國立臺灣大學獸醫專業學院臨床動物醫學研究所

碩士論文

Institute of Veterinary Clinical Sciences
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Master's Thesis

評估 15 週 LHOP 化療療程對多中心型淋巴瘤犬隻的療效

Evaluate the efficacy of a 15-week LHOP chemotherapy protocol including vincristine, L-asparaginase, doxorubicin, and prednisolone on dogs with multicentric lymphoma

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誌謝

從大學畢業後在檢驗科工作一年半，一路到去智遇工作四五年，其實都在釐清自己未來想過什麼樣的生活，文智學長既是我的老闆也是我的人生導師，在我出現回到學校就讀研究所的念頭時，一直鼓勵我繼續進修，同時在智遇的生活有很多姊姊們，第一天到智遇的時候虹吟學姊見到我緊張模樣還先跟我打氣加油，惟寧學姊在我遇到不懂的時候提供我建議陪我一起進步，希望我可以在成長的過程中變成跟他們一樣溫柔的大人。

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學校的一顆定心丸，雖然都說是自己養自己，但其實吃用住都還是在家裡，也感謝爸爸媽媽都把自己照顧得很好，讓我能自由選擇自己想要的生活。

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中文摘要

多中心型淋巴癌是犬隻最常見的惡性腫瘤，若不進行任何治療，存活時間可能不到六週。目前在小動物的淋巴癌治療上，主要是以 CHOP (C，環磷酰胺；H，阿黴素；O，長春新鹼和 P，類固醇)化療療程為基底的誘導治療。這個治療療程當中包含了四個週期的療程。獸醫師藉由縮短治療週期和搭配不一樣的藥物，希望能減少治療的花費和增加犬隻的生活品質。CHOP 化療療程為基底的化療從 25 週逐漸縮減成 15 週都有相似的治療效果。

在這項研究中，我們將 19 週的 LHOP (L，天冬酰胺酶；H，阿黴素；O，長春新鹼和 P，類固醇)化療療程縮短成 15 週希望能讓動物的生活品質更好和減少飼主的花費。然而在縮短治療時間減少了四次 vincristine 投予之後，結果顯示，縮短後的治療有統計上顯著較差的疾病無進展生存時間（p=0.006）和生存時間（p=0.018）。在 15 週 LHOP 化療療程和 19 週 LHOP 化療療程的中位疾病無進展生存時間分別為 265 天和 401 天，而中位存活時間分別為 420 天和 530 天。而兩組在年紀（p=0.851）、體重（p=0.934）、性別（p=0.093）、臨床分期（p=0.448）、亞分期（p=0.396）、免疫表型（p=0.344）、和總血清鈣（p=0.474）在統計上都沒有顯著差異。因此不建議將 19 週 LHOP 化療療程縮短為 15 週 LHOP。

關鍵字：犬隻，淋巴癌，化療，天冬酰胺酶

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ABSTRACT

Lymphoma is the most common malignant tumor in dogs. Without therapy, dogs with lymphoma may survive less than 6 weeks. In current clinical practice, the majority of veterinarians utilize a CHOP-based (C, cyclophosphamide; H, doxorubicin; O, vincristine; and P, prednisolone) chemotherapy protocol as an induction protocol to treat lymphoma. This protocol includes four cycles. To reduce costs and improve the quality of life for dogs, veterinarians have attempted to shorten the chemotherapy protocol and combine different drugs. The CHOP protocol shortened from 25 weeks to 15 weeks showed similar efficacy.

In our study, we shortened the 19-week LHOP (L, L-asparaginase; H, doxorubicin; O, vincristine; and P, prednisolone) to a 15-week LHOP to improve the patient's quality of life and reduce the cost of treatment. However, after omitting four doses of vincristine, the results showed that the 15-week LHOP had worse progression-free survival and overall survival time than the 19-week LHOP, with significant differences, indicated by *p*-values of 0.006 and 0.018, respectively. The median progression-free survival of the 15-week LHOP and 19-week LHOP were 265 and 401 days, respectively. The median overall survival time of the 15-week LHOP and 19-week LHOP were 420 and 530 days, respectively. No significant differences were found in age (p=0.851), body weight (p=0.934), gender ratio (p=0.093), clinical stage (p=0.448), substage (p=0.396),

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immunophenotype (p=0.344), and total serum calcium (p=0.474). Therefore, shortening the 19-week LHOP to a 15-week LHOP is not recommended.

Keywords: Canine, lymphoma, chemotherapy, L-asparaginase

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LIST OF ABBREVIATIONS

ABBREVIATIONS	
ALL	Acute lymphoblastic leukemia
CHOP	C, cyclophosphamide; H, doxorubicin; O, vincristine; and P, prednisolone
CBC	Complete blood count
CR	Complete remission
COP	C, cyclophosphamide; O, vincristine; and P, prednisolone
DFI	Disease-free interval
G-CSF	Granular colony-stimulating factor
IHC	Immunohistochemistry
L-ASP	L-asparaginase
LHOP	L, L-asparaginase; H, doxorubicin; O, vincristine; and P, prednisolone
MTD	Maximally tolerated dose
NTUVH	National Taiwan University Veterinary Hospital
OST	Overall survival time
PLI	Pancreatic lipase immunoreactivity
PR	Partial remission
PARR	PCR for antigen receptor rearrangement
PFS	Progression-free survival
PD	Progressive disease
QoL	Quality of life
SD	Stable disease
SHC	Sterile hemorrhagic cystitis
IACUC	Institutional Animal Care and Use Committee
VCOG	Veterinary Cooperative Oncology Group
WHO	World Health Organization

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Chapter 1 Introduction

Lymphoma is the most common malignant tumor in dogs, with an incidence ranging from 0.02% to 0.1% (1). Although there are many subtypes, such as mediastinal, alimentary, cranial nerve system, and cutaneous types, multicentric lymphoma comprises 80% of all cases of canine lymphoma (2, 3). The most obvious sign is the enlargement of peripheral lymph nodes, sometimes accompanied by clinical signs such as decreased activity, reduced appetite, and weight loss. Lymphoma can be initially diagnosed by fine needle aspiration cytology, followed by flow cytometry, PCR for antigen receptor rearrangement (PARR), or biopsy with immunohistochemistry (IHC) as second-tier diagnostic methods. Canine lymphoma is classified into B-cell, T-cell, and non-B non-T types based on cell surface antigens. CD79a and CD20 are B-cell lymphocyte-related markers, and CD3 is a T-cell lymphocyte-related marker (4). Approximately 80% of canine lymphoma cases are of the B-cell immunophenotype, while about 20% are of the T-cell immunophenotype. T-cell lymphoma is often associated with hypercalcemia and a worse prognosis (4, 5).

