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Prognosis of malignant lymphoma in dogs and correlation to thymidine kinase (TK1) - a retrospective study

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Prognosis of malignant lymphoma in dogs and correlation to thymidine kinase (TK1) - a retrospective study

Prognos vid malignt lymfom hos hund och korrelation till thymidinkinas (TK1) - en retrospektiv studie

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SUMMARY

Malignant lymphoma is the most common canine hematopoietic neoplasm, with an estimated incidence rate of 20-100 cases per 100,000 dogs. Malignant lymphoma can arise in any organ containing lymphoid tissue and is characterized by malignant proliferation of lymphoid cells. Lymphomas most commonly occur in middle-age to older dogs, where the age category six to ten years seems to be predisposed. Although any dog breed can be affected, middle-sized to large dog breeds are overrepresented.

There are many similarities between canine lymphoma and human non-Hodgkin lymphoma (NHL) and the canine lymphoma serves as a good large-animal model for the human NHL.

Thymidine kinase is a cellular enzyme involved in the salvage pathway of DNA-synthesis. Thymidine kinase 1 (TK1) is regulated by the cell cycle and is located in the cytoplasm. Moreover, TK1 is converting thymidine to thymidine monophosphate, and is related to DNA replication and cell proliferation, which makes it possible to use as a proliferation marker in malignant tumors. Thymidine kinase 1 activity is present in the early S-phase of cell division in normal cells. However, in abnormal cells the activity of TK1 is often much higher. Previous studies have shown that TK1 is useful in diagnosis and prognosis in humans and dogs with malignant lymphoma.

The challenging area with canine lymphoma is rarely to set the diagnosis, but to predict the prognosis and monitor the remission status in an objective way. Moreover, it is a challenge to detect relapse in treated patients. The purpose of the study was to compare initial blood parameters, for example TK and CRP, with clinical symptoms, clinical stage and prognosis. The aim was to improve the prognostication and thereby be able to select the right patients for further treatment.

The results of the study showed that there were no significant correlation between a higher initial TK value and a shorter survival time and poorer prognosis. Neither could a high CRP level, hypercalcemia, anemia, a high clinical stage, a T-cell lymphoma, B symptoms nor a high grade tumor be correlated to a shorter survival time. The most important thing for a longer survival time was, according to this study, treatment with the ADRIA-plus protocol. This treatment resulted in a significantly longer survival time than the treatment with prednisolone or doxorubicin alone. Further studies are needed to investigate if the above mentioned parameters are useful in prognostication in dogs with malignant lymphoma.

SAMMANFATTNING

Malignt lymfom är den vanligaste hematopoetiska neoplasin som drabbar hundar, med en incidensrat på 20-100 fall per 100 000 hundar. Malignt lymfom kan uppstå i alla organ som innehåller lymfoid vävnad, och karaktäriseras av malign proliferation av lymfoida celler. Lymfom drabbar framför allt medelålders och äldre hundar, med störst risk för hundar i åldersgruppen 6-10 år. Alla raser kan drabbas av lymfom, men medelstora och stora hundraser är klart överrepresenterade.

Det finns många likheter mellan malignt lymfom hos hund och humant non-Hodgkins lymfom (NHL) och hunden fungerar i detta fall som en bra stordjursmodell för humana NHL.

Thymidinkinas är ett cellulärt enzym som är involverat i "salvage pathway" i DNA-syntesen. Thymidinkinas 1 (TK1) regleras av cellcykeln och finns lokaliserat i cytoplasman. TK1 omvandlar thymidin till thymidinmonofosfat, och kopplas till DNA-replikation och cellproliferation, vilket möjliggör användandet av TK1 som en proliferationsmarkör hos maligna tumörer. TK1-aktivitet förekommer i tidig S-fas vid celledelning i normala celler. I onormala celler är däremot TK1-aktiviteten ofta mycket högre. Tidigare studier har visat att TK1 är användbar vid diagnostisering och prognostisering av malignt lymfom hos både människor och hundar.

Utmaningen med malignt lymfom hos hundar är sällan att ställa diagnosen, utan att förutspå prognos och monitorera remissionsstatus på ett objektivt sätt. Det är dessutom en utmaning att upptäcka sjukdomsåterfall hos redan behandlade patienter. Syftet med denna studie var att jämföra initiala blodparametrar, såsom TK och CRP, med kliniska symtom, klinisk gradering och prognos. Målet var att förbättra prognostiseringen och därmed möjligheten att välja ut rätt patienter som ska kunna genomgå behandling.

Resultaten i denna studie visade att det inte fanns någon signifikant korrelation mellan ett högre initialt TK-värde och en kortare överlevnadstid eller sämre prognos. Det fanns inte heller någon korrelation mellan en kortare överlevnadstid och ett högt CRP, hyperkalcemi, anemi, ett högt kliniskt stadium, T-cellslymfom, B-symtom eller en histologiskt höggradig tumör. I denna studie var den viktigaste faktorn för en längre överlevnadstid att hunden genomgick en behandling med ADRIA-plus-protokollet. Denna behandling ledde till en signifikant längre överlevnadstid jämfört med endast kortison- eller doxorubicinbehandling. Fler studier krävs för att vidare undersöka om ovan nämnda parametrar är värdefulla i prognostisering hos hundar med malignt lymfom.

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INTRODUCTION

Malignant lymphoma (also known as lymphoma or lymphosarcoma) is the most common canine hematopoietic neoplasm, with an estimated incidence rate of 20-100 cases per 100,000 dogs (Zandvliet, 2016). Malignant lymphoma can arise in any organ containing lymphoid tissue, and is characterized by malignant proliferation of lymphoid cells (Argyle *et al.*, 2009). Lymphomas most commonly occur in middle-age to older dogs. Dogs below four years of age are less likely to develop lymphoma, and the age category six to ten years seems to be predisposed (Simon *et al.*, 2008; Villamil *et al.*, 2009; Mirkes *et al.*, 2014). Although malignant lymphoma can affect any dog breed, middle-sized to large dog breeds are overrepresented. Some of the predisposed breeds are Bullmastiff, Boxer, Bernese mountain dog, Dogo de Bordeaux, Golden Retriever, German Shepherd, Labrador retriever and Scottish terrier (Villamil *et al.*, 2009; Vezzali *et al.*, 2010; Elliott *et al.*, 2013). Heritable factors may contribute to the increased frequency in these breeds (Elvers *et al.*, 2015; Tonomura *et al.*, 2015). Moreover, intact female dogs seem to have a decreased risk in developing malignant lymphoma. However, the risk does not differ between neutered female dogs and male dogs (Villamil *et al.*, 2009).

There are many similarities between canine lymphoma and human non-Hodgkin lymphoma (NHL), including clinical symptoms, molecular biology, treatment and treatment results. The dog's genome has been sequenced and the dog breeds represent closed gene pools (Berlin *et al.*, 2005). Furthermore, many dog owners want to treat their dogs diagnosed with malignant lymphoma. This makes the canine lymphoma a good large-animal model for the human NHL (Zandvliet, 2016).

There is no established cause for canine lymphoma to this day. Several predisposing factors have been suggested, for example living near industrial areas, exposure to chemicals, living near waste incinerators, radioactive or polluted sites and exposure to magnetic fields. Many other animal species have a species-specific leukemia virus, which makes it possible to believe that a canine "lymphoma virus" may exist. However, a viral etiology is still not generally accepted (Zandvliet, 2016).

Thymidine kinase (TK) is a cellular enzyme involved in the salvage pathway of DNA-synthesis. There are two different forms of TK; TK1 and TK2. Thymidine kinase 1 is regulated by the cell cycle and is located in the cytoplasm. Thymidine kinase 2 is located in the mitochondria and is expressed in all cells, without influence of the stage in the cell cycle (Arnér & Eriksson, 1995). Thymidine kinase 1 is converting thymidine to thymidine monophosphate, and is related to DNA replication and cell proliferation, which makes it possible to use as a proliferation marker in malignant tumors (He *et al.*, 2010). The TK1 activity is present in the early S-phase of cell division in normal cells. However, in abnormal cells the activity of TK1 is often much higher (Hengstschläger *et al.*, 1994).

Previous studies have shown that TK1 (*TK onwards*) is useful in diagnosis and prognosis in humans and dogs with malignant lymphoma. Furthermore, TK can be used in immunohistochemistry for RNA/protein expression in tissue specimens and for activity or

protein/peptide levels in serum from patients. In humans, TK is used in measuring several forms of blood malignancies, as well as solid tumors, for example breast and lung cancer. However, the results are poor on solid tumors in dogs. Canine solid tumors are hard to distinguish from normal controls because they tend to induce much less of a response in terms of systemic enzyme activity (von Euler & Eriksson, 2011; Leijon, 2013).

The challenging area of canine lymphoma is rarely to set the diagnosis, but to predict the prognosis and monitor the remission status in an objective way. Moreover, it is a challenge to detect relapse in treated patients. The purpose of the study is to compare initial blood parameters, for example TK and CRP, with clinical symptoms, clinical stage and prognosis. The aim is to improve the prognostication and thereby be able to select the right patients for further treatment. The aim is also to find a better method to select patients who may need more frequent monitoring after completed treatment.

The study will focus on the following set of questions:

- Can the initial TK level in serum be used to predict the prognosis and survival time for a patient diagnosed with malignant lymphoma?
- Can the initial CRP level, together with clinical symptoms and stage be used for prognostication?
- Are the initial calcium and hematocrit levels, as well as the clinical stage, immunophenotype, tumor grade and clinical symptoms correlated with prognosis and survival time?

The hypothesis is that the initial TK level in serum can be used to predict the prognosis and survival time of a dog with malignant lymphoma. Another hypothesis is that the CRP level may be used for prognostication, but only together with the clinical stage and symptoms and not as a single analysis. Furthermore, it is believed that hypercalcemia, T-cell lymphomas and a high clinical stage are correlated to a shorter survival time.

