoma.

A review of the veterinary literature with regards to the use of cancer makers reveals few markers of interest. To date, the measurement of serum markers in canine lymphoma had focused for the most part on serum thymidine kinase 1 (TK1) (13-16). The results published suggest that TK1 is prognostic and that its elevation could precede clinical recurrence in some cases (13). A variety of other markers have been studied, including several inflammatory proteins (17-21), alpha-fetoprotein (22-24), alkaline phosphatase (25, 26), lactate dehydrogenase (27, 28), and plasma DNA (29). However, no conclusions can be drawn yet on their potential clinical use based on the results on these studies. Moreover, the search for tumor markers is even more challenging, with only one studied target: P-glycoprotein (30). Despite their tremendous potential value, the use of cancer biomarkers in veterinary oncology therefore remains in its infancy, and awaits identification of a sensitive and specific lymphoma marker to become clinically applicable.

Using a translational proteomics approach, Lagüe *et al* have identified in several human cancers, including Non-Hodgkin's Lymphoma (NHL), a new promising serum

biomarker, valosin containing protein (VCP) (12). VCP proved to be a highly sensitive marker, being often increased in cancer patients who did not otherwise display increased levels of the "gold standard" serum markers for their cancer type (12). Furthermore, a growing body of research suggests that VCP expression correlates with prognosis in a range of human malignant tumors (31-37), making the protein a possible major target in the development of the next generation of cancer biomarkers.

The ubiquitously expressed VCP (also known as p97) is a prominent member of the highly conserved AAA+ proteins, which are known for their various cellular and molecular chaperone activities (38). VCP is an essential biochemical component of a wide-range of ubiquitin-linked cell biological processes, including the ubiquitin-dependent proteasome degradation pathway, Golgi, endoplasmatic reticulum (ER), and nuclear membrane reassembly, ER associated degradation (ERAD), cell cycle regulation and DNA repair (39-43). Within these processes, VCP acts as a molecular segregase by binding to distinct cofactor proteins and utilizes ATP-powered conformational changes in the assembly and disassembly of macromolecular machineries (44-46). Due to its participation in a remarkable number of vital cell biological processes, mutations in VCP have been linked with accumulation of misfolded, polyubiquitinated proteins that endanger the cell's viability and lead inevitably to apoptosis (47-49). Not surprisingly, a few studies have reported elevated levels of VCP in several human malignancies (12, 31-37) and this is most likely due to protein induced chromatin stress (PICHROS) (50) and protein damage-induced stress signals that are elevated in cancer cells (51). Having a high proliferative and metabolic rate, malignant cells seem more dependent on VCP for the clearance of abundant, misfolded, aggregate-prone, and potentially toxic proteins, stabilizing their genome and facilitating their survival (51).

Given that the molecular and genetic processes that drive lymphoproliferative cancers development in humans and dogs share many similarities (52-54), we hypothesize that VCP will be significantly increased in sera and tumor tissue of dogs with lymphoma compared to healthy dogs. We also hypothesize that VCP levels will vary according to the tumour's grade, rendering VCP measurements more useful for lymphomas with a high mitotic rate and significant necrosis. Our specific objectives are to: 1) study the expression of VCP in canine lymphoma and normal lymphoid tissue; 2) determine if VCP may be useful as a serum and tumor biomarker in canine lymphoma; and 3) determine any potential correlation between tumor and serum VCP expression in canine lymphoma.

MATERIALS AND METHODS

Lymphoma Tumor Samples and Cell Lines

Frozen and formalin-fixed lymphomas tumor samples and sera used for western blotting and immunohistochemistry were obtained from the Canine Comparative Oncology and Genomics Consortium (CCOGC) and from the Oncology Service at the Faculté de Médecine Vétérinaire, Université de Montréal. Tumor grades and phenotype were determined using the classification system established by Valli et al (55) by a board-certified veterinary pathologist. Healthy lymph nodes used as controls were obtained from cadavers of healthy dogs euthanized for reasons unrelated to illness, and were obtained from the Département de Pathologie et de Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal. All lymph nodes were aseptically removed during autopsy. A portion of the node was placed in formalin for the purpose of histopathological evaluation. The remainder of the lymph node

was trimmed to remove the capsule, connective tissue, and adipose tissue; placed in a Falcon™ 15 mL high-clarity polypropylene conical centrifuge tube and stored at -80^{0}C until processed.

The lymphoma cell lines used in this study (CL-1, 17-71, CLBL-1) were previously characterized and were cultured as described in Seiser et al. and Rütgen et al. (56, 57). Dr. Steven Sutter (North Carolina State University) provided the 17-71 and CL-1 cell lines and Dr. Barbara Rütgen (Central Laboratory, Department of Pathobiology, University of Veterinary Medicine Vienna) the CLBL-1 cell line. The cell lines were cultured in T75 flasks using cell media containing either 10% (CL-1 and 17-71) or 20% (CLBL-1) heat inactivated fetal bovine serum, RPMI, and 100 units/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of fungizone (Invitrogen, catalog numbers 12483-020, 61870-036, 15240-062, respectively). The cells were incubated at 37°C in humidified 5% CO_2/95% air.

Peripheral blood mononuclear cells (PBMCs) were obtained from clinically healthy dogs (n=5) by venipuncture and isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), as directed by the manufacturer. Briefly, whole blood was collected in 5ml heparinized tubes, layered on an equal volume of histopaque-1077 and centrifuged at 400g for 30 minutes for the recovery of mononuclear cells. PBMCs were cultured under the aforementioned conditions as CL-1 and 17-71 cells. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Université de Montréal and conformed to the Canadian Council on Animal Care (CCAC) Policy on Humane Care and Use of Laboratory Animals.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded, 3 μm lymphoma and normal lymph node sections using the VectaStain Elite Avidin-Biotin Complex Kit (Vector Laboratories, Inc., Burlingame, CA) as directed by the manufacturer. Sections were deparaffinized and probed with Anti-VCP [5] mouse monoclonal antibody (Abcam Inc., Cambridge, MA, catalog number ab11433) as directed by the manufacturer, except blocking was done with 5% normal serum in TBST for 1 hour at room temperature and incubation with the secondary antibody (biotinylated anti-mouse reagent, Vector Laboratories, Inc., dilution 1:500) was done for 30 minutes. Staining was done using 3,3'-diaminobenzidine peroxidase substrate kit (Vector Laboratories, Inc.) as directed by the manufacturer. Negative controls were prepared using the primary antibody described above that was pre-incubated for one hour at room temperature with VCP peptide (792-806, Abcam Inc., catalog number ab39788) in a 1:10 antibody:peptide ratio. Sections were counterstained with hematoxylin prior to mounting.

In order to evaluate the staining intensity distribution of VCP, four regions per slide (n=4 slides per tumor phenotype and grade) were selected at random and photos were taken at a 63x magnification. From these photos, a smaller field containing approximately 100 cells was selected at random and used for analysis. VCP expression on each slide was placed into four categories, 0 (negative), 1 (low), 2 (intermediate), and 3 (high), based on the scoring results for the level of nuclear and cytoplasmic staining. All slides were reviewed by two separate evaluators and a board certified pathologist, all blinded with regards to the phenotype and grade of the tumor. The grading system was inspired by Steinhardt, *et al* 2009 (58).

