“The efficacy of the melanoma vaccine in CMM and the potentials for application of the vaccine in other domestic animals”

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Abstract

Introduction: canine malignant melanoma (CMM) is a common, aggressive and potentially metastatic tumour that typically caries a grave prognosis. Melanoma in cats is relatively uncommon, but caries the same poor prognosis, in contrary to horses where many animals will be affected by it and generally have benign tumours. MSTs of 1-5 months in CMM treated with standard therapy protocols have been previously reported. Xenogeneic DNA vaccines used as a treatment in CMM have shown to induce both humoral and cellular immune responses and may cause regression of the tumour and to increase survival time.
Aim of the study: The aim of the study was to determine the efficacy of the xenogeneic human tyrosinase DNA vaccine as an adjunctive therapy in CMM and the therapeutic potential for the use of this vaccine in other animal species, mainly cats and horses. It was expected that this melanoma vaccine would considerably lengthen MSTs.
Materials and Methods: A total of 21 dogs have been examined since February 2011, 2 of which were misdiagnosed as CMM. Therefore 19 dogs (13 male and 6 females) were included in the statistical analysis; 13 oral -, 5 non-oral malignant melanoma and 1 metastatic melanoma, with the primary tumour unidentified. The following protocol was used; after confirmation of the tumour as a CMM and clinical staging using the WHO TNM system, the tumour was surgically removed, followed by local radiation therapy. The xenogeneic human tyrosinase vaccine was administered in 4 doses, every other week. Subsequently, the patients received physical examinations and radiographs/CTscan to a preset protocol, at 1, 3 and 6 months after the final vaccination, to check for signs of recurrence and metastases.
Results: The mean ST of all 19 dogs was 437 days from the start of treatment. Stage 1 and 2 dogs had significantly longer STs compared with stage 3 and 4 dogs ($P = 0.042$). The mean RFI was 468 days, the mean MFI was 268 days and the mean DFI was 326 days. No median ST times could be calculated because the 0.5 cumulative survival was not reached, as 12 of 19 dogs were still alive at the end of the study.
Discussion: The study data are not yet complete, therefore median ST and several other parameters were not yet available for interpretation, which made comparing the results to previously reported results difficult. Vaccinations generally occurred without difficulty or local irritation. One dogs developed a superficial bacterial dermatitis during the course of the study, which probably was unrelated to the vaccine therapy.
It must be considered positive that many dogs survived considerably longer than would be expected in those treated with conventional treatment modalities. More research is needed on the efficacy of the xenogeneic DNA vaccine in CMM and also on the therapeutic potential of xenogeneic DNA vaccines in feline and equine because it is very well possible that positive effects are found in further investigations.

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Introduction

Epidemiology and pathogenesis

Malignant melanoma (MM) is a common diagnosis in dogs, where it accounts for 7% of all malignancies, oral melanomas being the most common form [Smith et al 2002].
Epidemiological data show that melanocytic tumours are found in all vertebrate species (mammals, birds and fish). These tumours are most commonly found in humans, dogs, horses and some pig breeds. Incidence is lower in cats, cattle and goats [Baba 2007].

MM in dogs typically caries a grave prognosis. The disease often is discovered in a late stage, when excision of the tumour is no longer curative because metastasis already occurred to regional lymph nodes. Although MM in cats is uncommon, it carries the same poor prognosis as in dogs. This in contrary to grey horses, where melanocytic tumours are so common that almost every animal will be affected by it at some point [Smith et al 2002].

Normal melanocytes are dendritic cells arising from neuroectodermal melanoblasts that migrate to the epidermis and dermis during embryogenesis. These cells are localised in the basal stratum of the epidermis, interspersed with keratinocytes. The conversion of normal melanocytes (non-pigmented and isolated) to tumour cells is a process consisting of several steps; after initiation, promotion, transformation and metastasis take place [Smith et al 2002].

In animals, as well as in humans, there still are a lot of uncertainties concerning the aetiology of MM. A multifactorial aetiology may be admitted, in which there are risk factors that favour disease. First there is the hereditary factor that is scientifically documented at least in horses and pigs. These hereditary factors need intervention of an ecological factor for the clinical expression of the disease. Other risk factors in animals possibly are local irritating factors (demonstrated in hamsters), congenital neural crest abnormalities, melanin production imbalance and the presence of blue naevi [Baba 2007].
The aetiology of melanocytic tumours in dogs and cats is largely unknown. Ionizing sunlight cannot be pointed out as a causative factor in these species, because of tumour location (on haired skin and in the oral cavity) [Withrow and Vail 2007].
As more than 80% of grey horses acquire melanocytic tumours, it appears that there is a genetic predisposition to developing melanoma [Bellone 2010].

Clinical presentation in dogs

Canine malignant melanoma (CMM) is a very common disease in dogs. These tumours are most often located in the oral cavity (56%), on the lip (23%), skin (11%) en digits (8%) [Smith et al 2002]. Tumour location is highly prognostic [Smith et al 2002, Withrow and Vail 2007]. Melanocytic neoplasms in the oral cavity are almost always malignant as opposed to melanocytic tumours arising from the haired skin, of which >85% are behaviourally benign. In melanomas arising from the nail bed (subungual tumours) 50% is behaviourally malignant, so it can go either way [Withrow and Vail 2007].

Oral malignant melanoma (OMM) shows high growth rates, are locally invasive en metastasize in 70-90% of cases. Common sites for metastasis are the regional lymph nodes and the thoracic cavity, and the abdominal cavity to a lesser degree. Recurrence after surgical excision of the tumour is common. Symptoms that can occur are dysfagia, halitosis, ptyalism, bleedings and occasionally mandibula fractures [Smith et al 2002].

Melanocytic skin tumours are relatively common in the dog, accounting for 5-7% of all skin tumours in the dog, of which less than 5% is malignant [Smith et al 2002]. Older dogs (average age 9 years) that have darkly pigmented skin are affected more often [Withrow and Vail 2007]. Malignant cutaneous neoplasms are most often located on the head, ventral abdomen and scrotum. MM can also occur on the mucocutaneous junctions, such as the lips, preputium, vulva, anus, nose and palpebrae. MM arising from the nail bed are the most common digital neoplasm after squamous cell carcinoma [Smith et al 2002].
Melanocytic tumours are the most common ocular neoplasms in dogs. These ocular melanomas most frequently arise from the iris and ciliary body. Canine ocular melanoma most often is benign, but due to expansive growth it can eventually lead to loss of the eye. Visceral metastasis to the lungs, liver and other organs can occur, but the risk is relatively low [Grossniklaus 2000].

The diagnosis can usually be made with cytology but histology is required for establishing the degree of malignancy (mitotic rate and infiltrative growth are predictive factors). The prognosis for cutaneous melanocytic tumours with a high degree of malignancy is guarded; reported metastatic rates vary from 30-75%. The dog breed is also a predictive factor, melanomas in Doberman pinschers and miniature schnauzers are more likely to be benign and melanomas in miniature poodles are more likely to be malignant [Withrow and Vail 2007]. Amelanotic melanomas occur most often in the oral cavity of dogs (about one third is amelanotic), but can also occur at cutaneous sites. These agranular variants can represent a confusing histologic picture. Special immunohistochemical stains, such as melan A may be helpful in identifying melanomas in dogs. Melan A is specific for melanoma, but its sensitivity highly depends on the degree of differentiation [Withrow and Vail 2007].

The prognosis for dogs with OMM is guarded mainly due to the high metastatic propensity of the disease. Local disease often can be controlled by surgery and radiation therapy, but strategies to manage the high metastatic potential require further research. Local recurrence after surgical removal of the tumour is however common, varying from 22% after mandibulectomy to 48% following maxillectomy [Withrow and Vail 2007].

If surgery alone is performed in OMM the median survival time varies from 150 to 318 days. The 1-year survival rate is less than 35%. These survival times are only modestly increased when chemotherapy is added to the treatment protocol. Including surgery in the treatment

plan, however has a significantly better effect on tumour control and MST. For untreated dogs, the MST of OMM in dogs is only 65 days. Tumour size, clinical stage and ability of the first treatment to achieve local control are variables known to be of prognostic significance in dogs treated with surgery alone or surgery combined with other modalities. MST for dogs with tumours smaller than 2 cm diameter are 511 days compared to 164 days in tumours larger than 2 cm or lymph node involvement. Tumour recurrence has a clear negative influence on the median survival times. In OMM age, breed, sex, degree of pigmentation, microscopic appearance and DNA ploidy are not prognostic [Withrow and Vail 2007].

OMM responds well to hypofractionated radiation therapy, with complete response rate up to 70%. Local recurrence after complete response occurs in 15-26% of cases with a median RFI of 139 days. Metastasis is the most common cause of death, reported in 58% of cases with a median MFI of 311 days. With radiation therapy, median ST are 211 to 363 days with a one-year survival of 36-48% and 2 year survival of 21%. Local tumour control and survival times are considerably improved with rostral tumour location, tumour size, lack of evidence of bone lysis and post-operative radiation of microscopic tumour disease. Median survival times are significantly better in dogs with no risk factors present [Withrow and Vail 2007]. Unfortunately OMM has shown to be relatively resistant to most chemotherapeutics, with modest response rates (<30%) and the disadvantage of side effects [Withrow and Vail 2007].

Clinical presentation in cats

In cats melanomas are quite rare, they account for less than 1% of all feline oral neoplasms and about 0.5% of feline skin tumours. The ocular and cutaneous forms are more common compared to oral melanoma. The cutaneous melanoma is most frequently observed on the head, tail, distal extremities and lumbar region. The prognosis is generally poor. Local recurrence and regional metastasis occur in about 50% of cases [Smith et al 2002].

Ocular melanoma is the most frequently reported primary neoplasm in cats. Feline ocular melanoma most often originate from the anterior iris surface and a diffuse darkening of the entire iris becomes evident. Feline ocular melanomas have a greater metastatic propensity compared to canine ocular melanomas. The most common site of metastasis is the liver, followed by the lungs [Grossniklaus 2000]. The typical clinical presentation is the presence of a diffuse hyperpigmentation of the iris, which is slowly progressive. Occasionally a pigmented iridal nodule is seen or an amelanotic mass. In general the diagnosis is made clinically, but the diagnostic and prognostic value of FNA is worthy of further investigation [Withrow and Vail 2007].
In ocular melanomas no evidence of sex or breed predisposition has been found. Feline MM most often is observed in cats between the age of 8 and 12 [Smith et al. 2002]. Anterior uveal melanomas seem to be the most frequently occurring ocular melanomas in cats. Most cats are 9 years or older at the time of diagnosis. Malignant uveal melanomas have high metastatic rates, varying from 55-66% or even higher. However, the progress of hyperpigmentation to the extent that the eye has to be enucleated often takes months to years. It can then take up to 3 years before metastatic disease becomes evident [Withrow and Vail 2007].