LITERATURE REVIEW

Anatomy of the lymphatic system

The components of the lymphatic system are divided into two groups; the primary organs and the secondary organs. The primary organs consist of the thymus gland and the bone marrow. These organs regulate the production and differentiation of lymphocytes. Both T- lymphocytes and B-lymphocytes are derived from stem cells in the bone marrow. The B-cells mature in the bone marrow, while the immature T-cells are transported to the thymus for their final development. Mature B- and T-lymphocytes are released into the circulation and transported to the secondary lymphoid organs, where most of them will remain. The secondary organs include the lymph nodes, lymphatic vessels, aggregated lymphoid tissue and the spleen.

Within the secondary organs, the lymphocytes undergo antigen-dependent proliferation and differentiation into effector cells. The effector cells can either attend to the disposal of particular antigens or provide the memory cells that become temporarily inactive. While the primary

lymphoid organs are only involved in the immune function, the secondary organs are involved in the immunity, fat absorption and fluid regulation.

The lymphatic vessels link together the secondary lymphoid organs and makes a connection to the cardiovascular system. They provide a route where the lymph can flow from the body tissues to the heart. The lymph is a fluid collected from the interstitial spaces into lymphatic capillaries. The lymph is transported in the lymphatic vessels through the lymph nodes, where a filtration of the lymph takes place before it reaches the large ducts and enters the blood circulation in the upper chest.

The lymph nodes are encapsulated structures distributed throughout the body. They are comprised of several lymph nodules, which are compartments with several B-cells, T-cells and macrophages. Unfiltered lymph is delivered through several afferent vessels. The lymph is then filtered for antigens and particular matter, and an immune response may be generated if it is necessary. The filtered lymph leaves the lymph node through one or two efferent vessels.

The spleen is composed of two types of tissue; the red and the white pulp. The red pulp is mostly associated with blood storage and the destruction of old erythrocytes. The white pulp is formed of lymph nodules, explaining why the tissue has lymphogenic and phagocytic properties.

The aggregated lymphoid tissues are collections of non encapsulated lymphoid tissues of various size and organization, for example the tonsils in the oral cavity and Peyer’s patches in the lining of the small intestines. The main purpose of the aggregated lymphoid tissue is to prevent infections from developing at the mucosal surfaces, where microorganisms easily can enter the body (Dyce *et al.*, 2010).

Classification

There are different types of lymphomas, which can be named by their anatomical localization. These are listed in Table 1 (Argyle *et al.*, 2009).

Table 1. *Anatomic classification of lymphomas and some of the clinical symptoms they may cause*

Type of lymphoma:	Clinical presentation:
Multicentric lymphoma	Localized or generalized lymphadenopathy with or without hepatosplenomegaly. May cause respiratory distress and edema formation if the lymph nodes are reducing the venous flow.
Thymic lymphoma	Anterior mediastinal mass. May cause pleural effusion, leading to respiratory signs.
Alimentary lymphoma	With or without a palpable abdominal mass. May cause vomiting, diarrhea and weight loss.
Cutaneous lymphoma	Cutaneous tumors which rapidly progress to cause systemic disease.
Hepatosplenic lymphoma	Causes hepatosplenomegaly. May cause vomiting, diarrhea and weight loss.
Nasal lymphoma	May cause nasal discharge, epistaxis, epiphora and facial deformity.
Ocular lymphoma	May cause uveitis with or without hypema.

The most common anatomical form of lymphoma is the multicentric form, accounting for approximately 75 % of all canine lymphomas (Argyle *et al.*, 2009; Vezzali *et al.*, 2010). The diagnosis is often confirmed with a fine-needle aspirate (FNA) or a biopsy from an enlarged lymph node. There is also a histopathological classification of the canine lymphomas, where the tumors are divided into a low grade (also known as small-cell or lymphocytic lymphoma) and a high grade (also known as large-cell or lymphoblastic lymphoma). High grade lymphoma is the most common form in dogs and results in death within weeks from diagnosis, if appropriate treatment is not initiated (Marconato *et al.*, 2011). The low grade lymphoma is characterized by an indolent clinical course and is correlated with an average survival time of 4.4 years after diagnosis. The low grade lymphomas represents 5.3-29 % of all canine lymphomas (Flood-Knapik *et al.*, 2013). Furthermore, it is possible to classify the tumors as T-cell (CD3 positive) or B-cell (CD79 positive) lymphomas using immunophenotyping methods. B-cell lymphomas accounts for approximately 80-85 % of the canine lymphomas (Vezzali *et al.*, 2010) and are associated with a better prognosis and a longer median survival time than T-cell lymphomas (Argyle *et al.*, 2009; Elliott *et al.*, 2013).

Clinical staging enables a more accurate prognostication and can be done when the diagnosis is stated. The World Health Organization (WHO) staging scheme is the most used scheme and it is appropriate for dogs with multicentric lymphoma. This scheme takes into account the extent of the peripheral lymph node involvement, involvement of liver, spleen, bone marrow and nonlymphoid organs (table 2) (Owen *et al.*, 1980).

Table 2. *Clinical staging for multicentric lymphoma according to The World Health Organization (WHO)*

Stage	
I	A single lymph node affected, or lymphoid tissue in a single organ (excluding bone marrow)
II	Two or more lymph nodes affected in a regional area (i.e., cranial or caudal to the diaphragm) ± tonsils
III	Generalized lymph node involvement
IV	Involvement of liver and/or spleen (± stage III)
V	Manifestation in the blood and involvement of bone marrow and/or other organ systems (± stages I-IV)
Substage	
a	Without clinical signs
b	With clinical signs, i.e., fever, weight loss, hypercalcemia

Diagnosis

Clinical pathology

Most dogs diagnosed with malignant lymphoma show a wide range of non-specific abnormalities on the hemathological and clinical chemistry profile. A mild to moderate non-regenerative anemia is seen in many dogs. Both leukopenia and leukocytosis are described and the latter is usually combined with neutrophilia representing an inflammatory response. A mild, asymptomatic trombocytopenia is common (Gavazza *et al.*, 2008). Hypercalcemia is seen in 10-15 % of the dogs and is often associated with T-cell lymphoma. The hypercalcemia results from the parathyroid hormone-related peptide (PTH-rP) by CD4+ T-cell lymphoblasts (Ruslander *et al.*, 1997; Zandvliet, 2016). Hypercalcemia reduces the response to antidiuretic hormone (ADH), leading to polyuria (Zandvliet, 2016).

Dogs with malignant lymphoma often have a higher TK value than healthy dogs. The TK value also seems to correlate with stage and prognosis of the disease (von Euler *et al.*, 2004; Elliott *et al.*, 2013). Measurements of C-reactive protein (CRP) levels has a low sensitivity and specificity for diagnosing malignant lymphoma, and needs to be combined with clinical data to be useful as a diagnostic test (Mirkes *et al.*, 2014).

Elevated liver enzymes or kidney values may be due to the lymphoma affecting the liver or the spleen, but more often it is caused by reactive hepatopathy and dehydration as a secondary effect of the lymphoma. Proteinuria is a common finding in dogs with multicentric lymphoma, even though urinalysis is not routinely performed in the diagnostics. Bone marrow involvement (leukemia) is reported in up to 55 % of the dogs (Raskin & Krehbiel, 1989). However the bone marrow involvement cannot be detected accurately from peripheral blood counts. The most commonly used method today is a cytologic examination of a single bone marrow aspiration sample, which is proved sufficient for diagnosing bone marrow involvement (Martini *et al.*, 2015; Zandvliet, 2016).

Diagnostic imaging

Thoracic and abdominal radiographs often show unspecific findings which can be used to see if lymphoma is a differential diagnosis rather than to set the diagnosis. Thoracic radiographs can reveal thoracic lymphadenopathy, pulmonary infiltrates and a cranial mediastinal mass. Ultrasonography of the abdomen and lymph nodes is a very useful diagnostic tool, where one can assess lymph node size and architecture, as well as hepatic and/or spleen involvement (Crabtree *et al.*, 2010; Zandvliet, 2016). However, abdominal ultrasonography is not as useful in the diagnostics of alimentary lymphoma, because they tend to have non-specific, or absent, findings (Frances *et al.*, 2013). A computed tomography (CT) scan may be useful to evaluate the extent of a tumor but cannot be used to set a specific diagnose, for example differentiate a thymoma from a mediastinal lymphoma (Yoon *et al.*, 2004).

Cytology and histology

A cytological examination of a fine-needle aspirate from an enlarged lymph node is an easy, cheap and minimally invasive method to diagnose high grade canine lymphomas. However, cytology is sometimes insufficient for diagnosing low grade lymphomas or characterizing

atypical lymphoid proliferations (Sözmen *et al.*, 2005; Zandvliet, 2016). A histological examination of a biopsy may improve the diagnosis of a low-grade lymphoma. Furthermore, the histological examination of a biopsy will allow further classification of the lymphoma. Histological characterization of the lymphoma includes growth pattern, nuclear size and morphology, mitotic index and immunophenotype (Zandvliet, 2016).

Treatment

Generally, the aim of the treatment is not to cure the patient, but to give the dog a good quality of life striving to get the lymphoma in to remission for as long as possible. Without treatment, most dogs with lymphoma die within 4-6 weeks from diagnosis (Zandvliet, 2016). A systematic therapy is required to achieve the best possible outcome (Argyle *et al.*, 2009; Marconato *et al.*, 2011). In general, dogs diagnosed with multicentric lymphoma have the best response to treatment. However, only 10 % of the dogs with multicentric lymphoma who have been treated with cytotoxic agents survive more than two years from diagnosis (Marconato *et al.*, 2011). Dogs with thymic lymphoma often have a good initial response to therapy, but the remission period is often shorter than for the multicentric form. However, the alimentary form, as well as the cutaneous form and the hepatosplenic form, are often difficult to treat (Argyle *et al.*, 2009). There is a significant difference in prognosis between a T-cell lymphoma and a B-cell lymphoma, where the latter one is more susceptible to chemotherapy and is correlated to a better prognosis than the former one (Ponce *et al.*, 2004).

