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# Globo H 於犬惡性乳腺腫瘤之預後價值的評估與探討

# Prognostic Significance of Globo H Expression in Canine Malignant Mammary Gland Tumors

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# 中文摘要

Globo H 為一常見分布於癌細胞表面的醣類抗原，它在正常細胞的表現受限，因此可以做為辨識惡性細胞的標籤，並適合成為癌症免疫治療的標靶。Globo H 於人類醫學中已被廣泛研究，並發現它表現在多個不同的癌症，包含乳癌及肺癌，並被發現與這些惡性腫瘤中較差的預後具有顯著的相關性。執行在小鼠與人的免疫細胞中的分子實驗發現，globo H 具有協助癌細胞迴避免疫系統偵測的功能，對於癌細胞的生長、血管新生、轉移，皆扮演要角。針對 globo H 抗原的疫苗在人乳癌患者的臨床試驗中發現，疫苗的副作用不明顯，並於部分出現 globo H 抗體力價上升的患者中可見存活時間上升的趨勢。犬隻至今未有與 globo H 相關之研究，因而本研究欲調查 globo H 於犬隻惡性乳腺腫瘤的表現狀況，並調查 globo H 是否與惡性乳腺腫瘤的負面預後因子具有相關性。在 globo H 陽性的犬隻中，我們也於第一期臨床試驗中測試 globo H 疫苗在犬隻中的安全性。

在 49 個惡性乳腺腫瘤樣本中檢測 globo H 表現，其中 30（61%）個具陽性表現。與乳腺腫瘤預後因子比較，發現 globo H 的表現與臨床分期較早的案例具顯著相關性（$P = 0.007$），與大小較小的腫瘤具顯著的相關性（$P = 0.04$），並與病理惡性程度較低的腫瘤具顯著相關性（$P = 0.001$）。以上發現皆指向 globo H 陽性表現較常出現於預後較好的乳腺腫瘤，但無病存活時間和整體存活時間並未於統計上與 globo H 表現出現相關性。此研究結果發現 globo H 表現與犬隻乳腺腫瘤負面預後因子並無顯著相關性，與多篇人類腫瘤的研究結果牴觸。

五隻患有惡性乳腺腫瘤的犬隻參與 globo H 疫苗第一期臨床試驗，接受了 globo H 疫苗做為術後的輔助性治療。其中三隻犬隻於接受疫苗注射後出現了疫苗相關的副作用，最常見的副作用為精神沉鬱、厭食、及局部注射部位疼痛反應。副作用的嚴重程度大多輕微，僅有一隻犬隻因出現第三級的精神沉鬱而於後續治療中將疫苗注射的劑量減低，而所有的副作用皆為自限性的，都在三天內完全自行緩解。初步結果發現 Globo H 疫苗耐受度尚可。

關鍵字：globo H、免疫治療、犬乳腺腫瘤、預後因子、乳癌



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# ABSTRACT

Globo H, a tumor-associated carbohydrate antigen, is present on various epithelial tumors such as the breast, colon, ovarian, gastric, pancreatic, lung and prostate cancers in humans. It was realized that globo H had restricted presence on normal epithelial cells, making it an ideal target of immunotherapy. Preclinical studies showed that an antibody response could be successfully generated after vaccination of globo H conjugates. Phase II/III clinical trials found that human breast cancer patients with an elevated antibody response experienced prolonged progression free survival and overall survival times. The present study investigated globo H expression in canine malignant mammary gland tumors (MGT) and its prognostic significance in this type of cancer. Evidence of globo H facilitating cancer cells to evade immune surveillance was apparent *in vitro*, in addition to having associations with negative prognostic factors clinically in various cancers in humans, so we hypothesized that globo H expression would also have significant associations with the known negative prognostic factors in MGT. We also reported here the preliminary results from an accelerated phase I clinical trial of the globo H conjugate vaccine when used in dogs with malignant MGT.

Globo H expression was evaluated in 49 malignant MGT samples from 45 dogs. Globo H was positive in 30 (61%) tumors and negative in 19 (39%) tumors. The median tumor size was significantly larger for globo H negative than positive tumors ($P = 0.04$). Globo H expression differed for early and advanced staged patients; globo H positive tumors were more likely to be early staged tumors ($P = 0.007$). Histologically, globo H expression was significantly more likely to be negative in grade 3 tumors than lower graded tumors ($P = 0.001$). Breed ($P = 0.014$) and weight ($P = 0.013$) also differed significantly for dogs with globo H positive and negative tumors. Globo H expression was



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not associated with overall survival time and disease-free interval for the dogs.

Five dogs with malignant MGT received vaccinations of globo H-KLH with QS-21 as the post-operative adjuvant therapy. Four out of 5 dogs experienced various degrees of adverse events related to the vaccines. Two dogs experienced injection site reaction and 3 dogs became anorexic and lethargic after the second dose of vaccination. While most of the events were low grade and self-limiting, one dog experienced an episode of grade 3 lethargy after the second vaccination, requiring dose reductions in following vaccinations.

Contrary to the findings from human studies, our results showed that globo H expression was associated with several favorable prognostic factors in MGT such as histologic low grade, early stage and small tumor size. According to the molecular functions of globo H, it is suspected that presence of globo H on well differentiated secretory epithelial cells may create a microenvironment suitable for tumor development, facilitating malignant transformation of mammary gland tumor cells. With highly malignant MGTs, tumor cells begin to lose their differentiation, hence the mostly undetectable globo H expression in grade 3 MGTs in our study. More research is required to clarify the discrepancy found in the present study and previous studies. Overall, the case number is too limited to conclude on adverse effects of globo H vaccine but when injected in the initial dose range, it was well tolerated. Further study on safety and efficacy of the vaccine should be continued.

*Keywords*: globo H, immunotherapy, mammary gland tumors, canine, prognosis, human breast cancer



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# Chapter 1 Introduction

Globo H, a tumor-associated carbohydrate antigen, is now found to be expressed on a variety of malignancies in humans[1]. Its expression has not been explored in canine neoplasms before. Globo H has shown promise as a treatment target in human breast cancer (BC); not only because it is present on luminal borders of normal cells where systemic immune response is inaccessible, earlier studies have also demonstrated that an antibody response could be generated by vaccination of synthetic globo H conjugates[2, 3]. Canine malignant mammary gland tumors (MGT) were chosen as a target of exploration here because of the known similarities between canine MGT and human BC[4-6]. Moreover, there is currently a lack of known effective systemic treatment for metastatic MGT, globo H vaccination could potentially be used in metastatic MGT if the molecule's prevalence is high and the vaccine is deemed safe.

The present study consisted of two objectives; first, we investigated the proportion of globo H positivity among canine malignant MGTs then we attempted to identify any correlations between the expression of globo H and the known clinical or histologic prognostic factors of MGT. Second, a fully synthetic globo H conjugate was used as an adjuvant treatment modality in an accelerated phase I clinical trial for canine MGTs[7]. We hypothesized that, as in human malignancies, globo H would carry a negative prognostic impact on the disease progression and survival of canine MGT patients.



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# Chapter 2 Literature Review

## 2.1 Globo H

### 2.1.1 Defining Globo H

Cancer cells commonly present with aberrant glycosylation patterns on their surface glycoproteins or glycolipids. These aberrations have been associated with a change in conformation and functional activity of membrane molecules, thereby affecting many biological processes, namely those involved in immune response and oncogenesis. One such carbohydrate antigen is globo H, a hexasaccharide that was first identified as a ceramide linked glycolipid in a human breast cancer cell line, which then went on to be found to be present on a variety of epithelial tumors including colon, lung, prostate, ovarian, gastric, pancreatic and endometrial cancers. Globo H was first recognized when monoclonal antibodies with specificity for breast carcinoma cells and normal mammary epithelial cells in humans were discovered in 1983[8]. To characterize the antigen on breast cancer cells, murine spleen cells were immunized with cell membrane from the human breast carcinoma cell line MCF-7 then hybridized with mouse myeloma cells. The panel of hybridomas secreting monoclonal antibodies were tested for their range of reactivity. Two of the monoclonal antibodies, named MBr1 and MBr2, had a limited range of activity and reacted with a cell membrane structure common to ductal breast carcinoma cells and normal mammary ducts. They manifested 70% reciprocal inhibition in cross-inhibition tests which suggested that the target of the two monoclonal antibodies was likely the identity of the molecule or of the epitope. One year after the discovery of the monoclonal antibody MBr1, the antigen defined by this antibody was described[9]. Since the antigen was found to be soluble in chloroform/methanol or tetrahydrofuran phosphate buffer extract, it was assumed to be a glycosphingolipid. Through methylation analysis,



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NMR spectroscopy, direct probe mass spectrometry, and enzymatic degradation, the structure of the antigen was identified as Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer. It was clarified that the antigen was a unique globo-series having an H-like determinant at the terminus[10]. Interestingly, the monoclonal antibody has been shown to not cross-react with the normally present H antigen carried by the lacto-series structure which only differed in the configuration of the HexNAc 4-hydroxyl group. However, a degree of cross-reaction with ganglio-series H structure was seen, possibly based on the common structure of Fucα1 → 2Galβ1 → 3GalNAc. The ganglio-H structure is also limited in its expression and is mainly found in normal outer mammalian cells and tissues.

### 2.1.2 Globo H as a target of immunotherapy

For an antigen to be considered as a target for immunotherapy, its precise distribution among the malignant and normal tissues must be identified. Zhang et al. in 1997 studied the distribution of a series of tumor-associated antigens on frozen tissue sections from tumors and normal tissues using a panel of well-characterized murine monoclonal antibodies[1]. Among the antigens included in the study, the globo H hexasaccharide was found to be recognized by the antibody MBr1 in a variety of epithelial tumors, including breast, prostate, lung, pancreas, gastric, ovarian, and endometrial tumor cells. As for their presence on normal tissues, MBr1 was immunoreactive with the epithelia of lung, breast, stomach, pancreatic, uterus, ovary and prostate. Importantly, it was shown that reactivity of MBr1 with epithelia of normal tissues was restricted to the luminal surfaces of glandular tissues. Given that the expression of globo H on normal tissues is restricted to the luminal borders, where immune access is not easily achievable, it has been considered as a potential target for immunotherapy despite being broadly present in normal epithelial



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tissues.

Since the identification of globo H as a suitable target for the development of active immunotherapy, multiple trials have shown success in generating an antibody response to the vaccine targeting globo H[2, 3]. The first total synthesis of the hexasaccharide was made via the method of glycal assembly. The synthetic compound was able to bind MBr1 and inhibit its binding to MCF-7 cells. The compound was then conjugated with a carrier protein, keyhole limpet hemocyanin (KLH), and with bovine serum albumin (BSA) for immunological investigation. Vaccination of mice with 10 mcg of these synthesized epitope along with 10 mcg of immunological adjuvant QS-21 was performed. Serological responses as determined by enzyme-linked immunosorbent assay (ELISA) showed that both IgG and IgM antibodies were induced, with IgM titers being mostly higher than IgG titers, even after booster vaccinations. The sera from the immunized mice that contained anti-globo H antibodies were tested for their reactivity with MCF-7 cells. The anti-globo H antibodies showed direct reactivity with MCF-7 cell lines and did not react with globo H-negative melanoma cell lines at all. It was also observed that antibodies generated by globo H-KLH demonstrated stronger reaction than globo H-BSA-induced antibodies. The antibodies induced were also capable of inducing complement-mediated cytotoxicity and lysis of globo H positive tumor cells. This study confirmed that the synthetic carbohydrate antigen was immunogenic, and the antibodies generated were specific for globo H only. Following the success in the murine model, the vaccination of synthetic globo H-KLH plus QS-21 was initiated in globo H-positive cancer patients[3]. Expression of MBr1 positive antigens were noted on both primary and metastatic prostate cancer specimens, providing rationale for carrying out clinical trials in patients with prostate cancer. In the study that was conducted in 1999, 5 patients with progressive and recurrent prostate cancer received the conjugate vaccine that contained 30 mcg of globo H plus QS-21. Post-



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vaccination patient sera were analyzed with ELISA to estimate IgG and IgM titers against globo H. Of the 5 patients, all demonstrated consistent and strong IgM response, while 2 also showed concurrently high IgG response. Similar to the findings of the previous study using a mouse model, postvaccination sera showed specificity in that they recognized globo H positive tumor specimens and not melanoma specimens which were known to be globo H negative.

It was first observed in the early 1990s during trials of immunization with carbohydrate vaccines in patients with melanoma that despite IgM antibodies were detected in most patients and that disease-free interval and survival were extended in patients producing high-titer antibodies against the GM2 ganglioside, the induced antibody to GM2 was predominantly IgM with short duration whereas IgG response was inconsistent and there was no booster effect[11]. To overcome the limitation that carbohydrates generally exhibit poor immunogenicity, conjugation with a carrier protein and coadministration with an immunological adjuvant have been utilized. In the mouse model, the immunogenicity of the carbohydrate vaccine could be greatly augmented by binding to KLH and with QS-21 as the adjuvant; the IgM titer was higher, and the IgG response was also stronger and long-lasting. The vaccine used by Ragupathi et al. in 1999 for prostate cancer patients was prepared the same way and it also showed strong IgM responses and some had high IgG responses as well[3].

### 2.1.3 Clinical trials of active immunotherapy with globo H conjugates

Following promising results from previous trials, a phase I clinical trial of a fully synthetic globo H-KLH conjugate plus QS-21 vaccine was performed in patients with metastatic breast cancer without evidence of disease or with stable disease on hormone therapy[12]. The primary objective of the trial was to determine the toxicity of this



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vaccine in human breast cancer patients. The study also looked at the antibody response generated and whether the antibodies could mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) as a secondary aim. The patients were monitored for clinical toxicity and response during the trial. Toxicity profile was evaluated in 27 patients in this trial. The vaccine was well tolerated among the patients, with local skin reactions and mild flu-like symptoms being the most common side effects. Local reactions experienced by the patients include discomfort, erythema, and induration, occurrences ranged from 1 to 7 days in duration but most resolved within 3 to 4 days. Flu-like symptoms include myalgias, arthralgias, fever and fatigue also lasted from 1 to 7 days but often resolved within 2 to 3 days. Other more significant toxicity includes one patient that developed a grade 3 elevation in the amylase level of 281 units/liter prior to the $5^{th}$ injection. But there were no symptoms while abdominal ultrasound exam and serum chemistry test were all unremarkable. The amylase level returned to within normal limits two weeks later. Amylase level was monitored closely because globo H is expressed on normal pancreatic cells. Despite the grade 3 increase in the index, there was no apparent illness. Clinical pancreatitis has not been observed in any previous trials either. Since there was only one patient that had such a dramatic increase in amylase, other causes such as infection could not be ruled out. Other biochemical toxicities include grade 1 toxicity in glucose, alkaline phosphatase, serum aspartate aminotransferase, calcium, albumin, or potassium. It is difficult to differentiate between normal biologic fluctuations and toxicity related to the vaccine for these biochemical abnormalities for this patient.

