Research Project Veterinary Medicine University Utrecht

The effectiveness of a human tyrosinase DNA vaccine in dogs with CMM and the possible effects of this vaccine on unborn fetuses when administered during gravidity

S. Hoogendoorn, 3050688
July-October 2012

Project tutor:
Prof. Dr. J. Kirpensteijn

Faculty of Veterinary Medicine, Utrecht University,
Department of medical science of companion animals, Surgical Oncology

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Abstract

Canine Malignant Melanoma (CMM) is an aggressive tumor, which mostly affects the oral cavity, mucocutaneous junctions and digits in the dog. It metastasizes very quickly, mainly to the regional lymph nodes and lungs. Especially the metastases are poorly responsive to conventional therapy. The average median survival time of CMM patients after conventional treatment is 2 to 14 months, dependent of the severity of disease (12 to 14 months for stage I dogs, <5 months in stage II dogs, and 2 to 3 months in stage III and IV dogs).

This study investigates the effectiveness of the administration of a xenogeneic DNA vaccine, containing a human tyrosinase encoding sequence, in addition to the conventional therapies of surgery and radiation therapy. The hypothesis is that the use of this vaccine will increase both survival time (ST) and quality of life in CMM patients.

In this study 19 dogs were included since February 2011, of which 13 were male and 6 were female. Of these dogs, 5 were classified as stage I, 3 as stage II, 7 as stage III and 4 as stage IV. All dogs underwent surgical excision of the primary tumor (and affected lymph nodes if present), 6 x 6 Gy radiation therapy of the excision site and 4 x the administration of the Oncept ® vaccine.

During this study (from February 2011 to October 2012) 7 dogs died of which 4 from melanoma disease. The mean ST for all 19 dogs was 437 days, with the 0.5 cumulative survival not yet reached. Therefore, calculating a median ST was not possible yet. The ST for stage I+II patients was significantly longer than the ST for stage III+IV (P=0.042). Data such as Recurrence Free Interval (RFI), Metastasis Free Interval (MFI) and Disease Free Interval (DFI) were also estimated.

As the median is still not reached after 1.5 years, the Oncept ® vaccine seems to positively influence the ST of CMM patients. However, to make definite conclusions about the effectiveness of this vaccine, the median should be reached so that a median ST can be calculated. Further investigation on this subject is therefore recommended.

Introduction

Etiology

The malignant melanoma is an aggressive type of neoplasm that occurs frequently in dogs. Approximately 4% of all tumor types diagnosed in dogs concern the Canine Malignant Melanoma (CMM). Of the oral tumor types in the dog, the malignant melanoma is the most represented.[16,17,21] Differential diagnoses of oral CMM are squamous cell carcinoma, fibrosarcoma, epulides, odontogenic tumors and others.[19]

CMM is also well-represented in the types of tumors to affect the digits.[1-3,6,7,16,17,19-21] Dependent of the histological grade, size and localization, CMM metastasizes very quickly: in 32-40% of the patients diagnosed with digital CMM, these cells have also been found in other tissues. [19,21] In patients with oral CMM the percentage of metastases is even higher (approximately 80%).[7]

Pathophysiology

Two types of melanocytic neoplasms exist; these include the melanocytoma and the melanoma. The melanocytoma is a benign proliferation of melanocytes, and represent for 85% of all the melanocytic tumors in the dog. The melanoma is the malignant variant of melanocytic tumor.[1,6] The biologic behavior of the latter is extremely variable, depending on many different factors. CMM can be determined by its size, localization, stage and histologic parameters.[19]

The location of appearance differs between the benign and malignant tumor type of melanocytic origin. The melanocytoma can mostly be found in the skin of the head (especially eyelid and muzzle), trunk and paws. The malignant melanoma can also be found in the head region, but in the dog it affects the mucous membranes of the oral cavity more frequently than the skin. Other preferred locations of the malignant tumor are the skin of the limbs, digits (including the nail bed), scrotum, lip and trunk. The tumor size range from 0.5 to 10 cm in diameter. This tumor type has a predilection to metastasize to (regional) lymph nodes and other organs, most frequently the lungs.[1-3,6,21]

Most dogs diagnosed with melanoma are adults, at an average age of approximately 9 years. The predisposed breeds for both types of melanocytic tumor include the Scottish terrier, Airedale, Boston terrier, Cocker spaniel, springer spaniel, boxer, golden retriever and the Irish setter. Also the dog breeds with heavily pigmented skin, such as miniature schnauzers, standard schnauzers and Scottish terriers, seem predisposed for developing melanoma. There seems to be no gender predilection.[1,6,19]