Singel agent (mono) therapy

Glucocorticoids - Prednisone

Glucocorticoids are routinely used as a palliative treatment in dogs suffering from malignant lymphoma. The glucocorticoids induce apoptosis of lymphocytes and lymphoblasts (Smith & Cidlowski, 2010). Most dogs will experience a partial to complete response, but the remission duration is often short and lasts approximately two to four months (Squire *et al.*, 1973; Bell *et al.*, 1984; Argyle *et al.*, 2009). Moreover, dogs pretreated with glucocorticoids tend to have a lower probability to enter complete remission after chemotherapy treatment (Gavazza *et al.*, 2008; Marconato *et al.*, 2011).

Anthracyclines

The first anthracyclines were isolated from *Streptomyces spp.*, explaining why they are classified as antitumor antibiotics. Anthracyclines induce protein-linked breaks in the DNA and inhibit re-ligation of DNA cleaved by topoisomerase type II. Furthermore, the anthracyclines may also insert a part of their structure between two adjacent base pairs in the DNA strand. The anthracyclines may also form free radicals that can cause oxidative damage to cellular proteins by undergoing iron-catalyzed reduction. The anthracyclines are non-cell-cycle specific drugs which are used extensively in veterinary medicine. Frequently used anthracyclines are doxorubicin, dactinomycin and mitoxantrone (Argyle *et al.*, 2009).

If doxorubicin is used as a single agent treatment, the drug is given as a slow intra venous infusion every 21 days. Usually five doses are given over 15 weeks. The drug has a complete remission rate of 59-85 % and the remission duration is up to seven months (Simon *et al.*,

2008). The remission duration is shorter than after using a multidrug chemotherapy protocol and doxorubicin as a single agent treatment should only be used as a palliative treatment. Dogs with decreased heart contractility due to heart disease should not be treated with doxorubicin, because of its potential cardiotoxicity. Furthermore, it is very important that doxorubicin is given strictly intra venously. Extravascular injection causes severe tissue damage and necrosis that may require amputation or euthanasia (Argyle *et al.*, 2009).

Alkylating agents

The alkylating agents are the oldest group of antitumor drugs. These drugs consist of small molecules that bind covalently to electron-rich parts on biological molecules. The binding of the drugs to cellular DNA is believed to be the lethal event. Cell death may occur when the cell tries to replicate the damaged DNA. Repair enzymes may worsen the damage process by creating base deletions or misplacements of single or double strand breaks. The alkylating agents are non-cell-cycle specific. Many alkylating agents are frequently used in veterinary oncology, including mechlorethamine, melphalan, chlorambucil, cyclophosphamide and lomustine (Morris & Dobson, 2008).

Lomustine is given per os every 21 days until progressive disease or hepatotoxicity develops. Lomustine is more often used as a rescue treatment for relapsed multicentric lymphoma and for cutaneous lymphoma (mycosis fungoides) (Argyle *et al.*, 2009). Rescue protocols are used as an attempt to rescue a patient back to remission, if they have failed to achieve remission as a result of the first line therapy. The remission response rate of lomustine is 30-50 % and the median response duration is one to three months from the therapy start (Sauerbrey *et al.*, 2007; Argyle *et al.*, 2009). Most bioavailability data come from human studies and the oral chemotherapy drugs, such as lomustine, are designed for the human gastrointestinal tract. There is not much information about the absorption in veterinary patients to this day (Argyle *et al.*, 2009).

L-Asparaginase

L-asparaginase is an enzyme that inhibits the protein synthesis by the depletion of L-asparagine sources, which is necessary for transformed lymphoid cells to proliferate (MacEwen *et al.*, 1992). Unlike lymphoma cells, normal cells synthesize their own asparagine which explains why they do not get affected by the L-asparaginase (Argyle *et al.*, 2009). The problem with L-asparaginase treatment is the immunogenicity of the enzyme and the risk of developing anaphylactic reactions. Fatal anaphylactic reactions have been reported after intravenous injections. Hence, L-asparaginase is always given as an intramuscular or subcutaneous injection (MacEwen *et al.*, 1992; Argyle *et al.*, 2009).

Multidrug chemotherapy

Multi-agent therapy protocols are often injection protocols that combine cyclophosphamide, doxorubicin (Hydroxydaunorubicin), vincristine (Oncovin), prednisone (so-called CHOP protocol), and sometimes L-asparaginase (so-called L-CHOP protocol). These protocols result in a high response rate and long response duration and therefore form the basis for most of the current protocols used for treating high grade malignant lymphomas (Zandvliet, 2016). The

most frequent used protocol at the University Animal Hospital in Sweden is the ADRIA-plus protocol consisting of doxorubicin, L-asparaginase, cyclophosphamide, chlorambucil, hydroxyurea and prednisone, as described by Piek *et al.* (1999). The reported remission rate is 65-84 % with the ADRIA-plus protocol and up to 22 % of the treated dogs survive more than two years (Piek *et al.*, 1999).

Table 3. An overview of the ADRIA-plus protocol used at the University Animal Hospital in Sweden. The treatment is interrupted if the patient is at complete remission at week 19. Planned revisits week 24, 27, 30, 39 and 52

Drug	Week																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
L-asparaginase	■								■								
Doxorubicin		■		■			■			■				■			
Cyclophosphamide									■								■
Chlorambucil				■	■	■						■					
Hydroxyurea																	
Prednisone				■	■	■	■	■									
Dexamethasone		■															

Side effects

It is well known that humans going through chemotherapy suffer from many side effects. However, dogs are less sensible to these side effects than humans. Furthermore, a lower dose of cytotoxic drugs is used in the veterinary medicine to reduce the risk of side effects. Chemotherapy damages dividing cells. This means that both cancer cells and healthy cells are affected, particularly fast dividing cells. Some organ systems are more likely to suffer from side effects, due to their fast dividing cells. One of these organ systems is the bone marrow. If the bone marrow suffers from too much chemotherapy, the patient may develop anemia and an increased risk of developing infections and bleedings. Therefore, it is important to take a blood sample and control the erythrocyte, leukocyte and platelet levels prior to each treatment. Furthermore, another organ system often affected by side effects is the gastrointestinal tract, due to the constant exchange of the intestinal mucosa. This may cause nausea, inappetence and diarrhea.

Humans often suffer from hair loss as a side effect of the chemotherapy. Dogs rarely suffer from this side effect, except for certain breeds such as poodles and schnauzers which may develop focal hair loss. All breeds may experience a temporary increase in fur shedding during the treatment (Piek *et al.*, 1999; Nelson & Couto, 2008; Argyle *et al.*, 2009; Zandvliet, 2016). It is common to assess the hepatic and renal function before, during and after administration of chemotherapy drugs that are either toxic to these organ systems or are excreted via these organs (Morris & Dobson, 2008).

Acute Tumor Lysis Syndrome (ATLS)

Acute tumor lysis syndrome occurs in patients with lymphoproliferative malignancies, often after initiation of treatment. The massive tumor cell lysis causes a release of large amounts of potassium, phosphate, and uric acid. This may lead to acute renal failure due to deposition of uric acid and calcium phosphate crystals in the renal tubules. Healthy kidneys normally excrete these products, why a pre-existing renal failure makes the risk of ATLS higher. The treatment aims to clear high plasma levels of potassium, uric acid, and phosphorus, to correct the acidosis, and prevent acute renal failure by aggressive intravenous fluid therapy (Davidson *et al.*, 2004).

Drug resistance

The cancer genome is unstable and may result in spontaneous mutations, making some cells inherently resistant to chemotherapy. The heterogenous population of cells in a tumor varies in their resistance to chemotherapy. This depends on many factors, including cell cycle-phase, distance from vascular supply and genetically acquired properties.

The patient often shows a good initial response to chemotherapy. However, as soon as the chemosensitive cells are killed, the chemoresistant cells have a selective advantage, leading to progressive tumor growth despite of chemotherapy. Multi-agent therapy enables a maximal amount of killed tumor cells within the range of toxicity tolerated by the patient for each drug. The multidrug chemotherapy also provides a wider range of coverage of resistant cell lines. Furthermore, the development of new resistant cell lines is prevented or slowed down when using a multi-agent therapy protocol compared to a single-agent therapy (Morris & Dobson, 2008).

Tumor resistance to anthracyclines is primarily caused by overexpression of membrane P-glycoprotein (P-gp). On the other hand, resistance to the alkylating agents is caused by changes in the cellular metabolic systems. This mechanism makes the drug detoxification better and results in a water soluble, less toxic product. Resistance to L-asparaginase comes from an increased expression of the asparagine synthetase gene. Furthermore, formation of antibodies against L-asparaginase may also decrease its efficacy (Morris & Dobson, 2008).

Enzymatic function of thymidine kinase 1

Thymidine kinase is a key enzyme in the salvage pathway of nucleotide metabolism. This pathway recycles free deoxyribonucleosides originating from the DNA breakdown, and makes new DNA precursor deoxyribonucleoside triphosphates. Thymidine kinase catalyses the initial step of the salvage pathway, which is a transfer of γ -phosphoryl group from ATP to thymidine, leading to the formation of thymidine monophosphate (TMP). The intracellular TMP is then quickly phosphorylated into thymidine diphosphate (TDP) and further to thymidine triphosphate (TTP). TTP can then be used as a substrate for the incorporation of pyrimidines in the growing DNA-strand. It is not possible to form new DNA strands without TTP, meaning that TTP is crucial for further replication and cell division (Berenstein, 2004). Studies have shown that the serum TK1-activity is increased in dogs suffering from malignant lymphoma (von Euler *et al.*, 2004; Euler *et al.*, 2009). Moreover, these studies showed that the TK activity

in serum was significantly higher three weeks before and at the time of relapse, than the activity measured at complete remission (CR).