Without therapy, dogs with lymphoma may survive less than 6 weeks. If treated with prednisolone alone, the median overall survival time (OST) was 27 days (6). Consequently, various chemotherapies have been applied to treat canine lymphoma, aiming to improve survival time and quality of life (1). Using doxorubicin as a single-

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agent chemotherapy every three weeks for 4-6 cycles has shown a 70-80% response rate and a median OST of 169-182 days (7, 8). Further research has shown that multi-drug chemotherapy with doxorubicin can achieve a response rate of over 90% and a median OST of approximately 350 to 400 days (9-11). In current clinical practice, the majority of veterinarians utilize a 19-week CHOP chemotherapy protocol as an induction regimen. This protocol includes four cycles of cyclophosphamide, doxorubicin, and vincristine. Prednisone is prescribed at the beginning of the treatment. Veterinarians are continually seeking improved treatment methods to achieve longer survival times, better prognoses, and reduced side effects of chemotherapy in dogs.

L-asparaginase (L-ASP) is an enzyme derived from bacteria that inhibits the uptake of L-asparagine into lymphoid tumor cells, leading to a deficiency in protein synthesis and cell death. The depletion of asparagine by L-ASP starves the cancer cells, leading to apoptosis and tumor reduction. In human medicine, L-ASP has been successful in treating acute lymphoblastic leukemia, significantly improving survival rates in pediatric patients (12). In veterinary medicine, efforts have been made to incorporate L-ASP into multi-drug chemotherapy regimens for treating canine lymphoma. When L-ASP was added to COP (C, cyclophosphamide; O, vincristine; and P, prednisolone) therapy, there was no significant difference in median PFS compared to COP therapy without L-ASP. However, the group treated with L-ASP did show a longer median progression-free survival (PFS)

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of 25 weeks, and the group without L-ASP was with a median PFS of 12 weeks (13). Subsequently, L-ASP was used to replace cyclophosphamide for canine multicentric lymphoma or as a single agent for canine gastrointestinal lymphoma to achieve better outcomes (14, 15). Despite its efficacy, L-ASP can cause allergic reactions and pancreatitis in both humans and dogs, as demonstrated in previous research. Pre-treatment with steroids or diphenhydramine before L-ASP administration has been shown to reduce hypersensitivity reactions (16, 17). Recent studies have indicated no significant increase in canine pancreatic lipase immunoreactivity (PLI) concentrations before and after L-ASP treatment (18). Furthermore, the addition of L-ASP to the 19-week CHOP protocol did not result in additional bone marrow suppression or delays in the treatment regimen. Therefore, L-ASP is considered a relatively safe drug with fewer side effects, holding promise for the treatment of canine lymphoma.

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Chapter 2 Literature Review

2.1 Chemotherapy treatment with or without maintenance

To evaluate whether the maintenance period after a chemotherapy induction protocol affects the efficacy of canine lymphoma treatment, a retrospective study compared the PFS and OST of 6-month CHOP-based protocols with and without a maintenance period. The disease-free interval (DFI) and survival time of two groups revealed no significant differences (10). Recent study in L-ASP and CHOP-based protocol, 81% dogs (333/408) were discontinued after induction and 29% dogs followed by maintenance protocol with chlorambucil and actinomycin-D for two years. There was no significant difference between two groups in response rate, DFI and OST (19). Therefore, there was no advantage to include a maintenance period in the treatment of canine lymphoma. Based on these findings, it is recommended to discontinue the chemotherapy protocol after completing the induction protocol to decrease costs and minimize drug-related side effects. In veterinary medicine, the treatment to chemotherapy is often different from human medicine, and the focus was on closely monitoring the treatment process to ensure a good quality of life. It has been observed that without maintenance phase not only reduces the financial burden on pet owners but also leads to fewer adverse drug reactions, enhancing the overall well-being of the animals. Additional study had focused on the

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quality of life (QoL) for dogs undergoing chemotherapy, and most owners felt treatment was beneficial for canine patients (20). Even without a maintenance period, effective tumor control can still be achieved with minimal discomfort to the patient.

2.2 Shortening the multi-drug chemotherapy protocol

Without a maintenance period, the 25-week CHOP protocol was the main treatment for canine lymphoma. However, veterinarians were still trying to shorten the duration of chemotherapy, and hoping to maintain the same therapeutic effect even with a reduced treatment course. According to several studies, the 25-week CHOP protocol had a median PFS and OST of 282 days and 397 days, respectively, compared to the 19-week CHOP protocol, which had a median PFS and OST of 174 days and 275 days (10, 11). The 19-week CHOP protocol consisted of four identical treatment cycles, while the 25-week protocol administered chemotherapy biweekly for the final two cycles. Despite differences in treatment duration, both groups received the same total drug dosages and showed similar outcomes. These findings suggest that the 19-week protocol may be as effective as the traditional 25-week protocol for treating canine lymphoma.

In a study, a 15-week CHOP protocol omitted four doses of vincristine and did not include co-administration of any chemotherapy drugs at same week, resulting in a median PFS of 176 days and an OST of 311 days (21). The OST from 15 to 25 weeks CHOP

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protocol showed no significant differences, and could be a time and money-saving protocol choice for patients. In order to achieve the goal of shortening the treatment cycle as much as possible, the 12-week CHOP protocol was used for treating canine lymphoma. The median PFS was 243 days, but the median OST was not described due to the owner willingness and different protocol of re-induction (22). In another study that compared a 12-week CHOP protocol with 19-week CHOP protocol, the response rate was similar. The median PFS and OST of 19-week CHOP protocol was 245 and 351 days which was similar with previous CHOP-base chemotherapy protocol. However, the median PFS and OST of 12-week CHOP were 141 and 229 days. The statistically significant decrease in OST and PFS was observed in the 12-week protocol (23). Further investigation is needed to determine the efficacy of the 12-week CHOP protocol. The CHOP protocol was adjusted from 25 weeks to 12 weeks to treat canine lymphoma in the veterinary field. Shortening CHOP protocol could reduce chemotherapy-induced discomfort, decrease the cost of chemotherapy, and lower the cost of managing side effects.