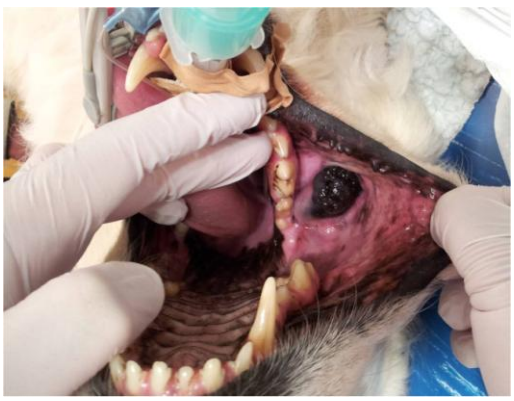


Figure 1: One of the patients in our clinic during this study. This dog had a buccal oral melanoma, stage IV. *Photograph: Prof. dr. J. Kirpensteijn*

Diagnosis

Melanocytomas clinically appear as solitary, well-circumscribed, dome-shaped, firm, alopecic, pedunculated or wart-like masses that range from 0.5 to 10 cm in diameter.
Whereas the malignant melanomas can also be solitary, firm, alopecic, pedunculated or wart-like, these masses are more often ill circumscribed: several shapes are possible (dome, plaque and polypoid) and also is ulceration. Also, melanocytomas are usually brown to black (melanotic), where the melanoma may appear as a melanotic or an amelanotic mass. The growth rate of the melanoma can be fast and secondary bacterial infections may occur.[2,6]

Diagnosis of both tumor types can be made by cytology or histology of a biopsy sample. Both tumors exists of round, oval, stellate or spindle-shaped cells, with a moderate amount of cytoplasm. The cells of a melanocytoma usually contain granules of brown to green-black pigment, whereas the cells of a malignant melanoma normally may have less pigment.
Also, more invasiveness, cellular pleomorphism and mitotic figures can be seen in malignant tumors than in benign ones. However, the differentiation between the benign melanocytoma and the malignant melanoma is not reliable. Besides, approximately 10% of histologically benign tumors behave in a malignant manner.[2,6]

To investigate the presence of metastases, biopsy samples of the regional lymph nodes are also needed. As the lungs are the predilected organs to be affected (especially in oral melanoma), thoracic radiography should be performed.

Therapy and prognosis

The conventional therapy of CMM consists of surgical excision of the primary tumor and, if present, the affected lymph nodes. Subsequently, radiation therapy (6-9 Gy weekly to every other week) of both surgical field and ipsilateral regional lymph node is indicated.[16,17,19,21]

When metastatic lesions are found in cancer patients, additional treatment is required. Unfortunately, the metastatic cells of CMM do not respond very well to chemotherapy. In humans the conventional chemotherapy used in HM patients (Human Melanoma) is decarbazine, whereas in dogs the common chemotherapeutic is carboplatin. In both human and canine patients chemotherapy had some effect in only 8-28% of the cases[16,17,19].

Due to the lack of response of the metastases to conventional therapy, the median survival time of CMM patients is short. The median ST varies from 2 to 14 months, depending on tumor stage: in stage I the median ST is approximately 12 to 14 months, in stage II less than 5 months and in stage III and IV 2 to 3 months.[16,17,19]

In patients with digital melanoma, which underwent digital amputation, the median ST was approximately 12 months, with 42-57% alive at 1 year, and 11-13% alive at 2 years. Of these dogs, the lymph nodes were not affected and there was no distant metastases present.[19]

The anatomical localization is an important prognostic factor for the survival time; it is predictive of local invasiveness and metastatic propensity.[19] The oral/mucosal melanoma behaves more aggressively; a higher degree of local invasiveness and distant metastases has been seen in patients with oral or mucosal CMM.