Cutaneous melanocytic tumours in the cat are rare, accounting for 0.8-2.7% of feline skin tumours [Withrow and Vail 2007]. These tumours in cats may be malignant or benign. Experimentally skin melanomas can be induced with the feline sarcoma virus, but this aetiology is unlikely to be associated with clinically observed cases of feline melanoma. Frequent locations of melanocytic tumours in cats are the eye lids, tumours on the nose and pinna and less often the extremities. Nonocular melanomas in cats have a similar clinical appearance compared to those in dogs, but unlike in dogs histologic criteria do not seem reliable in predicting the degree of malignancy and clinical outcome. Ocular melanomas are more malignant in behaviour than oral melanomas. In the cat dermal melanomas can be both malignant and benign. Reported metastatic rates of cutaneous melanomas in the cat vary from 5-50% [Withrow and Vail 2007].
Hypofractionated radiation therapy has been described in OMM in cats with response rates of 60% and a MST of 146 days (ranging from 66-224) [Withrow and Vail 2007].

Clinical presentation in horses

In horses 15% of all skin tumours are melanocytic. In contrary to CMM more than 90% is benign at first presentation, but two thirds of these tumours are potentially malignant and can metastasize [Smith et al 2002]. The majority of melanocytic tumours occur in grey horses before the age of 5, coinciding with change in coat colour. Oral melanomas in horses are rare and make up for 17% of all equine oropharyngeal tumours. The prevalence of melanomas increase with age, no sex predisposition has been observed, but some breeds are affected more often [Smith et al 2002]. Most equine melanomas are localized in the skin in the perineal region, ventral tail en external genitalia, but can also occur at more atypical locations. Clinical signs are variable and can differ greatly in severity [Smith et al 2002].

Different growth patterns are observed, some melanomas show low growth rates and never metastasize, others become more malignant over time and the last group show fast growth rates and signs of malignancy from the start [Smith et al 2002]. Many of the histologically benign dermal melanomas in horses may eventually metastasize, and so must be considered potentially malignant. The actual incidence of metastatic melanoma is unknown, but is most likely to occur in older grey horses, one study showing a median age of 16.5 years. The most common sites for metastasis are the lymph nodes, liver, spleen, skeletal muscle, lungs and blood vessels throughout the body [MacGillivray et al. 2002].

In horses there are 4 types of melanocytic tumours; melanocytic nevi, anaplastic malignant melanoma, dermal melanoma and dermal melanomatosis. Melanocytic nevi occur in younger both grey and non-grey horses, and are benign superficial lesions. Excision is generally curative. Anaplastic malignant melanomas have high metastatic rates and occur in older, non-gray horses. Equine dermal melanomas are discrete tumours with low metastatic rates, excision usually is curative. Equine dermal melanomatosis has a high incidence of metastasis and surgical excision is not feasible. Both dermal melanomas and dermal melanomatosis are found in the perineum, ventral tail, external genitalia in older gray horses [Valentine 1995, MacGillivray et al. 2002]. Melanomatosis however, occurs mostly in horses over 15 years of age. As these to tumour types are very similar in histology, it does not aid in predicting tumour behaviour or metastatic potential. However, sudden changes in growth rate are associated with malignant transformation [Valentine 1995, MacGillivray et al. 2002].

Classification, staging and prognosis

The World health organization (WHO) developed a tumour grading system for OMM in dogs. These tumours are considered extremely malignant with a highly invasive growth pattern and metastasize often. Using this TNM system based on tumour size (T), involvement of the regional lymph nodes (N) and metastasis at a distance (M), OMM can be categorized in different stages.

Tumour categories:

T1: Tumour ≤ 2 cm in diameter
T2: Tumour is 2-4 cm in diameter
T3: Tumour > 4 cm in diameter

Lymph node involvement:

N0: No signs for involvement of the regional lymph nodes,
N1: Histologic/cytologic evidence for involvement of the regional lymph nodes.

Signs of distant metastasis:

M0: No signs for metastasis at a distance
M1: Signs for metastasis at a distance [Bergman 2007].

The factors combined make up the following stages:

Stage 1: Tumour category 1, no signs of lymph node involvement or metastasis.
Stage 2: Tumour category 2, no signs of lymph node involvement or metastasis.
Stage 3: Tumour category 2 with lymph node involvement or tumour category 3 with no lymph node involvement, no signs of distant metastases.
Stage 4: Any tumour- or lymph node category with signs of distant metastasis.

For non-oral MM no standard staging system is available [Bergman 2007]. However, for digital melanoma the WHO TNM staging system for digital tumours has been used in previous study based on tumour size and local tumour invasion, with stage 1: tumour < 2 cm in diameter and superficial, stage 2: tumour 2-5 cm diameter and minimal tissue invasion, stage 3: tumour > 5 cm or invading the subcutis and stage 4: tumour invading fascia and bone [Henry et al 2005, Bergman 2007].

The median survival times for canine oral melanomas treated with surgery for stage I, II and III respectively are 18, 5-6 and 3 months [MacEwen et al. 1986]. However, more recently a median survival times were reported of 12-14 months for stage I melanoma treated with conventional therapies (surgery, radiation and/or chemotherapy) [Bergman 2007]. For stage II-IV OMM in dogs treated with standardised therapies median survival times of 1-5 months have been reported [Bergman et al 2006]. It seems important to add further staging variables to the staging system, to further categorize patients into stages and make the staging system more reliable. For instance, tumour size is unrelated to the patient size, so a 1.8 cm tumour is categorized the same way as in a Rottweiler [Bergman 2007]. Negative prognostic factors are stage, tumour size, signs for metastasis and several histologic criteria [Bergman 2007]. The MST is significantly improved if surgery and radiation therapy are successful in controlling local disease [Withrow and Vail 2007]. The median survival time for dogs with digital melanoma treated with surgery is about 12 months [Bergman et al., 2006].

Potentials for the treatment of MM with xenogeneic DNA tyrosinase vaccines

DNA vaccine in dogs with CMM

Conventional therapy protocols for CMM, consisting of surgical resection, radiation and/or chemotherapy only give minimal clinical improvements. Death mostly occurs as a result of systemic metastases [Bergman 2007, Bergman et al. 2003]. Carboplatin is the standard chemotherapeutic, but response is generally poor, gain in MST is low and systemic side effects occur [Bergman et al. 2003]. The study of Catchpole 2006 proposed a theory that immunosuppressive antigens (regulatory IL-10 and TGF-β1) can add to the symptoms occurring in OMM. In humans it has been shown that melanoma cells themselves are the source of the particularly immunosuppressive cytokine IL-10, leading to metastatic disease. In the evaluated patient researchers failed to detect T cell cytokines such as IL-2, IL-4 or IFN-Ɣ in the draining regional lymph node of the primary tumour, suggesting a lack in immune response to the tumour. This could partly explain the aggressive nature of the malignant melanoma [Catchpole 2006].

Different new therapies for MM have been developed, but xenogeneic DNA vaccines are the most promising when it comes to clinical efficacy [Bergman 2007, Bergman et al. 2003]. These vaccines are capable of inducing both humoral and cellular immune response and are relatively cheap and easy to produce. One of the DNA vaccines currently evaluated as a possible therapy in CMM is the tyrosinase vaccine, which interferes with antigens involved in melanoma differentiation. Tyrosinase is a melanosomal glycoprotein essential in melanin synthesis. Research has shown that vaccination with human DNA encoding proteins from the tyrosinase family can induce an anti-tumour response in mice in the form of antibody production and activation of cytotoxic T-cells [Bergman et al. 2003].

The research of Bergman et al (2003) on the survival of dogs receiving xenogeneic DNA vaccinations as an adjunctive therapy for OMM showed that the tyrosinase DNA vaccine is a safe and potentially effective therapy. No signs for systemic side effects were found, only minimal local toxicity can occur. The vaccine seems to have a strong positive effect on MST in stage II-IV CMM. Of the 9 dogs treated with the humane tyrosinase vaccine complete response was observed in 1 dog with lung metastasis, 2 with stage IV and bulky metastasis lived for more than 400 days and 2 dogs with stage II and III disease surviving more than 500 days with causes of death unrelated to the MM. A median survival time (MST) of 389 days was reported [Bergman et al. 2003].
A study on the efficacy of the murine tyrosinase vaccine as adjunctive therapy in canine digital melanoma showed a median survival time of 351 days from the start of treatment (the first vaccination) and 476 days from the time of diagnosis [Manley et al. 2011].

Tyrosinase expression melanoma and the potentials for application of the melanoma vaccine in other domestic animals

Tyrosinase is considered the rate limiting step in melanin production; it catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine. In normal tissue tyrosinase expression (in humans normally expressed in the epidermal melanocytes and pigmented epithelium in the eye) seems to be tightly regulated, with high expression in developing melanocytes and lower expression in mature or quiescent melanocytes. However, in neoplastic tissues the tyrosinase expression appears to be considerably higher in all malignant tumours. In humans, tyrosinase has proven itself a useful target for immunotherapy approaches in melanocytic tumours [Philips et al. 2012]. It is generally accepted that tyrosinase expression is specific for melanocytic cells and (amelanotic) melanomas [Burchill 1991].

By means of extensive research it is now accepted that tolerance to self-antigens (for instance on cancer cells) can be overcome by the use of active immunization strategies. The xenogeneic DNA vaccines appear to be especially capable in inducing an immune response to cancer cells, and can help in controlling dissemination of tumour. However, several steps are involved in the generation of an immune response. After the injection of the vaccine, the tyrosinase antigen is taken up by muscle cells, expressed on the cell surface and recognized by antigen presenting cells (APC). These APCs, mainly dendritic cells (DC) will take up the antigen, followed by antigen processing and migration to draining lymph nodes. Antigen presentation leads to induction of antibodies and a cytotoxic T-cell response. These immune cells must then travel to distant tumour sites and recognize and lysate tumour cells. Also, there should be a sufficient memory pool of effectors to challenge tumours carrying the same antigens over time. A break anywhere in this sequence can cause disease progression, a frustration frequently encountered in (newly developed) cancer immunotherapies. Immunotherapeutic strategies found to be effective *in vitro* often do not translate in a desired clinical response [Srinivasan 2004].