Images were taken using Axio Imager M.1 microscope (Zeiss), analyzed, and processed using Zen 2012 Digital Imaging for Light Microscopy software (Zeiss).

Western Blot Analysis

Lymphoma tumor samples, healthy lymph nodes, lymphoma cell lines, PBMCs and sera were used for western blot analysis. Lymphoma cell lines and PBMCs protein extracts were obtained using M-PER® Tissue Protein Extraction Reagent and Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Rockford, IL, catalog numbers 78510 and 78442, respectively) as described by the manufacturer. Lymphoma tumors and healthy lymph node protein extracts were obtained using RIPA buffer, PhosSTOP Phosphatase Inhibitor Cocktail Tablet and Complete Mini Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Indianapolis, IN, catalog numbers 04906845001 and 11836153001, respectively). Protein concentrations were quantified using the Bradford method (BIO-RAD Protein Assay, 500-0006). Samples (18μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to Hybond-P PVDF Membrane (GE Amersham, Piscataway, NJ). Blots were then probed at 4^{o}C overnight with primary antibody against VCP [5] mouse monoclonal antibody (Abcam Inc., Cambridge, MA, catalog number ab11433), Ubiquitin Lys48-Specific clone Apu2 (Millipore, Temecula, CA, catalog number 05-1307), and ACTB (C4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, catalog number sc-47778) as directed by the manufacturers. ACTB was used as the loading control. The blots were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit IgG antibodies (Promega, Madison, WI, catalog numbers W4021 and W4011, respectively), and then Immobilon Western Chemiluminescent HRP Substrate

(Millipore, catalog number WBKLS0500) was used to visualize the protein bands with Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Hercules, CA). To quantify the specific band intensity for further statistical analysis the software Image Lab™ 5.0 (Bio-Rad, Imaging software) was also employed.

Statistical methods

A ROC curve was performed on Western blotting data in order to establish the optimal cut-off value that would define high vs low VCP expression levels. K-48 polyubiquitinated protein and VCP expression in tumor and sera were analyzed by one-way ANOVA with Tukey's multiple comparisons test to identify differences between specific groups and an unpaired t test with Welch's correction for the VCP expression in PBMCs and lymphomas cell lines. Data were log-transformed if variances were significantly different between samples. Data are presented as means ± SEM. Differences were considered significant when $P < 0.05$. Analyses were done using Prism 6.0 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

VCP protein expression correlates with malignancy in canine B-cell lymphomas.

To study VCP expression in canine lymphomas, 22 B-cell and 18 T-cell tumors were analyzed by immunoblotting and compared to normal lymph nodes. VCP levels in low-grade B-cell lymphomas were found to be similar to normal lymph nodes. However with a reference value for VCP levels set by the low-grade tumor's immunoblotting band intensity, we observed that dogs with high-grade B-cell lymphomas displayed significant ($P < 0.05$)

increased levels of the protein (Figure 1A, 1C). Marginally increased levels of VCP were observed in the intermediate-grade B-cell lymphoma tumors compared to normal tissue, however the differences were not statistically significant (Figure 1A). VCP expression was also studied by immunohistochemical staining of 19 B-cell lymphomas slides (5 low-grade, 6 intermediate-grade, 8 high-grade) and 4 normal lymph nodes. This furthered showed that low-grade B-cell lymphomas express VCP at a level comparable to transient B-cells present in the mantle of lymphoid follicles, whereas VCP expression in intermediate and high-grade B cell lymphomas was more comparable to that found in maturing B-cells in germinal centers (Figure 2A-B). On the other hand, VCP expression in T-cell lymphomas did not show any significant pattern that would correlate with the grade of the tumor (Figure 1B). Immunohistochemistry performed on 15 T-cell lymphomas (3 low-grade, 5 intermediate-grade, 7 high-grade) appeared with almost the same staining pattern, grade confounded, and lesser staining intensity compared to intermediate and high B-cell tumors (Figure 2C).

VCP expression in PBMCs and canine lymphoma cell lines (CLBL-1, 17-71, CL-1)

We further used immunoblotting to compared the expression of VCP in 3 canine lymphoma cell lines to PMBCs. Analyses included the B-cell lymphoma-derived lines CLBL-1 and 17-71, and the T-cell lymphoma line CL-1. A significant increase ($P < 0.05$) in VCP expression was associated with the lymphomas cell lines compared to PBMCs (Figure 3A). Moreover, the protein was expressed at high levels in each cell line individually when compared to the blood isolated normal cells (Figure 3B).

K-48 polyubiquitinated protein expression in canine lymphomas

To determine if the variations of VCP protein expression within B-cell or T-cell tumors affect the levels of polyubiquitinated proteins within the cell, immunoblotting was done using 21 B-cell lymphomas and 18 T-cell lymphomas. Surprisingly, it was found that the differences were not statistically significant ($P > 0.05$) when compared between types, grades or to normal lymph nodes (Figure 4A-B). Increased VCP expression in intermediate and high-grade B-cell lymphomas therefore did not coincide with an expected decrease in K-48 polyubiquitinated protein.

Serum VCP protein expression in canine lymphomas

To assess serum VCP protein expression in canine lymphomas, sera from 22 healthy dogs were compared by immunoblotting with 20 B-cell and 18 T-cell lymphomas diagnosed animals. Analyses of immunoblot band intensity found VCP expression to be marginally increased in the sera of some dogs with cancer compared to healthy subjects, however the differences were not statistically significant ($P > 0.05$) (Figure 5). In addition, we further investigated any potential correlation between VCP expression levels in sera versus tumors (not shown). However, no correlation was detected ($r=0.062$).

DISCUSSION

VCP seems to be a cancer biomarker of interest in human oncology with several studies examining its expression in numerous types of tumor tissues and sera of cancer patients (12, 31-37, 59-62). A recent study has also indicated that VCP expression may be an

independent prognostic factor for the overall survival and disease recurrence in primary orbital MALT lymphoma, a type of B-cell lymphoma (37). In veterinary medicine, there are no published studies on VCP expression in lymphoma tissue, or any other cancer for that matter, and to our knowledge no publications exist on the expression of the protein in sera of dogs with lymphoma. In this report, we verified elevated VCP protein expression in canine lymphomas and we are the first to show that VCP is overexpressed in B-cell lymphomas, specifically in high-grade tumors. It remains to be determined if this increase in the protein's levels is a cause or a consequence of tumor development, what the biological significance might be, and what its relatedness might be to the tumors' grade. Additional analyses will therefore be required to answer these questions. It should however be noted that since VCP is involved in cancer cell proliferation and metastasis (63), indicating that it plays a crucial role for cancer biology, this could be an indication malignant B cell lymphomas require elevated levels of VCP to reduce PICHROS (protein induced chromatin stress) thus avoiding apoptosis. The exact mechanism of how malignant B-cell would produces such a high tension on their chromatin is unknown, but a possible explanation might be simply related to the higher growth rate and accumulation of genetic mutations specific to cancer cells (64). This would be entirely consistent with our finding that VCP expression in high-grade B-cell tumors is comparable to that found in the germinal centers of lymph nodes, where B-cells have a higher proliferation rate (65). Furthermore, low grade B-cell lymphomas, being characterized as slow-growing tumors, had, in immunohistochemistry trials, VCP levels comparable to those found in the mantle of lymph nodes, where cells are more differentiated and less proliferative (66). It therefore seems reasonable to propose that the increased VCP expression that we observed in B-cell lymphomas could be reflective of both malignancy and proliferative

activity, making VCP a potentially clinically useful tool for predicting tumor recurrence and patient prognosis.