The serologic response evaluated in these patients showed that a strong IgM response was generated among these patients, whereas IgG antibodies titers were consistently low throughout treatment. Significant increases in CDC and ADCC activity



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appeared in approximately 30% of the patients' generated antibodies. The IgM antibodies induced by vaccination demonstrated reactivity with globo H and not with other tumor-associated carbohydrate antigens, further confirming their specificity. The study concluded that vaccination with the globo H conjugate can successfully generate an antibody response with minimal toxicities. Also that the toxicities observed were consistent with those caused by the immunologic adjuvant used in the vaccines[13]. The increased CDC and ADCC observed also encouraged further clinical trials to evaluate its efficacy as a treatment modality.

A multicentered, international, randomized, double-blinded, placebo-controlled phase II/III trial followed to explore the efficacy of active immunotherapy with the globo H-KLH conjugate. The trial allowed hormone therapy and concurrent low-dose chemotherapy using cyclophosphamide was mandatory. The result showed that there was no difference between progression-free survival (PFS) or interim overall survival (OS) for patients receiving the vaccine or placebo. However, PFS and OS improved significantly in a group of patients that developed a globo H specific IgG response, defined as a titer $\geqq$ 1:160 at any time during treatment. Similar to previous findings, the vaccine injections were well tolerated with most adverse events as low grade injection site reactions[14]. Further definitive phase III study is still under development as the results from the previous trial indicated that a target population might exist for which higher antibody titers can be generated and a better therapeutic response can be achieved.

The application of globo H as a therapeutic target has not been limited to human BCs only. Recently, the vaccination of a unimolecular pentavalent carbohydrate containing globo H in advanced stage ovarian cancer patients has been performed in a phase I study. Ovarian cancer is an example where cancer cells are highly heterogeneous in their expression of cell surface antigens; and that a wide array of different surface antigens,



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including globo H, are expressed on the cell surface. it is postulated that to target multiple antigens on the cancer cells would induce a border immune response that lead to better therapeutic efficacies[15]. Other than for the purposes of treatment, the presence of naturally occurring anti-glycan antibodies was also investigated in ovarian cancer patients as a means for diagnosis. The anti-globo H antibody levels significantly differed between ovarian cancer patients and healthy controls, hinting that antibody levels may also be used as an adjuvant diagnostic tool to monitor for the disease[16]. The expression of globo H on different types of tumors, the function of this glycosphingolipid and its prognostic significance are all being investigated currently, further expanding its clinical applications in the future.

## 2.2 Canine mammary gland tumors

### 2.2.1 Overview of canine mammary gland tumors

Canine MGTs are the most common neoplasms in sexually intact female dogs. Well known risk factors for development of MGTs in dogs are age, hormonal exposure and breed of the dog[17]. Growth of the mammary glands is highly dependent on estrogen and progesterone. It has been observed that once the mammary glands have been exposed to these hormones, the changes they make to the mammary glands are irreversible. Hormones binding to their respective receptors induce growth and expansion of the mammary glands that can cause neoplastic transformation. It was also known that estrogen also exerts direct genotoxic effects that increase the risk of mutations. Hence several studies have agreed that the highest benefit of ovariohysterectomy (OHE) to prevent MGTs is when it is done before the first estrus; the risk reduction provided by OHE decreases when the bitch experiences more estrus cycles. Since the performance of



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OHE which prevent exposure to ovarian hormones is done at different timings in different areas of the world, the estimated incidence of MGT varies over different epidemiologic studies. It is commonly noted that the current incidence of MGT in the United States is lower than in other countries because OHE is often done at an early age. Recent epidemiological study performed in Northeastern Italy reported an annual incidence rate of 250 cases per 100,000 dogs[4], which is higher than reported for the United States and other European studies. The overall higher incidence rate may be explained by less common practice of early spaying. However, the same study and another epidemiological study conducted in Mexico recently both observed a particular increase in the incidence of malignant MGT over the years. This may be because of higher awareness of veterinarians of the disease and owners being more willing to pursue a diagnostic work-up for their dogs, leading to more confirmed diagnoses. The influence of environmental factors should also be considered as more studies are reporting on possible carcinogens that may directly affect estrogens and indirectly promote tumor development. The trend of increasing malignant MGTs emphasize that better prophylactic measures or more research to discover possible risk factors that have arose in the modern environment is indicated[18]. Age has been known to be an associated risk factor in the development of MGT. Middle-aged to older dogs are more likely to be affected by MGTs, with a tendency for malignant MGTs to be diagnosed in older dogs and benign MGTs diagnosed in middle-aged dogs. Purebred dogs are more susceptible to the development of MGTs. Both small and large breed dogs have been reported to be at increased risk, including poodles, Chihuahuas, dachshunds, Yorkshire terriers, Maltese and cocker spaniels in the small-breed category, German Shepherds, Pointers, Dobermans, Boxers and Samoyeds in the large-breed category. Genetic influences are suspected to be the reason for such observation. As in well known in human BCs, familial or inherited mutations of certain



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genes such as *BRCA1* and *BRCA2* significantly increase the risk of BCs. The exact role of *BRCA1* and *BRCA2* mutations in canine MGTs is not well understood at present as studies with conflicting results on the effect of these mutations on development of canine MGTs are reported.

### 2.2.2 Treatment and prognosis of canine mammary gland tumors

Surgery is considered the mainstay of treatment for canine MGTs but there is currently no standardized guideline in the surgery for different MGT presentations. MGTs commonly present as multiple tumors due to the nature of hormonal exposure to all of the mammary tissues. For the same reasons, MGTs could progress from benign lesions to malignant lesions under histopathology examination. Dogs previously diagnosed with a malignant MGT also suffer from a higher chance of developing new MGTs[19]. However, past studies have not proven that more aggressive surgery range (chain or regional) mastectomy provided better overall survival times or disease-free intervals[20]. According to these results, when encountering a single tumor of unknown histotype, a surgical excision that is wide enough to ensure complete removal of the tumor will suffice. Available evidence now does not support routine unilateral or bilateral chain mastectomies for prophylactic purposes despite the increased probability of new tumor development in these cases. Well established prognostic factors for MGTs include the tumor size, lymph node involvement and World Health Organization (WHO) stage. Tumor size smaller than 3 cm is found to have a better prognosis, likely associated with the ease to achieve clear margins with surgery. Primary tumor size loses its significance in predicting prognosis when the regional lymph node becomes involved. Lymph node involvement is associated with significantly worse outcome compared to when it is not present, likely indicating more advanced disease. WHO staging system as a prognostic



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factor reflects both the size of the primary tumor and the presence of metastasis, as stated above[17]. Recent studies looked at the impact of different histologic subtypes on progression and survival time have found that specific subtypes such as anaplastic carcinomas and carcinosarcomas were the most aggressive tumor subtypes, harboring the shortest survival times and highest metastatic rates. Simple tubulopapillary carcinomas, intraductal papillary carcinomas and carcinoma and malignant myoepithelioma were also generally more aggressive subtypes than complex carcinomas with a higher risk of death. Histologic grading was associated with overall survival time and with local recurrence and distant metastasis; dogs with grade III tumors had significantly shorter total survival times, whereas dogs with grade I and II tumors both experienced prolonged survival times. While these observations agree with aggressive nature described with grade III tumors, both higher local recurrence rate and distant metastatic rate were also recorded in these reports[21, 22].

Systemic treatment is recommended for some dogs diagnosed with MGTs; as those with more advanced stage disease or higher histologically graded tumors are likely to have already developed micro-metastasis which makes surgery alone insufficient as the sole treatment. However, despite MGT being a commonly encountered neoplasm in the female dogs, so far there is no solid evidence regarding the efficacy of chemotherapy in an adjuvant setting. For the past two decades, several studies reporting on uses of different chemotherapy regimens in canine MGTs have been published. Studies in favor of chemotherapy in the adjuvant setting has reported that drugs including cyclophosphamide, 5-fluorouracil and carboplatin provided benefit in prolonging disease-free interval and survival time[23, 24]. However, these studies suffer from a communal issue in that only limited case numbers were available and the population in each treatment arm were quite heterogeneous, making the level of evidence rather weak. Similarly, studies evaluating



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doxorubicin, docetaxel, gemcitabine and mitoxantrone stated they did not provide any significant survival advantage and also like other studies they do not have inadequate power in their evidence[25-27].

Other than traditional chemotherapy, adjuvant therapy focusing on anti-angiogenesis and immunomodulation utilizing a metronomic approach has shown promise. The expression COX-2 has been reported in MGTs and associated with disease progression and poorer prognosis due to its participation in angiogenesis. COX-2 inhibitors such as firocoxib used in clinical trials show a positive outcome. Thalidomide, owing to its anti-angiogenic and immunomodulatory properties, has also been tested in canine MGT with an observed clinical benefit[27, 28]. Further investigation with larger scale clinical trials is currently lacking, which can confirm their efficacies in canine MGTs. Since no treatment other than surgery has been determined with confidence to be effective for canine MGT, a significant proportion of dogs still suffer from metastatic disease and eventually die from it. New and effective treatment methods are much needed for high-risk canine MGTs.

### 2.2.3 Canine mammary gland tumors in comparison to human breast cancer

Spontaneous canine malignancies have acted as models for cancers in humans on numerous occasions. Canine MGTs and human BC share similarities among them from molecular to clinicopathologic aspects. In particular, the simple type carcinomas of the mammary glands in dogs have been found to exhibit most molecular homology compared to human breast carcinomas. On the other hand, a major difference between complex carcinomas of canines and human BC exists in that myoepithelial proliferation is common



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and an essential part of the complex type carcinomas. Simple type carcinomas are therefore considered more appropriate as spontaneous models for the basal-like subtype of human BCs. Complex type carcinoma also serves as a model for research in the role of myoepithelial cells because their growth is extremely rare in humans[5]. Epidemiologically, malignant canine MGTs also share similar features with human BCs, in that older age at the time of diagnosis, lower expression of hormone receptors, and higher proliferation indices occur in both species[6]. The vast similarities between dogs and women may be attributed to the sharing of the same environment and pollutants that may expose them both to carcinogens[4]. The prognostic factors reported for canine MGTs such as tumor size, nodal involvement and histologic grade are also strong prognostic indicators in human BC, further concurring with the idea of similar biology of the two cancers[6]. The expression of globo H has been extensively examined in human BC and other cancers, however no such research has been done in canine malignancies. Considering several studies on the similarities of the two have been published, we set out to find out whether similar expression of globo H exist on canine MGTs. As effective adjuvant therapies for high-risk MGTs are lacking, the expression of globo H could potentially be a therapeutic target if it is commonly expressed on the tumor.



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# Chapter 3 Materials and Methods

Dogs evaluated in the study were analyzed for the prognostic significance of globo H expression and investigated for the safety of a synthetic globo H vaccine used in dog patients in the study group.

The following information was taken into account when available whether the dog was included for globo H expression study or enrolled for the vaccination trial. Complete clinical history regarding MGT and the neutered status at the time of diagnosis were obtained. Clinical stage was determined using the modified World Health Organization (WHO) staging system[29]. All dogs were physically examined to record the number and size of lesions present at the mammary glands and any presence of regional lymphadenopathy, followed by standard staging tests include thoracic radiography to determine the initial staging. Computed tomography (CT) scans of the thoracic cavity were available for some cases for staging purposes. Fine needle aspiration was routinely performed prior to surgery to rule out the possibilities of tumors other than epithelial tumors at the site of mammary glands. All dogs underwent surgery to obtain a histopathologic diagnosis of MGT. Information regarding details of the surgery, including the intent of the surgery and the extent of the surgery were recorded for all cases. Histopathologic information including surgical margins were collected when available.

## 3.1 Data collection for evaluation of globo H expression and its prognostic significance

### 3.1.1 Study population

Medical records of dogs diagnosed with malignant MGTs of epithelial origin at



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NTUVH between January 2010 and March 2019 were reviewed. Dogs without histopathologic slides available for staining of globo H were excluded.

### 3.1.2 Histopathology and immunohistochemical examination

Samples of surgically resected mammary neoplasms were submitted for histopathologic examination at the Veterinary Pathology Center of National Taiwan University or other private veterinary pathology centers. Samples were fixed in 10X Neutral Buffered Formalin, routinely processed and diagnosed according to the WHO classification system of canine MGTs[21, 30].

Areas of the tumor representative of malignant MGT diagnosis were chosen for immunohistochemical (IHC) staining with a globo H antibody, clone VK9. The staining procedure was performed by Rapid Science Co., Ltd. Interpretation of the staining results was performed by pathologists at the Taipei Institute of Pathology. The results of globo H IHC staining were interpreted semiquantitatively as the percentage of positive cells. Any cell that presents with positive staining within the slide is considered positive. Intensity of staining was further categorized as weak (1+), moderate (2+), and strong (3+). A final H score (range 0-300) was then calculated by multiplying the percent staining by the intensity level. For the purpose of the present study, a H score > 0 was deemed a positive expression of globo H.

### 3.1.3 Statistical analysis

Statistical analysis was performed with SPSS Statistics v.25 (IBM, Somers, New York). The Pearson's Chi-square test or the Fisher's exact test was used to detect whether any significant differences existed between globo H positive and negative cases regarding



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the patients' breed, neutering status, location, stage, presence of ulceration, total number of surgeries received, type of surgeries received, any adjuvant therapies received, histologic grade, and type of the tumors. For categorical variables such as the patients' age, tumor size and weight, the Mann-Whitney U test was used. The clinical outcome of the cases was evaluated in the means of the disease-free interval (DFI) and the overall survival time (OST). DFI was defined as an event where a new lesion development or local recurrence or metastasis was detected. Dogs were censored for analysis of DFI if no tumor recurrence or distant metastasis had been detected at the time of death, at the time of writing or if the patient had been lost to follow up. OST was defined as the time from diagnosis of MGT to the time of death of the dog. Dogs that were lost to follow up, were still alive at the time of writing or died due to causes other than MGT were censored in the analysis of OST. The DFI and OST curves were generated according to the Kaplan-Meier method. The following factors were evaluated for their potential prognostic impact of DFI and OST via the log-rank test: age, body weight, breed, neutering status, tumor size, tumor location, total number of surgeries received, stage, presence of ulceration, type of surgery received, any adjuvant therapy received, tumor grade, histologic type and the expression of globo H. The difference was considered significant when $P < 0.05$. Multivariate Cox's proportional hazard regression analysis was conducted for factors significant in the log rank analysis for DFI and OST.

A spearman's rank correlation coefficient was calculated for the association between the globo H score and the grading of MGTs that were positive in globo H expression to specifically characterize the association between globo H expression and grading of MGTs. The rho value was calculated for evaluation of the degree of association between the two variables.



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## 3.2 Phase I trial of vaccination with a synthetic globo H conjugate

### 3.2.1 Patient selection and inclusion criteria

Dogs presented to the oncology treatment center at NTUVH from July 2018 onward were eligible for inclusion when fulfilling the following criteria: 1) patients surgically treated for MGT that were histopathologically diagnosed to be malignant MGTs, 2) positive globo H expression confirmed via IHC staining. The histopathologic exam and IHC procedure were the same as described above for the retrospective study of globo H expression. The patients were excluded from enrollment if suffering from concurrent diseases including chronic kidney disease of IRIS stage $\geqq$ 3, stage C degenerative valve disease, autoimmune diseases requiring the use of immunosuppressive medications. Serum total bilirubin $\geqq$ 1.5 – 3 times the upper limit excludes the patient from enrollment. Other hematologic and biochemical parameters must be evaluated and considered acceptable via the clinician. The patient was not allowed to use any concurrent non-steroidal anti-inflammatory drug (NSAID) or steroids. And the expected life expectancy must be assessed by the clinician to exceed more than three months to be enrolled in the prospective trial. All enrolled patients had written consent from their owners.