During malignant transformation of melanocytic cells, the expression of MHC II molecules is upregulated, greatly increasing the density of class II MHC molecules presenting antigens (such as human tyrosinase antigen) on the cell surface. Normal cutaneous melanocytes do not express MHC II molecules [Grosenbaugh et al. 2011].

The study of Phillips et al 2012 was the first to perform a comparative analysis of tyrosinase expression in canine en equine melanocytic tumours, to help understand the role of targeted immunotherapy in these target groups. It was revealed that in melanocytic tumours, in canines as well as horses, the tyrosinase expression was considerably higher compared to control tissues (pigmented and non-pigmented). In dogs no significant difference was found between benign vs. malignant tumours, nor between oral vs. non-oral locations and melanotic vs. amelanotic tumours [Philips et al. 2012].
In horses no significant difference in tyrosinase expression were found between gray vs non-gray horses nor between tumour histologic classifications, however the number of equine cases was small. Because of high expression levels in melanocytic tumours and very low expression in normal reference tissues tyrosinase-targeted immunotherapies may be considered in horses, as well as dogs. Further research is needed however to examine the tyrosinase expression in additional control samples and other tumour types to try define the expression patterns more accurately. Also, the prognostic significance of relative tyrosinase expression is worth investigating further. Evaluation of additional cases will be helpful in answering these questions [Philips et al. 2012].

The research from Phillips et al (2012) has shown that the tyrosinase expression is similar in canine and equine melanocytic tumours. All melanocytic tumours showed a high tyrosinase expression when compared to normal tissues and the expression was independent of tumour pigmentation. This would suggest that a DNA tyrosinase vaccine developed for the use in CMM possibly has positive effects when used in other animal species, such as the horse [Philips et al. 2012]. More research to determine the efficacy of the vaccine in dogs, but also in humans and domestic animals species appears to be very useful [Bergman et al. 2003].

Aim of the study

The purpose of the research on melanoma vaccinations is to determine the efficacy of the xenogenic human tyrosinase DNA vaccine in CMM and the possibilities for the use of this vaccine in other animal species, mainly cats and horses. The hypothesis which is investigated is: "The DNA vaccine against MM will significantly improve survival times and quality of life in dogs with this condition." A substudy which was investigated by literature research is: "What are the similarities and differences between CMM and melanomas in horses and cats, and whether it can be assumed that the melanoma vaccine also is effective in these target groups". An introduction has been provided in the previous paragraphs.

Materials and Methods

Experimental design and DNA vaccinations

During the study that started in February 2011 in the university clinic for small animals in Utrecht (UKG) a total of 21 dogs underwent treatment with the melanoma vaccine as an adjunctive therapy in CMM. These patients were monitored during the entire procedure. The 'protocol melanoma vaccine', a clear clinical guideline for CMM-patients set up especially for this study in the UKG, consists of 3 phases; the diagnostic-, treatment- and evaluation phase.
In phase I (the diagnostic phase) the tumour is identified as a MM using FNA or histology and the patients were clinically staged using the WHO TNM system. This staging system was designed for canine OMM, but has been applied to all canine melanomas in this study. FNAs are taken from the regional lymph nodes and a CT scan of the head (in OMM), thorax and abdomen and a basic blood analysis are performed.

At the time of diagnosis in 14% of OMM cases signs of metastasis are detected on thoracic radiographs. 13% of thoracic radiographs are false-negative for metastases in canine OMM [Todoroff & Brodey, 1979]. The sensitivity of CT scan is considerably higher, only 9% of the metastatic lesions detected using CT are also detected on thoracic radiographs. CT can detect lesions as small as 1 mm diameter. With X-rays lesions of 7-9 mm and larger are detected [Nemanic et al., 2006]. To aid proper staging of CMM patients it is therefore highly recommended that dogs are evaluated for distant metastasis using CT instead of radiographs.

In phase II, the treatment phase, surgical resection of the tumour (if possible, with wide surgical margins) and local radiation therapy took place. The tumour tissue was sent for histology. Within 7-10 days after surgery radiation therapy started, in 6 fractions of 6 Gy each, twice a week for 3 consecutive weeks. Not only the surgical field, but also the regional lymph node was often included in the radiation field.

Also, a total of 4 DNA vaccinations were administered, every other week. To minimise any possible discomfort, the first 2 vaccinations were combined with the other treatment modalities, under general anaesthesia. Sedation for the vaccinations only is not necessary. These xenogeneic tyrosinase DNA vaccines (Oncept®), Merial) were imported from the US, where research on the melanoma vaccine began. The vaccine containing the human DNA was administered in the semitendinosus of semimembranosus muscle using a transdermal system under high pressure, in doses of 500 μg, to be taken up and expressed by the canine host.

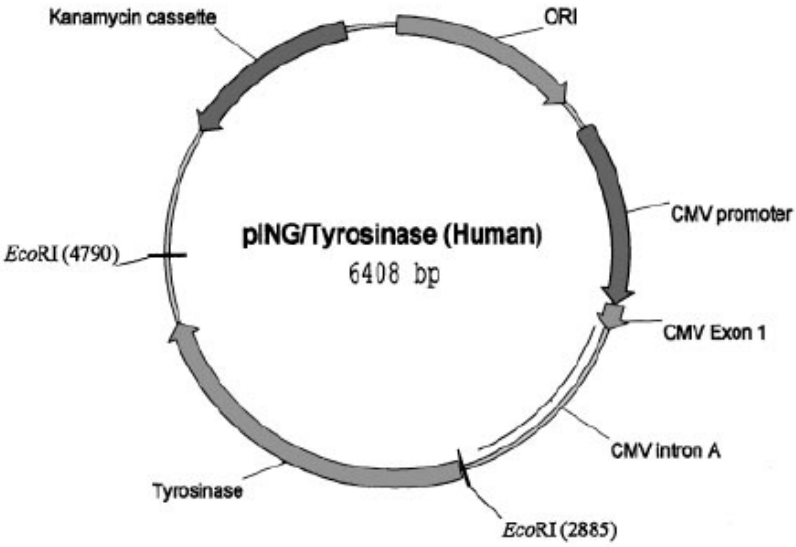

Figure 1; Bacterial pING human tyrosinase plasmid, used for vaccination in dogs with CMM *(Bergman et al., 2003)*

During phase III (the evaluation phase) the dogs were checked for signs of recurrence and metastasis by evaluating the general condition of the animal, physical examinations and thoracic radiographs, following a set protocol. During radiation therapy and vaccinations the animals were checked for signs of disease. One month after the final of 4 vaccinations a physical examination was performed. Three months after the final vaccination a physical examination and radiograph of the thorax were performed. Six months after the final vaccination the patients were re-evaluated by means of physical examination and CT scan to help the specialist and owner decide whether to start another round of vaccinations. During this evaluation phase contact was maintained by telephone or email. By the means of this extensive follow-up, data concerning signs of general illness, for local recurrence at the original tumour site, lymph node and/or distant metastases and survival time and/or time of death were gathered to be analysed using SPSS.

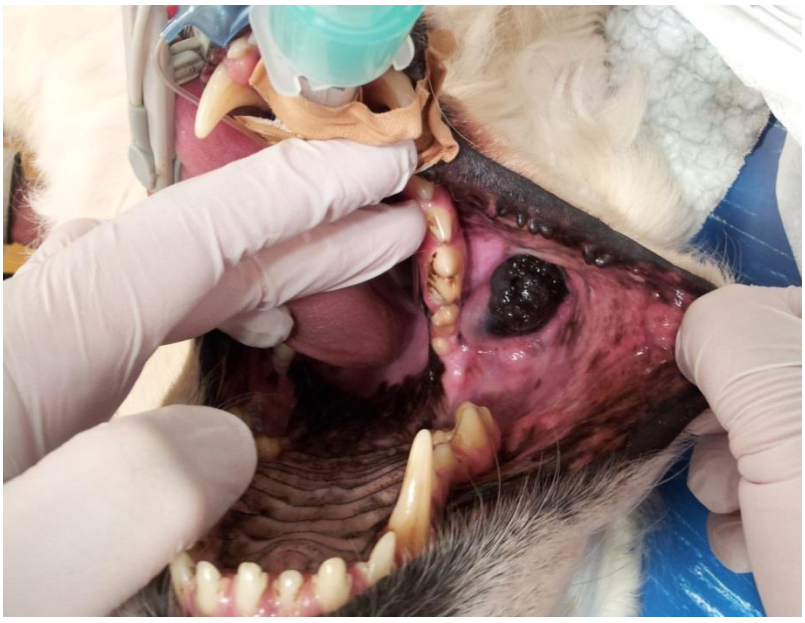

Figure 2
Golden retriever with an oral malignant melanoma. This dog also had an affected mandibular lymph node.

Animals

A total of 21 dogs took part in the study of the melanoma vaccine used in CMM in the University clinic for small animals in Utrecht (UKG) since February 2011. Data were collected from the beginning of this research onwards, concerning general patient information (such as breed, age, sex, weight), tumour location, - size and clinical staging (using the TNM system), signs for recurrence/metastasis, side effects etc.

In the course of the study it became evident that two dogs were misdiagnosed as malignant melanomas and therefore these patients were removed from the study data. Therefore 19 dogs were included in the statistical analysis, with 13 males and 6 females. A total of 12 dog breeds and 1 mixed breed dog were represented in this study, of which 13 dogs suffered from oral melanoma (3 of which were amelanotic), 3 had digital melanomas, 1 cutaneous melanoma and 1 scrotal melanoma. In 1 dog a metastatic melanoma was found in the mandibular lymph node, but the primary tumour could not be identified.

Before the start of treatment, all dogs were staged, 5 of which were stage 1 (26.3%), 3 stage 2 (15.8%) 7 stage 3 (36.8%) and 4 stage 4 dogs (21.1%). An overview is given in T*able 1*.