2.3 High dose intensity protocol

In human cancer research, there has been discussion on dose intensity for diffuse large B cell lymphoma, and patients with higher ratio of average relative dose intensity could have better outcome (24). However, it is important to note that chemotherapy

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dosages can be adjusted based on individual patient tolerance to treatment toxicity. Furthermore, bone marrow transplantation and G-CSF (granular colony-stimulating factor) are supportive care measures for high-intensity chemotherapy to achieve better outcomes (25).

A previous study conducted on small animals sought to investigate the effects of increasing doses of cyclophosphamide (from 200mg/m^2 to 250mg/m^2) and doxorubicin (from 30mg/m^2 to 37.5mg/m^2) in a 25-week CHOP protocol, aiming to prolong the PFS and OST. The median PFS and OST of the regular dose group were 197 and 270 days, respectively, while the high dose group showed 300 and 318 days (26). The results indicated that there was no significant difference in PFS and OST between the regular dose group and the intense dose group. However, the median PFS was longer in the high dose group, which included more dogs with substage b. The intense dose group did not show improved treatment efficacy and experienced more severe side effects due to the increased dosage. Therefore, the advantage of the high dose group was mild, and conventional doses would be favorable.

2.4 Co-administration of chemotherapy

Shortening the protocol and administering two drugs together was considered another way to maintain efficacy. However, in a 15-week CHOP protocol, co-

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administration vincristine and cyclophosphamide in chemotherapy. At the end of research, only 61% of dogs completed the treatment as intended, and average completing time was 18.9 weeks due to delays of treatment. The median PFS and OST were 140 days and 257 days for all dogs (27). The group experienced two additional delays, which may suggest that co-administration resulted in a maximally tolerated dose (MTD) protocol, leading to prolonged PFS and OST (284 days and 414 days) compared to the group with one or no delays (74 days and 183 days). Although the delayed group exhibited better OST, the patients experienced severe side effects and ultimately did not see a reduction in the duration of the protocol. Hence, co-administration was not advised unless we could evaluate the dosage to the MTD for each patient, aiming to enhance efficacy and minimize the frequency of toxicity.

2.5 Replacing cyclophosphamide with L-ASP

Cyclophosphamide is a foundational chemotherapy drug in the CHOP protocol and used for treating lymphoma in both human and small animal. It is an alkylating agent that alkylates or binds to DNA, leading to cross-linking of DNA and RNA strands and inhibition of protein synthesis (28). Acrolein, a metabolite of cyclophosphamide, may cause sterile hemorrhagic cystitis (SHC). Gastrointestinal side effects and bone marrow suppression are potential risks associated with cyclophosphamide treatment. To evaluate

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the efficacy of each cytotoxic drug in the CHOP regimen, real-time PCR was employed to quantify residual tumor cells after each treatment. The analysis revealed that cyclophosphamide had a lesser cytoreductive effect compared to vincristine and doxorubicin (29). Additionally, clinical research has shown that the relapse of canine lymphoma frequently occurred after the administration of cyclophosphamide (30). Considering the side effects and lower efficacy of cyclophosphamide, substituting it with other drugs could offer a new approach to treating canine lymphoma.

In a previous study conducted at the National Taiwan University Veterinary Hospital (NTUVH), a 19-week LHOP chemotherapy protocol that replaced cyclophosphamide with L-ASP resulted in a median OST of 344 days (14). The outcome was similar to retrospective CHOP-based protocol in NTUVH, and the toxicity of L-ASP was mild. The use of L-ASP could also lead to convenience in veterinary oncology due to its ease of administration, such as subcutaneous or intramuscular injections (31). Additionally, reducing the severe side effects of cytotoxic chemotherapy may result in fewer hospital visits for managing complications, and further improving the overall treatment experience for both pets and their owners.

Therefore, the 19-week LHOP chemotherapy protocol could be regarded as a first-line treatment option for canine lymphoma due to its similar efficacy, reduced side effects, and potential for improved quality of life. Future studies should continue to explore the

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long-term outcomes and potential combinatory effects of L-ASP with other chemotherapeutic agents to further optimize lymphoma treatment protocols.

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Chapter 3 Objectives

Our study aimed to conduct a prospective trial to shorten the duration of the LHOP chemotherapy protocol from 19 weeks to 15 weeks for canine multicentric lymphoma. Previous research has shown that higher dosages or co-administration of chemotherapy drugs did not lead to better outcomes. Therefore, by reducing the treatment duration without altering the dose intensity, we hope to improve the patient's quality of life and reduce the cost of treatment.

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Chapter 4 Material and Methods

4.1 Patient collection and evaluation

Between August 2021 and February 2024, the dogs diagnosed with multicentric large cell lymphoma, confirmed by fine needle aspiration cytology or histopathology, were recruited for this study at NTUVH (32). The Institutional Animal Care and Use Committee (IACUC) of National Taiwan University granted approval for this research (Approval No. NTU-110-EL-00117). Dogs that had previously received chemotherapy or corticosteroid treatment were excluded.

Prior to treatment, all dogs underwent a comprehensive evaluation that included a physical examination, complete blood count (CBC), serum biochemistry panel, thoracic radiography, and abdominal ultrasonography. Flow cytometry was performed on samples from fine needle aspiration to differentiate between B cell (CD21) and T cell (CD3) origin. Data such as breed, age, body weight, gender, neuter status, total serum calcium, immunophenotype, substage, and clinical stage were recorded for group analysis. The substage and clinical stage, as classified by the World Health Organization (WHO) staging system, were determined based on the results of these examinations (33).

Treatment response, initiation of therapy, disease progression, final follow-up, and death dates were recorded. Clinical pathologists from the Department of Clinical Pathology examined the peripheral blood smears of all dogs. If lymphocytosis or

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lymphoblasts were present in the peripheral blood smear, CD34, a marker for immature hematopoietic progenitor cell, was evaluated to distinguish between stage V lymphoma and acute lymphoblastic leukemia. CD34 is positive in most of ALL cases and negative in most of lymphoma cases (34, 35). A bone marrow examination, considered the gold standard for diagnosing leukemia, was not routinely performed.