Motivated by the increased values of VCP expression in high-grade B-cell tumors, we have carried out an experiment to measure its expression in PBMCs and cancerous cell lines 17-71, CLBL1, CL1. This finding confirms and adds to our previous discovery, as the B-cell lymphoma cell lines, 17-71 and CLBL1 had increased VCP expression than normal blood mononuclear cells. Corroborated with the previous argument, this may indicated that malignant cells are dependent on VCP overexpression, making this protein a potential pharmaceutical target in treating lymphoma in dogs.

Despite our expectations, we found that there was no correlational pattern between the accumulation of K-48 polyubiquitinated proteins and tumor's grade. The western blot analysis revealed, surprisingly, statistically insignificant levels of polyubiquitinated proteins in all lymphoma types. However, taking in account that cancerous cells, compared to their normal counterparts, have a higher mitotic rate (64), making them more prone for DNA damage and misfolded protein accumulation (REF), an accumulation of "signaled for degradation" proteins should have been observed in high-grade tumors where cellular division is significant. This finding suggest that high-grade B-cell lymphoma cells are likely react to an increase in polyubiquitinated proteins, associated with increased proliferative related mutations, by up-regulating VCP expression. Due to the role VCP has in the maturation of autophagic vesicles and in the ERAD (67, 68), the two major systems for protein elimination, the degradation of proteins is accelerated, returning them to a level that is comparable to that found in lower-grade tumors and normal cells. To answer these questions, addition work will be required in

order to make a clear correlation between VCP's overexpression and the decrease in polyubiquitinated proteins in cancerous cells.

Unexpectedly, we failed to identify any relevant increase of VCP protein in the sera of dogs with lymphoma, and also failed to identify a correlation between serum and tissue levels of VCP in canine lymphomas. This is in contrast to the study of Laguë *et al* where VCP serum levels were significantly increased in human patients with NHL (12). It is often the case that there is no correlation between protein expression in cancer tissue and circulating levels of the protein (69). The reason for this observation is possibly due to the impossibility of the protein to access the circulating system or just a simple matter of dilution. Perhaps the VCP production by tumor cells in the selected dogs for this study was not strong enough to give significant western blot detection. Moreover, our analysis were limited by the relatively small sample size available, however it should be noted that there is a possibility that VCP's expression pattern in human lymphoma is different than in its canine counterpart. Further experiments will be required to explore these novel theories.

In this study, the VCP expression levels were analyzed by western blotting and immunohistochemistry. We reported a clear correlation between VCP expression and canine B-cell lymphomas of high-grade. Moreover, using either one or both methods, several studies have reported the same positive correlation in hepatocellular carcinoma (35), gastric carcinoma (34), gingival squamous cell carcinoma (61), esophageal carcinoma (60) and several other human cancers (12, 31-33, 36, 59, 62) . Taken together with the other results, this shows the reliability of immunohistochemical and western blot method for the evaluation of VCP in canine malignancies.

In summary this study shows for the first time that VCP expression, as determined by western blot and immunohistochemistry, is a new malignancy marker for canine high-grade B-cell lymphoma. This represents the first step towards establishing VCP as a valuable clinical tool for diagnosis, predicting tumor recurrence and prognosis, which could eventually apply to the stratification of dogs with lymphoma for standardized treatment protocol. Based on our results, we conclude that VCP expression positively correlates with malignancy in canine B-cell lymphomas and might represent a novel way to explore effective treatment modalities for lymphoid malignancies.

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Sabin Filimon, performed research, collected and analyzed data, and wrote the paper. Mayra Tsoi, Charlène Rico collected data. Marie-Ève Nadeau designed and performed research. Marilène Paquet analyzed data. Derek Boerboom designed research, analyzed data and wrote the paper.

GRANT SUPORT

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CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest.

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FIGURE LEGENDS

Figure 1. VCP protein expression in canine lymphoma tumors. A) Analysis of the levels of VCP in low (n=6), intermediate (n=7), and high-grade (n=9) B-cell lymphoma tumors by western blot. Data are shown as means (columns) ± SEM (error bars). The results showed that the level of VCP is increased in high-grade tumors compared to low grade ones: *:$P<0.05$. B) Using the same method, we further examined the expression of VCP in low (n=4), intermediate (n=5), high (n=9) T-cell lymphomas tumors. Data are shown as means (columns) ± SEM (error bars). No significant statistical difference was recorded: $P>0.05$. C) Representative western blot images form the analyses done in A and B. The first two lanes represent the levels of VCP in normal lymph nodes, followed by the expression levels of the protein in B-cell and T-cell lymphomas. β-Actin (ACTB) was used as a loading control.

Figure 2. Western blotting analysis of VCP in PBMCs and lymphoma cell lines (17-71, CL-1, and CLBL-1). A) Detection of the expression of VCP in PBMCs (n=7), used as control, and canine lymphoid tumor cell lines (n=3). Data are shown as means (columns) ± SEM (error bars). Significant difference from control ($P<0.05$) is indicated with and asterisk (*). B) Western blot showing that the lymphoma cell line demonstrated markedly increase VCP expression levels compared to control PBMCs. β-Actin (ACTB) was used as a loading control.

Figure 3. Immunohistochemical analysis of VCP expression in B-cell and T-cell lymphomas compared to lymph nodes . A) Photomicrograph of a normal lymph node

expressing different levels of VCP in the germinal center compared to the mantle. The dotted line delineates the two histological areas. B) Sections from representative low, intermediate, and high-grade B-cell lymphoma tumors. Note positive correlation of the increasing staining pattern with the tumor grade. C) Sections from representative T-cell tumors, exhibiting weak VCP staining and no significant increase in the protein levels among the low, intermediate, or high grades. Original magnification, 400X (A-C).