Table 1

Patient	Breed	Date of birth	Sex	Tumour type/location	TNM stage
Brumby	Shiba Inu	26-5-2005	Male	Oral melanoma	2
Borka	Labrador retriever	1-4-2001	Male	Oral amelanotic melanoma (>1)	4
Dino	Beagle	15-9-2002	Male	Oral amelanotic melanoma	2
Bobby	Beagle	23-6-1998	Male neutered	Oral amelanotic melanoma	3
Luna	Leonberger	4-10-2002	Female neutered	Oral melanoma	1
Joyce	Border collie	18-9-1998	Female	Oral melanoma	3
Woody	Nova scotia duck tolling retriever	14-1-2003	Male neutered	Multiple cutaneous melanocytoma	4
Endo	Airedale terrier	17-5-1999	Male	Oral melanoma	3
Patrick	Irish terrier	6-6-2004	Male	Scrotal melanoma	1
Julius	Labrador retriever	12-9-2001	Male	Digital melanoma	3
Raya	Newfound-lander	7-1-1999	Female neutered	Oral melanoma	2
Peggy	Spitz dog (= keeshond, middenslag)	31-5-2003	Female neutered	Oral melanoma	3
Charley	Mixed breed	1-1-2000	Male	Oral melanoma	4
Milo	Golden retriever	21-7-2006	Male neutered	Digital melanoma	1
Duke	Golden retriever	27-10-2003	Male	Digital melanoma	1
Snuf	Beagle	13-2-1999	Female	Oral melanoma	3
Micha	Border collie	11-9-2000	Male	Oral melanoma	1
Leon	Giant Schnauzer	27-6-2003	Male neutered	Mandibular lymph node metastasis *	3
Britt	Golden retriever	21-6-2002	Female neutered	Oral melanoma	4**

* Primary tumour was not found
** In this dog 2 lesions in the thoracic vertebrae were found, presumably these are metastatic lesions but this is no certainty. This dog also had an affected mandibular lymph node (see *Figure 2*)

Statistical analysis

SPSS was used to analyse the patient data collected during this study. Data on the TNM stage, date of birth, date of the first clinical symptoms, date of diagnosis and the start of therapy (defined as the first vaccination) were recorded. Also the dates of local recurrence, metastasis and survival times were recorded.

The RFI (recurrence free interval) is defined as the time from surgical removal of the tumour to the time the first signs of recurrence were noticed (often first discovered by the owner). The MFI or metastasis free interval is the time until the first signs of metastasis have occurred (taking into consideration that this only applies to animals were no signs of metastasis have been found in the start of the study, unless these tumours were removed). Metastasis generally is only detected using the diagnostic methods such as radiographs or CT scans (with possible exceptions such as in cutaneous - or regional lymph node metastases. Not all animals have undergone these examinations at this point, so the MFI dates are not complete. The DFI (disease free interval) is defined as the time until the first signs of disease (defined as either recurrence or metastasis) occurred. Kaplan-Meier curves for RFI, MFI and DFI and ST were made with SPSS. SPSS was also used to determine the 95% confidence intervals for mean– and median survival times.

Results

Table 2; *Overview of patients, age, TNM stage (1-4), dates of first symptoms, - diagnosis and start of therapy*

Patient	TNM stage	Date of birth	Date the first clinical symptoms appeared	Date of diagnosis	Date of first vaccination
Brumby	2	26-5-2005	20-12-2010	31-12-2010	25-2-2011
Borka	4	1-4-2001	20-1-2011	26-1-2011	25-2-2011
Dino	2	15-9-2002	26-11-2010	1-2-2011	22-3-2011
Bobby	3	23-6-1998	10-1-2011	20-1-2011	28-3-2011
Luna	1	4-10-2002	23-2-2011	2-3-2011	11-4-2011
Joyce	3	18-9-1998	7-4-2011	19-4-2011	2-5-2011
Woody	4	14-1-2003	18-4-2011	3-5-2011	13-5-2011
Endo	3	17-5-1999	31-8-2011	16-9-2011	4-10-2011
Patrick	1	6-6-2004	26-4-2011	19-10-2011	27-10-2011
Julius	3	12-9-2001	13-6-2011	27-9-2011	27-10-2011
Raya	2	7-1-1999	1-12-2011	1-1-2012	25-1-2012
Peggy	3	31-5-2003	27-12-2011	26-1-2012	22-2-2012
Charley	4	1-1-2000	11-7-2011	11-7-2012	14-3-2012
Milo	1	21-7-2006	5-3-2012	14-3-2012	2-5-2012
Duke	1	27-10-2003	9-4-2012	19-4-2012	3-5-2012
Snuf	3	13-2-1999	31-5-2012	31-5-2012	5-6-2012
Micha	1	11-9-2000	15-4-2012	15-4-2012	22-6-2012
Leon	3	27-6-2003	13-5-2012	30-5-2012	27-6-2012
Britt	4*	21-6-2002	25-6-2012	5-7-2012	20-7-2012

Table 3; Overview of patient RFI, MFI, DFI and ST dates.

Also included is the dates were censored (1) or not (0). In survival times '0' is used to state that the animal was still alive at the end of this study, or that the dogs died of illness unrelated to the melanoma.

Patient	RFI date	Censor RFI	MFI date	Censor MFI	DFI date	Censor DFI	ST date	Censor survival
Brumby	17-9-2012	0	10-5-2011	1	10-5-2011	1	17-9-2012	0
Borka	15-5-2012	1	15-5-2012	1	15-5-2012	1	25-7-2012	1
Dino	10-9-2012	0	6-6-2012	0	10-9-2012	0	10-9-2012	0
Bobby	10-8-2011	1	30-6-2011	1	30-6-2011	1	10-8-2011	1
Luna	11-9-2012	0	7-11-2011	0	11-9-2012	0	11-9-2012	0
Joyce	5-12-2011	0	19-8-2011	1	19-8-2011	1	5-12-2011	1
Woody	19-9-2012	0	19-7-2012	1	19-7-2012	1	19-9-2012	0
Endo	2-1-2012	0	2-1-2012	0	2-1-2012	0	2-1-2012	0
Patrick	25-1-2012	0	25-1-2012	0	25-1-2012	0	25-1-2012	0
Julius	17-9-2012	0	18-7-2012	0	17-9-2012	0	17-9-2012	0
Raya					23-3-2012	1	30-3-2012	0
Peggy	15-5-2012	1	22-2-2012	0	15-5-2012	1	13-6-2012	1
Charley			28-3-2012	1	28-3-2012	1	24-9-2012	0
Milo	28-8-2012	0	2-5-2012	0	28-8-2012	0	28-8-2012	0
Duke	10-9-2012	0	3-5-2012	0	10-9-2012	0	10-9-2012	0
Snuf	19-9-2012	0	3-7-2012	1	19-9-2012	0	19-9-2012	0
Micha	19-9-2012	0					26-9-2012	0
Leon	25-9-2012	0			25-9-2012	0	25-9-2012	0
Britt	26-9-2012	0			26-9-2012	0	26-9-2012	0

Kaplan Meijer curves

Graphs are made for the survival times, calculated from the start of treatment, from the date the first symptoms appeared. Curves for the group total, but also for separate TNM stages are presented (all stages separately but also pooled '0' = stage 1+2 vs. '1' = stage 3+4). Because this study is not completed (and many dogs are still alive at this point, the mean survival times calculated using SPSS only represent the results so far (estimates) and will rise when more data become known.

Survival curves

Figure 3

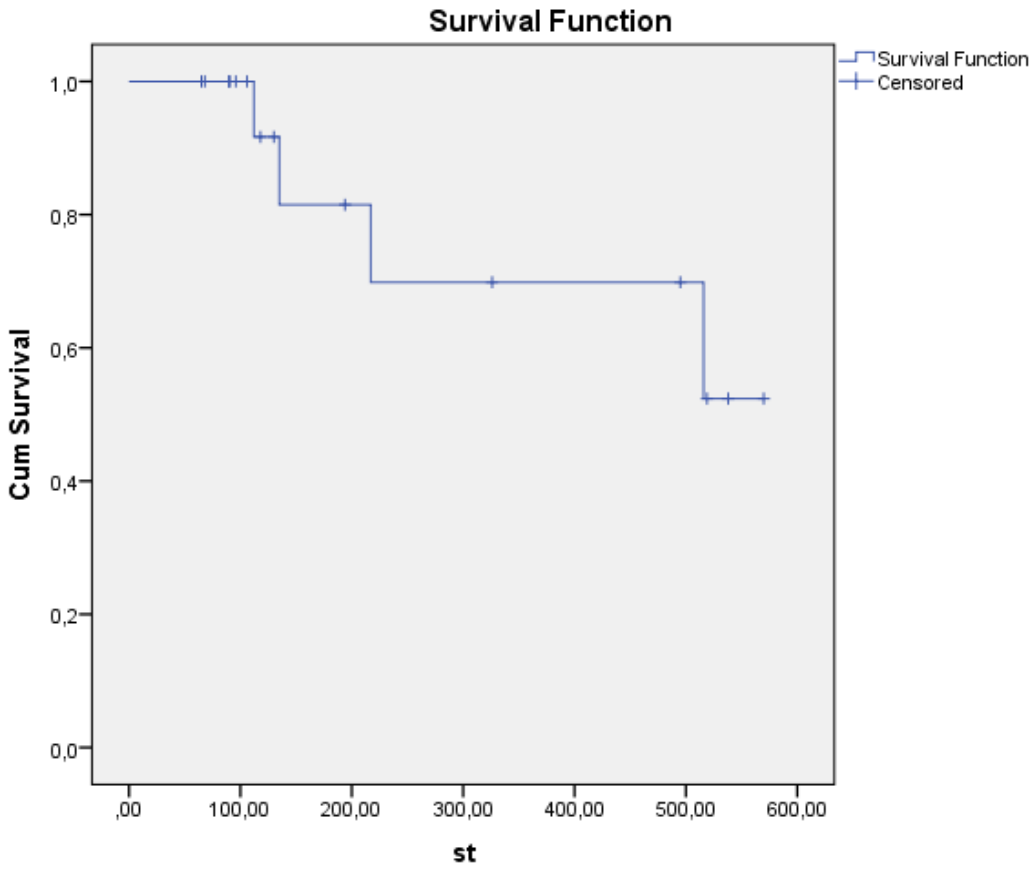

Figure 3 shows the Kaplan Meijer curve of the cumulative survival time from the start of treatment (defined as the date of the first vaccination). The mean survival time for the total study group was 437 days, with a 95% confidence interval (CI) of 321 to 553 days. At this point the median survival time could not be calculated, because the 0.5 survival point was not reached.
During the course of this study a total of 4 dogs died because of local tumour progression and/or distant metastasis, 3 of which were stage III and 1 stage IV dog with survival times of 112, 135, 217 and 516 days. Also 3 dogs have died with causes of death unrelated to melanoma early in this study (survival times of 65, 90 and 90 days).