C-reactive protein and lymphoma

C-reactive protein (CRP) is an acute phase protein produced in the liver. The serum CRP is typically increased in response to inflammation or infection (Merlo *et al.*, 2007). Interleukin 6 (IL-6) is a cytokine that regulates acute phase proteins, and is the main inducer of the synthesis of CRP. Furthermore, IL-6 plays a central role in normal B-cell maturation and in proliferation of some B-cell malignancies, including human NHL. Studies have shown that the serum IL-6 levels correlates with B symptoms (fever, weight loss and night sweats) in human NHL. Furthermore, these studies showed that the serum CRP levels correlated with the serum IL-6 levels (Legouffe *et al.*, 1998). Therefore, the human medicine has been using serum CRP concentrations as a prognostic factor for patients with lymphoid tumours, and as a marker for remission and relapse (Legouffe *et al.*, 1998; Elahi *et al.*, 2005; Merlo *et al.*, 2007).

When it comes to veterinary medicine, Merlo *et al.* (2007) found that the serum CRP concentration is not a useful marker for relapse in dogs with multicentric lymphoma. However, dogs often have high serum CRP concentrations at the time of diagnosis (Merlo *et al.*, 2007; Nielsen *et al.*, 2007). Furthermore, Merlo *et al.* (2007) found that chemotherapy itself does not affect serum CRP concentrations. Another study showed that the serum CRP concentration may be useful to determine complete remission status after treatment with cytotoxic drugs. However, the CRP concentration is not reliable to use in monitoring progression of the disease, due to the big individual variation between dogs' mean CRP concentrations (Nielsen *et al.*, 2007).

MATERIAL AND METHODS

A list of all dogs who had received the diagnosis malignant lymphoma in the last seven years (2010-2017) at the University Animal Hospital in Sweden was created from medical records. The study initially included 129 dogs with malignant lymphoma. However, due to inadequate information in the medical record, 19 dogs were excluded from the study (two dogs due to a prior diagnosis and treatment of the lymphoma at another animal hospital with no copy of the initial medical record, seven dogs due to the lack of a definitive diagnosis and ten dogs due to no record of the euthanasia or death date). To obtain missing information about the date of euthanasia or death, 42 owners were phone called. Those who not answered the phone call received both a text message and a phone message. Complementary information was obtained from 32 of these owners. The remaining ten owners either did not answer or declined the participation in the study. To be included in the study, the date for diagnosis and euthanasia or death were required.

Each one of the 110 dogs was evaluated on the basis of the information collected at the first examination including history, physical examination, diagnostic imaging and laboratory examinations: complete blood count, serum biochemical profile, FNA and immunophenotyping. All information was collected from the medical records. The collected information were: breed, sex, age at diagnosis, weight, body condition score (BCS), symptoms (polyuria/polydipsia, fatigue and inappetence), blood analyzes (hemoglobin [Hb], hematocrit

[Hct], TK, CRP and calcium), information about the tumor (clinical stage, immunophenotype, diagnostic method and malignancy grade) and information about the treatment (type of treatment/protocol, pre-treatment with prednisolone or not, time to progression and overall survival time from first day of treatment).

The serum TK levels were analyzed by the use of two different methods, either the radio enzyme assay (TK-REA) or the TK-activity assay described by Stålhandske *et al.* (2013).

Staging was not complete in all cases. Patients with multicentric lymphoma who did not undergo any diagnostic imaging were classified as stage 3, if no splenomegaly or hepatomegaly were suspected during abdominal palpation.

The breed distribution was calculated and compared to the Swedish dog register from the Swedish Board of Agriculture, to see if any breed seemed to be predisposed in developing malignant lymphoma. Furthermore, the dogs were categorized in four different groups depending on their sex and the distribution of males, neutered males, females and neutered females was calculated. Mean values and median values were calculated for the weight and age of the dogs. The Hct values were categorized in 4 different groups; within reference value (>0.37 L/L), mild anemia ($0.30-0.37$ L/L), moderate anemia ($0.20-0.29$ L/L) and severe anemia (<0.19 L/L). The Hb values were categorized into two groups; within the reference value and below the reference value, after which the distribution was calculated. Moreover, the distribution of dogs suffering from hypercalcemia was calculated as well as the distribution of B- and T-cell lymphomas.

The dogs were categorized into two groups with different cut-off values on their initial TK levels in serum. The cut-off values follows, for the respective groups; 0-11.0, 11.1-20.0, 20.1-40.0 and 40.1-100 and 0-15.0, 15.1-40.0 and 40.1-100. Dogs pretreated with prednisolone before the blood sample were excluded from the TK groups. Furthermore, the dogs were categorized into different groups depending on their initial treatment method. The groups were; no treatment, ADRIA-plus protocol, prednisolone \pm L-asparaginase, CCNU (lomustine), doxorubicin and other treatment.

The TK, CRP, calcium and Hct levels, as well as the clinical stage, immunophenotype, tumor grade and clinical symptoms, were evaluated to see if they correlated with prognosis and overall survival time in days from treatment. Dogs that did not receive any treatment were excluded from the survival analysis, due to not having any date of euthanasia or death. Dogs that lacked certain test results were only excluded from that specific calculation concerning the missing data. The survival distributions were estimated nonparametrically using the Kaplan-Meier method. Furthermore, the significance was evaluated using the log rank test. A p-value < 0.05 was regarded as significant and a p-value < 0.01 was regarded as highly significant. All numerical calculations were performed in Excel with an add-in called ExcelSurvival to draw the Kaplan-Meier curves.

At the end of the study, six dogs were still alive at day 129, 130, 175, 204 and 468 from the first day of treatment. One dog with an indolent lymphoma did not receive any treatment under

the duration of the study. Five dogs in the study were euthanized of other reasons than the lymphoma.

Background data

Breed

Fifty different breeds were represented in the study (table 4). Out of these, 25.5 % (28/110) were mixed breeds. The most frequent pure breed was Golden Retriever (8/110), followed by Rottweiler (6/110) and Border Collie, Flat Coated Retriever, Nova Scotia Duck Tolling Retriever and Bernese Mountain Dog (4/110). When compared to the total population of dogs in each breed in Sweden (Jordbruksverket, 2016), the breeds Nova Scotia Duck Tolling Retriever, Bernese Mountain Dog, Doberman and Great Dane seems to be overrepresented in the study.

Table 4. Table showing all breeds that are represented in the study and the number of dogs in each breed. The Columns show the percentage of each breed in the study (a) and the percentage of each breed in Sweden (b), together with the ratio between them (a/b)

Breed	Number of dogs	Percentage in study (a)	Percentage in Sweden (b)	Ratio (a/b)
Mixed breed	28	25.5	26.58	0.96
Golden Retriever	8	7.3	2.67	2.72
Rottweiler	6	5.5	1.74	3.13
Border Collie	4	3.6	1.29	2.81
Flat Coated Retriever	4	3.6	0.85	4.28
Nova Scotia Duck Tolling Retriever	4	3.6	0.45	8.02
Bernese Mountain Dog	4	3.6	0.54	6.74
Miniature Schnauzer	3	2.7	1.43	1.91
Finnish Hound	2	1.8	0.42	4.42
German Shepherd Dog	2	1.8	3.31	0.55
Great Dane	2	1.8	0.31	5.83
Doberman	2	1.8	0.32	5.75
Rhodesian Ridgeback	2	1.8	0.44	4.13
English Springer Spaniel	2	1.8	0.87	2.10
West Highland White Terrier	2	1.8	0.35	5.23
American Staffordshire Terrier	1	0.9	0.92	0.99
Staffordshire Bull Terrier	1	0.9	0.66	1.37
Labrador Retriever	1	0.9	3.08	0.30
Boxer	1	0.9	0.55	1.65
Miniature Pinscher	1	0.9	0.46	2.0
Border Terrier	1	0.9	0.64	1.43
Bouvier des Flandres	1	0.9	0.06	15.16

Rough Collie	1	0.9	0.51	1.79
Norwegian Elkhound	1	0.9	0.84	1.09
Miniature Poodle	1	0.9	1.06	0.86
Standard Poodle	1	0.9	0.55	1.65
Whippet	1	0.9	0.39	2.33
Catalan Sheepdog	1	0.9	0.02	53.96
Saint Bernard Dog	1	0.9	0.14	6.38
Phalène	1	0.9	0.12	7.37
Bloodhound	1	0.9	0.01	79.36
German Spaniel	1	0.9	0.58	1.57
Beagle	1	0.9	0.38	2.40
Cavalier King Charles Spaniel	1	0.9	1.23	0.74
Polish Lowland Sheepdog	1	0.9	0.04	24.53
Gordon Setter	1	0.9	0.11	8.65
Briard	1	0.9	0.14	6.70
Irish Glen of Imaal Terrier	1	0.9	0.01	106.51
Dachshund	1	0.9	0.90	1.01
Clumber Spaniel	1	0.9	0.02	37.83
Dandie Dinmont Terrier	1	0.9	0.03	34.89
Miniature Bull Terrier	1	0.9	0.04	23.74
Norfolk Terrier	1	0.9	0.11	8.45
Scottish terrier	1	0.9	0.05	19.89
Irish Setter	1	0.9	0.35	2.58
Fox Terrier, smooth	1	0.9	0.16	5.84
Australian Shepherd	1	0.9	0.35	2.61
Curly Coated Retriever	1	0.9	0.04	22.55
Giant Schnauzer	1	0.9	0.20	4.48
Coton de tuléar	1	0.9	0.17	5.28

Total number of breeds: 50

Total number of dogs: 110

Sex, weight and age

The sex distribution of the study follow as, 30.9 % (34/110) male dogs, 20 % (22/110) neutered male dogs, 36.4 % (40/110) female dogs and 13.6 % (15/110) neutered female dogs. The mean age was 7.5 years (90.25 months) and the median age was 7.3 years (87.5 months, range 10-162 months). The mean weight was 28.2 kg and the median weight was 27.2 kg (range 2.3-80 kg).