4.2 The 15-week LHOP protocol

The 15-week LHOP protocol consisted of 4 cycles, each combined with vincristine, L-asparaginase, and doxorubicin weekly, followed by a one-week rest after doxorubicin (Table 1). Prior to each treatment, a physical examination was conducted to assess the response to the treatment, and a CBC was done to assess for bone marrow suppression. If neutropenia (less than 3,000 cells/μL) or thrombocytopenia (less than 100,000 cells/μL) was detected, chemotherapy would be delayed for 5-7 days to manage chemotherapy-induced side effects. Otherwise, diphenhydramine was administered before L-asparaginase and doxorubicin to prevent allergic reactions.

Following the completion of the 15-week LHOP chemotherapy regimen, patients would undergo follow-up evaluations to monitor for any signs of recurrence. Follow-up appointments were scheduled monthly for the first three months, and then every three months thereafter. Owners were instructed on how to palpate their dog's peripheral lymph

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nodes to check for swelling, and if any abnormalities were found, they were advised to promptly return for a check-up.

4.3 Treatment response assessment

The effectiveness of the 15-week LHOP chemotherapy regimen was evaluated based on the Veterinary Cooperative Oncology Group (VCOG) consensus criteria for the assessment of peripheral lymph nodes (36). Complete remission (CR) was defined by the absence of pathological lymph nodes, along with a normal echogenic spleen and liver. Partial remission (PR) required a reduction in the size of pathological lymph nodes by at least 30% but less than 100%. Stable disease (SD) was characterized by a size reduction of less than 30% or an increase of less than 20% in the dimensions of lymph nodes. Progressive disease (PD) was indicated by more than a 20% increase in the size of lymph nodes and an increase of at least 5 mm compared to the nadir. The development of new lesions and infiltration in new locations were also considered indicators of PD.

The PFS began with the initiation of treatment and ended upon disease progression or the patient's death. The OST also started with the commencement of treatment and concluded at the time of the patient's death. Dogs were censored from the remission duration analysis under the following circumstances: (1) if there was an absence of relapse

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by the study's conclusion; (2) if they became untraceable during the remission period; or (3) if they experienced demise prior to any relapse. Similarly, dogs were censored from the survival analysis if they were (1) untraceable; (2) deceased due to reasons not associated with lymphoma; or (3) they remained alive by the end of the study.

4.4 Relapse protocol

A second cycle of 15-week LHOP chemotherapy was administered as re-induction when lymphoma relapsed, except that doxorubicin was replaced with mitoxantrone (5 mg/m^2). Mitoxantrone, an antineoplastic antibiotic similar to doxorubicin, intercalates into DNA and disrupts topoisomerase-II-mediated DNA repair mechanisms. It was used to treat canine multicentric lymphoma and is associated with fewer side effects than doxorubicin, such as myocardial damage (37-39). Mitoxantrone is used to minimize cumulative cardiotoxicity following a total administered dose of 150 mg/m^2 of doxorubicin (40).

Rescue therapy was administered with alkylating agents for chemotherapy because they enter cells by diffusion and are not substrates for multiple-drug-resistance export systems (41). If the tumor progressed during the induction or re-induction of the 15-week LHOP chemotherapy, alkylating agents would be administered to minimize the effect of

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cross-resistance. Cyclophosphamide with a dosage of 250mg/m^2 every week was used as a first-line drug in the CHOP-based protocol, but it served as a rescue chemotherapy agent in our study. Lomustine, with a dosage of 60 mg/m^2 every three week, was used to treat refractory canine lymphoma as a single agent, and the response rate was 27% (11/41) (42). In addition to its efficacy in treating refractory canine lymphoma, lomustine has shown promise in managing central nervous system infiltration lymphoma due to its ability to penetrate the blood-brain barrier. This characteristic makes it a valuable treatment option for cases where the disease has spread to the central nervous system, offering new hope for dogs with this challenging condition. The treatment options remaining vary depending on the individual patient.

4.5 Historical comparison groups

Medical records of dogs diagnosed with multicentric lymphoma who received the 19-week LHOP at NTUVH from September 2018 to February 2024 were retrospectively reviewed, comparing them with dogs that received the 15-week LHOP protocol chemotherapy which mentioned above. The 15-week LHOP protocol used the same chemotherapy drugs and dosages as the 19-week LHOP (Table 2), with the exception of reducing the number of vincristine administrations by four times compared to the 19-week LHOP, resulting in a shorter protocol. Data on breed, gender, neuter status, age,

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body weight, clinical stage and substage, immunophenotype, total serum calcium, treatment response, dates of therapy initiation, disease progression free survival, final follow-up, and mortality were reviewed and analyzed.

4.6 Statistical analysis

The Student's *t*-test was used to compare the age and body weight difference among the 15-week LHOP and 19-week LHOP protocol. The Fisher's exact test was used to analyze the differences in gender, clinical stage and substage, immunophenotype by flow cytometry or IHC, serum total calcium, and response rate between each group. Kaplan-Meier analysis was used to evaluate PFS and OST, with log-rank test for the difference between treatments. A *p*-value less than 0.05 was considered statistically significant. Statistical software package (IBM SPSS Statistics version 29) was used for statistical analysis.

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Chapter 5 Results

5.1 Study population

There were eighteen dogs diagnosed as multicentric lymphoma and treated with the 15-week LHOP chemotherapy protocol in the study. The dogs included 5 Welsh Corgis, 3 mixed breeds, 3 Poodles, 2 Dachshunds, 2 French Bulldogs, and one each of Beagle, Pomeranian, and Shih Tzu. The median age was 9.5 years, ranging from 3 to 15 years old. The median body weight was 10.9 kg, ranging from 4.2 to 18.4 kg. Among the dogs, there were 12 male dogs, with 7 castrated and 6 female dogs, with 4 spayed. According to the WHO staging system, three dogs were in WHO stage V, 12 in stage IV, 2 in stage III, and 1 in stage II. Eight dogs exhibiting clinical signs were classified as substage b, while the remaining ten dogs were classified as substage a. Seventeen dogs were diagnosed with B-cell lymphomas, while one dog was diagnosed with a T-cell lymphoma. Hypercalcemia was diagnosed in the dog with T-cell lymphoma before receiving the 15-week LHOP protocol. However, the total serum calcium levels returned to the normal range after chemotherapy treatment.