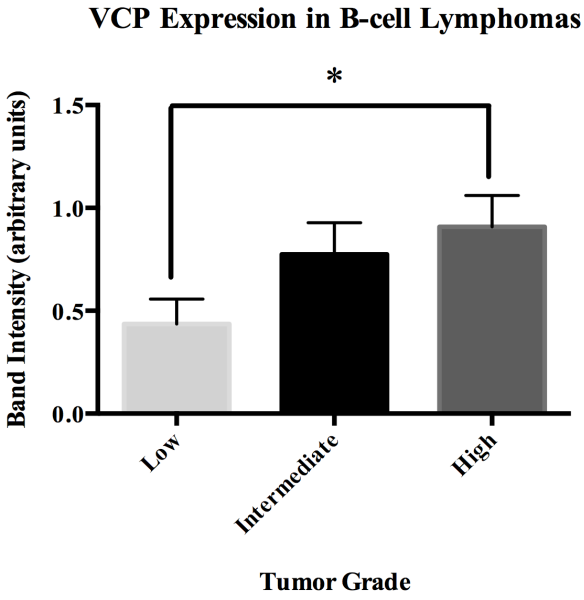
Figure 4. K-48 Polyubiquitinated protein expression in canine lymphomas tumors. A), B) Western blot analysis of the expression levels of K-48 polyubiquitinated protein in low (n=6), intermediate (n=6), and high-grade (n=9) B-cell lymphomas tumors and low (n=4), intermediate (n=5), and high-grade (n=9) T-cell lymphomas tumors. Data are shown as means (columns) ± SEM (error bars). No significant statistical difference between groups was detected: $P>0.05$. C) Representative western blot images from the analyses shown in A and B. The first two lanes represent the levels of K-48 polyubiquitinated in normal lymph nodes, followed by the expression levels of the protein in B-cell and T-cell lymphomas. β-Actin (ACTB) was used as a loading control.

Figure 5. Serum VCP expression in canine lymphomas. Western blot analysis of the levels of VCP expressed in the serum of healthy dogs (n=22) compared to dogs diagnosed with high grade B-cell (n=9) and T-cell (n=9) lymphomas, intermediated grade B-cell (n=6) and T-cell (n=5) lymphomas, and low grade B-cell (n=5) and T-cell (n=4) lymphomas. Data are shown as means (columns) ± SEM (error bars). No significant statistical difference was recorded: $P>0.05$.

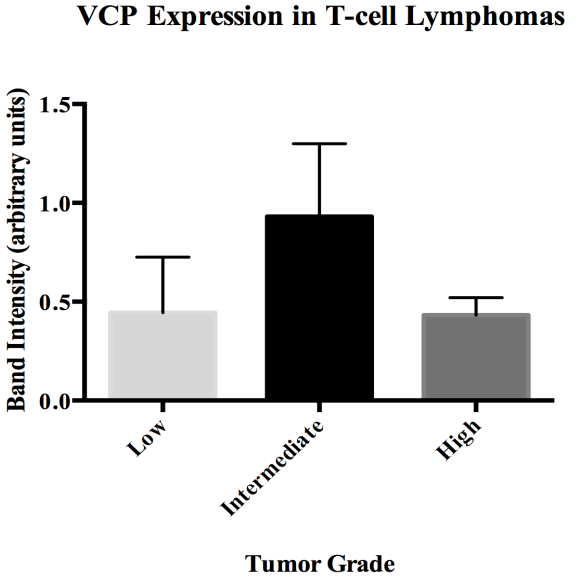
A

FIGURE 1

B


VCP Expression in B-cell Lymphomas
*
Band Intensity (arbitrary units)
1.5
1.0
0.5
0.0
Low
Intermediate
High
Tumor Grade

C


VCP Expression in T-cell Lymphomas
Band Intensity (arbitrary units)
1.5
1.0
0.5
0.0
Low
Intermediate
High
Tumor Grade

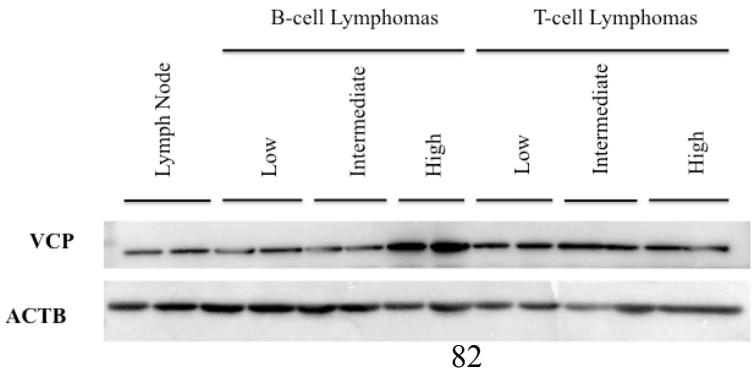
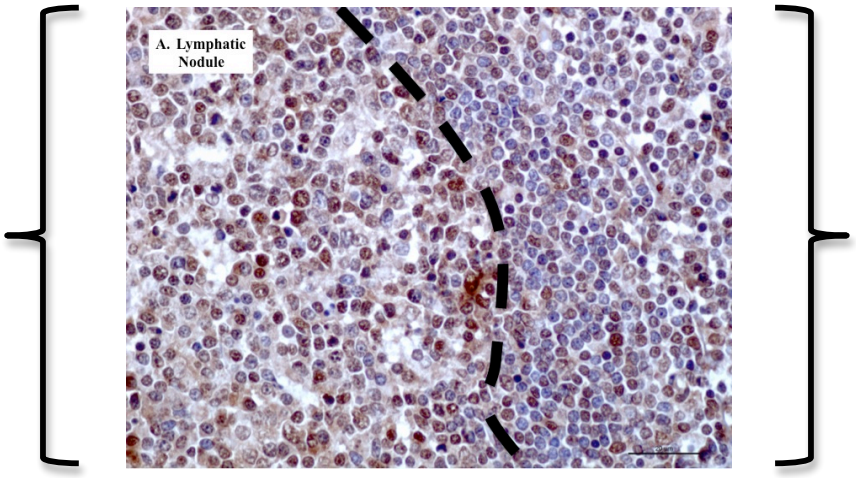
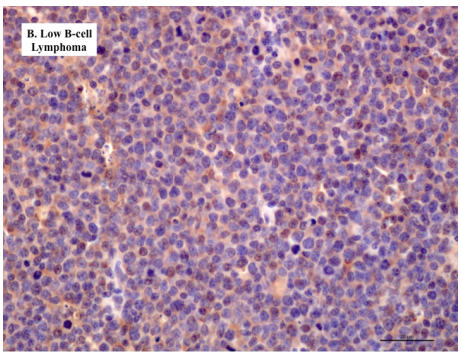

B-cell Lymphomas
T-cell Lymphomas
Lymph Node
Low
Intermediate
High
Low
Intermediate
High
VCP
ACTB