Blood analyzes

Hypercalcemia was seen in 6.67 % of the dogs (6/90). The initial calcium value was not analyzed in 30 dogs. The TK values were categorized in two different ways, either in three or four groups, with the distribution shown in figure 1. The initial TK value was only analyzed in 40 dogs. When it comes to CRP, 37 % of the dogs (17/46) had values below 30 mg/L and 63 % (29/46) had values above 30 mg/L. The group of dogs with a Hct within the reference value (>0.37 L/L) accounted for 69.3 % (70/101) of all the dogs that had an initial Hct value and the dogs with mild anemia (0.30-0.37 L/L) accounted for 22.8 % (23/101), while 7.9 % (8/101) of the dogs had a moderate anemia (0.20-0.29 L/L). No dogs suffered from severe anemia (<0.19 L/L) in this study. When focusing on the Hb value, 70 % (70/100) of the dogs had values within reference, while 30 % (30/100) of the dogs had values below reference.

Distribution of TK values

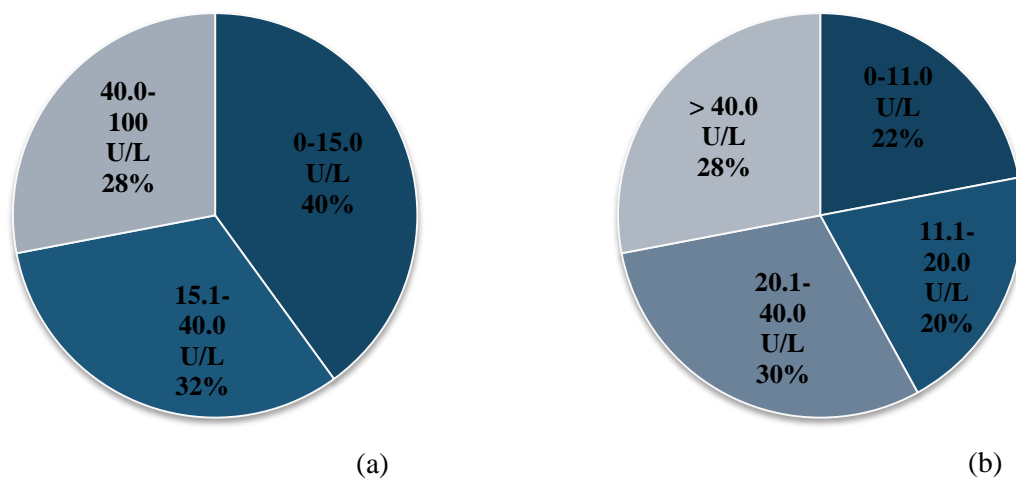


Figure 1a & b. Distribution of dogs when categorizing their TK values into two groups with three (a) or four (b) cut-off values. There were, in total, 40 dogs with an initial TK value in the study.

Immunophenotype

Immunophenotyping was performed in 30 of the 110 dogs. The remaining 80 dogs had an unknown immunophenotype. Out of these 30 tumors, 53.3 % (16/30) were B-cell lymphomas, 36.7 % (11/30) were T-cell lymphomas, 6.7 % (2/30) were mixed B- and T-cell lymphomas and 3.3 % (1/30) were nullcell (non-B-non-T-cell) lymphomas.

Tumor grade

The histological grade of the tumor was known in 97 dogs, showing that 10.3 % (10/97) of the dogs had a low grade lymphoma, while 89.7 % (87/97) of the dogs had a high grade lymphoma.

Clinical stage

No dogs in the study were at stage 1, 7.3 % (8/110) of the dogs were at stage 2, 34.5 % (38/110) of the dogs were at stage 3, 31.8 % (35/110) of the dogs were at stage 4 and 26.4 % (29/110) dogs were at stage 5 at the time of diagnosis.

Treatment method

In the study, 30 % of the dogs (33/110) did not receive any kind of treatment, whilst 70 % of the dogs were treated in different ways. Out of these, 31.8 % (35/110) were treated with the ADRIA-plus protocol (consisted of doxorubicin, L-asparaginase, cyclophosphamide, chlorambucil, hydroxyurea, and prednisone) and 15.5 % (17/100) were treated with prednisolone, with or without L-asparaginase injections. On the other hand, 9.1 % (10/110) of the dogs were treated with CCNU (lomustine) and 8.2 % (9/100) of the dogs were treated with doxorubicin as a single agent treatment. Five dogs, 4.5 %, received another treatment.

RESULTS

TK levels in serum

Figure 2 and 3 shows the correlation between the TK value in serum and the overall survival time from the first day of treatment. The cut-off values are different in figure 2 and 3, with four different groups in the former one and three different groups in the latter one. A statistical significant difference exists between the group with TK values from 0 to 11.0 U/L and 11.1 to 20.0 U/L ($p = 0.026$). This indicates that a TK value from 0 to 11.0 U/L in serum is correlated with a shorter survival time than a TK value from 11.1 to 20.0 U/L. When comparing the other groups, no significant difference exists ($p > 0.05$).

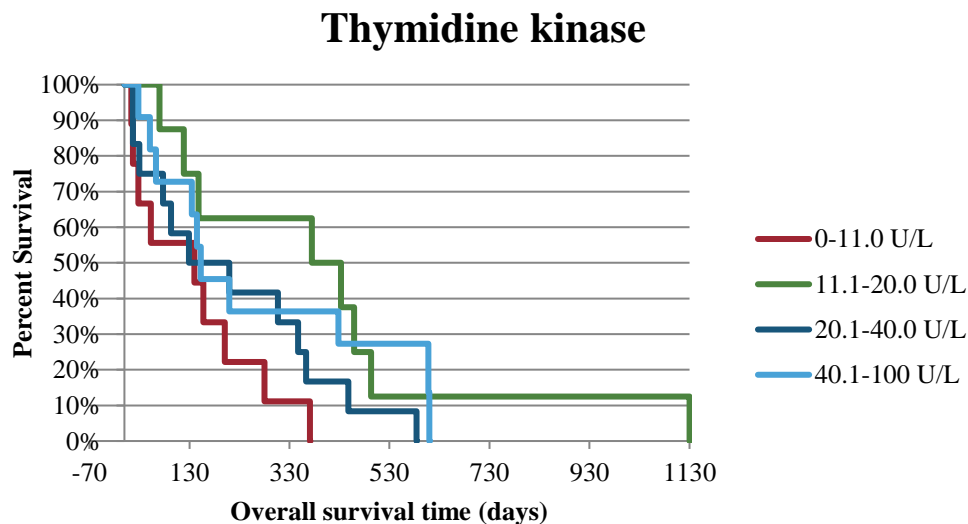


Figure 2. Correlation between TK values in serum and overall survival time from the first day of treatment. A statistically significant difference between the group from 0-11.0 U/L and 11.1-20.0 ($p = 0.026$) exists. When comparing the other groups, no statistical significance exists. The total number of dogs in each group, 0-11.0 U/L, 11.1-20.0 U/L, 20.1-40.0 U/L and 40.1-100 U/L, are 9, 8, 12 and 11 respectively. At the end of the study, one of the dogs in the group from 40.1-100 U/L was still alive at day 468.

Thymidine kinase

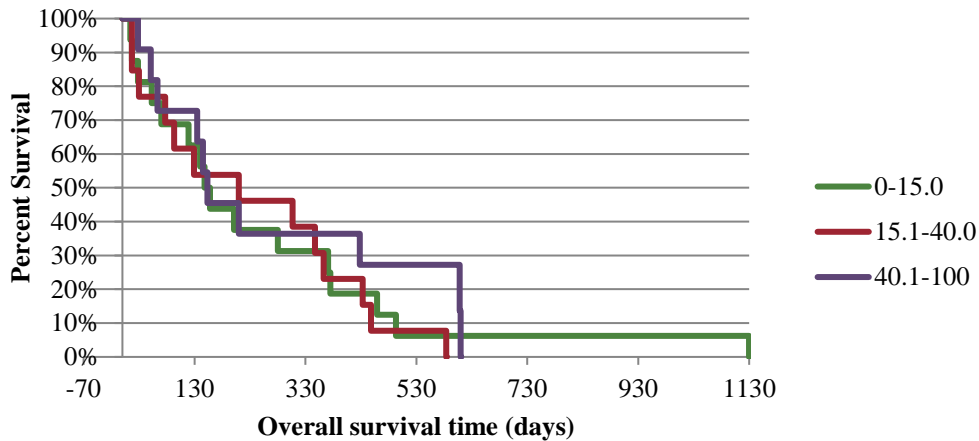


Figure 3. Correlation between TK values in serum and overall survival time from the first day of treatment. There are no statistically significant differences between the groups ($p > 0.05$). The total number of dogs in each group, 0-15.0 U/L, 15.1-40.0 U/L and 40.1-100 U/L, are 16, 13 and 11 respectively. At the end of the study, one of the dogs in the group from 40.1-100 U/L was still alive at day 468.

CRP levels in serum

Figure 4 shows the correlation between the initial CRP level in serum and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

C-reactive protein

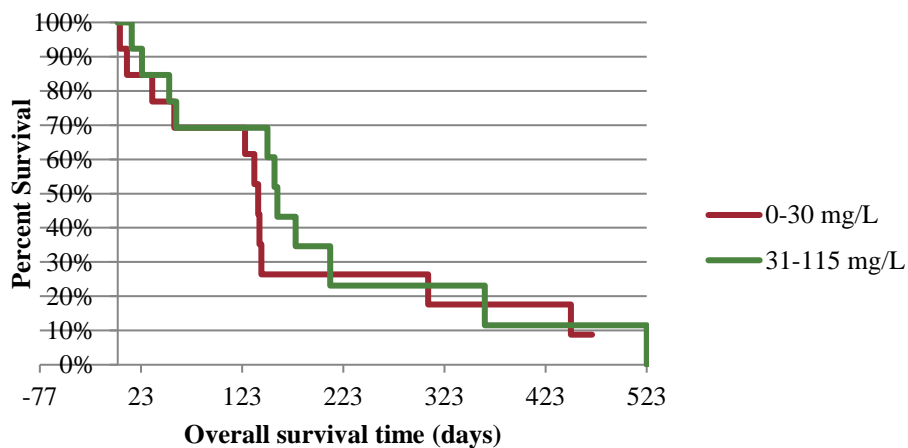


Figure 4. Correlation between CRP values in serum and overall survival time from the first day of treatment. No statistically significant differences between the groups exists ($p > 0.05$). There are 13 dogs in each group. At the end of the study, two of the dogs in the group from 0-30 mg/L were still alive at day 130 and 468. Two of the dogs in the group from 30-115 mg/L were still alive at day 129 and 204.