A one hundred percent response rate was observed in the study, with seventeen dogs exhibiting CR, and only one dog exhibiting PR. Three dogs died without evidence of tumor progression and were censored from the PFS analysis. Four dogs were censored in

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the OST analysis. Two dogs died from other diseases (one with renal disease and the other with heart disease), and the other two dogs were still alive at the end of the study. The median PFS and median OST were 265 days, ranging from 91 to 437 days and 420 days, ranging from 91 to 663 days, respectively.

Twenty dogs with multicentric lymphoma, treated with the 19-week LHOP chemotherapy protocol at NTUVH between September 2018 and February 2024 were retrospectively analyzed. The dogs included 7 mixed breeds, 3 Maltese Terriers, 2 Chihuahuas, and one each of Beagle, Welsh Corgi, Dachshund, French Bulldog, Golden Retriever, Standard Poodle, Pug, and Yorkshire Terrier. The median age was 9.5 years, ranging from 4 to 14 years old. The median body weight was 9.55 kg, ranging from 2.2 to 37.7 kg. Among the dogs, there were 8 male dogs, with 3 castrated and 12 female dogs, with 10 spayed. According to the WHO staging system, two dogs were in WHO stage V, 15 in stage IV, and 3 in stage III. Seven dogs exhibiting clinical signs were classified as substage b, while the remaining thirteen dogs were classified as substage a. Seventeen dogs were diagnosed with B-cell lymphomas, while three dog were diagnosed with a T-cell lymphoma. Hypercalcemia was not diagnosed in any dog before receiving the 19-week LHOP protocol.

The response rate of the 19-week LHOP was one hundred percent, with eighteen dogs exhibiting CR, and two dogs exhibiting PR. Two dogs showed no relapse, one dog

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was untraceable, and six died without evidence of tumor progression. These cases were censored from the PFS analysis. Eight dogs were censored in the OST analysis. Five dogs died from other diseases (four with heart disease), one dog was untraceable, and the other two dogs were still alive at the end of the study. The median PFS and median OST were 401 days, ranging from 28 to 1435 days and 530 days, ranging from 71 to 1435 days, respectively.

5.2 Comparison of the protocols

The 15-week LHOP chemotherapy protocol involved a shortened treatment period and a reduction of 4 doses of vincristine. There was no significant difference in age and body weight between the two groups, with *p*-value of 0.851 and 0.934, respectively (Table 3). The ratio of males to females showed no difference with *p*-value of 0.093, and there were no significant differences in clinical stage (p=0.448), substage (p=0.396), immunophenotype (p=0.344) and total serum calcium (p=0.474). No statistics were computed because response rate, including CR and PR, was a constant in two groups. The median PFS and OST were significant difference between the 15-week LHOP and 19-week LHOP, with *p*-values of 0.006 and 0.018, respectively. (Figure 1 and 2)

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Chapter 6 Discussion

Reducing the treatment duration from a 19-week to a 15-week protocol was more convenient for the pet owners. The 15-week LHOP protocol lowered the overall cost of treatment due to fewer weeks of therapy and fewer blood exams before each treatment. We did not record the toxicity of the 15-week LHOP. However, reducing four doses of vincristine from the 19-week LHOP could potentially reduce side effects and toxicity associated with bone marrow suppression and gastrointestinal issues. Nonetheless, shortening the treatment duration may impact the effectiveness of the therapy, and may lead to significant differences in PFS and OST between the two groups. The 19-week LHOP protocol demonstrated better efficacy and a lower risk of relapse, with longer median PFS and OST of 401 days and 530 days, respectively. These results indicated that the 19-week LHOP chemotherapy was better for treating canine multicentric lymphoma as a first-line treatment.

In a previous study, 134 dogs with naïve canine multicentric lymphoma were treated with a 15-week CHOP protocol without higher dose protocol or the co-administration of any chemotherapy drugs. The overall response rate was 98%, with a median PFS of 176 days (ranging from 17 to 1478 days) and a median OST of 311 days (ranging from 25 to 1770days) (21). Similar outcomes compared to other CHOP-based protocols and a higher number of dogs with stage V, substage b, and hypercalcemia in the study suggested that

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the 15-week CHOP protocol was suitable for the treatment of canine lymphoma. Therefore, based on previous studies, reducing the number of vincristine doses by four was believed not to affect the effectiveness of the treatment. This was why the 19-week LHOP protocol was modified to a 15-week LHOP protocol.

However, in this study, reducing the vincristine doses by four led worse PFS and OST. Compared to vincristine and doxorubicin in the CHOP-based protocol, cyclophosphamide works as a non-specific agent in the cell cycle, leading to the belief that it is less likely to result in cross-resistance (41). However, other mechanisms can still cause treatment resistance, such as changes in drug metabolism, activation of DNA repair pathways, and the development of resistance mutations (43). Vincristine works by breaking down cytoplasmic microtubules, leading to cell cycle arrest only at the G2 to M phase transition. Vincristine had strong cytoreductive activity in canine lymphoma (29). While reducing four doses of vincristine in the LHOP protocol had a greater impact than in the CHOP-based protocol, further investigation is still needed to understand the relationship between the drugs and resistance.

Vincristine resistance is related to increased P-glycoprotein (P-gp) and multidrug-resistant protein 1 (MDR1), which are linked to the *ABCB1* gene (44). A previous study attempted to evaluate the resistance of vincristine with the *ABCB1* gene to understand the different responses of canine transmissible venereal tumors, but the results did not show

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a high expression of the *ABCB1* gene (44). Therefore, further investigation into the decreasing vincristine doses between 15-week LHOP and 19-week LHOP protocols was deemed necessary.