The DNA vaccine

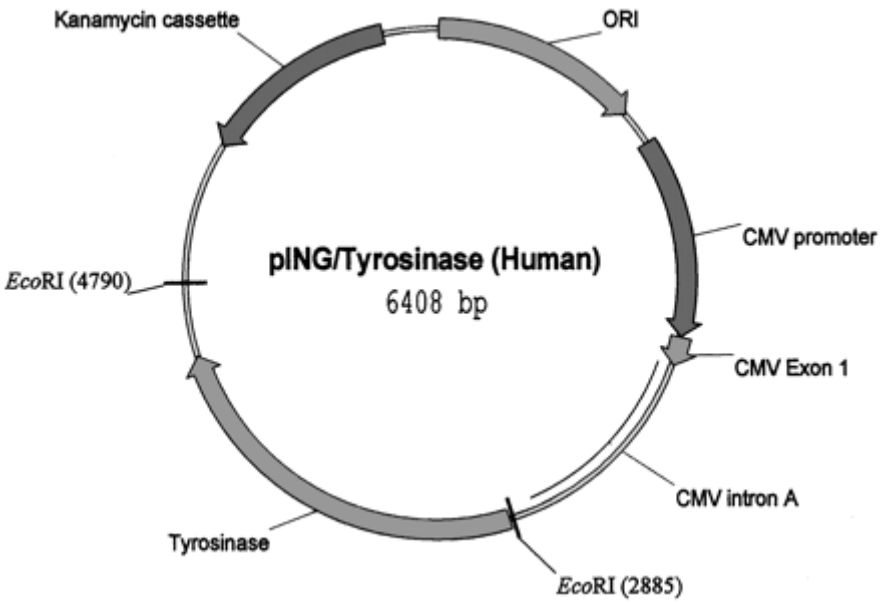
Recently, a novel immunotherapy modality was introduced using a xenogeneic DNA vaccine. This vaccine contains a sequence encoding human tyrosinase (Figure 2), a melanosomal glycoprotein which is essential in melanin synthesis. In the tyrosine-melanin pathway, it catalyses the first reaction. Without tyrosinase, this reaction cannot occur and no melanin can be produced.[16]

Several studies using mice showed that no immune response occurred after administration of syngeneic tyrosinase. However, the immune system seemed to be triggered in C57BL/6 mice when using xenogeneic (human) tyrosinase instead of syngeneic tyrosinase. In these mice both humoral and cellular response were present against this foreign protein.[16]

When dogs firstly were administrated with a xenogeneic DNA vaccine encoding tyrosinase, DNA of mice was used.[21] Now, a sequence encoding human tyrosine is more common in use, as it is more preferable because of great similarities with the canine analoge.[16] The gene encoding human tyrosinase exists of 5 exons, and is localized on chromosome 11 (11q14-q21).[17] This sequence, when transcribed and translated in the canine cells, results in the production of a tyrosinase protein that is 85% homologue to canine tyrosinase, while it is still foreign enough to initiate an immune response. Because of the great similarity of human tyrosinase and canine tyrosinase, the immune system will also attack cells containing canine tyrosinase (i.e. melanocytes). This immune response seems to be highly effective against especially the malignant cells, while the normal cells are much less affected. The different presentation of proteins in malignant melanocytes compared to normal melanocytes is probably the explanation for this phenomenon.[20]

Bergman et al (2006) describes a Kaplan–Meier median survival time (KM MST) for all stage II–IV dogs treated with human tyrosinase encoding DNA - of 389 days.[17]

Figure 2: A pING plasmid with a sequence encoding human tyrosinase.[16]



Aim of study

The aim of this study is to investigate the effects of this DNA vaccine on survival time and quality of life, when administered in addition to conventional therapy to dogs with CMM. The hypothesis is (based on the results of previous studies): "Administration of a xenogeneic DNA vaccine, containing a sequence encoding human tyrosinase, to dogs with CMM in addition to conventional therapy will increase both the survival time and quality of life, compared to the use of conventional therapy alone".
As CMM has many similarities with HM in humans, especially in its biologic behavior, this study is a good therapeutic model for immunogenic therapy in humans.

Material and Methods

In this study 19 dogs (13 male, 6 female) of different breeds were included from February 2011 to September 2012. Of these dogs, 11 had oral melanoma and 8 had non-oral (e.g. cutaneous or digital) melanoma (Table 1). Most dogs had been diagnosed with CMM by their own veterinarian, but all dogs were also thoroughly examined in our clinic before they could participate in the study. These examinations included:

- Physical examination
- Full hematological and biochemical profile (hematocrit, leucocytes including differentiation, sodium, potassium, alkaline phosphatase, bile acids, ureum/creatinin ratio, total protein, albumin)
- Histologic or cytologic confirmation of the primary tumour
- Cytology of the regional lymph nodes (by Fine Needle Aspiration Biopsy)
- Full-body CT scan (to screen for metastases)