Calcium levels in serum

Figure 5 shows the correlation between the initial calcium level in serum and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

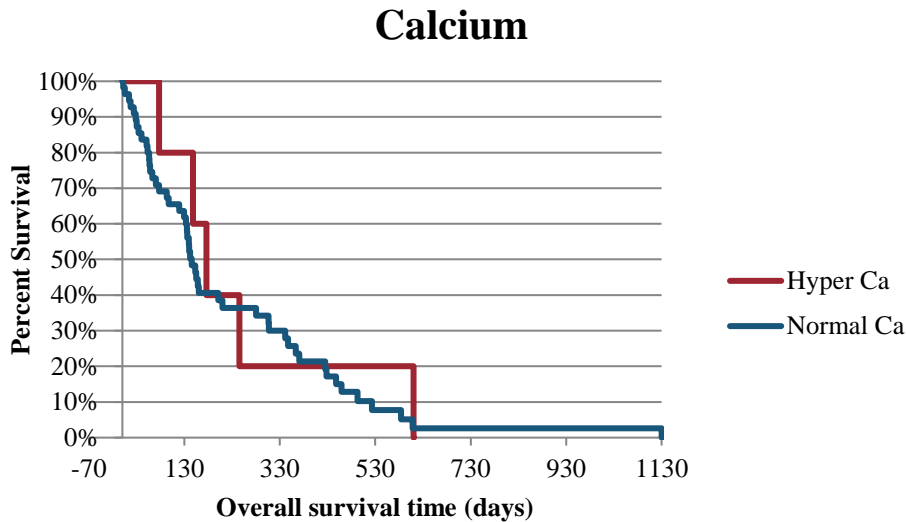


Figure 5. Correlation between calcium levels in serum and overall survival time from the first day of treatment. No statistically significant differences between the groups exists ($p > 0.05$). The total number of dogs in each group, hypercalcemia and normal, are 5 and 55 respectively. At the end of the study, five of the dogs in the group with normal calcium levels were still alive at day 129, 130, 175, 204 and 468.

Hematocrit level

Figure 6 shows the correlation between the initial hematocrit level and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

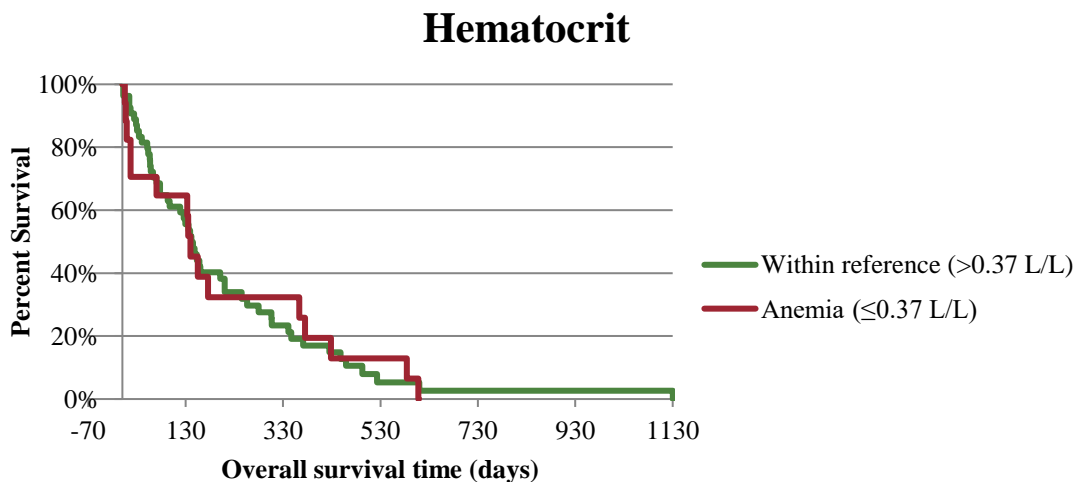


Figure 6. Correlation between the Hct level (anemia) and overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$). The total number of dogs in each group, within reference and anemia, are 54 and 17 respectively. At the end of the study, four of the dogs in the group with a normal Hct level were still alive at day 130, 175, 204 and 468, and one of the dogs in the anemia group was alive at day 129.

Clinical stage

Figure 7 and 8 shows the correlation between the clinical stage at the time of diagnosis and the overall survival time from the first day of treatment. In figure 8, stage 3 and 4 are combined to one single group. No statistically significant differences between the groups exists ($p > 0.05$).

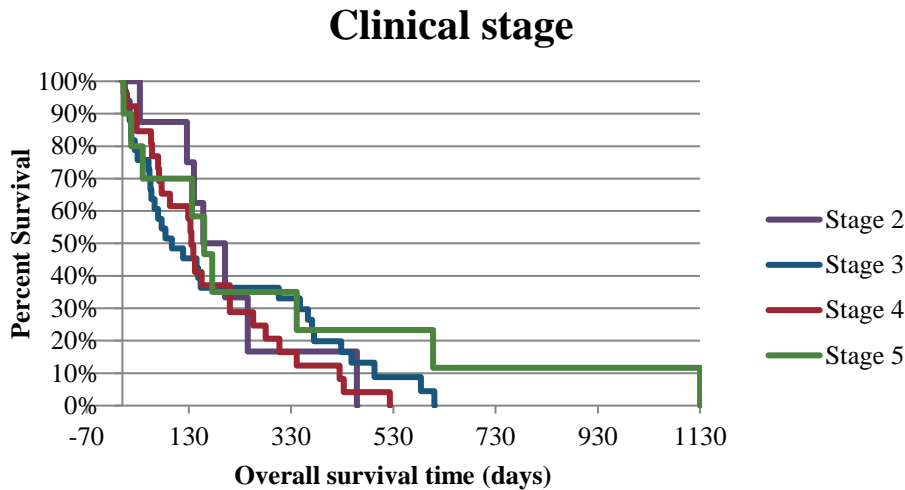


Figure 7. Correlation between the clinical stage and overall survival time from the first day of treatment. There are no statistically significant differences between the groups ($p > 0.05$). The total number of dogs in each group, stage 2, stage 3, stage 4 and stage 5, are 8, 33, 26 and 10 respectively. At the end of the study, five dogs were still alive; one in stage 2 at day 175, two in stage 3 at day 204 and 468, one in stage 4 at day 129 and one in stage 5 at day 130.

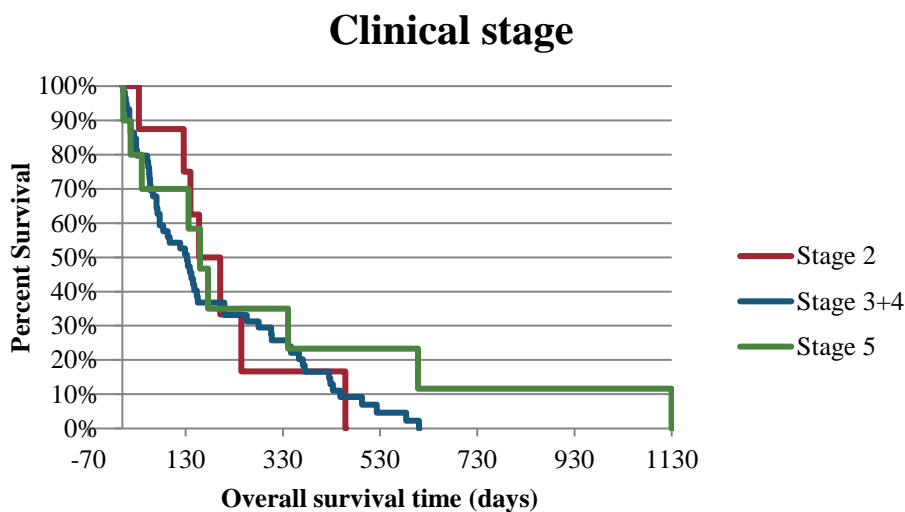


Figure 8. Correlation between the clinical stage and overall survival time from the first day of treatment. There are no statistically significant differences between the groups ($p > 0.05$). The total number of dogs in each group, stage 2, stage 3+4 and stage 5, are 8, 59 and 10 respectively. At the end of the study, five dogs were still alive; one in stage 2 at day 175, three in stage 3+4 at day 129, 204 and 468 and one in stage 5 at day 130.

Immunophenotype

Figure 9 shows the correlation between the immunophenotype of the tumor and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

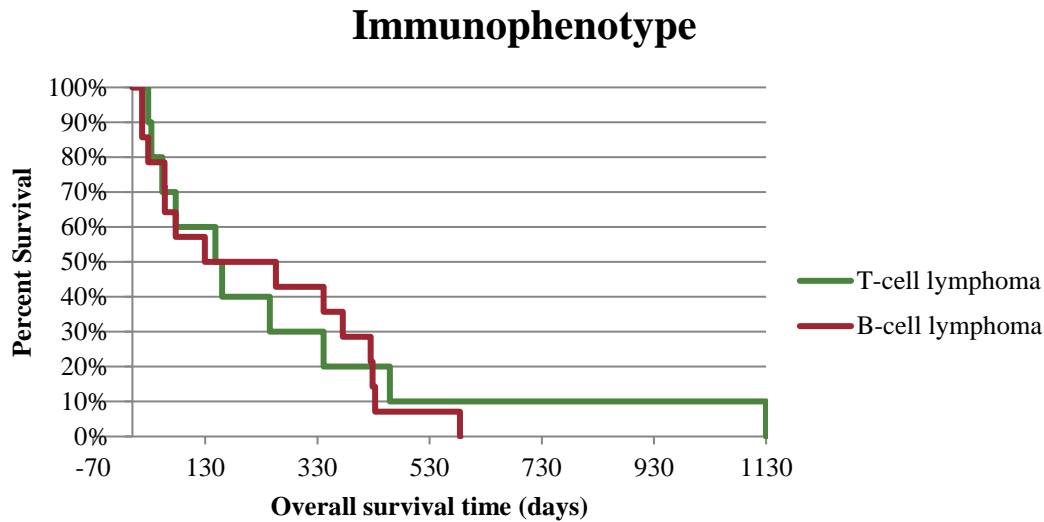


Figure 9. Correlation between the immunophenotype of the tumor and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$). The total number of dogs in each group, B-cell lymphoma and T-cell lymphoma, are 14 and 10 respectively.