The mechanism of L-ASP differs from that of cytotoxic drugs in CHOP-based protocol. Altering the combination of drugs and changing the frequency of L-ASP administration from every five weeks to every four weeks may lead to different results compared to previous study with shortened CHOP-based protocols. Although L-ASP is not involved with the *MDR-1* gene, the immune response to L-ASP was difficult to evaluate, and antibodies against L-ASP could significantly reduce its activity and negatively impact outcomes (16). In the field of human cancer treatment, monitoring L-ASP activity has been clinical valuable and replacing it with a PEGylated version or L-ASP from *Erwinia chrysanthemi* can reduce antigenicity (45, 46). In our study, we did not measure the concentration of antibodies over different weeks, which was related to treatment efficacy. Therefore, developing an L-ASP antibody evaluation protocol and assessing the reasons for resistance to chemotherapy in the future may enhance treatment efficacy. To refine the 15-week LHOP protocol to achieve efficacy comparable to the 19-week protocol while maintaining the benefits of reduced treatment duration.

Although reducing four times of vincristine injection may lead to shorter PFS and OST in canine lymphoma, reducing four times of blood tests can reduce overall blood

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testing costs by 25% the same tests were performed before each treatment. The cost of chemotherapy drugs varies in different countries and hospitals. For instance, reducing four times of vincristine in a dog weighing ten kilograms can result in a 16% reduction in treatment costs at NTUVH. Based on a rough estimate, completing the 19-week LHOP at NTUVH would likely cost around $3,300. Reducing the duration to 15 weeks could decrease the total cost by 20%, providing significant financial relief for pet owners.

Regarding the side effects, according to literature reducing the CHOP protocol duration from 19 weeks to 15 weeks, it reduced the administration of 4 times of vincristine and decreased the side effects of chemotherapy. Gastrointestinal side effects were observed in 80% (24/30) of dogs in the 19-week CHOP study, primarily grade 1 and grade 2, with 7% being grade 4 (11). Although the 15-week CHOP study focused on the total number of occurrences of gastrointestinal side effects, no grade 4 gastrointestinal side effects were recorded (21). The incidence of grade 4 neutropenia was 10% in the 19-week CHOP protocol, compared to 7% in the 15-week CHOP protocol. Although the difference was not statistically significant, the 15-week CHOP protocol not only reduced the treatment duration but also decreased the chances of discomfort. Therefore, in our experiment, we also hypothesize that reducing four doses of vincristine can reduce the discomfort experienced by animals during the treatment process.

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In human medicine, non-Hodgkin's lymphoma is similar in many respects with canine lymphoma and does-intense protocols were indicated for poor prognosis and relapse patient (47). Myeloablative high dose chemotherapy with stem cell transplantation was a treatment for patients less than 60 years old with aggressive relapse lymphoma indicated 46% response rate which was higher than standard dose with 12% response rate (25). However, several studies showed inferior outcomes with high dose protocol, further prospective trial was still needed to perform.

For canine patients, stem cell transplantation was also a strategy for high dose chemotherapy. Three cyclophosphamide dose levels were tested, and the dose were 300, 400 and 500 mg/m^2, respectively (48). In cases of lymphoma complete remission, bone marrow collection followed recombinant canine G-CSF administration, and two weeks later, bone marrow infusion was performed 48 hours after high-dose cyclophosphamide. The highest dose group had the longest survival time, with a median survival time of 139 weeks. The most common side effect of chemotherapy was neutropenia, but with bone marrow infusion, this issue was resolved, and gastrointestinal side effect could be controlled with supportive care. The results indicated that high dose cyclophosphamide was safe and durable and may lead to a better outcome. In human oncology, the dose-dense CHOP chemotherapy shortening 21-day cycle to 14-day cycle and supporting G-CSF in every cycle could support produced better clinical outcomes (25). G-CSF can help

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manage side effects such as neutropenia, allowing for more consistent treatment schedules. Higher dose intensity, which means increasing the frequency of treatment cycles, has shown better outcomes in human oncology with bone marrow transplantation or G-CSF injection. In the small animal field, higher dosages of cytotoxic drugs could indicate better outcomes, but bone marrow transplantation is less common in canine patients. While high-dose and intensive chemotherapy regimens have not demonstrated significant improvements in PFS and OST in small animals, ongoing research into intense dose chemotherapy and supportive care strategies holds promise for enhancing the efficacy and tolerability of lymphoma treatment.

Canine gastrointestinal large cell lymphoma located in the stomach and small intestine, is aggressive and carries a poor prognosis. A study involving 30 dogs with gastrointestinal lymphoma, treated through various methods such as surgery alone, surgery with chemotherapy, chemotherapy alone, and supportive care only, revealed a median OST of 13 days for all cases (49). In another study, primary gastrointestinal lymphoma (13/18) and multicentric lymphoma with gastrointestinal involvement (5/18) were evaluated. Eighteen dogs were treated with a 20-week multi-drug chemotherapy protocol, resulting in a median OST of 77 days (50). Although 63% of dogs (10/16) had a T-cell immunophenotype, indicating a poor prognosis in canine multicentric lymphoma. A study on non-indolent T-cell lymphoma, which excluded gastrointestinal, skin,

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hepatosplenic, and low-grade T-cell lymphoma, found that out of the 70 dogs treated with doxorubicin-based chemotherapy, 64.3% achieved CR and 8.6% achieved PR, with a median PFS of 175 days and an OST of 237 days (51). Compared to multicentric B-cell lymphoma, T-cell lymphoma showed a worse response rate and outcomes.

Although CHOP-based protocol with L-ASP as induction showed no significant benefit for canine lymphoma, a study using continuous L-ASP treatment for canine malignant lymphoma involved 52 dogs (44%) resulting in a median PFS of 216 days and an OST of 236 days (52). The results were similar to the short doxorubicin-based protocol in the same study. However, it didn't administrate L-ASP regularly to patients. To achieve a better and longer treatment response for canine gastrointestinal lymphoma, a recent study replaced multi-drug chemotherapy with weekly administration of L-ASP (15). Weekly continuous administration of L-ASP had not previously recorded for canine lymphoma, as multi-drug chemotherapy had shown a good response rate for canine multicentric lymphoma. However, good treatment response for canine gastrointestinal lymphoma was hard to achieve. However, with weekly administration of L-ASP for canine gastrointestinal lymphoma at a dose of 400 IU/kg subcutaneously, the median PFS was 50 days ranging 2 to 214 days and the median OST was 147 days ranging 2 to 482 days, respectively. The shorter interval between L-ASP administrations appears to enhance its therapeutic efficacy, potentially reducing the development of resistance and

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improving overall outcomes. The findings suggested that weekly continuous administration of L-ASP could be a promising treatment strategy for canine gastrointestinal lymphoma, providing a viable alternative to traditional multi-drug chemotherapy protocols. In our study, we administered L-ASP every four weeks as an alternative short-interval treatment approach. However, monitoring asparaginase levels and antibodies should be conducted in the future for evaluating efficacy.