### 3.2.2 Study design

An accelerated phase I trial was designed with the intent of reducing the number of patients receiving suboptimal dosages[7]. Additionally, the globo H conjugate vaccine has been determined to be safe in humans. The globo H conjugate used in the present study consisted of 2 components; the first being globo H covalently linked to KLH, the



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second being QS-21, the saponin-based adjuvant. The vaccine was administered as a mixture of these two components. The initial dose received by the patients depended on their body weights. For the group of patients with body weight less than or equal to 10 kg, the starting dose was 10 μg for globo H-KLH, 30 μg for QS-21. For patients with body weight larger than 10 kg and less than or equal to 30 kg, the starting dose was 20 μg globo H-KLH, 60 μg QS-21. For patients with body weight larger than 30 kg, the starting dose was 30 μg globo H-KLH, 100 μg QS-21.

The accelerated dose escalation study was performed according to the following protocol: there was only one patient per cohort; where there was one patient per dose level, and the dose was increased for the next cohort until one patient exhibited dose-limiting toxicities (DLT) or 2 patients exhibited grade 2 toxicity during their first course of treatment. Toxicity was graded according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Effects (VCOG-CTCAE)[31]. If a patient experienced a DLT episode, the dose escalation strategy would be changed to the traditional method of escalation using a "3 + 3" cohort design[32], wherein the dose would be decreased to that used for the previous cohort and the maximally tolerated dose was then determined based on the number of patients experiencing a DLT. For the group of patients with body weight less or equal to 10 kg, the dose was increased by 100% in the second cohort, then 50% in the third cohort, until the maximal dose is reached. For patients with body weight larger than 10 kg and less than or equal to 30 kg, the dose was increased by 50% for the second cohort where the maximal dose would be reached. The maximal dose was defined as 30 μg globo H-KLH/100 μg QS-21, as this dose was found to be safe and effective in generating an antibody response in human patients. Hence for the group of patients with body weight larger than 30 kg, the starting dose was already the maximal dose, so no dose escalation was made for this group of patients. DLT was



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defined as a grade 3 or higher toxicity in any category except the hematologic category; where the DLT was defined as the occurrence of grade 4 toxicity.

### 3.2.3 Treatment plan and follow up

The vaccine of globo H conjugate was administered as subcutaneous injections. A total of 8 injections were planned on weeks 1, 2, 3, 5, 9, 13, 17, and 25. Before the first injection, a full physical check up to determine the patient's overall condition and accurate staging were performed. Hematologic parameters examined include a complete blood count, peripheral blood morphology, a routine biochemistry panel including albumin, alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, creatinine, blood urea nitrogen (BUN), glucose, total protein, electrolytes examined include sodium, potassium, chlorine, calcium and phosphorus. Chest radiography consisting of ventrodorsal, right and left recumbency views were performed as standard means to detect any metastasis. Abdominal ultrasonography was performed as a general survey and any suspected metastatic lesions within the abdominal cavity. CT of the thoracic and abdominal cavity was performed to accurately characterize any possible lesions within the thoracic and abdominal cavity that were not readily visible upon traditional radiography and ultrasonography. Urinalysis was also performed as a baseline value to detect for further changes following initiation of vaccination with the globo H conjugate. The patients undergoing the trial would be presented to us according to the treatment schedule (Table 7).

### 3.2.4 Safety and response assessment

Toxicity profile was assessed based on physical examination and blood examination



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when patient was presented to us according to the vaccination schedule. Any adverse events encountered were recorded and graded according to VCOG-CTCAE. Contact with owner via phone calls in between the visits were made when owners observed patients showing any discomfort. Hospitalization or extra visits to the hospital were advised when owners described toxicities at least grade 2 in severity.

The response to vaccination was not the primary objective of the study but was also assessed. The response was designated complete response (CR) when all target lesions disappeared, partial remission (PR) when there was at least a 30% decrease in the sum of diameters of all target lesions, progression disease (PD) when there was at least a 20% increase in the sum of diameters of target lesions or appearance of new lesion(s), stable disease (SD) when there was neither sufficient shrinkage to qualify for PR nor sufficient increase to quality for PD. Target lesion(s) was defined as a palpable cutaneous lesion(s), or measurable lesion(s) on abdominal ultrasonography, chest radiography, and abdominal and thoracic CT. Calipers were used to measure palpable cutaneous lesions; the PACS system was used to measure lesions present on imaging diagnostics. The longest axis of each lesion was measured and recorded when assessing the target lesions.



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# Chapter 4 Results

## 4.1 Globo H expression study

### 4.1.1 Patient characteristics

Forty-five patients with malignant MGTs were included in the study for the examination of globo H expression. Thirty-six dogs out of 45 (80%) were purebred dogs. Breeds encountered included 5 (14%) Maltese, 5 (14%) Dachshunds, 4 (11%) Miniature Schnauzers, 4 (11%) Miniature/Toy Poodles, 3 (8%) Shiba, 2 (6%) each of Huskies, Labrador retrievers, French Bulldogs, and 1 (3%) each of a Golden retriever, Tervuren, Jack Russell Terrier, Samoyed, Chihuahua, Beagle, Border Terrier, Pug and Yorkshire Terrier. Mixed breed dogs accounted for the remaining 9 (20%) patients. The median body weight of the population was 8.2 kg (range, 2.1 to 29.5 kg).

The median age at diagnosis was 11 years (range, 4 to 16 years). All patients were females. All dogs were spayed except for two patients (4%). More than half (29/45, 64%) of the spayed dogs were spayed at least one year before its MGT surgery. Twelve dogs (27%) were spayed concurrently with its MGT surgery. Two dogs (4%) were spayed within 1 year of its MGT surgery but not concurrently with the surgery.

### 4.1.2 Tumor characteristics and disease staging

Clinically, MGTs were present in the cranial glands only in 5 dogs (11%), in caudal glands only in 19 dogs (42%), and in 21 dogs (47%) they occurred in both cranial and caudal glands. Tumor size was recorded as the longest diameter of the largest presenting mass and was available for 43 dogs; the median size was 3.5 cm (range, 0.5 to 15 cm). Fourteen dogs (31%) presented with a single tumor at the time of diagnosis, the other 31 dogs (69%) had multiple tumors. Five patients (9%) had ulcerated tumors at the time of



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diagnosis.

The stage at the time of diagnosis was determined for all 45 dogs. Twenty dogs (44%) were in stage 1, 5 dogs (11%) were in stage 2, 5 dogs (11%) were in stage 3, 12 dogs (27%) were in stage 4, and 3 dogs (7%) were in stage 5. The detection of distant metastasis was done via radiography in 33 dogs (73%), and CT in 11 dogs (24%). In 1 dog the staging method was not specified. Lung metastasis was confirmed in 2 dogs via radiography and in 1 dog via chest CT. For the dogs in stage 4, lymph node metastasis was confirmed via histopathology in 6 cases, and via cytology in 5 cases. In 1 dog lymphadenopathy was seen in the axillary lymph node on CT imaging and a stage 4 was designated.

### 4.1.3 Histologic diagnosis and globo H expression

Out of the dogs that had more than one surgery to remove MGTs, four had multiple samples available for staining so a total of 49 slides from formalin-fixed, paraffin-embedded tissue specimens from these 45 patients were immunohistochemically stained with globo H antibody. Epithelial originated tumors including carcinomas (27/49, 55%) and adenocarcinomas (22/49, 45%) were diagnosed in the 49 malignant MGTs. Histologic subtypes were available for 48 MGTs. Eighteen cases (38%) presented with more than one type of histologic patterns. The complex and solid subtypes were among the most commonly presented subtypes, both accounted for 18% of all subtypes. Of the 49 tumors evaluated; 11 (22%) were classified as grade 1, 11 (22%) were classified as grade 2, and 27 (55%) were grade 3 tumors. For four tumors that were classified as grade 3, the grading was not initially available on the pathologic report and was later designated based on the histopathologic descriptions. For the four dogs that had more than one samples available for review; one had a grade 2 tumor the first time, grade 3 tumor the second time, one had a grade 2 tumor the first time, a grade one tumor the second time, the remaining two dogs



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had grade 3 tumors for both samples.

Globo H expression was evaluated for these 49 tumors; positive expression was detected in 30 out of 49 (61%) tumors (Figure 1Figure 2), whereas negative expression was detected in rest of the 19 tumors (39%, Figure 3). Among the dogs with two tumor samples from different MGTs available, one dog had one negative tumor and one positive tumor, one dog had two positive tumors, and the other two dogs had both tumors with negative globo H expression. Among the 19 MGTs without globo H expression, 2 (11%) were grade 1 tumors, 1 (5%) was a grade 2 tumor, and 16 (84%) were grade 3 tumors. For the 30 tumors expressing globo H, 9 (30%) were grade 1 tumors, 10 (33%) were grade 2 tumors and 11 (37%) were grade 3 tumors. A statistically significant difference ($p$ = 0.001) was found between the lower graded tumors (defined as grade 1 and grade 2 tumors) and grade 3 tumors for their expression of globo H (Table 1). Globo H expression was significantly more likely to be negative in grade 3 tumors than lower graded tumors. When comparing globo H expression between grade 3 and grade 1 tumors, and between grade 3 and grade 2 tumors, significant differences were also detected (P = 0.033 and 0.01, respectively). There was no significant difference detected between globo H expression in adenocarcinomas or carcinomas. Seven out of 19 (37%) globo H negative tumors were adenocarcinomas and the other 12 (63%) were carcinomas. For the 30 tumors that were globo H positive, adenocarcinomas and carcinomas each accounted for half of the tumors.

The globo H score in the positive tumors ranged from 0.5 to 140, the median score was 4.25. A statistically significant differences ($p$ = 0.045) was detected when looking for correlations between the globo H score and the grading of tumors. A negative correlation coefficient (rho = -0.374) was apparent, indicating lower graded tumors were associated with higher globo H scores.



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### 4.1.4 Treatment and follow up

All 45 patients received some form of surgery for treatment, diagnosis or both. Thirteen dogs (29%) underwent two mammary gland surgeries. Three dogs (7%) had more than three mammary gland tumor surgeries performed. For the patients that received more than one surgery, the following lists the primary surgery that obtained the pathologic diagnosis and does not include further surgeries received for treatment purposes. Lumpectomies were performed in 13 cases (29%), regional mastectomy was performed in 21 cases (47%), chain mastectomy was performed in 7 cases (16%), incisional biopsy/tru-cut was performed 2 cases (4%), single mastectomy was performed in 4 cases (8%), and bilateral chain mastectomies were performed in 2 cases (4%). For the total of 49 surgeries that had pathologic slides available for review, 46 of them had surgical margins evaluated. Thirty out of 46 (65%) had clean margins and the other 16 (35%) had dirty margins. Out of the 21 surgeries without local recurrences or metastases, 19 (90%) had clean margins, 2 had dirty margins (10%). Local recurrences developed in 2 cases (7%) following clean surgical margins, whereas it developed in 5 cases (31%) following dirty surgical margins. Two cases (7%) following clean margins and 4 cases (25%) following dirty margins developed both local recurrences and metastases. Three dogs (10%) developed MGTs in separate mammary glands following clean margin surgeries. Overall, 9 (56%) local recurrent events and 9 (56%) metastatic events occurred following dirty margin surgeries, 4 (13%) local recurrent events and 6 (20%) metastasis events occurred following clean surgical margins. Dirty margins were significantly more likely to be followed by local recurrences ($p = 0.006$) and distant metastases ($p = 0.002$) than clean margins.

More than half of the population (27/45, 60%) did not receive any adjuvant therapy following surgery. Twelve patients (27%) received traditional chemotherapy as an



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adjuvant therapy. Adriamycin (7/12, 58%) was the most commonly used chemotherapeutic agent. One patient received a combination of mitoxantrone, vinorelbine and cyclophosphamide. One patient received six doses of carboplatin for its MGT but had also received metronomic chemotherapy before this for its liver neuroendocrine tumor. Two patients received metronomic chemotherapy. One patient received ten cycles of vinblastine and cyclophosphamide for its mast cell tumor while its MGTs were already present but had not yet been histopathologically diagnosed.

The median follow up duration of the population was 152 days (range 14 – 2158 days). Within the study period, 21 dogs (47%) did not experience local recurrence, development of MGT at a different mammary gland, or metastasis to lymph nodes or lungs. Ten dogs (22%) developed local recurrence, 3 (7%) of which also had metastasis. Three dogs (7%) developed new MGTs at different mammary glands from the previously diagnosed location. Three dogs (7%) did not have all presenting tumors removed and also had metastatic disease. Eight dogs (16%) developed metastasis and did not have further local lesions detected. Twenty-four (53%) dogs were still alive at the time of writing. Fifteen dogs (33%) were confirmed dead, 9 (20%) were presumed to be MGT related as no post-mortem examination has been performed. Six dogs (13%) were lost to follow up but had all been bearing disease at the last time of follow up. Thirty-six dogs (80%) were censored for the analysis of overall survival time. The median OST of the population was not reached; whereas the median OST for the nine dogs with MGT as the presumed cause of death was 85 days. Three dogs (7%) were censored for the analysis of DFI because an event has not occurred at the time of their death due to other reasons. Two dogs (4%) were censored due to loss of follow up. Fifteen dogs (33%) were disease-free at the end of data collection. The median DFI for the whole population was 123 days.



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### 4.1.5 Prognostic factors

The following variables were evaluated for their impact on DFI and OST: age, breed, body weight, neutering status, tumor location, tumor size, number of malignant MGTs, presence of ulceration, disease staging, number of MGT surgeries received, type of surgeries received, histologic margins, any adjuvant therapy received, grading and histologic type.

Tumor size was significantly associated with OST (Figure 4); dogs with tumor size larger than 5 cm in diameter had a median OST of 164 days, whereas the median OST was not reached for dogs with tumors less than 3 cm or between 3 and 5 cm in diameter. The survival time difference between dogs with tumors larger than 5 cm and dogs with tumors less than 3 cm was statistically significant ($P = 0.001$). The median DFI was 243 days for dogs with tumors < 3 cm in diameter, 89 for dogs with tumors 3 – 5 cm in diameter, and 149 for dogs with tumors > 5 cm. The differences between tumors < 3 cm and > 5 cm was almost reaching significance ($P = 0.056$, Figure 5). The presence of ulceration significantly associated with both DFI and OST. The median DFI was 219 days for dogs whose tumors were not ulcerated, whereas the median DFI was 13 days for dogs with ulcerated tumors. The difference reached statistical significance ($P = 0.003$, Figure 6). The median OST was not reached for dogs without ulcerated tumors. The median OST was 65 days for dogs with ulcerated tumors. The difference was statistically significant ($P < 0.001$, Figure 7).