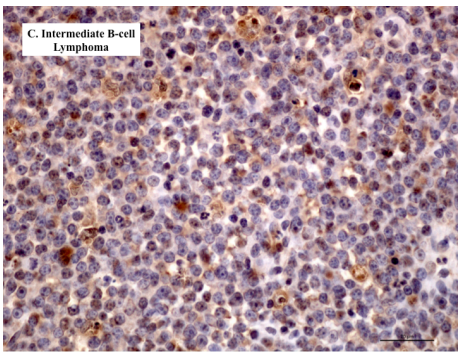
FIGURE 2

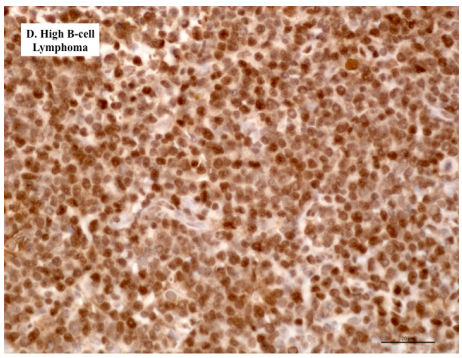
Germinal Center

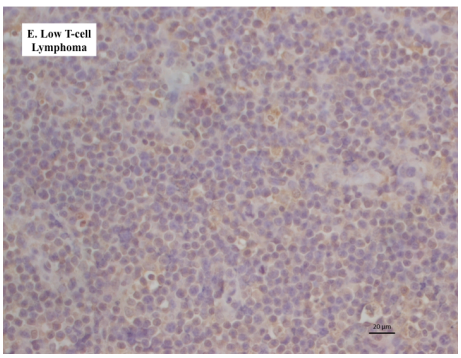
Mantle

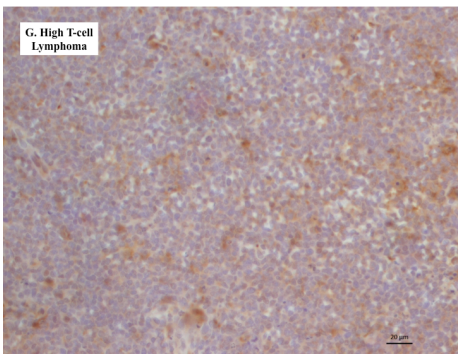












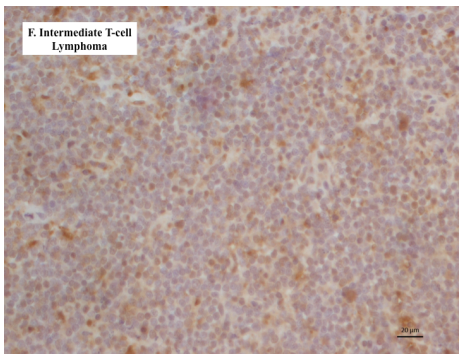
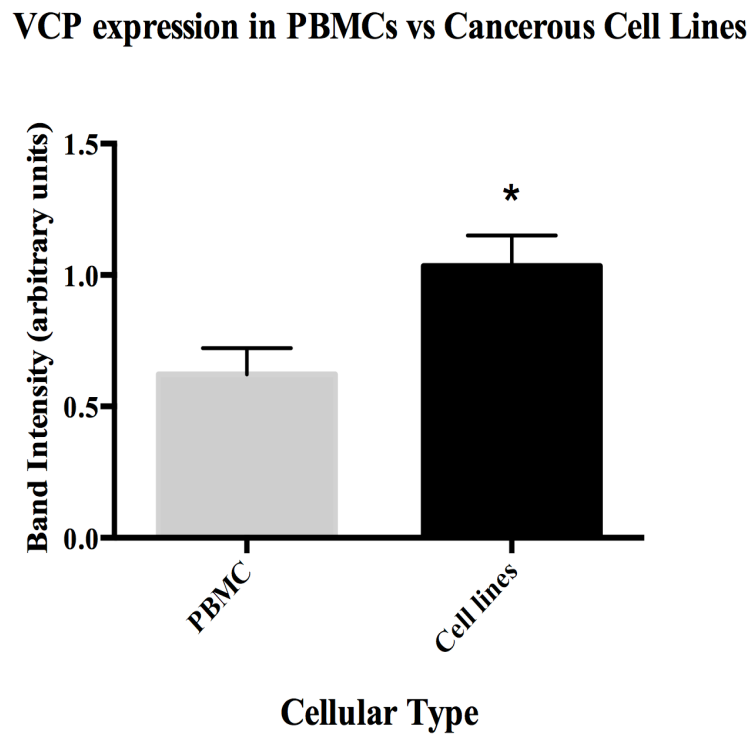


FIGURE 3


VCP expression in PBMCs vs Cancerous Cell Lines
Band Intensity (arbitrary units)
1.5
1.0
0.5
0.0
*
PBMC
Cell lines
Cellular Type

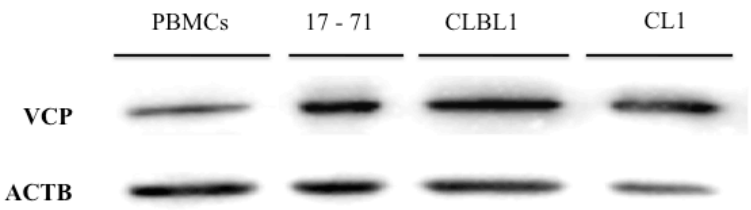
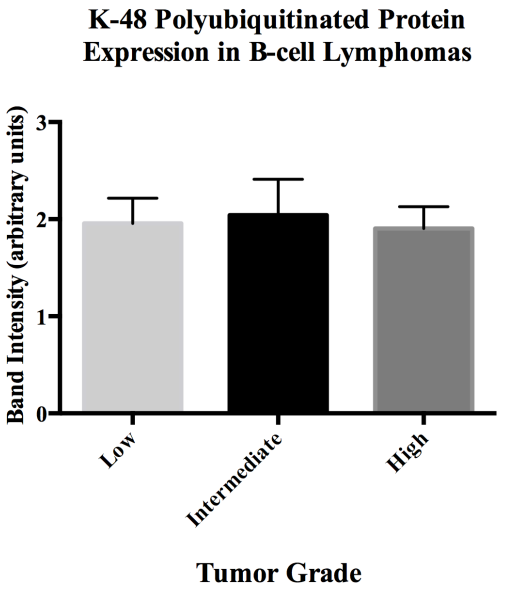
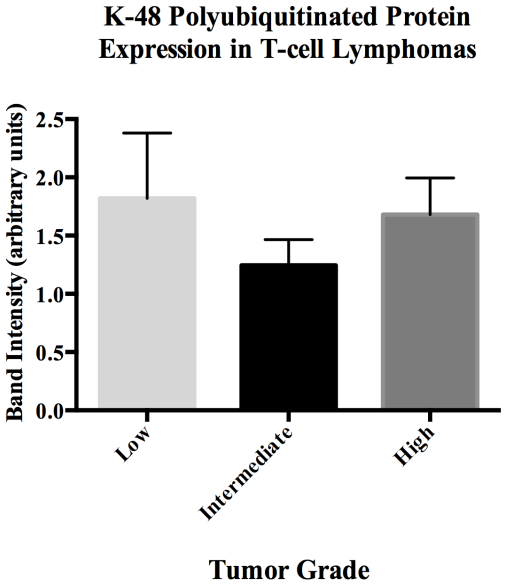

PBMCs
17 - 71
CLBL1
CL1
VCP
ACTB

FIGURE 4


K-48 Polyubiquitinated Protein
Expression in B-cell Lymphomas
Band Intensity (arbitrary units)
3
2
1
0
Low
Intermediate
High
Tumor Grade