In small animal studies, the relationship between the L-ASP activity and anti-asparaginase antibodies production has not yet been established. However, in human medicine, therapeutic drug monitoring provides a means of assessing a patient's current depletion status and can be used to better evaluate the potential benefit of treatment adjustments (16). The presence of anti-asparaginase antibodies has been associated with multiple doses of L-ASP administration and may lead to treatment resistance, a major challenge for lymphoma treatment in both humans and small animals. To determine if the antibody reaction is the same in dogs as in humans, a study was developed using ELISA to detect circulating antibodies in dogs treated with L-ASP.

According to human research, antibodies are produced after multiple L-ASP injections with over 50% occurrence. Increasing treatment interval is associated with a higher incidence of anti-asparaginase antibodies. A study tried to investigate the L-ASP and the antibody of canine lymphoma treating with L-ASP to monitor the relationship

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and treatment efficacy (45, 53). To establish positive and negative control, three dogs received three or four doses of L-ASP over several months as positive, while three dogs with lymphoma that had not been treated with L-ASP served as negative controls. Since there is no established canine antibody against L-ASP, the study aimed to distinguish between positive and negative anti-asparaginase antibodies responses. Then, 10 dogs had their first L-ASP administration were monitored for eight weeks, with three dogs showing elevated antibody levels. Of the seven dogs that received a second dose of L-ASP for re-induction or rescue therapy, four showed elevated anti-asparaginase antibodies. Although no significant difference in L-ASP activity was observed between dogs with positive or negative anti-asparaginase antibodies, L-ASP might still be influenced by IgG-mediated mechanisms. Due to the small sample size, further investigation into the relationship between L-ASP activity and antibody production is needed. To reduce the effect of anti-asparaginase antibodies, PEGylation was considered, as canine anti-asparaginase antibody induced by treatment with native E. coli L-asparaginase cross-reacts only weakly with the PEGylated drug in the study.

In children with acute lymphoblastic leukemia (ALL), the correlation with the L-ASP activity level and antibody were evaluated (54). During the induction phase of ALL treatment, L-ASP was administrated in eight doses for every three days. In the re-induction phase, L-ASP was given in four doses starting from day eight, every three days.

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In the induction phase, low L-ASP activity was noted in 7 out of 47 (15%) children, but only 3 of these 7 had anti-asparaginase antibodies, making the relationship unclear. However, children with higher anti-asparaginase antibodies levels were associated with shorter PFS and OST. During the re-induction phase, low L-ASP activity was found in 73% of patients with positive anti-asparaginase antibodies. Anti-asparaginase antibodies were more common during the second course of L-ASP treatment. Despite the different frequencies of L-ASP administration in humans and canines, further investigation into the outcomes and the relationship between L-ASP and antibody production is necessary.

In human medicine, several strategies have been developed to reduce the risk of anti-asparaginase antibody production, such as pretreatment with glucocorticoids, administering an intense and continuous dose, and substituting with an alternative form of L-ASP (16). However, the administration of steroids may mask the presence of subclinical hypersensitivity, which could affect the efficacy of L-ASP treatment and patient outcomes. Therefore, regular monitoring could be beneficial and could potentially be demonstrated in small animal models in the future. In a study involving children with ALL, continuous high doses of L-ASP were administered to forty children during the induction phase, while a lower frequency of L-ASP doses was given to nineteen children during the re-induction phase (55). It was observed that daily high-dose administration resulted in higher enzyme activities. Daily administration of L-ASP also induced less

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allergic reactions, as the continuous exposure to large quantities of the antigen may overwhelm the immune response. This suggests that continuous high-dose L-ASP administration could be a new strategy for treating small animals. In previous canine research, dogs could be treated with PEGylated-ASP as an alternative treatment to decrease immunoreaction for depletion of ASP activity, and there are several asparaginase formulations that could be tried. Consequently, we still have some strategies for addressing the problem of anti-asparaginase antibody.

6.7 Limitation

The limitations of the study included the small number of dogs currently treated with the 15-week LHOP protocol and the lack of a standardized treatment approach following relapse. Additionally, in diagnosing stage V lymphoma, CD34 positivity determined by flow cytometry of the blood is used to identify acute lymphoblastic lymphoma, but bone marrow biopsy was not used as a diagnostic method, which may have led to an underestimation of the proportion of acute lymphoblastic lymphoma.

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Chapter 7 Conclusions

The shorter PFS and OST of the 15-week LHOP protocol compared to the 19-week LHOP protocol was significant. The reasons for the worse outcomes, potentially related to the omission of four doses of vincristine or the presence of anti-asparaginase antibodies, still need further investigation. Therefore, we still need to monitor asparaginase levels to evaluate treatment efficacy, and there are strategies for addressing the problem of anti-asparaginase antibodies. Overall, ongoing research and modifications in chemotherapy protocols aim to optimize treatment for canine lymphoma, focusing on extending survival, improving quality of life, and minimizing side effects.