Table 1: General information of the CMM patients

Patient	Date of birth	Gender	Breed	Weight (kg)	Date of death
Duke	27-10-2003	M	Golden Retriever	50,5	
Luna	4-10-2002	F	Leonberger	41,0	
Micha	11-9-2000	M	Border Collie	21,5	
Milo	21-7-2006	M	Golden Retriever	33,4	
Patrick	6-6-2004	M	Irish terrier	18,5	18-02-2012
Brumby	26-5-2005	M	Shiba Inu	15,1	
Dino	15-9-2002	M	Beagle	14,8	
Raya	7-1-1999	F	New-foundlander	56,0	23-03-2012
Bobby	23-6-1998	M	Beagle	15,5	10-08-2011
Endo	17-5-1999	M	Airedale terrier	27,5	02-01-2012
Joyce	18-9-1998	F	Border Collie	18,2	05-12-2011
Julius	12-9-2001	M	Labrador Retriever	32,8	
Leon	27-6-2003	M	Riesen-schnauzer	46,0	
Peggy	31-5-2003	F	Keeshond	21,3	13-06-2012
Snuf	13-2-1999	F	Beagle	17,8	
Borka	1-4-2001	M	Labrador Retriever	39,8	25-07-2012
Britt	21-6-2002	F	Golden Retriever	31,6	
Charley	1-1-2000	M	Crossbreed	11,3	
Woody	14-1-2003	M	Nova Scotia Duck Tolling Retriever	22,7	

After the examinations, all patients were staged according to the TNM scale of the World Health Organization. This scale describes the different stages of disease, depending on the primary tumor size, the affected lymph nodes and the metastasis in the patient (Table 2a and 2b). Of the patients during this study, 5 were classified as stage I, 3 as stage II, 7 as stage III and 4 as stage IV dogs (Table 3).

Table 2a + 2b: The TNM scale of the World Health Organization[21]

T: Primary tumor (size in diameter)	T_1 : < 2 cm, superficial T_2 : 2-5 cm, minimum invasion T_3 : >5 cm, or invading the subcutis T_4: tumor invading fascia or bone
N: Regional lymph nodes	N_0: no evidence of tumor found N_1: Moveable ipsilateral N_2: Moveable contralateral or bilateral N_3: Fixed nodes (with or without tumor cells found)
M: Distant metastasis	M_0: None found M_1: Distant metastasis found

Stages	**T**	**N**	**M**
I	T_1	N_0	M_0
II	T_2	N-*	M_0
III	$T_{3, 4}$	N+*	M_0
IV	Any T	Any N	M_1

*N- includes any N which is histologically negative. N+ includes any N which is histologically positive.

The patients got the following therapy:

- Surgical excision of the primary tumour (some of the patients underwent surgery at their own veterinarian)
- Surgical excision of affected lymph nodes
- Radiation therapy of both surgical field and ipsilateral regional lymph node (6 x 6 Gy)
- Administration of the melanoma vaccine (4x)

The radiation therapy existed of a dose of 6 Gy each, twice a week for three weeks. The surgical field was radiated with lateral margins of 3 cm, and a minimum of 85% of the dose should penetrate the tissue for a distance of at least 3 cm as well. Furthermore, the regional lymph nodes were radiated with lateral margins of 1 cm, to prevent metastases being formed. A minimum of 85% of the dose should reach the total lymph node.

The four vaccinations were administered every 2 weeks, intramuscularly (biceps femoris, semitendinosus or semimembranosus muscle) using a transdermal device. After vaccination, the patients were evaluated to a standardised protocol with several check-ups. At the first check-up (1 month after vaccination) the dogs were only investigated by physical examination. At the second check-up (3 months after vaccination) also thoracic radiographs were taken, and at the third check-up (6 months after vaccination) a full body CT-scan was indicated. However, some owners did not want a CT-scan to be performed on their dogs (e.g. Julius and Snuf), because of the risk of anaesthesia.
At this last check-up, a new vaccination cycle could be advised to the owners, dependent of the clinical outcome.
During this period of the study, 14 dogs only had 1 cycle of vaccinations. The reasons differ amongst these patients. The owners of two dogs (Duke and Milo) decided not to continue vaccinating their dogs because of financial reasons. Four dogs (Micha, Britt, Leon and Snuf) were only just included in the study, and two of them had already planned to have a next cycle. One dog (Charley) already had metastases in the lungs at the onset of the vaccinations, which were increased in size after vaccinations. Therefore, the owner decided not to continue this treatment for his dog. The rest of these dogs (7; Raya, Bobby, Endo, Joyce, Patrick, Peggy and Borka) had died before they could have a second cycle of vaccinations.

Four dogs (Luna, Brumby, Julius and Woody) had two cycles of vaccinations, and one dog (Dino) had three cycles of vaccinations.