Figure 4

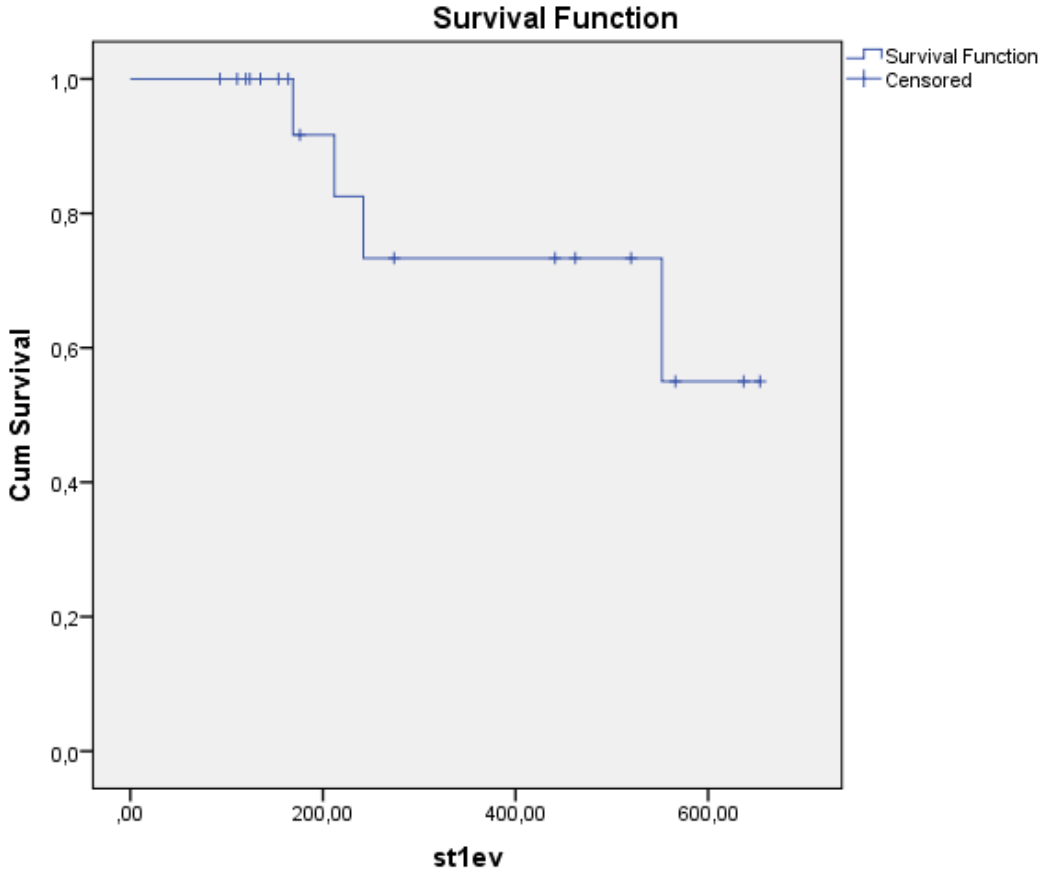

Figure 4 shows the cumulative MST calculated from the date the first symptoms appeared, which was 517 days, with a 95% CI of 403 to 630 days. Also here, no median survival time could be calculated.

Figure 5

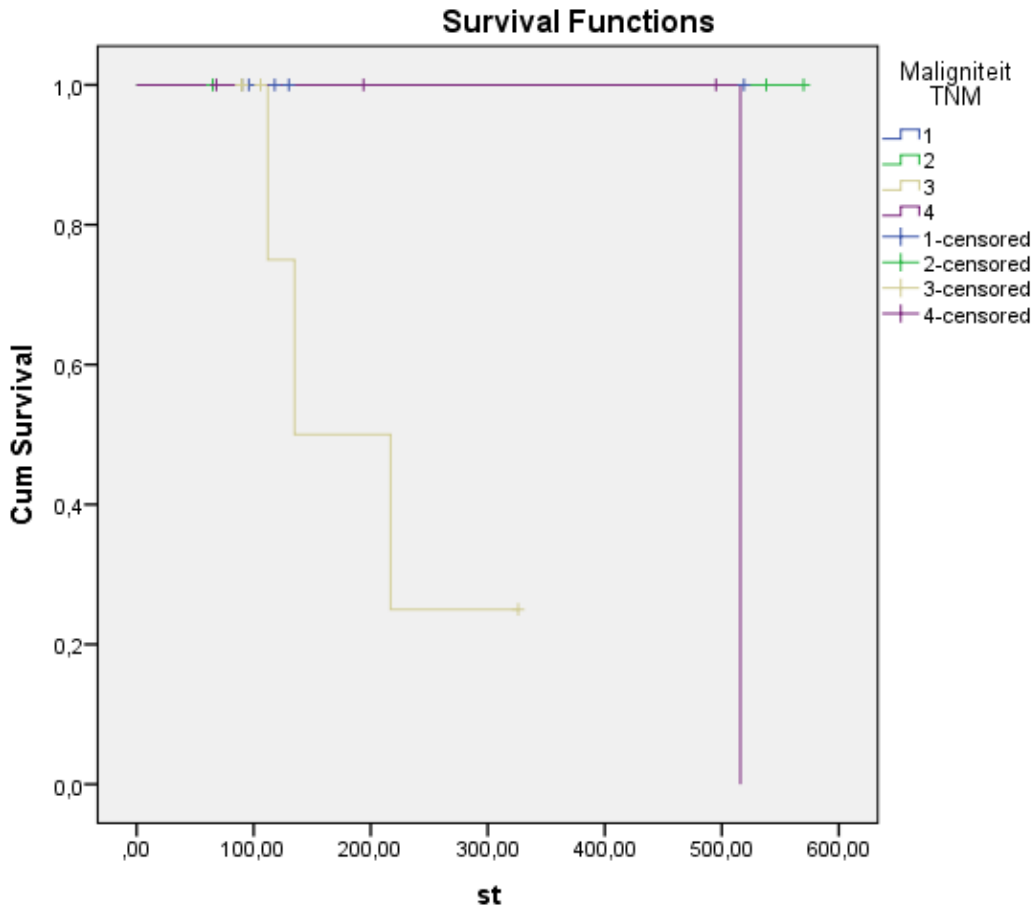

Figure 5 shows the Kaplan Meijer survival time curves for the separate TNM stages. A Mantel-Cox test of equality was performed for the separate stages, but no significant difference was found (P = 0.063). However, the graph shows that stage 1 and 2 seem to do much better compared to stage 3 and 4. It seems that stage 3, however did worse than stage 4, which is not expected based on previous studies.

Figure 6

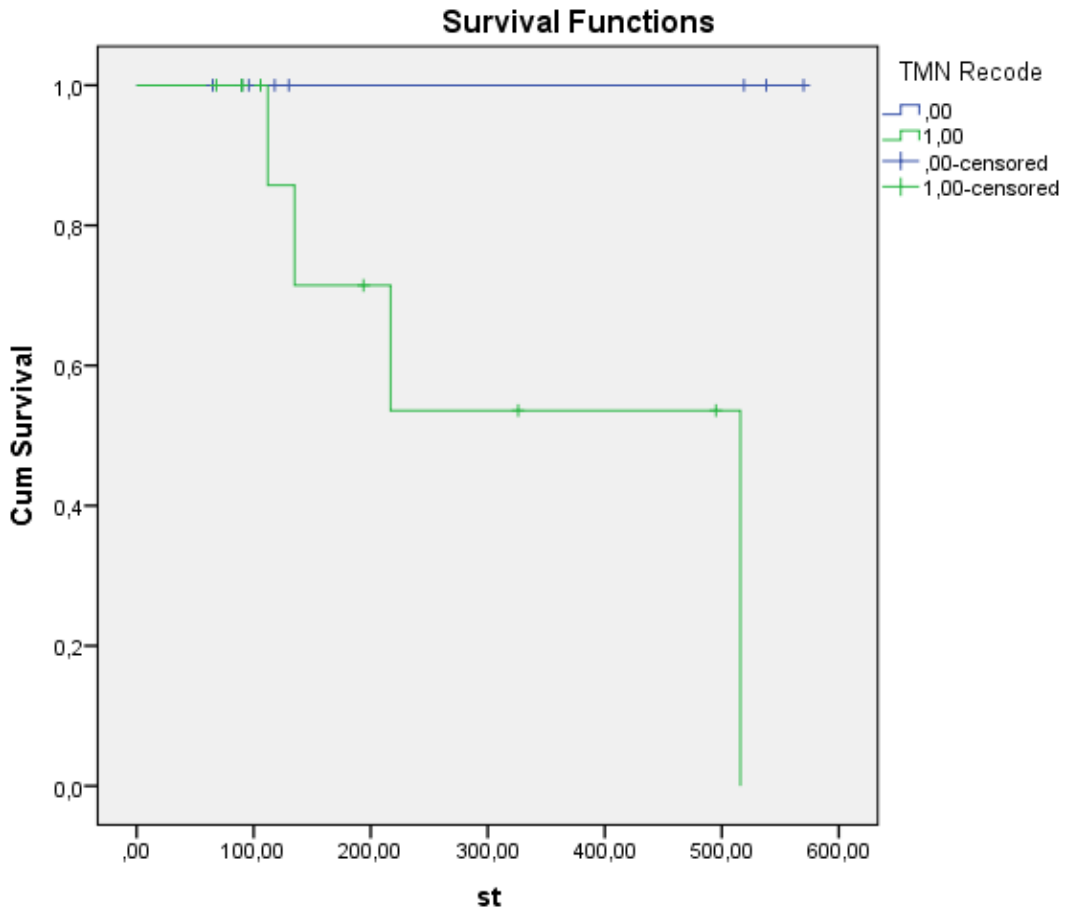

Figure 6 shows the pooled survival times stage 1&2 (0) vs. 3&4 (1). The Graph shows a considerable difference between groups. A test for equality of survival distributions (Log Rank Mantel-Cox) for the pooled TNM levels was performed, showing a significant difference between groups (P = 0.042). The median survival time for group 1 was days. For group 0 no median survival time could be calculated, because all dogs were still alive at the end of the study.