K-48 Polyubiquitinated Protein
Expression in T-cell Lymphomas
Band Intensity (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Low
Intermediate
High
Tumor Grade

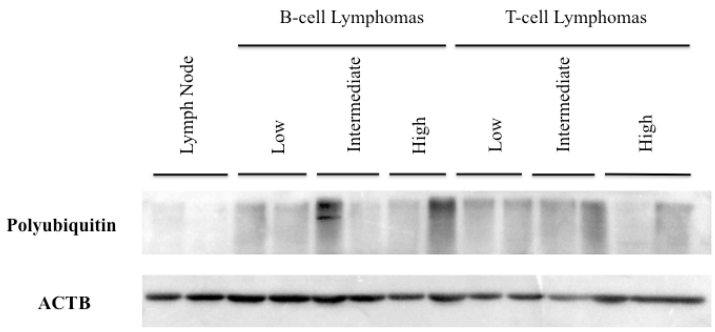
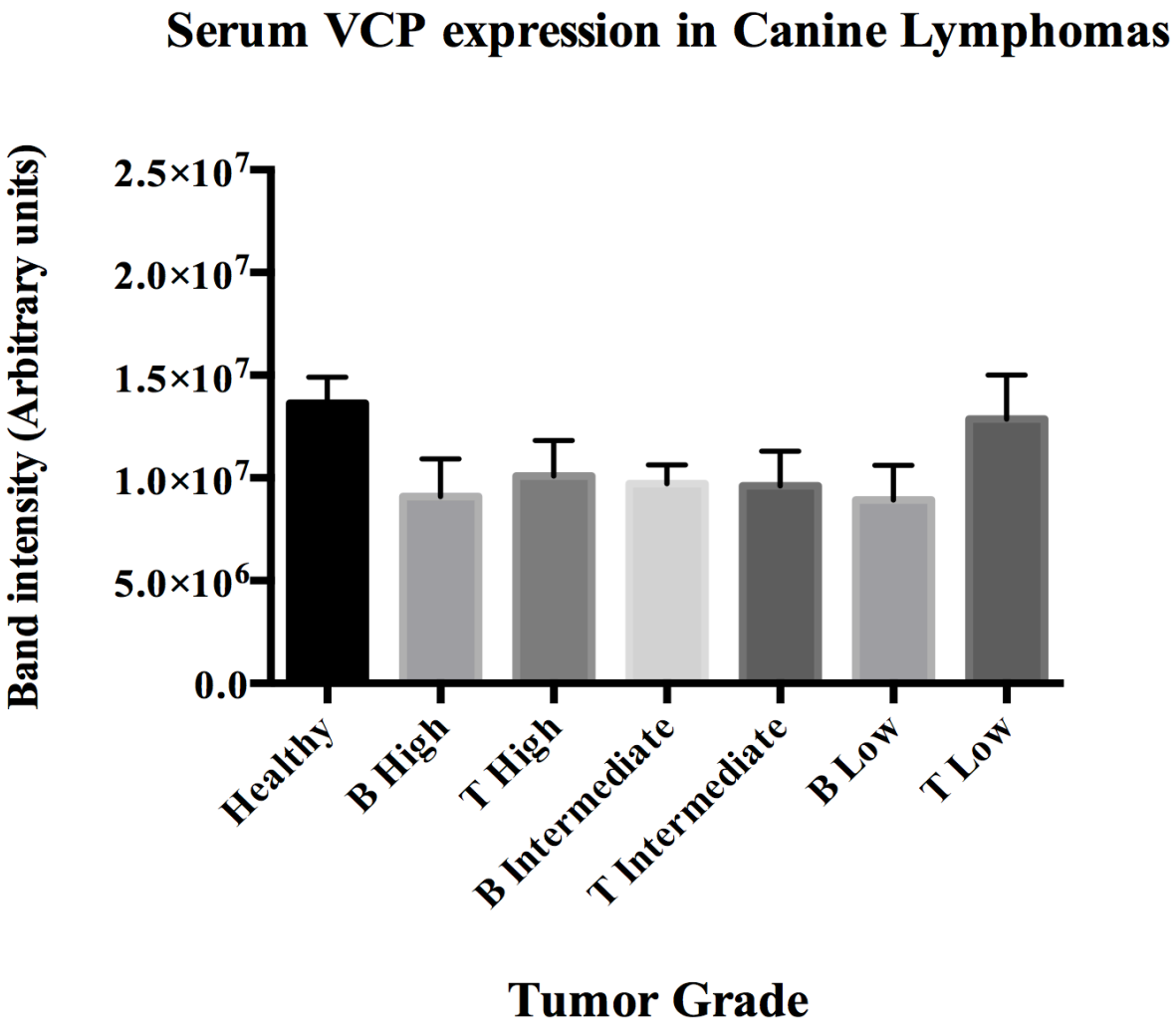

B-cell Lymphomas
T-cell Lymphomas
Lymph Node
Low
Intermediate
High
Low
Intermediate
High
Polyubiquitin
ACTB

FIGURE 5


Serum VCP expression in Canine Lymphomas
Band intensity (Arbitrary units)
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
Healthy
B High
T High
B Intermediate
T Intermediate
B Low
T Low
Tumor Grade

General discussion

Lymphoma is one of the most common cancers in dogs and the most common hematopoietic malignancy. Annually, anywhere between 24 to 107 new cases per 100 000 dogs will be reported (22, 23), however it is believed that the true incidence to be in the thousands (24). Besides its high occurrence within the population, the disease is also known as a particularly aggressive cancer with a range of development rates (30). Although the genetic changes that lead to the disease's initial development can take decades, the cancer grows quickly and spreads to other parts of the body within a matter of months (30). For the animals undergoing chemotherapy, a high rate of remission can be recorded (22), unfortunately the overall survival 2 years after the initial diagnosis, remains under 10% (122). The low success of our treatments is mainly due to inexistent screening tools, prognostic indicators, and presence of residual disease after treatment. Despite this, few studies have focused on the validation of cancer biomarkers in veterinary medicine, valuable tools that could be useful in the earlier recognition of disease and its relapse, better diagnosis, and even as future therapeutic targets.

Medical advancements in the treatment of canine lymphoma will not only improve the quality and length of life of our companions, but also may further characterize an appropriate model in comparative oncology. Canine lymphoma provides a logical tool for translational research for human oncology. For example, the lymphoid neoplasm incidence in humans is reported to be as high as in dogs, more specifically 386 000 new cases world wide in 2012 (123, 124). Furthermore, high-grade NHL was deemed comparable clinically and histologically with its canine counterpart (125). The lymphoproliferative disorder is

advantageous for study in a canine model because of the short remission times and the standard surgical removal of tumors followed by the histopathological analysis as a routine part of therapy (126), facilitating rapid data accrual. Moreover, veterinary oncologists have at their disposal the same chemotherapeutical drugs used in human medicine (most commonly vincristine, cyclophosphamide, L-asparaginase, and doxorubicin) proving that the treatment protocols could be very similar. (125). Finally, recent sequencing of the dog genome revealed around 19 300 protein-coding genes almost all having a human homologue (127). These considerations, taken together with the limited level of heterogeneity within the canine breeds, make the dog a powerful spontaneous model to identify possible heritable risks and genetic factors that are of pathological and clinical significance in lymphoma.