Tumor grade

Figure 10 shows the correlation between the histological tumor grade (low/high) and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

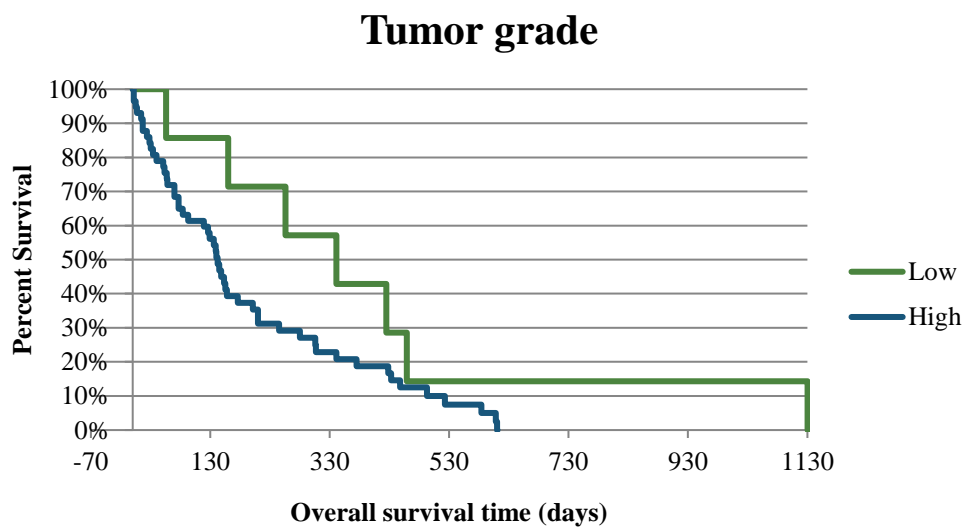


Figure 10. Correlation between tumor grade (low/high) and overall survival time from the first day of treatment. No statistically significant difference between the groups exists ($p > 0.05$). The total number of dogs in each group, low and high, are 8 and 57 respectively. At the end of the study, five of the dogs in the group with high grade lymphomas were still alive at day 129, 130, 175, 204 and 468.

Symptoms

Figure 11 shows the correlation between initial symptoms and the overall survival time from the first day of treatment. No statistically significant difference between the two groups exists ($p > 0.05$).

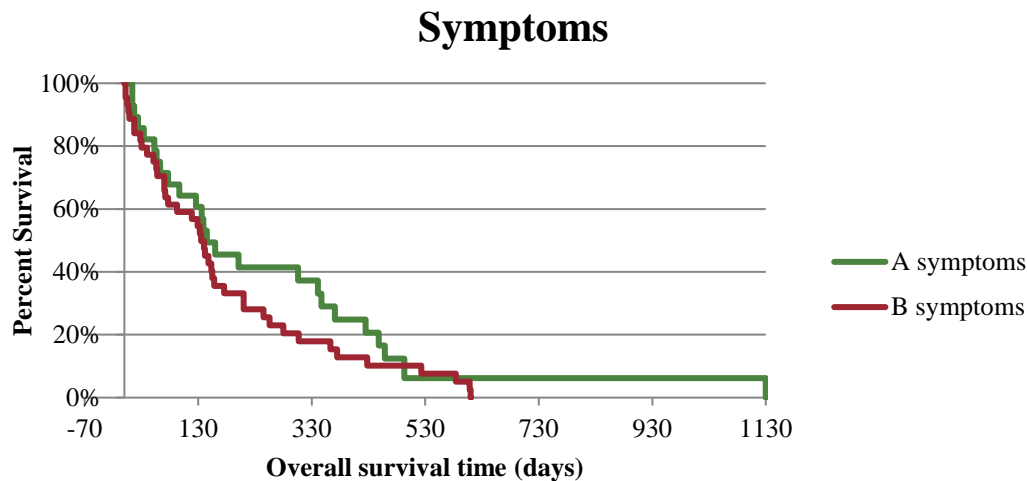


Figure 11. Correlation between symptoms and overall survival time from the first day of treatment. The dogs are divided into two groups, "A symptoms", which means no clinical symptoms, and "B symptoms" which means that the dogs have at least one of the following symptoms; polyuria, polydipsia, fatigue, inappetence, vomiting, diarrhea, weight loss or anemia. No statistically significant difference between the groups exists ($p > 0.05$). The total number of dogs in each group, A symptoms and B symptoms, are 28 and 44 respectively. At the end of the study, three dogs in the group with A symptoms were still alive at day 130, 175 and 468 and two dogs in the group with B symptoms were alive at day 129 and 204.

Treatment method

Figure 12 shows the correlation between the primary option of treatment and the overall survival time from the first day of treatment. A highly statistically significant difference between the group of dogs treated with prednisolone \pm asparaginase compared to the group treated with the ADRIA-plus protocol exists ($p < 0.001$), as well as the group treated with doxorubicin ($p = 0.004$) and the group treated with CCNU ($p = 0.0044$). A statistically significant difference between the group treated with doxorubicin as a single agent therapy compared to the group treated with the ADRIA-plus protocol exists ($p = 0.024$). No statistically significant difference between the group treated with CCNU and the group treated with the ADRIA-plus protocol exists ($p > 0.05$).

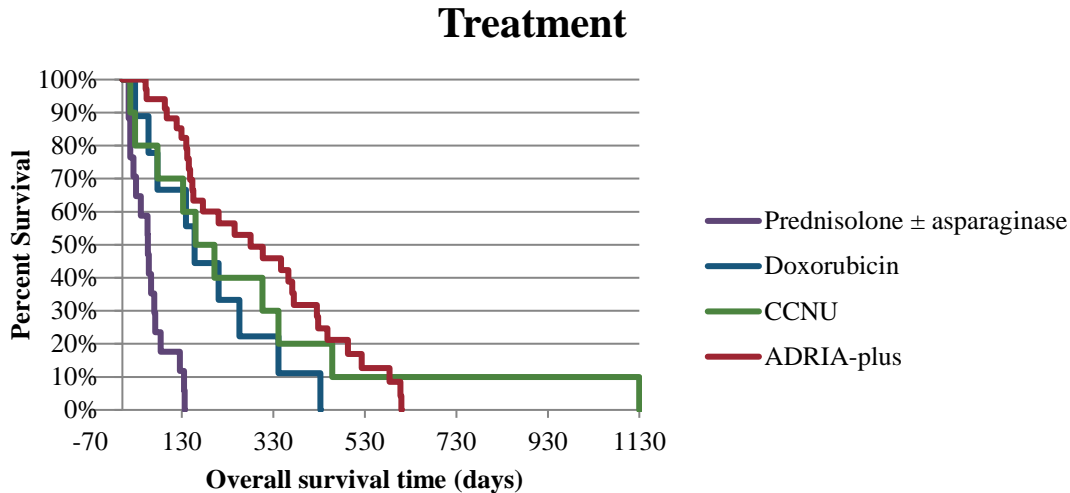


Figure 12. Correlation between the primary option of treatment and the overall survival time from the first day of treatment. A highly statistically significant difference between the group treated with prednisolone ± asparaginase and the group treated with the ADRIA-plus protocol ($p < 0.001$), as well as with the group treated with doxorubicin exists ($p = 0.004$) and the group treated with CCNU ($p = 0.0044$). A statistically significant difference between the group treated with doxorubicin as a single agent therapy compared to the group treated with the ADRIA-plus protocol exists ($p = 0.024$). No significant difference between the group treated with CCNU and the group treated with the ADRIA-plus protocol exists ($p > 0.05$). The total number of dogs in each group, prednisolon ± asparaginase, doxorubicin, CCNU and ADRIA-plus, are 17, 9, 10 and 34 respectively. At the end of the study, five of the dogs in the ADRIA-plus group were still alive at day 129, 130, 175, 204 and 468.

DISCUSSION

Age, weight, breed and sex

As stated in the introduction, lymphomas most commonly occur in middle-age to older dogs and the age category six to ten years seems to be predisposed (Simon *et al.*, 2008; Villamil *et al.*, 2009; Mirkes *et al.*, 2014). In this study the mean age is 7.5 years (90.25 months) which is consistent with the mean age in above mentioned studies. Earlier studies also claim that middle-sized to large dog breeds are overrepresented (Villamil *et al.*, 2009; Zandvliet, 2016), which agrees to the high mean weight in this study (28.2 kg).

According to some researchers, the following breeds are predisposed; Bullmastiff, Boxer, Bernese mountain dog, Dogo de Bordeaux, Golden Retriever, German Shepherd, Labrador retriever and Scottish terrier (Villamil *et al.*, 2009; Vezzali *et al.*, 2010; Elliott *et al.*, 2013). The most common breed in this study is Golden Retriever (8/110), followed by Rottweiler (6/110) and Border Collie, Flat Coated Retriever, Nova Scotia Duck Tolling Retriever and Bernese Mountain Dog (4/110). When compared to the total population of dogs in each breed in Sweden, the breeds Nova Scotia Duck Tolling Retriever, Bernese Mountain Dog, Doberman and Great Dane seems to be overrepresented in this study. Several breeds only have one affected dog in this study. A few of these are uncommon breeds, such as Irish Glen of Imaal Terrier and Bloodhound, which makes them seem to be overrepresented due to their high ratio (table 4).

However, they are not counted as overrepresented in this study because that one affected dog may be caused by a statistical fluctuation.