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Figures

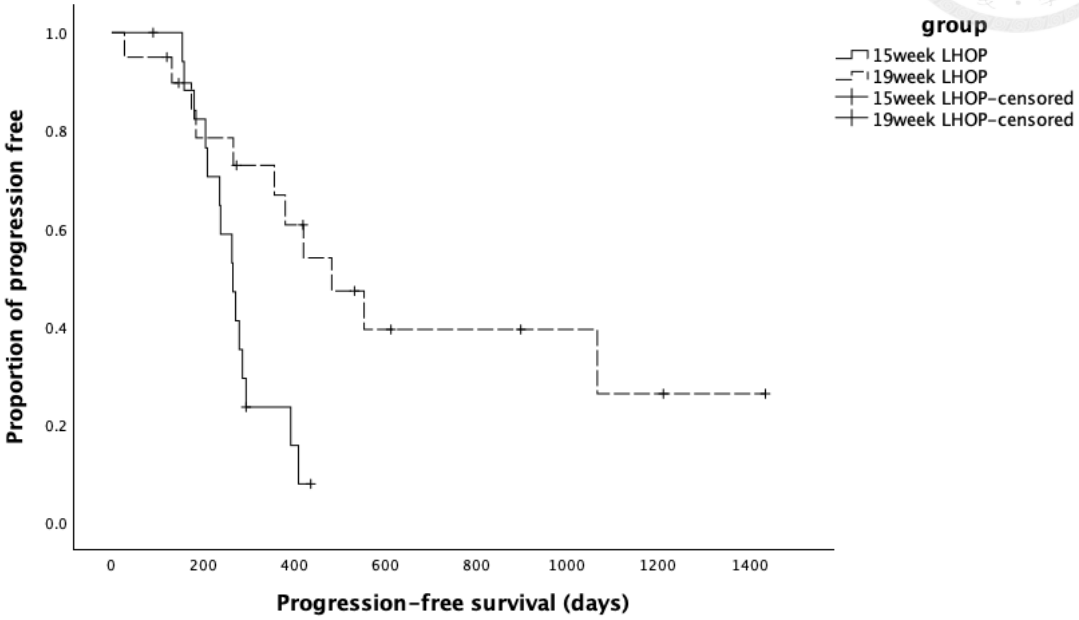

Figure 1. Kaplan-Meier curves represent the progression-free survival duration for dogs with lymphoma.

Dogs treated with the 15-week LHOP chemotherapy are represented by a solid line, and those treated with the 19-week LHOP chemotherapy are represented by a dashed line. Tick marks denote censored patients.

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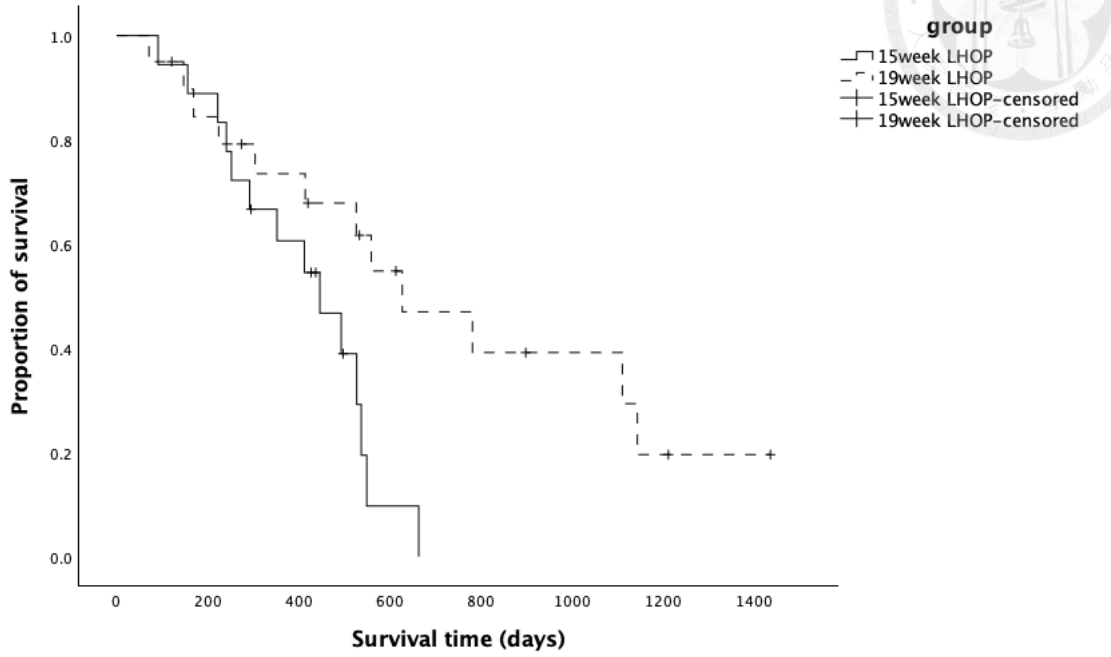

Figure 2. Kaplan-Meier curves represent the survival time for dogs with lymphoma. Dogs treated with the 15-week LHOP chemotherapy are represented by a dotted line, and those treated with the 19-week LHOP chemotherapy are represented by a dashed line. Tick marks denote censored patients.

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Tables

Table 1. The 15-week LHOP protocol.

	week														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Vincristine (0.7 mg/m² IV)	●				●				●				●		
L-asparaginase (400 U/kg IM)		●				●				●				●	
Doxorubicin (30 mg/m² IV)			●				●				●				●
Prednisolone (mg/kg/day PO)	2	1.5	1	0.5											

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Table 2. The 19-week LHOP protocol.

	week																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Vincristine (0.7 mg/m² IV)	●		●			●		●			●		●			●		●	
L-asparaginase (400 U/kg IM)		●					●					●					●		
Doxorubicin (30 mg/m² IV)				●					●					●					●
Prednisolone (mg/kg/day PO)	2	1.5	1	0.5															

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Table 3. Comparisons on signalment, negative prognostic factors, response, progression-free survival, and survival time between the 15-week LHOP and the 19-week LHOP treatment groups.

	15-week LHOP (n=18)	19-week LHOP (n = 20)	*p*-Value
Mean Age (years)	9.3	9.5	0.851
Mean Body weight (kg)	11.1	11.3	0.934
Sex			0.093
Female	6 (33.3%)	12 (60%)	
Male	12 (66.7%)	8 (40%)	
Clinical stage V	3 (16.7%)	2 (10%)	0.448
Substage b	8 (44.4%)	7 (35%)	0.396
T cell	1 (5.6%, 1/18)	3 (15%, 3/20)	0.344
Hypercalcemia	1 (5.6%, 1/18)	0 (0%)	0.474
Overall response (CR + PR)	18 (100%)	20 (100%)	-
Median progression free survival (days)	265 (91-437days)	401 (28-1435days)	0.006
Median survival time (days)	420 (91-663days)	530 (71-1435days)	0.018

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