Recurrence free intervals

Figure 7

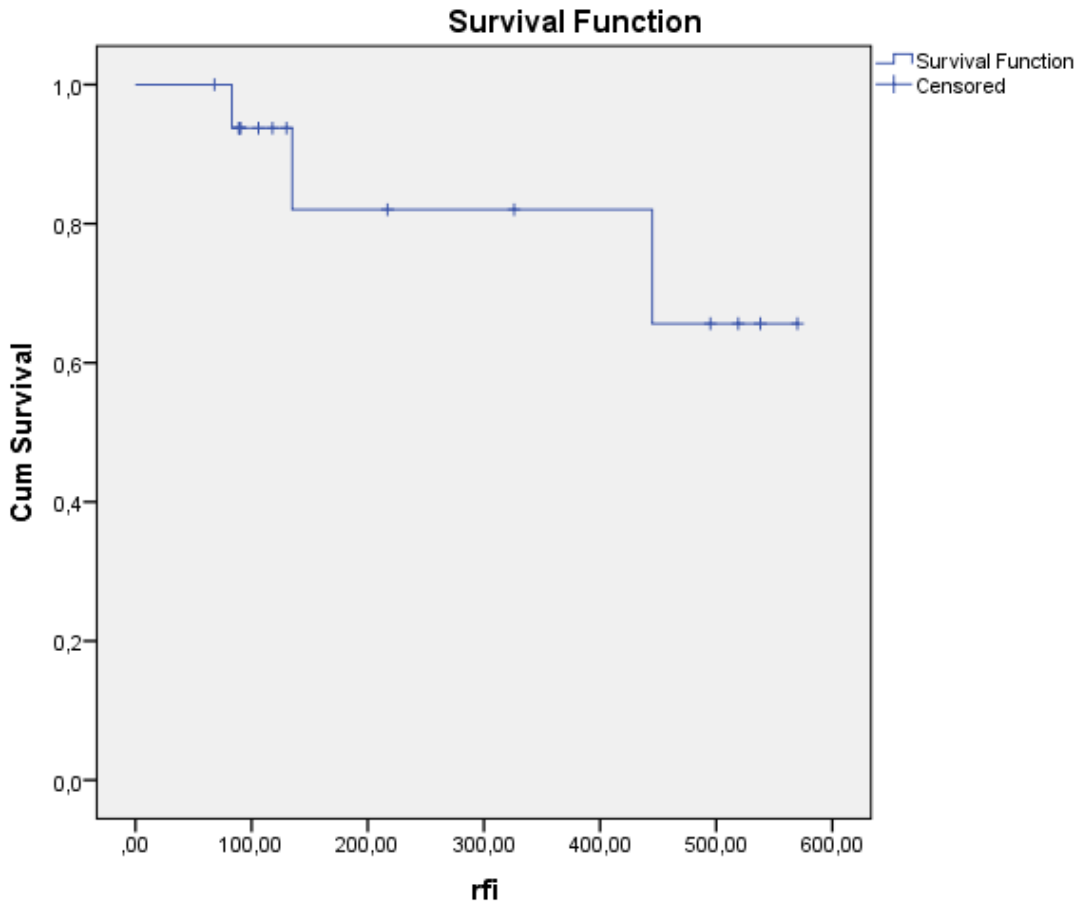

Figure 7 shows the survival curve of the RFI (for the total study group). The mean RFI is 468 days, with a 95% CI of 361 to 575 days. No median RFI could be calculated at this point. During this study a total of 3 dogs showed signs of local recurrence, 2 of which stage III and 1 stage IV dog with times till recurrence of 83, 135 and 445 days.

Figure 8

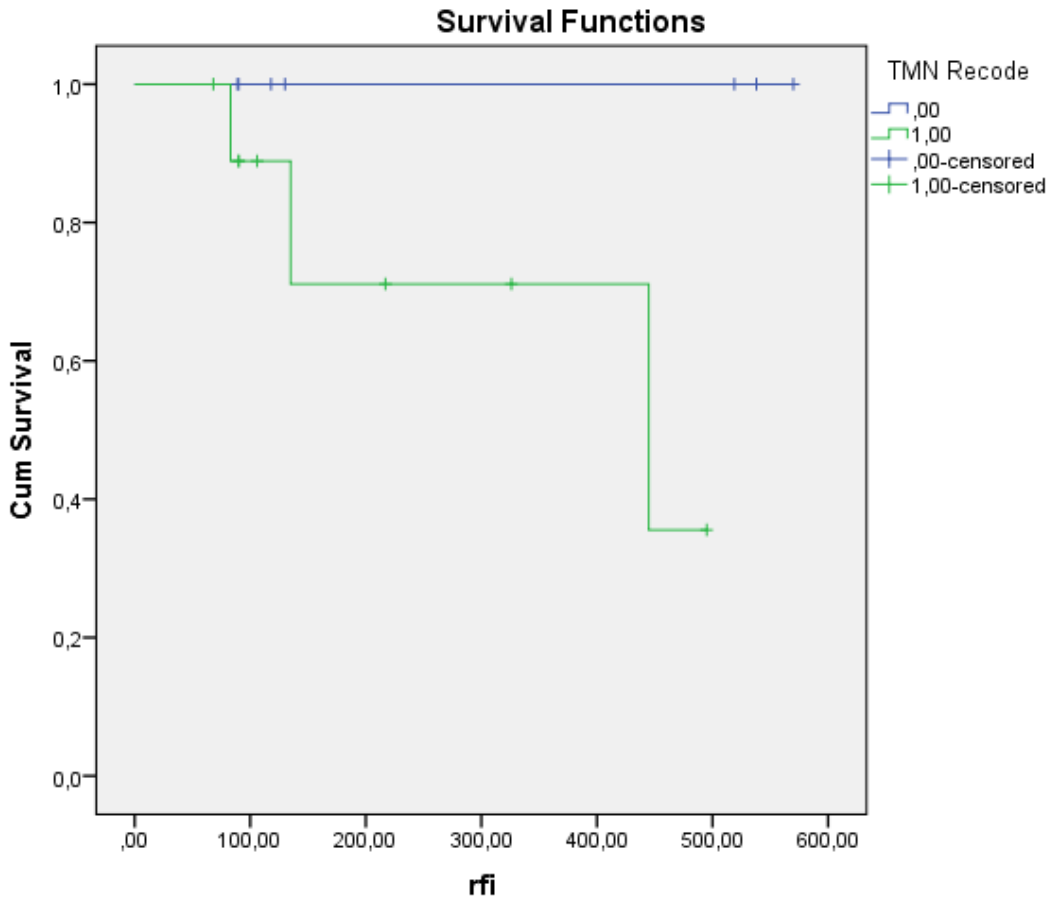

Figure 8 shows the recurrence free interval (RFI) for the pooled groups '0' and '1'. Also this plot appears to show a difference between the 2 groups (group 1 doing worse than 0), however no significant difference was shown (P = 0.096).

Metastasis free intervals

Figure 9

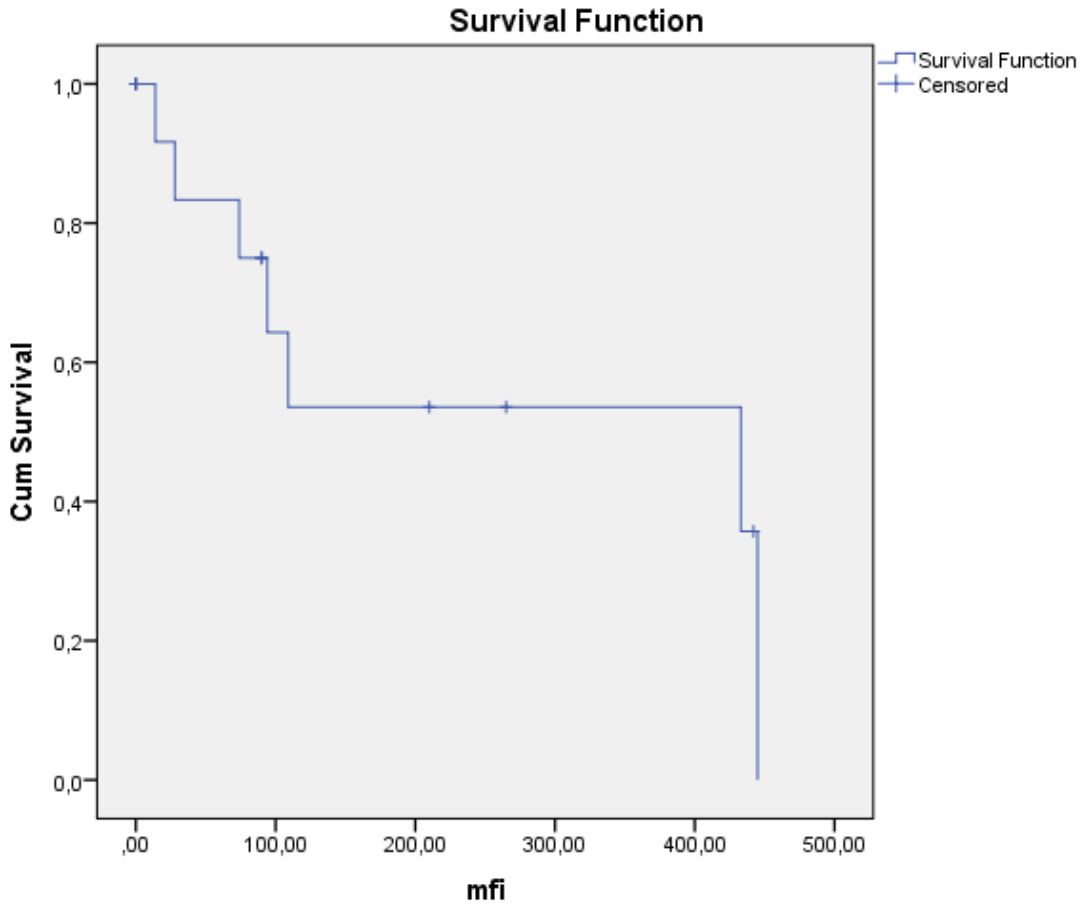

Figure 9 shows the cumulative survival curve for the metastasis free interval (MFI). The mean MFI is 268 days, with a 95% CI of 145 to 390 days. The median MFI was 433 days, with a 95% CI of 16 to 850 days. A total of 7 dogs showed signs of metastasis, of which 1 stage II, 3 stage III and 3 stage IV dogs with MFIs of 14, 28, 74, 94, 109, 433 and 445 days.