Stage of disease was evaluated in the means of whether disease remained local (stage 1 to 3) or metastasis has occurred (stage 4 and 5) due to insufficient case number in each stage. The median DFI was 243 days for dogs with local disease only. The median DFI was 13 days for dogs with metastatic disease. The difference reached statistical significance ($P = 0.029$, Figure 8). The median OST was not reached for dogs with local



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disease, whereas it was 164 days for dogs with metastatic disease. A statistically significant difference was detected ($P = 0.008$, Figure 9).

For adjuvant therapies received, only the group that did not receive any adjuvant therapy was evaluated against a group that received Adriamycin as their adjuvant therapy due to low case numbers in other groups of adjuvant therapy. The median DFI for Adriamycin group was 89 days, the median DFI for dogs that received no adjuvant therapy was 243 days; the difference was not significantly different ($P = 0.069$, Figure 10). The median OST was not reached for dogs that received no adjuvant therapy. The median OST for dogs that Adriamycin as 164 days. A statistically significant difference was reached ($P = 0.028$, Figure 11).

The median DFI and OST were not significantly different between dogs with tumors of different grading. For dogs with grade 1 tumors, median DFI was not reached; the median DFI was 184 days for grade 2 tumors and 149 days for grade 3 tumors ($P = 0.232$). The median OST for dogs with grade 1 tumors was 307 days whereas it was not reached for dogs with grade 2 and grade 3 tumors ($P = 0.482$). The dogs' age, body weight, breed, neutering status, number of malignant MGTs, and tumor location were not associated with DFI or OST.

Only dogs that received lumpectomies, regional mastectomies or chain mastectomies were included for the impact of the type of surgery on OST and DFI due to a low number of cases that had other types of surgeries performed. The type of surgery received did not significantly affect the DFI and OST of the population ($P = 0.458$ and 0.35, respectively). On the other hand, histologic margins were significantly associated with DFI ($P < 0.001$, Figure 12). Dogs with a dirty surgical margin had a median DFI of 0 days, whereas dogs with clean margins had a median DFI of 243 days. The median OST was not reached for both dogs with clean and dirty surgical margins; the difference



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between the two did not reach a statistically significant difference ($P = 0.063$, Figure 13).

The variables that exhibited significant differences via the log rank test, including tumor size, stage and ulceration for OST and stage, ulceration and histologic margin for DFI, were further included in the Cox proportional hazard regression model to test their significance in a multivariate model. For DFI, only dirty histologic margin retained a significant impact on DFI ($P = 0.026$, Table 5). It was found that none of the variables for OST retained significance under multivariate analysis (Table 6).

### 4.1.6 Globo H: its association with other prognostic factors and survival

Clinical variables such as body weight, tumor size, age, breed, neutering status, disease staging, number of malignant MGTs, tumor location, presence of ulceration, type of surgery received, any adjuvant therapy received, histologic grading, histologic margin, histologic type and subtype were evaluated between globo H positive and negative tumors to detect any significant differences between the two groups.

Among the clinical variables, it was found that dogs with globo H positive tumors (median body weight was 7.3 kg) weigh significantly less than dogs with globo H negative tumors (median body weight was 15.7 kg, $P = 0.013$). The median tumor size for globo H positive tumors was 2 cm, and the median tumor size for globo H negative tumors was 4.5 cm, reaching a statistically significant difference ($P = 0.04$, Table 2). Twenty-seven out of 30 (90%) globo H positive tumors were from purebred dogs, whereas only 11 out of 19 (58%) globo H negative tumors were from purebred dogs. The difference reached a statistical significance ($P = 0.014$).

For the 19 tumors that were globo H negative, 8 (42.1%) were staged as local disease only, the other 11 (57.9%) were staged as metastatic disease. For the 30 tumors that were globo H positive, 24 (80%) were staged as local disease and the other 6 (20%) were with



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metastatic diseases. A significant difference was detected ($P$ = 0.007); globo H positive tumors were more likely to be early staged tumors (Table 3).

The median OST and DFI did not differ significantly between dogs with globo H positive and negative tumors. The median OST for dogs was 555 days for dogs with globo H positive tumors and was not reached for dogs with negative tumors ($P$ = 0.73). The median DFI for dogs with globo H positive tumors was 219 days; for dogs with globo H negative tumors it was 483 days. The difference did not reach statistical significance ($P$ = 0.691). Globo H expression did not significantly differ with age, neutering status, number and location of malignant MGTs, presence of ulceration, type of surgery received, histologic margin and any adjuvant therapy received (Table 1 and Table 3).

## 4.2 Preliminary results from phase I clinical trial of globo H vaccine

### 4.2.1 Patient and tumor characteristics

Five patients were included in the phase I trial. Summary of each dog's status is listed in Table 8. The median age of the dogs was ten years (range, 7 to 13 years). Three of the dogs were spayed at least one year before MGT occurrence, where the other two were spayed concurrently with its mammary tumor surgery.

All five patients were referred to the oncology treatment center at NTUVH after surgery either from private practices or from the surgery department of NTUVH. The purpose and extent of surgery were decided by the surgeons. Dog 1 had undergone a lumpectomy at the left 4$^{th}$ mammary gland and was diagnosed as a grade 1 simple tubular carcinoma. Dog 3 had three mammary tumor surgeries in total, the first surgery consisted of a lumpectomy at the left third mammary gland that revealed a grade 2 simple type



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carcinoma, the second surgery was a left chain mastectomy 2 months later that revealed an adenoma at the location of the left 5th mammary gland, the third surgery was 5 months later and involved a single mastectomy at the right 4th mammary gland that revealed a grade 1 mixed type carcinoma. Dog 4 had a previous mammary tumor excision without a reviewable pathologic report. This patient had remaining gross lesions, one present on the midline of the second mammary glands, the other present on the left flank lateral to the mammary glands, both of which were diagnosed as malignant MGTs based on cytologic findings. Dogs 2 and 5 underwent a regional mastectomy and were diagnosed as grade 2 cribriform carcinoma and grade 3 solid type carcinoma, respectively. The surgical margins were clear for the most recent surgeries for all patients except dog 1 where the margin was not evaluable. The regional lymph node was excised while performing regional mastectomy for dog 2 and 4, which confirmed lymph node metastasis for dog 4.

Upon completing imaging examinations at entry into the study, stage 5 was confirmed for dog 4 that exhibited lung metastasis on CT. Dog 4 also had medial iliac lymphadenopathy and splenomegaly. Lymphadenopathy was suspected to be caused by tumor metastasis whereas splenomegaly was thought related to its previous babesia infection. All other dogs had no lesions detectable on CT, chest radiography or abdominal ultrasound and were determined to remain at stage 1.

### 4.2.2 Treatment and follow up

Dogs 1, 4, and 5 belonged in the under 10 kg body weight group. Dog 1 received the baseline dose for the group which was 10 μg globo H-KLH/ 30 μg QS-21. Dose escalation was performed for dogs 4 and 5. Dog 4 and 5 received 20 μg/ 60 μg and 30 μg/ 100 μg globo H-KLH, respectively. Dogs 2 and 3 were within the 10 to 20 kg body weight group. Dog 2 received the baseline dose of 20 μg/ 60 μg globo H-KLH for the group whereas



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dog 3 received the escalated dose of 30 μg/ 100 μg.

Dogs 1, 2, and 3 completed all 8 injections of globo H vaccine. Dog 5 has completed 6 vaccinations at the time of writing and will continue the protocol. Dog 1 developed a new mammary gland tumor at its right 2nd mammary gland that was subsequently excised and diagnosed as an adenoma 253 days from its initial diagnosis. Dog 2 had multiple soft tissue nodules detected within the lung fields that were consistent with metastasis 139 days from diagnosis, 100 days into the treatment. Dog 4 had gross lesions that continued to enlarge from the day of diagnosis and reached PD 29 days into the treatment. The dog discontinued treatment with globo H vaccine after four doses and went on to receive metronomic chemotherapy. All five dogs were alive at the time of writing. Dogs 3 and 5 have not experienced recurrence or metastasis so far. The median DFI for this group of patients was 253 days. The median OST was not reached.

### 4.2.3 Vaccine-related toxicity

A total of 34 doses of globo H vaccine were injected. Out of these 34 doses, adverse events were noticed after 18 doses. Dog 2 experienced no toxicity thought related to the vaccines during the treatment period. This dog developed hematuria 131 days into the treatment, 32 days post the 6th vaccination. Urinary tract infection was diagnosed upon a positive urine culture and considered irrelevant to the vaccines. Dog 4 experienced no adverse events in the doses that it received. Dogs 1, 3, and 5 all experienced adverse events related to the vaccinations. Three dogs (dog 1, 3 and 5) experienced administration site conditions; grade 2 injection site reaction reflected as pain upon palpation, swelling, and erythema of the site. The response was more severe for dog 5 where the swelling extended to the hind limb ipsilateral to the vaccinated side. The reaction resolved within three days for both dogs. One of the dogs received tramadol for pain management. The



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other dog received no extra management. Dogs 1, 3, and 5 experienced various degrees of anorexia and lethargy; dog 1 had grade 1 anorexia for 1 to 2 days post-vaccination but remained normal in activity; dogs 3 experienced grade 1 lethargy and grade 2 anorexia lasting 1 to 2 days post-vaccination; dog 5 had grade 3 lethargy, showing signs including marked generalized weakness, inability to urinate and to consume food or water. The condition resolved spontaneously by the third day after vaccination.

No abnormalities in hematology and serum biochemistry that occurred were thought to relate to the vaccinations. Dog 4 had thrombocytopenia before receiving vaccinations but was attributed to its previous Babesia infection and the resulted splenomegaly. Dog 1 had elevated liver enzymes, including grade 1 ALT, grade 3 AST and grade 2 total bilirubin elevations on the day scheduled for the $5^{th}$ vaccination. The patient exhibited no clinically-evident abnormalities. The abnormality was thought irrelevant to the vaccine and the vaccine was administered as planned. Dog 5 had grade 2 elevated ALT before the treatment and continued so throughout the treatment period. Dog 4 had decreased specific gravity of its urine before receiving vaccinations, so the subsequent mild increase in creatinine was considered to related to its primary renal insufficiency and not related to vaccinations.

The adverse events experienced by the patients were observed to be most severe after the second vaccination, whereas no adverse events occurred after the first vaccination for any of the dogs. The severity of the adverse events also decreased after the third vaccination but was still observed for dog 1, 3, and 5. Dog 5 was the only patient that required a dose reduction of its vaccine as a grade 3 adverse event was encountered. Dogs 3 and 5, both of which received the escalated dose for their range of body weight, experienced higher graded adverse events including lethargy and anorexia compared to the other dogs in their range of body weight.



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# Chapter 5 Discussion

## 5.1 Globo H as a prognostic indicator

Several cancer cell surface glycans and glycosphingolipids, including globo H, that display altered glycosylation patterns, have been associated with cancer cell migration, invasion, angiogenesis and metastasis. Tumor-associated gangliosides were shown to induce immunosuppression that aid cancers to escape from host immune surveillance through multiple mechanisms, including inhibition of antigen processing and presentation, and suppression of T cell proliferation and cytokine production such as IFN-γ and IL-4[33]. The aberrant glycosylation patterns of these glycan structures have mostly been associated with a negative prognosis, as evading immune surveillance and enhanced angiogenesis are key tumor survival and progression tactics. Earlier studies looking into the relationship between human breast carcinoma and the presence of globo H found that globo H expression was significantly associated with a premenopausal status. Diagnosis of BC in premenopausal women was a negative prognostic indication with a worse 15-year survival rate[34], indicating that the presence of globo H may have prognostic values.

Since globo H is widely expressed on a variety of cancers, whether it shared similar prognostic indication when present on different types of cancers were also investigated. Small cell lung cancer was examined with the monoclonal antibody MBr1 which led to the discovery of an association between short survival and the presence of globo H, concluding that the molecule was relevant to tumor aggressiveness[35]. In another study evaluating globo H expression in human thyroid tumor, it was found that the presence of globo H was associated with the presence of other prognostic indicators, such as higher frequency of extrathyroidal invasion and BRAF V600E mutation in the papillary thyroid cancer subgroup. There was also a correlation between more advanced disease and globo



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H, but a statistically significant difference was not reached. It was deduced that globo H might have prognostic value in this type of cancer as well[36]. To investigate the potential prognostic significance of globo H expression on canine malignant MGT, we first attempted to identify clinical and histologic variables that were associated with prognosis in our population and then assessed the association of globo H expression with these variables. The present study was the first canine study looking into the association between globo H and MGT.

In the present study, several well-defined prognostic factors in canine MGT were found, including larger tumor size, advanced staging, and the presence of ulcerated tumors, were significantly associated with a negative prognosis. Larger tumor size was associated with a shorter OST but not significantly associated with DFI. Tumor size has been widely reported to be a prognostic indicator in canine MGT[6, 20, 22, 37-39]. A tumor size ≥ 5 cm in diameter has been associated with a higher risk of lymph node metastasis, distant metastasis and local recurrence, and a lower survival rate at two years after surgery. Additionally, a strong association between tumor size and malignancy was also found; larger tumors were significantly more likely to be malignant in a previous study. This may be explained by a higher growth rate in malignant tumors and it has also been proposed that malignant MGTs arise from benign tumors in a prolonged clinical process. A tumor larger in size also marks increased difficulty for wide and complete excision of the tumor, hence may be responsible for the increased risk of local recurrence and shorter survival rate. The reason why DFI was not associated with tumor size in our study may be because of low number of samples, so the significance of the association was not reflected statistically. Results of the present study showed that tumor sizes differed significantly between globo H positive and negative tumors, with larger tumors more likely to be globo H negative ($P = 0.004$).



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Being in an advanced disease stage was associated with both DFI and OST in our study. Similarly, previous studies have found a significantly longer 1- and 2-year survival rate for dogs without evidence of metastasis[39-41]; lymph node involvement has consistently been an indicator of unfavorable prognosis. Being in stage 4 or 5 were not analyzed separately in our study for their prognostic significance due to insufficient case number in these two stages. As for globo H expression, similar to the findings regarding tumor size, a positive globo H expression was more likely to occur in tumors without metastasis. Both results suggested that globo H expression may be negatively correlated with the advancement of TNM stages.

Globo H expression was not found to be associated with the use of adjuvant therapy, which was associated with OST in our study, and with the presence of ulceration, which was associated with DFI in our study. But an association was found between the grading of tumor and globo H expression ($P = 0.001$). Globo H expression was significantly more likely to be negative in grade 3 MGTs compared to grade 1 and 2 MGTs. Despite grading not being identified as a significant prognostic indicator in the current study, grading was associated with survival time, local recurrence and distant metastasis in several studies. Grade 3 tumors had a significantly shorter survival time compared to grade 1 and 2 tumors[22, 39, 42]. In contrast to the speculation that globo H expression would also be a negative prognostic indicator in canine MGT as in human malignancies, the results so far have proven otherwise.