An important advance in the study of potentially novel veterinary cancer biomarkers came from the expansion of the field of proteomics. The idea behind this large-scale study of proteins is that malignant cells can increase the production of substances that are present in very low or undetectable concentration in the tissue or sera of healthy animals, but can prove valuable markers in cancer patients. This was the case of VCP, which was identified in several studies as a highly sensitive indicator of malignancy, often increased in sera and tumor tissue of cancer patients who do not otherwise display increased levels of the conventional markers for their cancer type (17, 18, 112-120, 128). More specifically, these studies determined that higher VCP levels are an indicator of poor prognosis in pancreatic ductal adenocarcinoma (114), pancreatic endocrine neoplasms (118), colorectal carcinomas (115), non-small-cell lung carcinoma (113, 128), hepatocellular carcinoma (119), gastric carcinoma (116), esophageal carcinoma (112), follicular thyroid cancer (120), gingival squamous cell carcinoma (117) and prostate cancer (18).

VCP, a type II member of the AAA+ ATPase family, is a highly conserved molecular chaperone that acts as a ubiquitin segregase that remodels multimeric protein complexes by extracting polyubiquitinated proteins for recycling or degradation by the proteasome (96). Recent descriptive studies have shown that the protein is associated with anti-apoptotic functions and metastasis, making it an important factor for the survival and division of cancerous cells (112-117, 119, 120). These observations, taken together with the elevated VCP's levels in the aforementioned human carcinoma, seem to suggest that cancers cells rely on intact VCP functions, identifying it as a potential valuable tool for cancer detection and monitoring.

One of the major goals of this thesis was the examination of VCP as potential biomarker for canine lymphoma. Biomarkers can be used for purpose of screening, diagnosis, prognosis, predicting drug efficacy, as well as an indicator of cancer recurrence (129). Despite their extensive use in human oncology, there are currently no markers used in veterinary medicine. Aberrant expression of VCP in canine lymphomas may potentially be a useful biomarker capable of predictive prognosis and cancer recurrence. As such, in this study, we are the first to show that VCP expression positively correlates with malignancy in B-cell lymphomas. Our research provides a new focus on the potential this protein has as a diagnostic biomarker for canine lymphoma. However, this hypothesis requires further investigation and some critical experiments, such as comparing VCP expression across several canine carcinoma and normal tissue, are missing in this work that would otherwise strengthen this conclusion.

Having identified a significant increase of VCP in high-grade B-cell lymphoma and intrigued by the possibility that the protein could be a marker of malignancy in canine

lymphoproliferative disorders, we wished to establish the commonality of VCP overexpression in lymphoma cell lines. Thus, in second part of our research we performed immunoblotting analysis of three lymphomas cell line, CLBL1, 17-71, and CL1. Preliminary evidence from our laboratory indicates that lymphoma cell lines had increased VCP expression compared to PBMCs. In accordance with the observations of other studies (93, 106), this work adds to the hypothesis that VCP may be required for tumorigenic transformation and metastatic growth, making the protein a promising novel cancer therapeutic target.

Our research has provided new evidence in establishing VCP as a malignancy and possible new diagnostic biomarker in veterinary medicine. However our data also conflicts with findings of other studies. One previous study identified elevated serum VCP levels in a clinically significant proportion of human patients with NHL (17). Here, we investigated VCP expression in sera of dogs with lymphoma and attempted to make a correlation between sera and tumor levels of the protein. Our analysis did not identify significantly higher levels of VCP in the sera of sick dogs despite a clear increase in VCP expression in high-grade B-cell lymphoma tumors relative to low-grade tumors. Furthermore, the study of Laguë (2012) found increased VCP levels in sera of several different cancer patients, such as granulosa cell tumors, ovarian carcinoma, breast cancer, and colon cancer, indicating the potential use of VCP as highly sensitive serum marker for several human cancers (17). The reasons we were not able to find the same high VCP serum levels as in human patients remain unclear. It is possible that dogs with lymphoma that have a tumor burden sufficient enough to permit VCP to enter the circulatory system do not live very long, succumbing to the disease before the protein's levels are detectable. Unfortunately, we did not have information on the complete

staging of each case; as such comparison of tumor burden between specimens could not be conducted. Another possible reason for this statistically undetectable serum VCP levels might be due to tumor viability. Necrosis, apoptosis, and cell fragility are hallmarks of large tumors, increasing the leakage of proteins into the blood. It is therefore tempting to speculate that our dogs where not as far advanced into their disease to allow statistically significant amounts of VCP to accumulate in their sera. Once again, due to lack of histopathological data, the amount of necrosis and apoptosis among different tumor types were not compared in this study. Finally, there is a distinct possibility that there are genetic differences between humans and dogs, which somehow prevent VCP reaching the circulatory system, even if the protein is overexpressed in high-grade tumors. While our data suggests that serum VCP levels may not be useful as a screening method for canine lymphoma, further studies of circulating VCP in dogs with cancer, including a larger sample of dogs with complete medical files, longitudinal studies with before and after treatment analysis, and improved detection methods, such as ELISA, is warranted.

In another study analyzing the critical role of VCP in the pathogenesis and progression of human non-small cell lung carcinoma (NSCLC), the authors show that VCP is significantly overexpressed in cancerous cells (128). Moreover, they observed, an accumulation of ubiquitinated proteins in NSCLC cell lines and tissues as compared to the normal controls (128). These results seem in contradictions with ours since, surprisingly, we were not able to detect any significant increase of polyubiquitinated proteins in lymphoma tissue. Although, this difference might be simply explained by the clinical and pathological distinction between the two cancers and the genetic differences between the two species, further analyses are required to elucidate the mechanisms behind the accumulation of ubiquitinated proteins or the

lack of thereof. Boyault *et al.* have demonstrated such a mechanism in which a balance between histone deacetylase 6 (HDAC6) and VCP might determine the degradation or accumulation of ubiquitinated proteins within the cell (130). As such, an increase in VCP, as the one observed in high-grade B-cell lymphomas, would lower the levels of polyubiquitinated proteins (130). On the other hand, an increase of HDAC6 and a subsequent decrease of VCP in the cells seem to favor the accumulation of polyubiquitinated proteins (130).

Differences between our results and those presented in the aforementioned studies might also reflect the limitations of the present research. Among them we can mention: the small N, the unknown normal expression of VCP in dog sera and tissue, the use of western blot as means of quantification, and the lack of a pure B-cell population as normal control. All these reasons combine to make immunoblotting quantification and immunohistochemistry staining difficult to interpret and in some situations, such as data analysis, impossible to run statistical tests that require normal distribution and variance. Some of these problems can be addressed in future studies by screening of a much larger cohort in order to gain statistical power and determine the spectrum of VCP expression in healthy animals and those with cancer. Large scale measurement will require the development of a high-throughput ELISA assay design for VCP, as western blots present certain pitfalls in terms of densitometry quantification and technical practicality that could have a major influence on the reported results (131). Finally, although normal lymph nodes might represent a good control for tumoral tissue, they do not contain a true population of normal lymphocytes. The existence of different other types of cells might influence the measured intensity of western blot's protein bands, thus justifying the use of a pure population of B-cell lymphocytes as control in future studies.