Figure 10

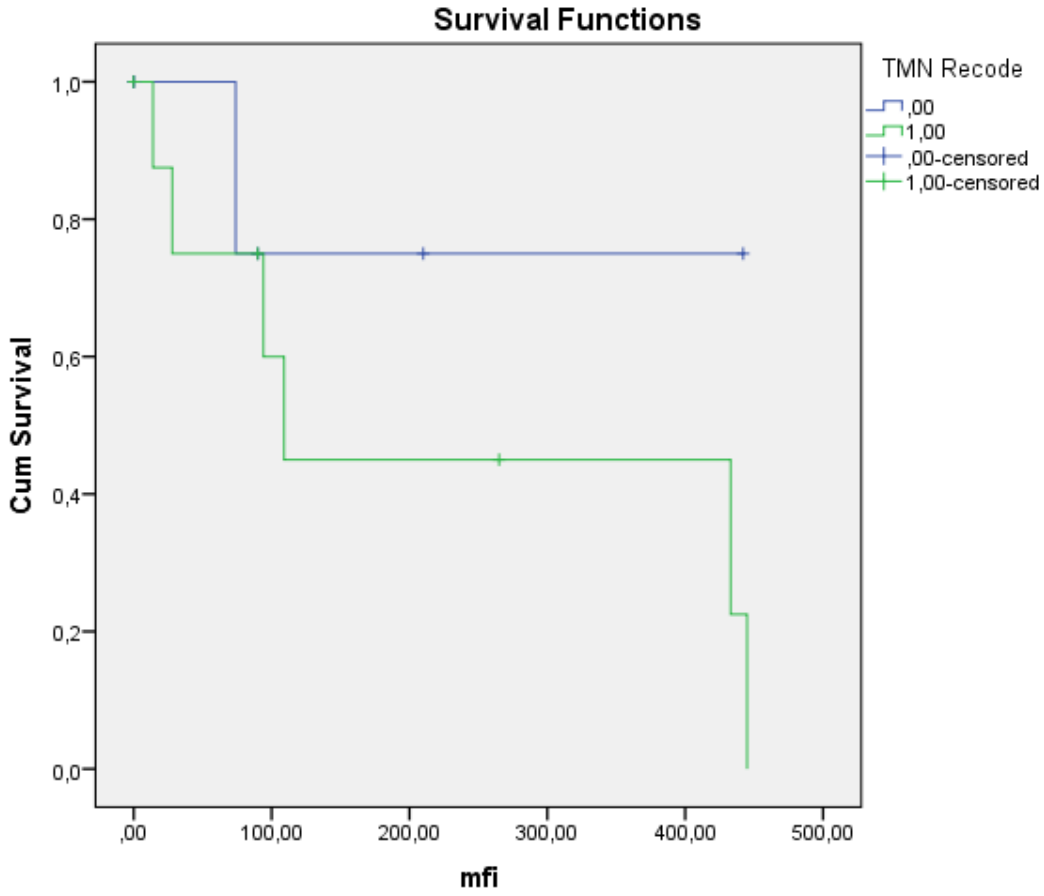

Figure 10 shows the Kaplan Meijer curves for the metastasis free intervals for the pooled groups ’0’ and ‘1’. The mean MFI for group 0 was 350 days, with a 95% CI of 194 to 506 days. The mean MFI for group 1 was 233 days, with a 95% CI of 82 to 384 days. The median MFI could not be calculated for group 0. The median MFI for group 1 was 109 days, with a 95% CI of 72 to 146 days. Also no significant difference between groups was shown (P = 0.365).

Disease free intervals

Figure 11

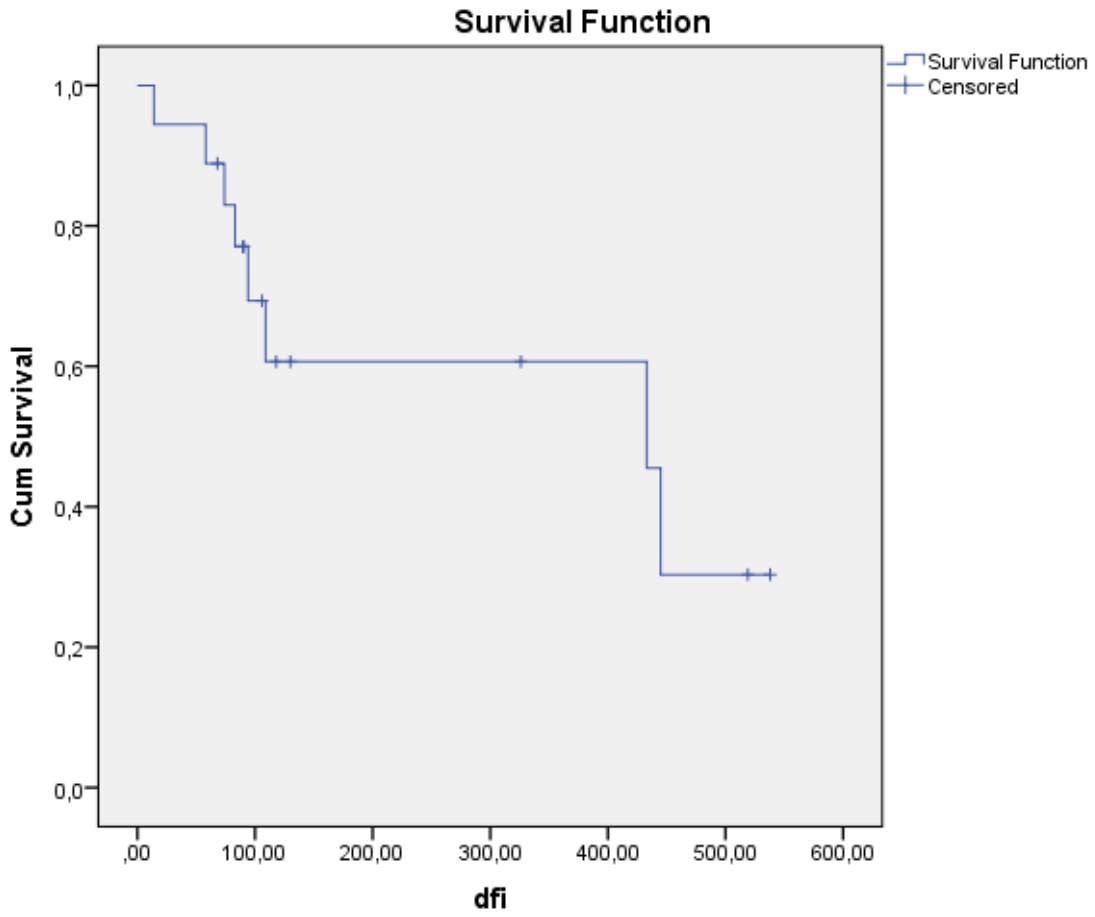

Figure 11 shows the Kaplan Meijer curves for the cumulative disease free interval (for the group in total). The mean DFI was 326 days, with a 95% CI of 218 to 435 days. The median DFI was 433 days, with a 95% CI of 78 to 788 days. A total of 8 dogs were censored because of progression of disease, defined as either signs of metastasis or local recurrence (or both), 2 of which were stage II, 3 stage III and 3 stage IV, with DFIs of 14, 58, 74, 83, 94, 109, 433 and 445 days.

Figure 12

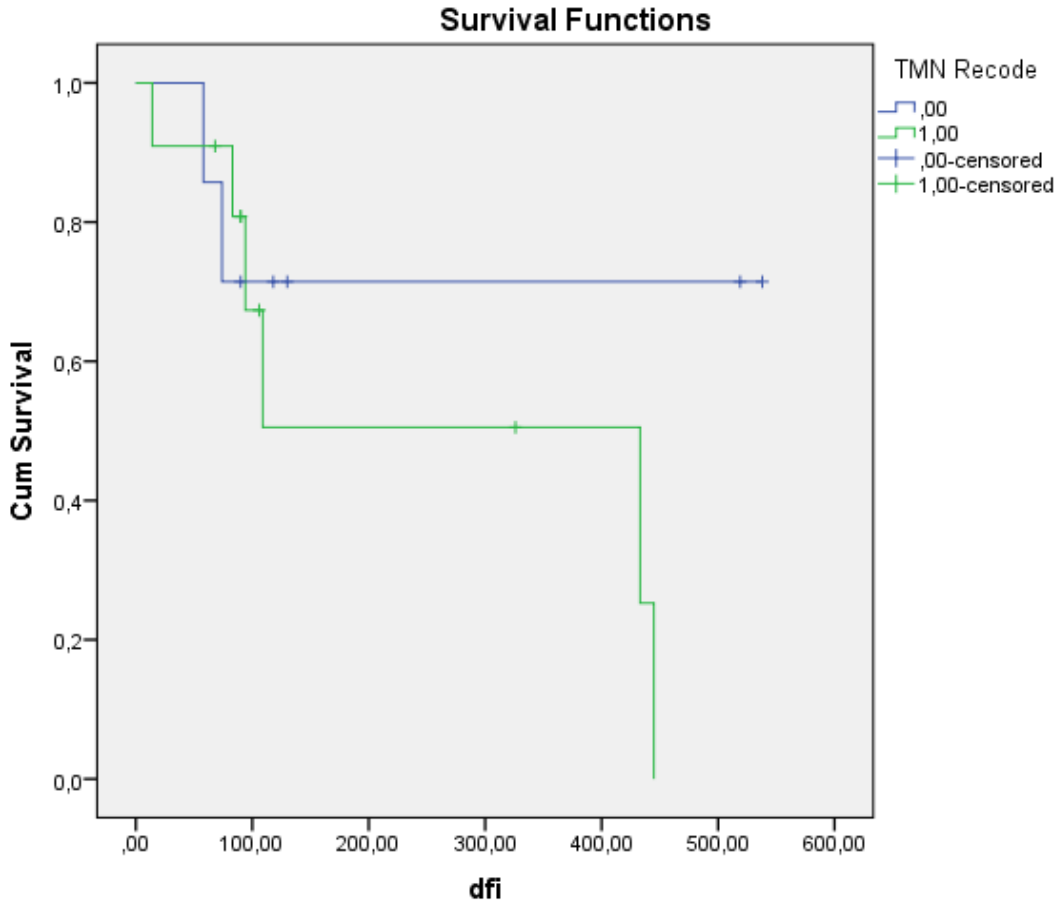

Figure 12 presents the Kaplan Meijer curves of disease free intervals of pooled groups 0 and 1. The mean DFI for group 0 was 403 days, with a 95% CI of 245 to 561 days. No median DFI could be calculated for group 0. The mean DFI for group 1 was 262 days, with a 95% of 120 to 405 days. The median DFI was 433 days, with a 95% CI of 114 to 752 days. Again, no significant difference between groups was found (significance level 0.240).

Side effects

Vaccinations generally occurred whithout difficulty or local irritation. One dog developed a superficial bacterial dermatitis during the course of the study, which was probably unrelated to the tyrosinase vaccinations.