Since the presence of globo H has corroborated with multiple clinical and histologic prognostic factors in various malignancies in humans as mentioned above, the molecular functions of globo H have thus been investigated. Studies done on globo H have demonstrated *in vitro* that globo H ceramide can inhibit activation and proliferation of human and murine immune cells. When human peripheral blood cells were cultured with



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cells from the MCF-7 cell line expressing globo H, expression of globo H on lymphocytes could be detected after 3 days, suggesting uptake of globo H by lymphocytes. Globo H-treated splenocytes and CD4+ T cells failed to respond to stimulation with plate bound antibodies against CD3/CD28 and had reduced proliferation. Globo H had otherwise no effect on proliferation when no anti-CD3/CD28 stimulation was performed. As for B cell proliferation, CD19+ B cells treated with globo H also had reduced proliferation upon stimulation with LPS or LPS+IL-4+CD40L. This differed from previous studies on other gangliosides that discovered they were able to cause only immunosuppression. Furthermore, the acquisition of globo H ceramide by activated splenocytes and CD4+ T cells decreased the production of IFN-γ, IL-2 and IL-4, leading to suppressed Th1 and Th2 responses and a favorable environment for cancer growth. Varying composition of carbohydrate moiety of glycosphingolipids on different tumors is thought to determine the balance of Th1/Th2 activities. It has been previously reported for tumors such as renal cell carcinoma that its surface gangliosides shifted the immune response toward Th2 to help evade host immune system, however conflicting reports that stated suppression of both Th1 and Th2 response also existed. As for globo H, the evidence so far suggests that both Th1 and Th2 responses were suppressed, which may have supported tumor progression[33, 43].

In the same study, it was also found that the IL-2 receptor, CD25, which was crucial for T cell proliferation was reduced in expression after splenocytes and CD4+ T cells were treated with globo H. Since it was known that the Notch1 signaling modulated IL-2 receptor expression on T cells, its expression was further investigated in the study. It was found that Notch1 signaling was reduced when treated with globo H ceramide. Therefore, it was suggested that the end result of inhibition of immune cell proliferation and differentiation by globo H might be attributed to suppression of the Notch1 signaling.



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Further investigation into the molecular mechanism of Notch1 suppression showed that Notch1 signaling itself was reduced at the transcriptional level by ID3 and at the protein level via ubiquitination by Itch. Overall, these findings suggested that the presence of globo H played a role in adjusting the tumor microenvironment and favored the survival of tumor cells by inhibiting immune function[43]. Globo H displayed another function that was promoting angiogenesis from the tumor microenvironment. It has been shown *in vitro* that globo H taken up into human umbilical vein endothelial cells were able to stimulate migration and differentiation of endothelial cells. Promotion of angiogenesis was also observed *in vivo*, thereby providing evidence of the association between globo H, enhanced angiogenesis and tumor growth[44].

The apparent discrepancies of globo H expression observed in canine MGTs and human malignancies are suspected to be because of the following reasons: the monoclonal antibody, MBr1, that initially identified globo H was able to bind to structures present on the cell membrane of both normal and neoplastic breast tissues, especially the ductal cells. Though human BC and canine MGT vary histologically, tubule formation is an important criterion when grading MGTs. Low grade MGTs tend to have more tubule formation and are better differentiated. It has also been reported that tubular carcinomas had a significantly higher 2-year survival rate compared to non-tubular, infiltrative and/or permeative carcinomas[41]. As globo H was commonly expressed in normal ductal cells and ductal carcinomas, the formation of these secretory structures, corresponding to tubule formation in dogs, may be essential for its expression. Additionally, it was also discovered in one study that MBr1-positive breast carcinoma cells had a higher expression of transferrin receptors; transferrin receptors were traditionally linked to a higher histological grading and expression of Ki-67, hinting an unfavorable prognosis. However, the study found transferrin receptor expression to be significantly associated



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with a better outcome. It was explained that the expression of cellular membrane receptors, such as transferrin, estrogen, or progesterone, are markers of a better-differentiated population. When considering the tendency of globo H to be expressed on ductal cells and on cells retaining membrane receptors, it may be that globo H is formed when complete differentiation of the epithelial cells has taken place. Better differentiated cells are usually thought to be not as aggressive in behavior when malignant. This theory would correspond positively to our finding that smaller tumors more commonly expressed globo H, whereas larger tumors may be less well differentiated and more malignant. This same study also did not find significant associations between known prognostic factors such as nodal status, tumor size, grading and MBr1 reactivity[8, 34].

Similarly, another earlier study found that the expression of MBr1-defined antigen was not significantly different between nodal positive or negative patients. The incidence of disease relapse was also not different between the MBr1 reactive and unreactive groups. Overall, no significant prognostic value of the antigen was noted. Furthermore, it was found that the incidence of MBr1 staining was lower in metastatic lesions compared to their respective primary tumors. As metastatic lesions are generally considered to be more heterogeneous and less differentiated, this supports our speculation that globo H may be better expressed on terminally-differentiated cells with secretory functions. This study has also investigated the antigen reactivity in the corresponding nonneoplastic tissues of each patient with BC and discovered that there was a high degree of concordance in its expression; only a few cases were reactive in the nonneoplastic tissues and unreactive in the neoplastic tissues, and no cases were reactive in neoplastic tissues and unreactive in nonneoplastic tissues[45]. Considering such high proportion of globo H presentation on the corresponding nonneoplastic breast tissues, it may also be possible that the presence of globo H, as we know now, first provided its immunosuppressive effects on the



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surrounding microenvironment and facilitated the growth of the oncogenic cells, eventually leading to the development of cancer. Another phenomenon that supports this hypothesis is that from the results of our study, the globo H score of the globo H positive tumors was generally low; 27 (90%) tumors scored less than 50, whereas 1 each of a grade 1 and 2 tumors had scores between 50 and 100. And only 1 grade 3 tumor had a score larger than 100. It is possible that in a mammary gland tumor when malignant transformation takes place from a benign tumor, it starts to change its phenotype so the number of cells that express globo H decreases. An investigation into globo H expression should be conducted for the corresponding nonneoplastic mammary gland tissues for canines to test this hypothesis.

The monoclonal antibody used in our study was a clone designed for the human species. It is currently unknown whether the antibody matched well with the molecule present on canine cells. It has been documented that some degree of cross-reaction still existed for the antibody with other gangliosides carrying the H antigen. Since the globo H score for the tumors in the present study were mostly low, another possible reason may be that this antibody only identified the antigens with similar structure but not the most prevalent cell surface glycan present on canine MGT cells. Interestingly, the tumor that exhibited a high globo H score (140) in our study was a grade 3 comedocarcinoma. This dog had a relatively small sized tumor at diagnosis but quickly developed metastatic disease and had a short overall survival time, contrasting with all other cases. It cannot be excluded that technical issues hindered majority of the cases to show the actual amount of globo H expression.

Another possibility to consider is that species differences exist for the role of globo H. The prognostic value of the globo H and its molecular function may be different among the two species. A larger population of canine malignant MGTs may be required establish



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the prevalence and prognostic indication of this molecule in this species and in this type to tumor.

It is worth mentioning that in our study, the use of adjuvant chemotherapy, namely Adriamycin, has a statistically negative impact on survival. Prior reports of canine MGT have not had a consistent result regarding the use of adjuvant chemotherapy, the major issue being selection biases in those that receive and do not receive post-operative chemotherapy. Although further analysis regarding the population that received chemotherapy was not performed due to limited case numbers, it is also common practice in our cancer treatment center to only offer chemotherapy for the patients perceived as high risk, thus contributing to the selection bias. The true effect of chemotherapy should be evaluated under circumstances where both arms of patients are under similar clinical conditions.

Two host factors, breed and body weight, are variables that remain ambiguous in their prognostic values in canine MGT but are associated in the development of MGT. These two factors were also found significantly associated with globo H expression in the present study. Obesity has been associated with decreased risk of development of BC in teenage women but with an increased risk in postmenopausal women. On the other hand, obesity in dogs during 9 to 12 months of age brought an increased risk in later MGT development and being underweight had a protective effect. It has been postulated that obesity is linked to increased serum estrogen levels because of reduced concentration of sex hormone-binding globulins. Also, adipose tissues may act as a source of increased estrogen production via aromatase-mediated conversion of androgens[17, 37]. However, despite connections to the development of MGT, obesity has not been directly associated with prognosis, including disease-free interval and survival time. Most importantly, the body condition score of each dog was not stated in our study. The association between



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globo H expression and weight cannot be concluded without acknowledging their body condition scores. What this finding reflected more was perhaps that larger breeds of dogs were associated with less globo H expression. As for the significant association that was found between purebreds and positive globo H expression, one explanation for this was that purebreds accounted for the majority of the study's population. The breeds included in our study matched previous descriptions of miniature and toy breeds being frequently presented. Another factor to take into consideration is that smaller breeds are more suitable for our urban environment, therefore a selection bias cannot be excluded. Genetic influence is evident in the development of MGT according to several studies[46], whether it also influences the expression of globo H requires a larger scale study to investigate.

## 5.2 Vaccine-related toxicity

The globo H conjugate vaccine was considered well tolerated as only 1 dog experienced a dose-limiting toxicity, in which lasted only 3 days and was self-limiting, and that this dog received the escalated dose. Since none of the other dogs experienced dose-limiting toxicities, further examinations into the efficacy, such as the antibody titers produced, should be performed to determine the lowest effective dose in order to prevent clinical toxicities.

Since the discovery of cancer-specific glycans, different vaccine adjuvants and carriers have been tested to find the most suitable one because carbohydrates alone generally exhibit poor immunogenicity[47]. Adjuvants, therefore, play an important role in boosting immunity and altering immune responses to carbohydrate antigens. Carrier proteins such as diphtheria toxoid, KLH and bovine serum albumin are commonly used for carbohydrate vaccines in humans to improve immunogenicity. The combination of



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globo-H-KLH and QS-21 was one that successfully induced antisera globo H-positive cell lines. QS-21 is a saponin-based adjuvant purified from the *Quillaia Saponaria* tree bark. Saponins are immunomodulators and can induce strong Th1 and Th2 responses, production of interleukin-2, interferon-gamma, as well as cytotoxic T lymphocytes. QS-21 demonstrated induction of highly antigen-specific antibody responses and CD8+ T cell response in mice, as well as promoting a balanced production of IgG1 and IgG2a, a phenomenon that was not quite observed in other adjuvants such as aluminum hydroxide. When well purified, QS-21 is considered generally safe. It is widely used for its high potency[48, 49]. However, the adverse events experienced by human BC patients administered with globo-H-KLH/QS-21 in early clinical trials for also fitted the toxicity profile reported for QS-21, including injection site reaction, fever, arthralgias, and myalgias. The adverse reactions associated with QS-21 in canine patients that have been reported include pain or nodule formation at the injection site, anorexia, apathy, vomiting, diarrhea, itching, dyspnea, coughing, anaphylaxis and facial and forelimb edema[50]. When administered intravenously, hemolysis has also been reported. The adverse events observed so far in our canine trial showed moderate differences to those of the human patients; injection site reaction occurred in 3 out of 5 (60%) dogs, whereas fever, arthralgias and myalgias were not reported. However, the occurrence of these events may have been underestimated as it was more difficult to observe these reactions in canine patients. Body temperatures were only measured on the days of vaccinations, so it was unknown if fever was concurrently present when injection site reactions were observed. Nevertheless, the lethargy and anorexia observed in our patients but rarely reported for human patients may reflect that canine patients become lethargic and anorexic as a mean to express their discomfort which may include arthralgias or myalgias. Dogs 3 and 5 received escalated dose of vaccine for their respective body weight, both experienced



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higher graded adverse events compared to other dogs within the same body weight range that received lower doses of vaccines. Higher doses of QS-21 that caused more severe adverse events were suspected to be one of the reasons.

The most severe adverse events all occurred after the second injection in the current study. Previous studies on the canine leishmania vaccine utilizing QS-21 have reported similar trends in occurrence of adverse events. It was observed in that study 15 (37%) and 23 (56%) dogs experienced local injection site reaction after the second and the third injections, respectively[51]. The events all resolved spontaneously within 2 to 8 days, similar to the findings from our study. But since no patient showed discomfort after the first injection, a hypersensitivity reaction may play a role in these adverse reactions. Previous studies on canine vaccine safety utilizing 50 μg QS-21 as an adjuvant found that the adverse events such as pain, anorexia, apathy and local swelling decreased with the number of injections given. Administering the vaccine at a different site of subcutaneous space were thought to help reduce pain reactions by decreasing sensitivity to pain[50]. In the present study, two dogs received the first two vaccinations at the same subcutaneous site, and both developed local swelling and pain reaction after the second injection. The remaining three dogs received their vaccinations at alternating sides of the flanks, but one dog still experienced pain, lethargy and anorexia following the second injection, notably with the greatest severity out of all the episodes. These observations indicate that apart from the toxicities with the use of QS-21, other components of the vaccine may be responsible for inducing an immune response.

## 5.3 Limitations

Several limitations exist in the present study. Firstly, regarding our study population,



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canine MGTs are known to be a highly complex and diverse group of tumors. Our sample size was insufficient to clarify the relationship between each subtype of MGT and globo H expression, especially as we hypothesize that globo H expression is related to tubular formation, the effect of different subtype of MGTs to globo H expression should be looked into.

The follow-up duration of the present study was possibly not long enough to observe the development of metastasis or local recurrences in the cases that remained alive and disease free at the end of the study period. Whether any correlations exist between globo H expression and clinical outcome require a longer time of follow up. No necropsies were performed in any of the cases. The cause of death was presumed and may have been inaccurate. The retrospective nature of data collection has its limitations in that some information was not recorded properly, causing difficulties in obtaining complete and accurate data. One of the major limitations regarding data collection would be the recorded size of MGTs, as this was one of the tumor characteristics that was found to be significantly related to globo H expression. The size of MGT was manifested as their longest diameter, however this could cause bias as the diameter does not reflect accurately the volume of the tumor, which should be the variable that is assessed regarding its relation to globo H expression. Moreover, it was also difficult to obtain data on the duration in which the MGT has been present prior to definitive diagnosis. Since MGT has been shown to demonstrate histologic malignant transformation, knowing how long the MGT has been present and its size change in between may be helpful in understanding whether globo H expression does decrease with increasing malignancy.

Secondly, the histopathologic slides were not uniformly reviewed by a single pathologist. The information presented within the pathologic reports differed among pathologists. Hence it was difficult to analyze the relationship between different



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histologic characteristics and globo H expression. Canine MGTs commonly present heterogeneity within one tumor, but the areas with globo H expression was not reviewed by a pathologist to confirm the area with positive expression concur with its final pathologic diagnosis. The current definitions of globo H positivity involves any cell that showed reactivity to the antibody, however, it was possible that with the heterogeneity present within MGT, the positive cells were not in fact malignant MGT cells.

Thirdly, the immunohistochemical staining of globo H on canine histopathologic slides were performed for the first time, the sensitivity and specificity of the procedures were not known and there was no positive control. Despite being positive, the H score recorded in our cases were mostly low [36]. This reflects that the staining methods may not be optimized yet or perhaps there were structural differences between canine globo H and human globo H, or possibly the expression of globo H is generally low in this species or this kind of tumor.