Villamil *et al.* (2009) stated that intact female dogs seem to have a decreased risk in developing malignant lymphoma compared to neutered females. However, in this study there are more intact female dogs than neutered females, 36.4 % and 13.6 % respectively. This may be explained by the high number of intact female dogs in Sweden. In 2012, the prevalence of neutered dogs in Sweden was 22 % (Svenska Kennelklubben, 2012) compared to 64 % in the United States in the year of 2007 (Trevejo *et al.*, 2011). This may explain why the result in this study does not agree with the result in the study made by Villamil *et al.* (2009).

Thymidine kinase

Previous studies have shown that TK is useful in diagnosis and prognosis in humans and dogs suffering from malignant lymphoma (von Euler *et al.*, 2004; von Euler & Eriksson, 2011; Selting *et al.*, 2016). Dogs with malignant lymphoma often have a higher TK value than healthy dogs. The TK value also seems to correlate with stage and prognosis of the disease (von Euler *et al.*, 2004; Elliott *et al.*, 2013). However, in this study, no correlation between a high initial TK value in serum and a shorter overall survival time is shown. On the other hand, in figure 2 there is a significantly shorter survival time in the group with a lower TK value compared to a group with a higher TK value. This result contradicts the results from the above mentioned studies. The eight dogs in the group with an initial TK value from 0-11 U/L are shown in table 5. The results may be explained by the fact that two out of eight dogs in this group had a high grade T-cell lymphoma. This type of tumor is correlated to a shorter survival time and a poorer prognosis (Ponce *et al.*, 2004; Calvalido *et al.*, 2016). Moreover, T-cell lymphomas often have lower TK values than B-cell lymphomas (Elliott *et al.*, 2013). Furthermore, one of the dogs only received prednisolone as treatment and the owners wanted to euthanize the dog before becoming more affected by the disease, explaining the short survival time of only 14 days. One dog with a high grade B-cell lymphoma has a survival time of 17 days. One explanation to this short survival time could be that the dog suffered from a more aggressive form of B-cell lymphoma called Burkitt's lymphoma, which is correlated to a poor prognosis and shorter survival time (Ponce *et al.*, 2004). However, in this case the mitotic activity was low. Another explanation could be that the tumor had a low proliferation rate, why resistance may occur due to the majority of cells being in G₀ phase (Jerkeman *et al.*, 2004). The small number of dogs (n = 8) in this group may also contribute to the result.

Furthermore, the TK levels were analyzed by the use of two different methods, either the radio enzyme assay (TK-REA) or the TK-activity assay described by Stålhandske *et al.* (2013). The latter analyze method was stopped from use at the date of 2017-01-01. The results in this study may have been affected by the fact that two different analyze methods were used and the results were compared without taking account of the method.

Table 5. A summary of all dogs with initial TK values from 0-11 U/L. The table is showing breed, initial TK value, clinical stage, tumor grade, immunophenotype, treatment and overall survival time in days from start of treatment

Breed	TK	Stage	Grade	Immuno-phenotype	Treatment	OS (days)
Golden Retriever	<2.5	5	High	Unknown	ADRIA-plus	140
Giant Schnauzer	4.1	3	High	T	ADRIA-plus	53
Labrador Retriever	<2.5	2	High	Unknown	CCNU	201
Flat Coated Retriever	2.7	4	High	T	CCNU	28
West Highland White Terrier	<2.5	3	High	B	CCNU	17
Catalan Sheepdog	3	2	High	Unknown	Doxorubicin	158
German Shepherd Dog	5.2	3	Unknown	Unknown	Pred only	14
Irish Setter	8.6	3	Low	Unknown	ADRIA	371

Another aspect that may affect the result is that all dogs that not received any kind of treatment were excluded from the survival analyzes because they did not have an overall survival time from first day of treatment. Dogs with indolent lymphomas often have low TK values and a long survival time. If these dogs were included in the survival analyzes, the results could have been different.

Furthermore, the difference between figure 2 and figure 3 shows that it is important to define the cut-off values before the start of the study. In figure 2 a significant difference between two of the groups exists, which is absent in figure 3 with different cut-off values.

Tumor grade and immunophenotype

Earlier studies show that the low grade lymphomas represents 5.3-29 % of all canine lymphomas (Flood-Knapik *et al.*, 2013). In this study, the prevalence of low grade lymphomas is 10.3 % (10/97) which is consistent with literature. On the other hand, when it comes to the survival analysis, no significant difference is seen between tumor grade and survival time in this study. At first sight, it looks like the low grade tumors have a longer survival time but no significant difference exists when calculating the p-value ($p = 0.299$). This may be affected if more data is added. Another solution would be to categorize the tumors in three different groups instead of two. In this way, all intermediate tumors will have their own group and may lead to a bigger difference between the low and high grade tumors.

The immunophenotype was only recorded in 30 out of 110 dogs in this study and the results shows that 53.3 % (16/30) of the dogs have a B-cell lymphoma. According to Vezzali *et al.*

(2010) B-cell lymphomas accounts for approximately 80-85 % of the canine lymphomas. It is possible that the low percentage of B-cell lymphomas in this study is caused by the low number of immunophenotyped tumors.

Anemia, hypercalcemia and clinical symptoms

According to Gavazza *et al.* (2008) a mild to moderate non-regenerative anemia is seen in many dogs suffering from malignant lymphoma. In this study, 30.7 % (31/101) of the dogs have a mild or moderate anemia, none of the dogs have a severe anemia. No correlation between anemia and a shorter survival time is found.

Hypercalcemia is seen in 6.67 % (6/90) of the dogs. According to Ruslander *et al.* (1997) and Zandvliet (2016), hypercalcemia is seen in 10-15 % of the dogs and is often associated with T-cell lymphoma and thereby a poorer prognosis. No correlation between hypercalcemia and a shorter survival time can be seen in this study.

Earlier studies states that dogs with B symptoms have a poorer prognosis and shorter survival time than dogs with A symptoms (von Euler *et al.*, 2004). In figure 11, there is a visual difference between the curves. However, the p-value is not significant ($p = 0.21$). This may be due to the number of dogs in each group ($n = 44$ and $n = 28$, respectively). A larger set of data could possibly affect the result.

Limitations of the study

This study has several limitations. First of all, this is a retrospective study, which means that all desired data is not always available. One of the advantages of a prospective study is that the protocols can be made in advance and it is easier to obtain the right set of data. Another problem linked to this study design is that no standardized staging was done. Some of the dogs went through all diagnostic methods and others only confirmed the diagnosis through FNA or biopsies, but no further staging was done. This makes it much more difficult to evaluate if the dogs were at stage 3, 4 or 5 at the time of diagnosis. This is why figure 8 was drawn, where stage 3 and 4 were put in the same group. Another negative aspect of a retrospective study is that it is challenging to count the progression free survival time, which is a better measure of time when evaluating the prognosis. In a retrospective study, the best way to find out the prognosis is to count the overall survival time. The owners often remember when their dog passed away if there is no information about it in the medical record. When choosing overall survival time as a measure of time in the Kaplan-Meier plot, you have to take into account that it is the owner's decision when the dog is going to die. Sometimes it depends on the owner's economy, other times on ethical reasons or on logistic reasons, if the owner cannot take the dog to the veterinary hospital as often as a treatment would require. It also depends on whether the owner contacts the veterinarian as soon as the lymph nodes are slightly enlarged or if they wait until the lymph nodes are very large and the dog finds difficulties breathing. In a prospective study where progression free survival time is used as a measure of time in the Kaplan-Meier plot, these factors will be eliminated. On the other hand, it requires a schedule with planned revisits in advance at decided days. Furthermore, a problem with this study design is that no standardized analyze methods were decided before the start of the study. This causes a difficulty

to compare the test results between different patients in the study and may affect the results in the survival analysis.

Another limitation in this study is the low number of dogs in each group. The problem in this study is that 33 dogs out of 110 could not be counted in the Kaplan-Meier survival curves because they did not receive any kind of treatment and therefore had no overall survival time from the first day of treatment. If a similar study is done, a count of the overall survival time from diagnosis would be preferable. Thereby it would be possible for these dogs to be included in the Kaplan-Meier curves as well.

Furthermore, a limitation in this study is that there is no standardized treatment method. The dogs are treated in several different ways, and the treatment methods have significant differences in the survival time. This is thought to affect the results considerably. A dog with a high grade T-cell lymphoma with clinical symptoms and a high initial TK value in serum, treated with the ADRIA-plus protocol, may have a longer survival time than a dog with a B-cell lymphoma without any clinical symptoms and a lower initial TK value due to treatment with prednisolone or doxorubicin as a single agent therapy.

One thing that may have affected the results in this study is that the oncologists at the University Animal Hospital in Sweden have a good knowledge of the latest research which states that for example a high initial TK value, a high clinical stage and severe symptoms correlates with a bad prognosis (Zandvliet, 2016). This may lead to the advice of euthanasia directly, without trying to treat the lymphoma. Furthermore, this could mean that the patients who have the poorest prognosis are not involved in the study data because they were euthanized right after diagnosis.

No multivariate analyzes are performed in this study due to the lack of significant p-values in the univariate analyzes.

One dog in the study has a survival time of 1130 days which deviates considerably from the other dogs. This dog has a low grade small T-cell lymphoma. This can be explained by the fact that the small clear-cell lymphoma is correlated to a good prognosis and a longer survival time. Its clinical behaviour seems to be similar to that of the indolent human lymphomas, with survival time measured in years (Ponce *et al.*, 2004).

CONCLUSION

In conclusion, the results in this study are not consistent with the hypotheses. No correlation between a higher TK or CRP value and survival is found. Neither could hypercalcemia, anemia, a high clinical stage, a T-cell lymphoma, B symptoms nor a high grade tumor be correlated to a shorter overall survival time. The most important thing for a longer survival time is, according to this study, treatment with the ADRIA-plus protocol, which leads to a significantly longer survival time than the treatment with prednisolone or doxorubicin. Further studies are needed to investigate if the above mentioned parameters are useful in prognostication in dogs with

malignant lymphoma. In the future, prospective studies with progression free survival time as a measure of time and standardized treatment protocols would be preferable.

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