Discussion

Efficacy of the melanoma vaccine in CMM

The aim of this study was to determine the efficacy of the melanoma vaccine, as an adjunctive therapy in CMM. In dogs with OMM treated with surgery alone median survival times of 18, 5-6 and 3 months for stage I, II and III respectively have been reported [MacEwen et al 1986]. For dogs with CMM treated with standard treatment protocols (consisting of surgery combined with radiation and/or chemotherapy) median survival time of 12-14 months was reported for stage I disease [Bergman 2007]. For stage II-IV OMM treated with standardised therapy protocols median survival times of 1-5 months were reported [Bergman et al 2006]. The first study (Bergman 2003) on the survival of dogs receiving xenogeneic DNA vaccinations as an adjunctive therapy for OMM shows a median ST of 389 days, with several stage III and IV dogs with survival times greatly exceeding those expected with conventional treatment modalities [Bergman et al. 2003]. Therefore it was to be expected that this study would also show positive results.
The efficacy of this study was 'measured' by a statistical analysis of the patient data, which unfortunately are not complete at this point. The median survival times cannot yet be determined because the 0.5 cumulative survival was not reached. Because 12 of 19 dogs were still alive at the time of the statistical analysis only estimates (results so far) of mean survival data are available for interpretation. The mean survival time for all dogs together was 437 days from the start of treatment and 517 days from the date the first symptoms appeared. One stage III dog (not censored for disease/death) has lived for 326 days. One stage IV dog (not censored also) has lived for 495 days. Many other dogs are still in the early treatment or evaluation phase, so it is not certain whether these dogs will do well. In this study, no significant differences were found for survival time between the separate TNM stage patient groups. This could be due to the relatively small number of patients in each group. Pooled groups (stage 1+2 vs. 3+4) did show a significant difference between groups in the survival time curve ($P = 0.042$). This demonstrates that stage 1 and 2 have a better chance of living longer while being treated with the melanoma vaccine compared to stage 3 and 4 dogs, which was expected based on previous studies.

For OMM in dogs treated with radiation therapy a median RFI of 139 days has been reported based on previous study. Metastasis is reported to be the most common cause of death, with a median MFI of 311 days [Withrow and Vail 2007].
The mean recurrence free interval, or RFI in this study was 468 days. No median RFI could be calculated at this point. No significant difference between pooled groups ($P = 0.096$) or separate TNM stages was found.
The mean MFI was 268 days, with a median MFI of 433 days. The mean MFI for stage 1+2 vs. 3+4 respectively were 350 days vs. 233 days. However, no significant difference between groups was found ($P = 0.365$). The median MFI for stages 3+4 together was 109 days, for stage 1+2 no median MFI could be determined.
The DFI or disease free interval (defined as the time in days until signs of either recurrence or metastasis occurred) was also calculated. The mean DFI in this study was 326 days, with a median DFI of 433 days. For stages 1+2 together, the mean DFI was 403 days, vs. 262 days for stage 3+4. However no significant difference was found ($P = 0.240$). The median MFI for stage 1+2 could not yet be determined, for stage 3+4 it was 433 days.

In the future this study will continue so more survival data will be collected on these dogs and more patients will be added to this study. Previous studies have generally used median survival times to 'measure' the efficacy of a certain therapy. Since there are very little median survival times, MFI, RFI and DFIs available at this point, it is rather difficult to compare the results from this study with previous research. However, the current data set seems to provide positive results with the adjunctive DNA vaccine compared to conventional treatment modalities. It is however difficult to quantify the results at this point and more research is required in the future. It must be regarded as positive however, that so many of the study group have managed to survive up till now.
During the course of this study a total of 4 dogs died because of local tumour progression and/or distant metastasis (all stage 3 or 4), with STs of 112, 135, 217 and 516 days. Also 3 dogs have died with causes of death unrelated to melanoma in the early time of this study (STs of 65, 90 and 90 days)

The median MFI found in this study was 433 days, compared to 311 days mentioned in previous study [Withrow and Vail 2007], this seems like a positive result. It is however difficult to say whether the research conditions were similar, which makes comparing these 2 results a little tricky.
Due to the (relatively) small patient numbers and highly variable results (resulting in high standard deviations) of therapy most 95% CI for survival time and other parameters were rather wide. Also, often no significant difference was found between separate TNM stages.

No control group was used in this study. Therefore, the results of this study could only be compared with results of previous research. Obviously this is not an ideal situation, but because recent studies have proven positives effects as result of adjunctive vaccine therapy it would be unethical to set up a study with negative controls. And this would also be difficult because the owners had to pay for their dog's treatment.
To examine the relation between parameters such as age, sex, breed, tumour type etc. and the results of this study it would be possible to perform a cox hazard analysis. However, with small patient numbers the chance that a significant difference is found is very low. Perhaps this can be further investigated in the future.

Discussion on the therapeutic potential of the melanoma vaccine in the horse and cat.

The other purpose of writing this paper was to evaluate the therapeutic potentials of the tyrosinase DNA vaccine in other domestic animals. To provide an answer to this question more information is necessary. First it would make sense to define the need for such a therapeutic DNA vaccine in the treatment of MM in other domestic animals, in this case horses and cats. MM is not very common in cats, with ocular melanomas being the most common form. However, feline MM does carry a poor prognosis. In horses melanocytic tumours are very common (in several forms) and are generally benign, but malignant melanoma also occurs. The need for such a vaccine seems to be higher in dogs and humans, but nevertheless horses and cats can suffer from MM and therefore it would be worth wile to examine the therapeutic possibilities of this vaccine in other animals. In dogs the therapeutic vaccine is safe to use and seems to be highly promising when it comes to clinical efficacy. Also other xenogeneic DNA vaccines have been investigated, such as the murine tyrosinase vaccine, with promising results.

In CMM treated with the xenogeneic human tyrosinase vaccine, the inserted DNA has to be taken up by muscle cells, expressed on the cell surface and then recognized, taken up and presented by APCs for an immune response in the form of antibodies and cytotoxic T-cells to occur. Then a cross-reaction with the host's tyrosinase (presented by tumour cells on the cell surface with MHC II molecules) occurs, hopefully leading to tumour regression. A break anywhere in this sequence can cause tumour progression.

To determine the therapeutic potential of the human tyrosinase vaccine in other animals more information needs to be collected concerning similarities and differences between canine melanomas and melanomas occurring in other domestic animals. Also data on tyrosinase expression in melanocytic tumours vs. normal cells, degree of similarity between tyrosinase types in these animals (so that a cross-reaction can occur) are required. Unfortunately these data are far from complete at this point.
In humans the tyrosinase antigen has proven to be a useful target for immunotherapy. Tyrosinase expression has also been evaluated in dogs and horses (on a small scale) and this research has shown that tyrosinase expression is high in melanocytic tumours while expression is very low in normal tissue. In dogs tyrosinase expression is high in all melanocytic tumours, regardless of malignancy, tumour location and pigmentation. In horses tyrosinase expression was also high in melanocytic tumours, regardless of coat-colour and tumour grade.
Therefore also in horses the tyrosinase antigen is a potential target for immunotherapy. To the authors knowledge, up to this point, no research was performed on the tyrosinase expression in feline melanocytic tumours. However, it is very well possible that if further investigated, similar results will be found in cats (and other domestic animals). Since tyrosinase expression in melanocytic tumours seems to be similarly high in humans, dogs and horses the tyrosinase vaccine could well be effective in species other than currently investigated.

The immune response which occurs in dogs treated with xenogeneic tyrosinase DNA vaccines is based on a cross-reaction with the hosts 'own' tyrosinase expressed on melanoma cells by MHC II molecules. This cross-reaction only occurs when treated with a tyrosinase antigen slightly different from the hosts tyrosinase, different enough to be recognized by the immune system as foreign, but also similar enough to the hosts tyrosinase for a cross-reaction to occur. Previous studies have shown that administering tyrosinase DNA from the same species does

not lead to an immune response and cross-reaction. Whether the xenogeneic human tyrosinase DNA vaccine is a suitable candidate for use as a therapeutic melanoma vaccine in horses and cats is uncertain. This xenogeneic melanoma vaccine, as well as the murine tyrosinase DNA could be further investigated in the future.

Unfortunately often vaccines that have proven to be effective *in vitro* are not effective *in vivo*. A break anywhere in the sequence of events necessary to generate an adequate immune response can cause disease progression, a frustration frequently encountered in (newly developed) cancer immunotherapies. However, based on the current knowledge there is no reason why in animals other than humans and dogs xenogeneic vaccines would not be effective, but more research is required in the future.

Also, special permission was granted for the use of the human tyrosinase DNA vaccine in dogs, in the Netherlands gene therapy is subject to strict regulation. Patients treated with xenogeneic DNA vaccines become GGO's, or genetic modified organism. Strict safety precautions have to be followed. Vaccinations can only take place in a specially designated (approved) space en a preset protocol must be followed. Approval for the use of a xenogeneic tyrosinase vaccine must first be granted, before gene therapy can be performed in cats, horses and other domestic animals.

Conclusion

The data collected in this study are not complete, but nevertheless the results so far are promising. Previous research has shown that treatment with the xenogeneic human tyrosinase vaccine may have positive effects on MST. Although median STs of this study are not available at this point, the mean STs are positive and seem to be higher than could be expected using the conventional treatment modalities. However, no control group was used in this study. Stage 1 and 2 dogs had better STs, but several stage 3 and 4 dogs also had good STs. In addition, it is to be regarded positive that 12 of 19 dogs have managed to survive until the end of this study, also 3 dogs died with causes of death unrelated to CMM. In the future more data will be collected, and more dogs will take part in the continuing study, so a better comparison can be made with previous research. The vaccine itself appears to be a safe therapy with minimal side effects.

Xenogeneic tyrosinase vaccines, such as the human tyrosinase could also be a potentially effective therapy in MM in other domestic animals, such as cats and horses. Research has shown that tyrosinase expression is much higher in melanoma cells compared to normal tissue, regardless of pigmentation in humans, dogs and horses. For a xenogeneic tyrosinase vaccine to be effective, a series of events has to occur, eventually leading to both a humoral and cellular immune response to the host tyrosinase expressed by melanomas on the cell surface, hopefully leading to tumour regression. The xenogeneic tyrosinase has to be slightly different so the host immune system will recognize it as foreign antigen, but must also be similar enough to cause a cross reaction. More research is needed on the tyrosinase expression of melanomas in horses and cats, the type of xenogeneic tyrosinase that would be most suitable to cause a cross-reaction in the host to further investigate the therapeutic potential of tyrosinase DNA vaccines in these species.

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Picture front page: http://www.vcaspecialtyvets.com/katonah-bedford/departments-doctors/doctors/philip-j-bergman/11186

Figure 2: picture of one of the CMM patients, made by Prof. Dr. J. Kirpesteijn