Lastly, any globo H score larger than zero was considered positive in the present study as this study was performed as a preliminary investigation into the expression of globo H in canine MGT. In contrast, the positive threshold for globo H expression was set as 50 in recent clinical trials in humans. So far, no investigation has been made to examine the association between globo H score and tumor characteristics of any kind. Neither has any correlations between globo H vaccine efficacy and globo H score been identified. Since most cases in our study had scores less than 50, a different positive threshold may be needed to truly reflect the prevalence of globo H expression in canine MGT. In order to establish a more appropriate positive threshold for globo H expression in dogs, a larger number of cases would be required for analysis. It is also worth investigating further the connections between globo H expression capacity and tumor/patient characteristics. The globo H scores recorded in the current study also



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showed 2 groups of extreme values, such as the low end was as low as 0.5 and the high end being 140. Therefore, it may be feasible to categorize the scores into a low, medium and high groups. This categorization was not performed in the present study because we had very limited case number exhibiting medium to high scores. However, this method may be considered in the future when case numbers are sufficient to detect whether differences in tumor characteristics and clinical outcome exist among different groups.

Possible adverse events were identified during the phase I trial of globo H conjugate vaccinations, however, we had too small a sample size to accurately determine all the possible adverse effects of the vaccine. Most of the dogs were not assessed by a veterinarian within the duration of adverse events from the vaccines, therefore the details of the events were mostly informed by the owners which could be biased and inaccurate. Hematologic and biochemical parameters and physical conditions were not documented during the episodes of discomfort, it was possible that abnormalities were left undetected. The purpose of the phase I trial was to identify the highest dose that have acceptable adverse reactions; but the sample was too small to draw any conclusions.



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# Chapter 6 Conclusion and prospects

The present study stands as a pilot investigation into the tumor-associated carbohydrate antigen, globo H, in malignant MGTs in dogs. The findings of the present study contrasted the findings from human studies, hence our hypothesis of globo H being a negative prognostic indicator in canine malignant MGT was rejected. This discrepancy cannot be definitively explained without further study. It can only be speculated that globo H may play a role in mammary gland tumors undergoing a malignant transformation. We suggest that further study into this topic may begin via performing IHC staining of globo H on the nonneoplastic mammary gland tissues of each patient with malignant MGT to establish the amount of globo H expression in corresponding nonneoplastic mammary gland cells. Additionally, benign MGTs may also be assessed for their globo H expression in order to understand the changes in globo H expression when MGTs are undergoing transformations in its malignancy. Since MGTs are highly hormone-dependent neoplasms, the correlation between the expression of hormone receptors such as estrogen and progesterone, and globo H expression may also be investigated to establish any connection.

The globo H vaccine appears well tolerated in dogs with malignant MGTs. However, due to the limited case number, a definitive conclusion regarding safety cannot be made. Further randomized and controlled studies should be performed to evaluate the safety and efficacy of the vaccine. Since MGTs are usually multicentric and frequently occur in previously unaffected mammary glands, a systemic adjuvant such as immunotherapy may be effective in preventing further new growths of MGT.



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Table 1 Analysis of association between globo H expression and surgical/histologic variables via Fisher's exact or Chi-square test

| Variables | | Globo H expression | | *P*-value |
|---|---|---|---|---|
| | | Number of positive tumors (%) | Number of negative tumors (%) | |
| **Grading** | 1 and 2 | 19 (39) | 11 (22) | 0.001* |
| | 3 | 3 (6) | 16 (33) | |
| **Histologic type** | Adenocarcinoma | 15 (31) | 7 (14) | 0.367 |
| | Carcinoma | 15 (31) | 12 (24) | |
| **Type of surgery received** | Lumpectomy | 5 (10) | 5 (10) | 0.129 |
| | Regional | 12 (24) | 10 (20) | |
| | Chain | 8 (16) | 1 (2) | |
| | Incisional/trucut | 0 (0) | 2 (4) | |
| | Single mastectomy | 3 (6) | 1 (2) | |
| | Bilateral chain | 2 (4) | 0 (0) | |
| **Histologic margin**[a] | Clean | 22 (45) | 8 (16) | 0.061 |
| | Dirty | 7 (14) | 9 (18) | |

[a]Three tumors did not have margins available

*Statistically significant difference



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Table 2 Analysis of association between globo H expression and continuous clinical variables via the Mann-Whitney U test

| Variables | Positive globo H expression | | Negative globo H expression | | *P*-value |
|---|---|---|---|---|---|
| | N | Median | N | Median | |
| **Body weight**[a] (kg) | 30 | 7.27 | 18 | 15.7 | 0.013* |
| **Tumor size**[b] (cm) | 29 | 2 | 17 | 4.5 | 0.04* |
| **Age** (years) | 30 | 11 | 19 | 12 | 0.442 |

[a]One dog had no body weight recorded

[b]Three dogs had no tumor size recorded

*Statistically significant difference



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Table 3 Analysis of association between globo H expression and clinical characteristics via the Fisher's exact or Chi-square test

| Variables | | Globo H expression | | *P*-value |
|---|---|---|---|---|
| | | Number of positive tumors (%) | Number of negative tumors (%) | |
| **Breed** | Purebred | 27 (55) | 11 (22) | 0.014* |
| | Mixed | 3 (6) | 8 (16) | |
| **Stage** | Local disease | 24 (49) | 8 (16) | 0.007* |
| | Metastatic disease | 6 (12) | 11 (22) | |
| **Adjuvant therapy**[a] | None | 19 (46) | 8 (20) | 0.158 |
| | Adriamycin | 4 (10) | 3 (7) | |
| | Metronomic | 1 (2) | 1 (2) | |
| | Others | 1 (2) | 4 (10) | |
| **Neutering status (timing in relation to diagnosis)** | Neutered at least one year prior | 18 (37) | 14 (29) | 0.273 |
| | Neutered concurrently | 10 (20) | 2 (4) | |
| | Within one year but not concurrently | 1 (2) | 2 (4) | |
| | Intact | 1 (2) | 1 (2) | |
| **Number of MGT**[b] | Single | 11 (23) | 5 (10) | 0.379 |
| | Multiple | 19 (40) | 13 (27) | |
| **Ulceration**[b] | Yes | 2 (4) | 2 (4) | 0.624 |
| | No | 28 (58) | 16 (33) | |
| **Location**[b] | Cranial | 4 (8) | 1 (2) | 0.63 |
| | Caudal | 12 (25) | 10 (21) | |
| | Both | 14 (29) | 7 (15) | |

[a]Excluded dogs that received globo H vaccines as their only adjuvant therapy

[b]One dog lacked available information

*Statistically significant difference



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Table 4 Univariate analysis of prognostic factors associated with OST and DFI in dogs with malignant MGT

| Variables | | N | OST | | DFI | |
|---|---|---|---|---|---|---|
| | | | Median (days) | *P*-value | Median (days) | *P*-value |
| **Age (years)** | ≤ 10 | 19 | NR | 0.92 | 243 | 0.872 |
| | > 10 | 26 | NR | | 184 | |
| **Body weight (kg)[a]** | ≤ 10 | 24 | 307 | 0.162 | 555 | 0.264 |
| | > 10 | 20 | NR | | NR | |
| **Breed** | Purebred | 36 | NR | 0.929 | 219 | 0.897 |
| | Mixed | 9 | NR | | 147 | |
| **Tumor size (cm)[b]** | < 3 | 19 | NR | | 243 | |
| | 3 – 5 | 10 | NR | 0.006* | 89 | 0.056 |
| | > 5 | 14 | 164 | | 149 | |
| **Location[a]** | Cranial | 5 | 555 | | 555 | |
| | Caudal | 18 | NR | 0.256 | 243 | 0.143 |
| | Both | 21 | 307 | | 149 | |
| **Ulceration[a]** | Yes | 39 | NR | < 0.001* | 219 | 0.003* |
| | No | 5 | 65 | | 13 | |
| **Number of MGT[a]** | Single | 13 | 555 | 0.417 | 190 | 0.605 |
| | Multiple | 31 | NR | | 243 | |
| **Grading** | 1 | 10 | 307 | | NR | |
| | 2 | 10 | NR | 0.482 | 184 | 0.232 |
| | 3 | 25 | BR | | 149 | |
| **Histologic type** | Adenocarcinoma | 20 | NR | 0.633 | 219 | 0.983 |
| | Carcinoma | 25 | NR | | 190 | |
| **Margin[a]** | Clean | 28 | NR | 0.063 | 243 | <0.001* |
| | Dirty | 16 | NR | | 0 | |



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Table 4 Univariate analysis of prognostic factors associated with OST and DFI in dogs with malignant MGT (continued)

| Variable | | N | OST | | DFI | |
|---|---|---|---|---|---|---|
| | | | Median (days) | *P*-value | Median (days) | *P*-value |
| **Number of surgeries receiced** | 1 | 29 | 555 | 0.994 | 219 | 0.891 |
| | 2 | 13 | NR | | 184 | |
| | ≧3 | 3 | 204 | | 483 | |
| **Type of surgery received** | Lumpectomy | 8 | NR | 0.350 | 72 | 0.458 |
| | Regional | 21 | NR | | 184 | |
| | Chain | 9 | 307 | | 190 | |
| **Stage** | Local | 30 | 164 | 0.008* | 243 | 0.029* |
| | Metastatic | 15 | NR | | 13 | |
| **Adjuvant therapy** | None | 27 | NR | 0.028* | 243 | 0.069 |
| | Adriamycin | 7 | 164 | | 89 | |
| **Neutering status (timing in relation to diagnosis)** | Neutered at least one year prior | 29 | NR | 0.981 | 190 | 0.588 |
| | Neutered concurrently | 12 | 307 | | NR | |
| **Globo H[c]** | Positive | 16 | NR | 0.74 | 483 | 0.691 |
| | Negative | 28 | 555 | | 219 | |

[a]One dog has unavailable information

[b]Two dogs have unavailable information

[c]One dog with both globo H positive and negative tumors was excluded

*Statistically significant difference

Abbreviations: NR, not reached



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Table 5 Multivariate analysis of variables associated with DFI of dogs with MGT

| Variables | | N | Hazard ratio | 95% CI | *P*-value |
|---|---|---|---|---|---|
| **Stage** | Local disease | 32 | 1.049 | 0.378 – 2.912 | 0.927 |
| | Metastatic disease | 17 | | | |
| **Ulceration** | Present | 4 | 3.021 | 0.738 – 12.361 | 0.124 |
| | Absent | 44 | | | |
| **Margin** | Clean | 30 | 3.371 | 1.157 – 9.823 | 0.026* |
| | Dirty | 16 | | | |

*Statistically significant difference



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Table 6 Multivariate analysis of variables associated with the OST of dogs with MGT

| Variables | | N | Hazard ratio | 95% CI | *P*-value |
|---|---|---|---|---|---|
| **Stage** | Local disease | 22 | 4.794 | 0.782 – 29.382 | 0.090 |
| | Metastatic disease | 11 | | | |
| **Tumor size** | Present | 20 | 2.942 | 0.415 – 20.878 | 0.280 |
| | Absent | 13 | | | |
| **Adjuvant therapy**[a] | Clean | 26 | 2.125 | 0.355 – 12.715 | 0.409 |
| | Dirty | 7 | | | |

[a]Comparing between dogs that received no adjuvant therapy and those that received Adriamycin as the adjuvant therapy



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Table 7　Protocol of phase I clinical trial of globo H vaccine

| Week | 1 | 2 | 3 | 5 | 9 | 13 | 17 | 25 | 29 | 33 |
|---|---|---|---|---|---|---|---|---|---|---|
| Vaccine injection | X* | X | X | X | X | X | X | X | | |
| Tumor measurement | X | X | X | X | X | X | X | X | X | X |
| Blood exam | X | | X | X | X | X | X | X | X | X |
| Urine | X | | | X | | | | | | X |
| Biopsy | X | | | | | | | X | | |
| Computed tomography | X | | | | | X | | X | | |
| Ultrasound | X | | | X | X | | | | | |
| X ray | X | | | X | X | | | | | |

*X indicates that the event is scheduled to be performed at the time



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Table 8 Summary of clinical and MGT-related characteristics of dogs included in phase I trial

| N | Breed | Age (years) | Neutering status | Surgery | Margin | Histological diagnosis | Grade | Stage[b] | Survival |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Miniature poodle | 9 | $S_{MGT}$ | Lumpectomy | Unknown | Carcinoma, simple, tubular | 1 | 1 | Alive |
| 2 | Samoyed | 13 | S | Regional + LN | Clean | Carcinoma, cribriform type | 2 | 1 | Alive |
| 3 | Husky | 11 | S | Lumpectomy | Clean[a] | Carcinoma, simple type | 1 | 1 | Alive |
| 4 | Jack Russell Terrier | 10 | $S_{MGT}$ | Regional + LN | Clean[a] | Carcinoma, simple, cystic-papillary | 3 | 5 | Alive |
| 5 | Dachshund | 7 | S | Regional | Clean | Carcinoma, solid type | 3 | 1 | Alive |

[a]Dogs underwent multiple mammary tumor surgeries; information presented here refer to the one closest to entering the trial
[b]The stage at the time of first entry into the trial
Abbreviations: S, spayed at least one year before mammary tumor surgery; $S_{MGT}$, spayed at the time of mammary tumor surgery; LN, regional lymph node extracted



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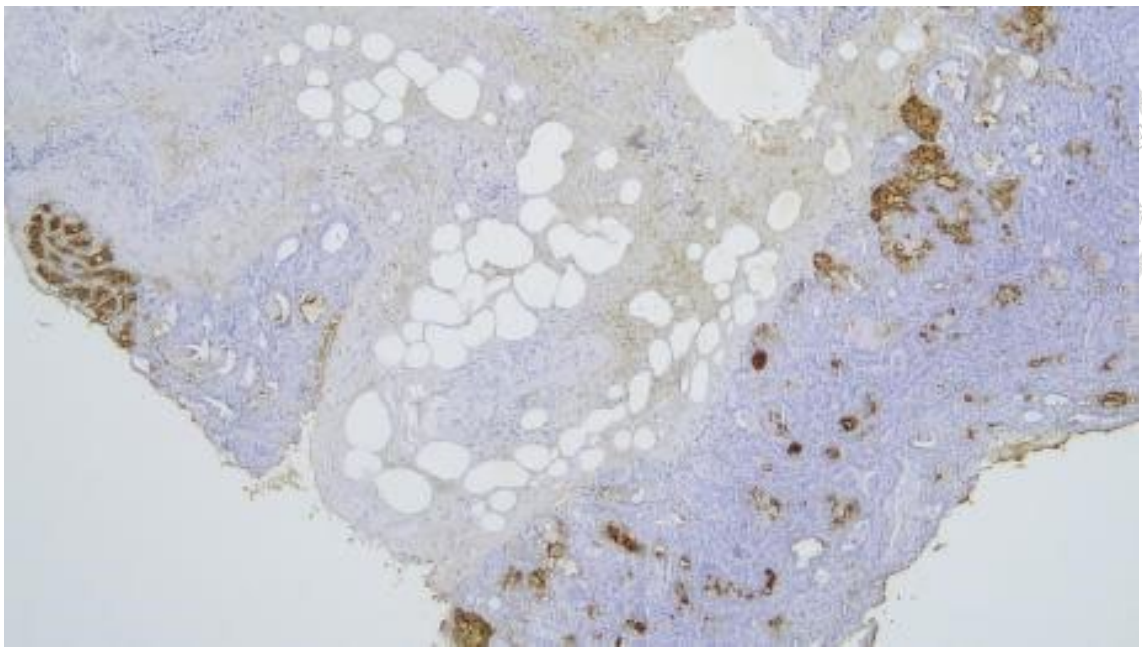