The fact that this study included for analysis only normal dogs and dogs with lymphomas is both a strength and limitation. The strength of analyzing VCP only in lymphoma is that the resulting protein overexpression can be considered to be sensitive to this type of tumor. However, it is also a limitation in that VCP might not be specific only to lymphoma, but present increased levels in other canine cancers, as determined for human malignancies by Laguë *et al* (17). Furthermore, we are mindful that certain inflammatory chronic diseases (CID) may cause VCP levels to increase, as it occurs with essentially all serum markers described to date (132). An ideal study design should also include samples from dogs with chronic inflammatory diseases such as, chronic arthritis, renal disease, mild liver disease, and/or inflammatory bowel disease. These conditions are frequently encountered as concomitant illnesses in cancer patients and the question of whether or not VCP is elevated in the CID population has not been fully answered. Incidence of CID is at its highest in dogs over 10 years of age, the same population that is likely to be often diagnosed with cancer (32). Therefore, it would be wise to clarify if there is a relationship between CID and VCP before using this marker to diagnose or make prognosis in canine lymphoma.

The authors also consider that it would have been best if the study had age and treatment-matched dogs. Since old age is significantly related to chronic diseases and previous medical conditions that required treatment, this study would have been better served if age and treatment-specific VCP values/cut-offs could have been reported. Understanding if VCP levels are altered by advanced age or treatment protocols could be an important objective for a further study. Furthermore, a better study design would also include a pre and post-treatment analysis of the protein levels. This would serve to establish VCP as a future recurrence and treatment follow-up marker.

Another limitation of this study is the absence of sufficient clinical data from CCOGC to establish any potential correlation between VCP levels and clinical features of dogs with lymphoma. An interesting correlation that could have brought more insight to the recorded VCP levels is the association between the protein's expression and the tumor size at the time of the excision. Moreover, additional information on recurrence would have allowed us to determine if higher VCP levels correspond with the end of the remission. Although we did received the survival time of each dog from the moment of diagnosis, no survival related correlation was performed since the files did not mentioned if the dog succumbed to its disease or was euthanized at the request of the owner. This problem can be addressed in future studies by asking the exact reason of death and eliminating the euthanasia cases.

Only a very limited number of anti-canine antibodies are commercially available as the dog model system is only recently gaining acceptance. Thus, short of generating new antibodies, the only way to study protein expression in canine samples is to validate currently available antibodies. Many of these are anti-mouse, anti-rat, anti-rabbit, and anti-human antibodies and, considering that VCP is a highly conserved essential AAA+ ATPase protein (93), they had a good chance of working in the canine model. We approached antibody validation by making sure a single band of the expected size, 97 kDa, was obtained in preliminary western blotting. This, undoubtedly, contributed to the reliability of VCP measurement in canine malignancies by western blot and immunohistochemistry.

Finally, another slight limitation to this study is that the control dogs (normal dogs) came form a university-wide announcement. An ideal control population would have been recruited from the same population as its cases. Therefore, ideally all subjects would have

been recruited from CCOGC. However, it is not unusual for preliminary studies to have control samples that are not collected through the exact avenues as the cases.

Further studies should focus on the sensitivity of this marker in a broader canine population. A larger case-control study in populations affected by canine lymphoma needs to be conducted. Pre-treatment blood draws should be collected when a cancer case is first suspected. Cases also should be followed-up long-term and serial VCP measurements should be taken (at regularly scheduled follow-up visits) after chemotherapy treatments are completed, to determine if their values decrease in the period after treatment. The same procedure should be followed during remission in order to detect any association between VCP levels and the return of the disease. By conducting the study in this manner, further correlations that could establish VCP as a promising prognosis biomarker could be made. Furthermore, important confounding factors such as, the affect of chemotherapeutic drugs on VCP's levels, could be eliminated.

If a larger case-control study were to verify VCP as a biomarker for canine lymphoma, then studies could also be conducted in other types of cancers. For example, a study could be conducted in dogs that have mammary cancer, another type of malignancy very often encountered in veterinary medicine (21). One study has already shown that women with breast cancer express higher VCP levels, without displaying increased levels of specific serum tumor markers for their cancer type (17). Since the cancer cells seem to be dependent on intact VCP functions for tumor growth and metastasis (128), it is not unlikely that the protein could be elevated in more than one type of canine cancer.

Determination of the efficacy of this marker in a separate large group, using healthy controls could represent the main goal of a future study. If the marker is again decided to be

sensitive and specific enough to warrant further study, a separate group of dogs with benign tumors such as, infiltrative lipoma, histiocytoma, basal cell tumor, cysts, fibroma, etc., should be studied in order to determine the usefulness of VCP in discriminating between dogs with cancerous and non-cancerous lumps.

Finally, this study was designed to determine the potential use of VCP as sensitive cancer biomarker for canine lymphoma, not to elucidate causes or pathways. There are a number of additional studies that could be performed to further investigate the roles of VCP in canine lymphoma. For instance, inhibition of VCP expression in tumor cell lines with the VCP inhibitor Eeyarestatin 1 (Eey1) (133) could allow long-term chemosensitivity and proliferation assays as well as other studies of tumor aggressiveness (e.g. colony formation) to be performed. As the protein seem to have a low expression in normal tissues, one could hypothesize that systemic blocking of VCP in cancer patients would have only limited side effects and may prove an effective method of limiting tumor cell invasion and metastasis. Similarly, stable blocking of VCP in PBMCs may provide further insight into its cellular role and the side effects if used as a future therapeutic target. Assessment of this methodology in canine models may provide an excellent platform for the development of a new anti-lymphoma treatment and to obtain a better understanding of the functions VCP has within the cell.

The extent to which VCP will be a clinically useful cancer biomarker, beneficial in the prognosis and monitoring of high-grade canine lymphomas, will be fascinating to learn as the field moves forward. The contribution made by this thesis defines the base of a pyramid of investigations into the area of cancer biomarkers in veterinary medicine. Many questions remain about the specific nature and potential functional role of VCP in tumorigenesis.

Therefore, more work remains to be done for a more complete characterization of VCP and establishing a direct causal link between its overexpression and canine lymphoma.

Conclusion

The present study has examined, for the first time, VCP expression in sera and tumor tissues of dogs with lymphomas via both immunohistochemistry and western blotting. Statistically significant differences were detected in VCP expression between high-grade and low-grade B-cell lymphomas, but not in T-cell lymphomas or serum samples relative to controls. In conclusion, we report that VCP levels correlate with malignancy in canine B-cell lymphoma. Although further studies will be necessary to substantiate the role of VCP as a potential new prognostic biomarker, these findings represent the first step towards an important advancement in the field of veterinary tumor biomarkers.

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