Figure 1. The image is showing an example of positive IHC staining of globo H in a MGT slide under low power field (50X) examination



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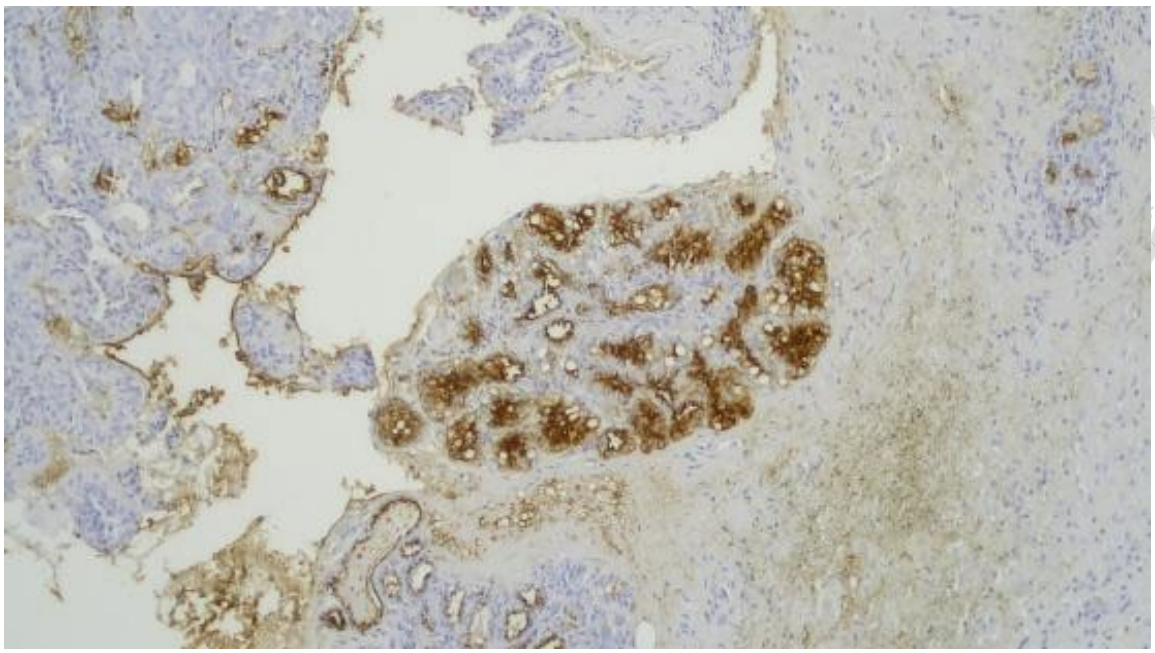

Figure 2. The image is showing an example, the same sample as shown in figure 1, of positive IHC staining of globo H in a MGT slide under high power field (200X) examination



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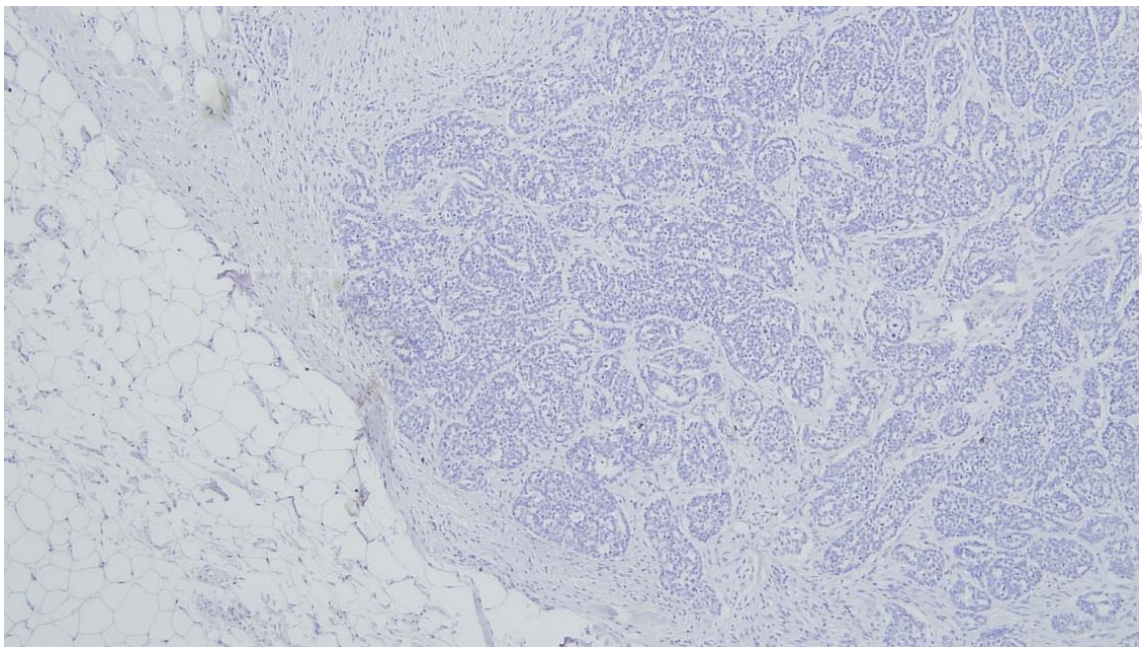

Figure 3. The image is showing an example of negative IHC staining of globo H in a MGT slide under low power field (50X) examination



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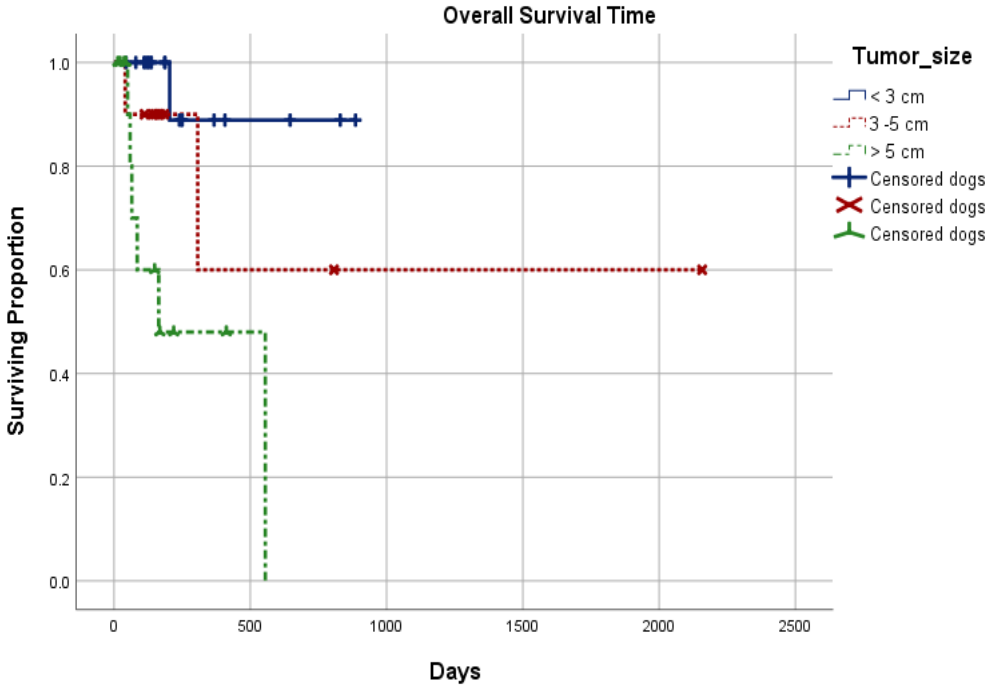


Figure 4. The Kaplan-Meier curve is showing OST of dogs with different tumor sizes. The median OST was not reached for patients with tumors < 3 cm (solid line, n = 19) and was 164 days for patients with tumors > 5cm (dotted and dashed line, n = 14). The difference was statistically significant (P = 0.001).



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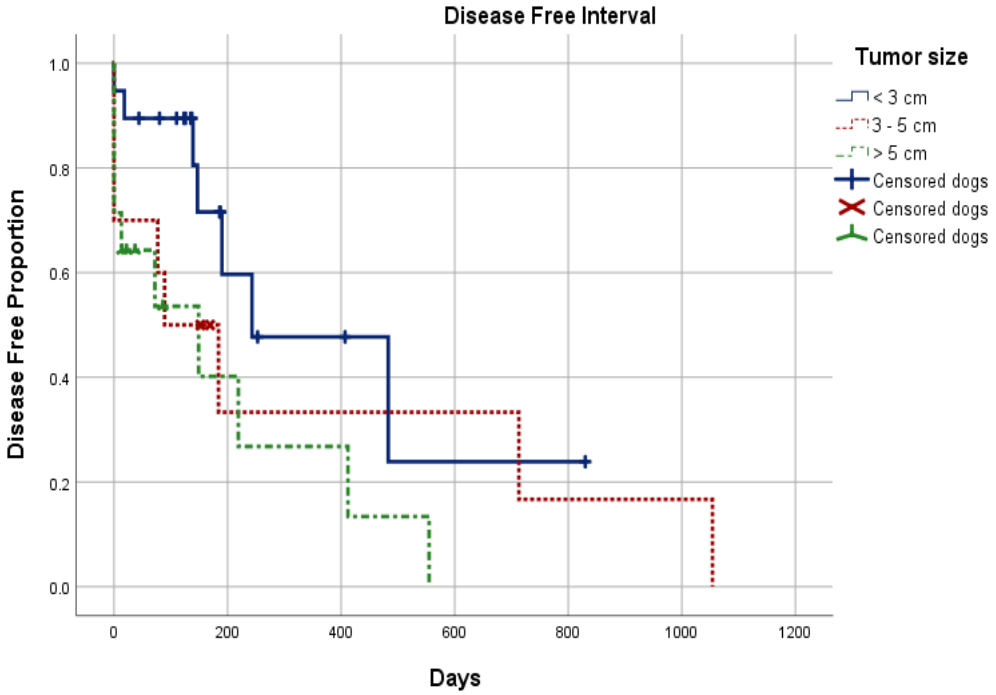


Figure 5. The Kaplan-Meier curve is showing DFI of dogs with different tumor sizes. The median DFI was 243 days for patients with tumors < 3 cm (solid line, n = 19) and 149 days for patients with tumors > 5 cm (dotted and dashed line, n = 14). A statistically significant difference was not reached (P = 0.056).



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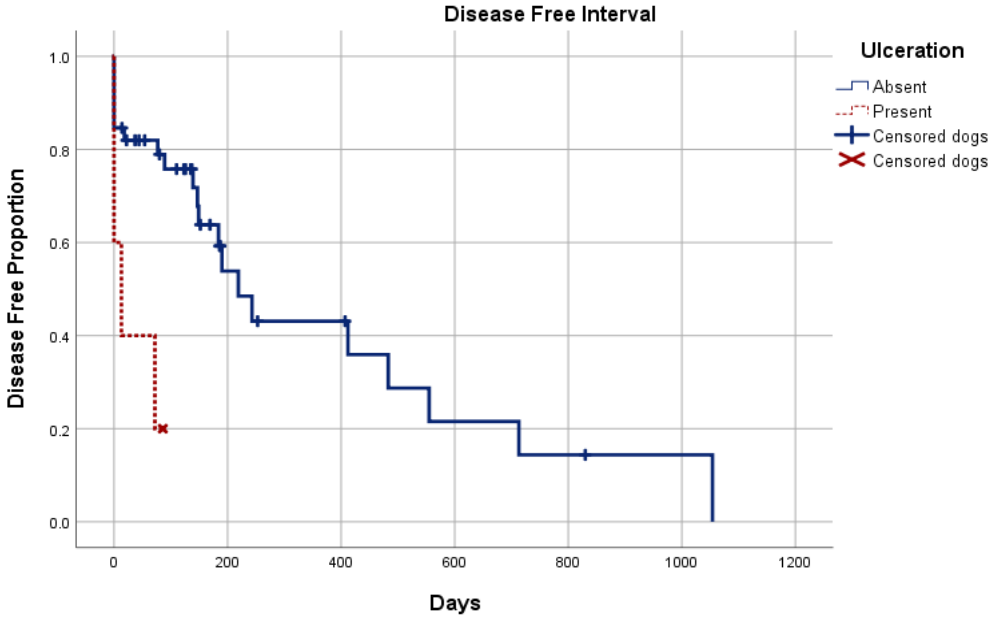


Figure 6. The Kaplan-Meier curve is showing DFI of dogs with ulcerated and non-ulcerated tumors. The median DFI for dogs with ulcerated tumors (dotted line, n = 5) was 13 days. The median DFI for dogs with non-ulcerated tumors (solid line, n = 39) was 219 days. The difference was statistically significant (P = 0.003).



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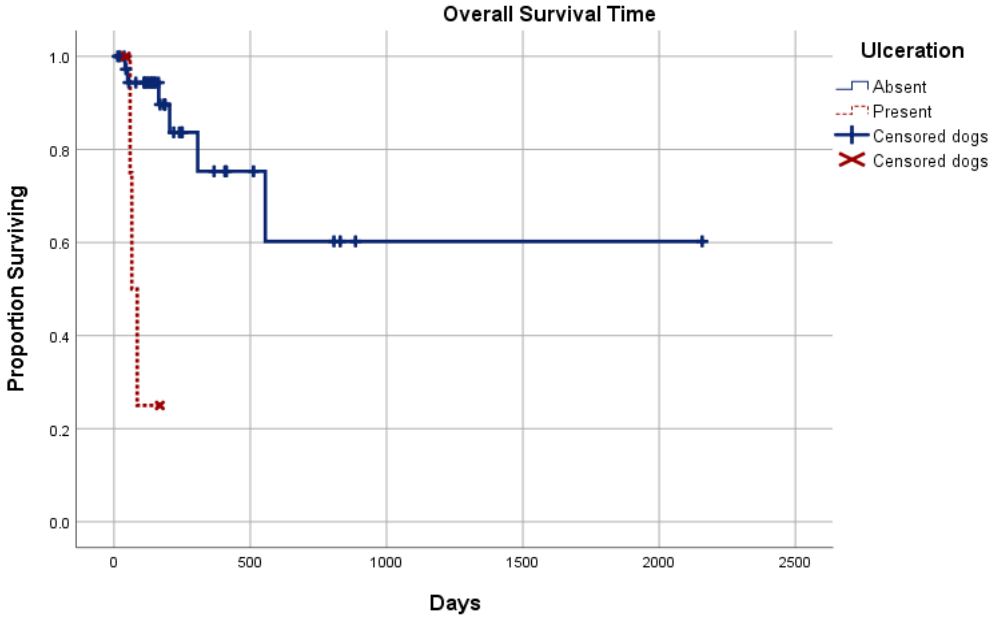


Figure 7. The Kaplan-Meier curve is showing OST of dogs with ulcerated and non-ulcerated tumors. The median OST for dogs with ulcerated tumors (dotted line, n = 5) was 65 days. The median OST for dogs with non-ulcerated tumors (solid line, n = 39) was not reached. The difference was statistically significant ($P < 0.001$).



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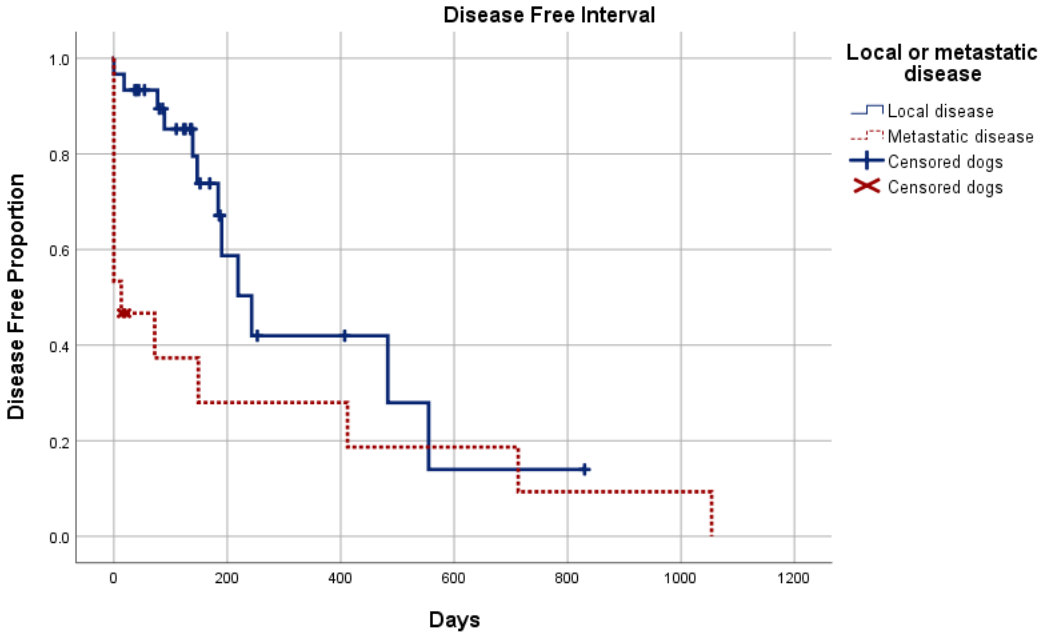


Figure 8. The Kaplan-Meier curve is showing DFI of dogs with localized and metastatic diseases. The median DFI for dogs with localized disease (solid line, n = 30) was 243 days. The median DFI for dogs with metastatic diseases (dotted line, n = 15) was 13 days. The difference was statistically significant (P = 0.029).



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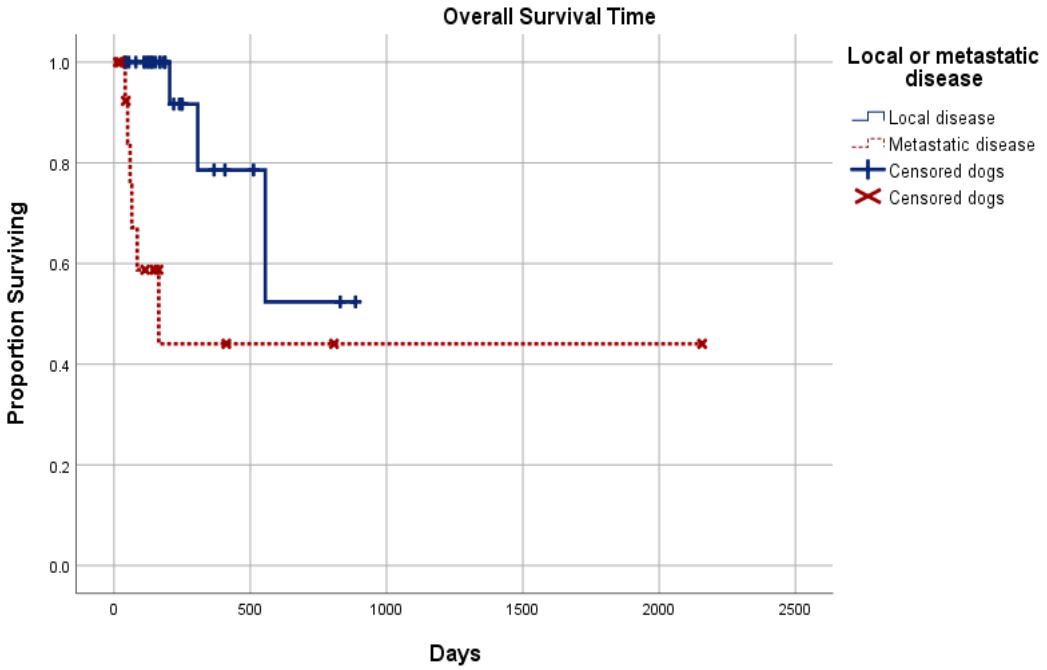


Figure 9. The Kaplan-Meier curve is showing OST of dogs with localized and metastatic diseases. The median OST for dogs with localized disease (solid line, n = 30) was not reached. The median OST for dogs with metastatic diseases (dotted line, n = 15) was 164 days. The difference was statistically significant (P = 0.008).



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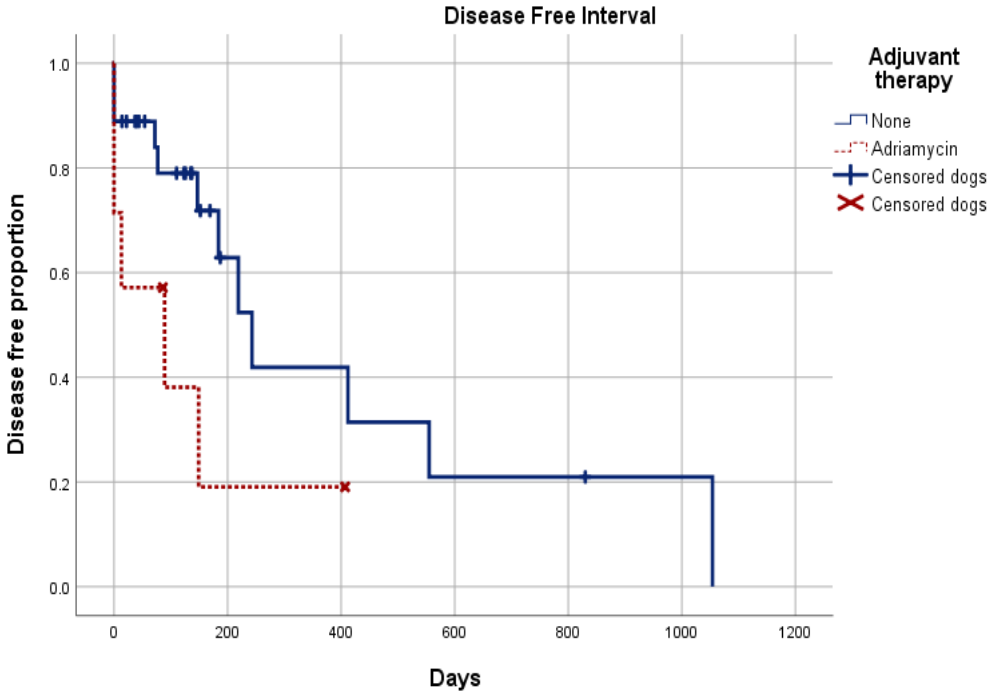


Figure 10. The Kaplan-Meier curve is showing DFI of dogs that received no adjuvant treatment and those that received Adriamycin. The median DFI for dogs receiving no adjuvant (solid line, n = 27) was 243 days. The median DFI for dogs receiving Adriamycin (dotted line, n = 7) was 89 days. The difference was not statistically significant (P = 0.069).



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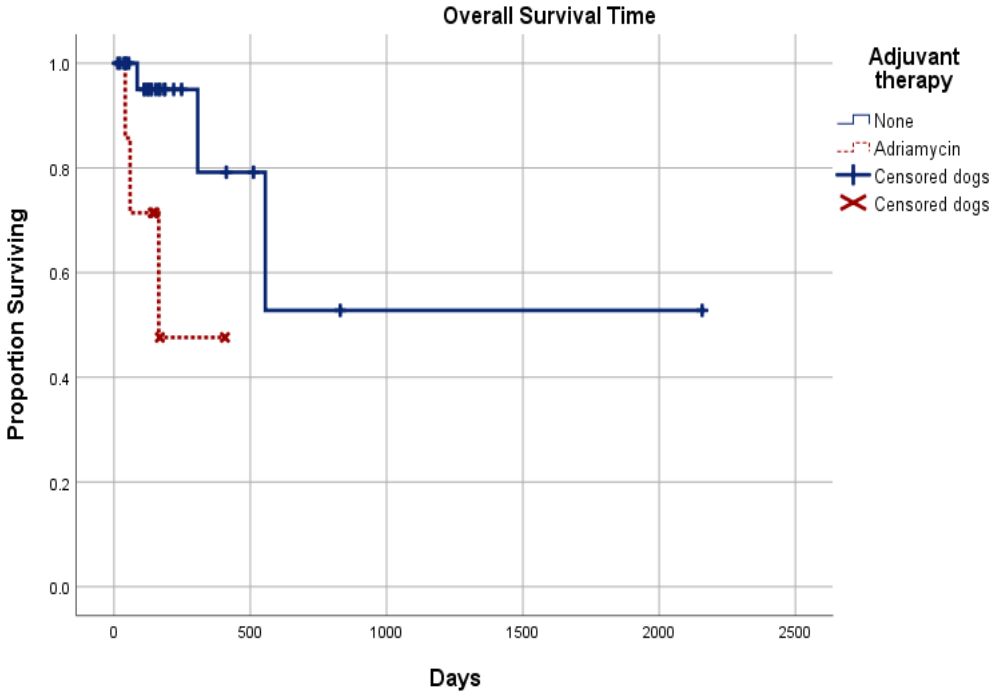


Figure 11. The Kaplan-Meier curve is showing OST of dogs that received no adjuvant treatment and those that received Adriamycin. The median OST for dogs receiving no adjuvant (solid line, n = 27) was not reached. The median OST for dogs receiving Adriamycin (dotted line, n = 7) was 164 days. The difference was statistically significant (P = 0.028).



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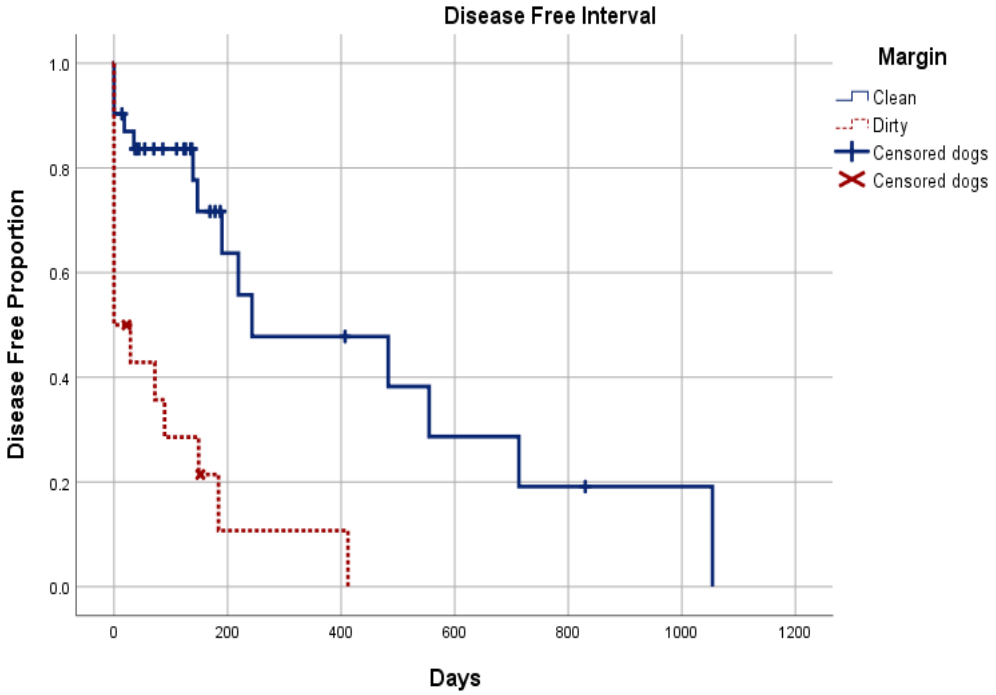


Figure 12 . The Kaplan-Meier curve is showing DFI of dogs with clean and dirty histologic margins. The median DFI for dogs with clean histologic margins (solid line, n = 30) were 243 days. The median DFI for dogs with dirty histologic margins (dotted line, n = 16) were 0 days. The difference was statistically significant ($P < 0.001$).



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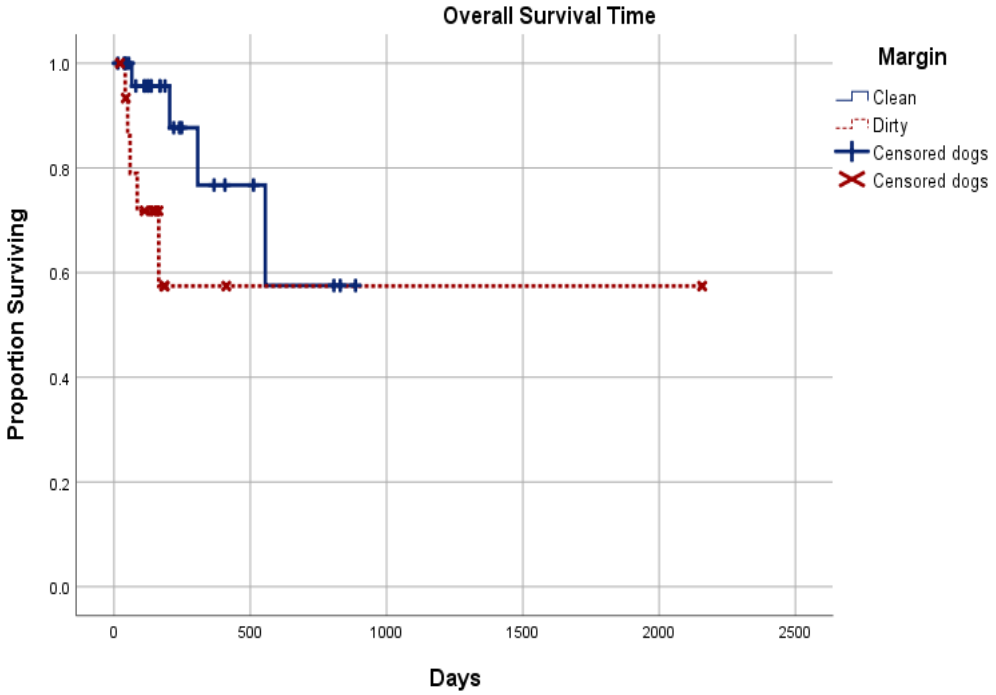


Figure 13 Kaplan-Meier curve is showing OST of dogs with clean and dirty histologic margins. The median OST of dogs was not reached for both clean (solid line, n = 30) and dirty histologic margins (dotted line, n = 16). The difference was not statistically significant (P = 0.063).



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