Table 3: Staging of the patients

Patient	Type of melanoma	Location	Tumor size (cm)	T	N (location)	M (location)	Stage
Duke	Digit	Digit V, right front	1,5 x 1,5	T_1	N_0	M_0	I
Luna	Oral	Left maxilla	1,5	T_1	N_1-	M_0	I
Micha	Oral	Left maxilla	1,5 x 1,5	T_1	N_3-	M_0	I
Milo	Digit	Digit V, left rear	1	T_1	N_0	M_0	I
Patrick	Cutaneous	Scrotum	1	T_1	N_0	M_0	I
Brumby	Oral	Mucosa upper lip	4x3x2	T_2	N_1-	M_0	II
Dino	Oral (amelanotic)	Right mandibula	3	T_2	N_1-	M_0	II
Raya	Oral	Hard palate	5	T_2	N_0	M_{0***}	II
Bobby	Oral (amelanotic)	Mucosa left cheek	0,2x0,2 x0,3	T_1	N_1-		III
Endo	Cutaneous	Upper lip	3x3	T_2	N_3+ (mandibular)	M_0	III
Joyce	Oral	Mucosa left upper lip	1,5x1,3 x0,8	T_1	N+ (mandibular)	M_{0***}	III
Julius	Digit	Digit II, right rear	0,75	T_1	N_3+	M_0*	III
Leon	Digit Cutaneous	Digit left front Scrotum, eyelid**	?	T?	N_3+ (mandibular)	M_0	III
Peggy	Oral	Left mandibula	3x2	T_2	N_3+ (mandibular, both sides)	M_0	III
Snuf	Cutaneous	Lip	1,5x1,5x1	T_1	N_3+ (mandibular)	M_0	III
Borka	Oral (amelanotic)	Left maxilla	2	T_1	N_1-	M_1 (cheek, P4)	IV
Britt	Oral	Mucosa right cheek	4x4,5x5	T_2	N_3+ (mandibular)	M_1 (thoracal vertebrae)	IV
Charley	Oral	Right mandibula	3	T_2	N_0	M_1 (lungs)	IV
Woody	Cutaneous	Nasal planum	0,2x0,2x0,2	T_1	N_0	M_1 (lip)	IV

*The owner of Julius did not want to perform a CT-scan because of the risks of anesthesia. Therefore, Julius underwent only a thoracic radiograph.
**Leon appeared to have a CMM lymph node metastasis with an unknown primary tumor. However, in 2010 he had a few tumors of melanocytic origin, which were removed. These tumors had no histopathologic signs of malignancy.
*** These patients developed metastases after onset of the study.

Results

To determine the tumor progression analysis, different dates were reported. These include: the date of first clinical symptoms, the date of diagnosis, the date of first treatment, the date of recurrence or metastasis of the tumor, and the date of last contact. All this information led to different variables: RFI (Recurrence Free Interval), MFI (Metastasis Free Interval), DFI (Disease Free Interval) and ST (Survival Time).

The RFI represents the time in which the dogs had no recurrence of the tumor after surgical excision since the date of diagnosis. In the same way, MFI represents the time in which the dogs had no metastases of the primary tumor since the date of diagnosis. The DFI stands for the period the dogs had no clinical signs, recurrence or metastases of their melanoma. Survival Time represents the period from vaccination until death. Most dogs were still alive at the end of this study, so their ST represents the period from vaccination until the date the analysis is performed.

These data have been censored (Censor Survival) for their death, either because of their melanoma, or because of another cause. Patients who died during the study because of melanoma were censored as '1', whereas patients who died due to another cause were censored as '0'. In this study, Kaplan-Meier analyses were made of the mean RFI, MFI, DFI and ST, using the SPSS software.

During this study a total of 7 dogs died, of which 4 because of local tumor progression and/or distant metastasis (all stage III or IV). Their survival times (ST) were 112, 135, 217 and 516 days. The other 3 dogs died or were euthanized because of unrelated disease*. The latter have been censored in the results.

The estimated mean ST for all 19 dogs was 437 days (Figure 3), with an standard error of 59 days and a 95% confidence interval of 321.2 to 552.8 days. The estimated mean RFI, MFI and DFI were 468, 268 and 326 days respectively (Figure 4 to 6).

*Endo died because of heat stress in Africa, Patrick was euthanized because of increased exercise intolerance and Raya was euthanized because of a chronic gastritis.

Figure 3: Kaplan-Meier ST plot for dogs with CMM treated with the melanoma vaccine. The estimated mean ST was 437 days.

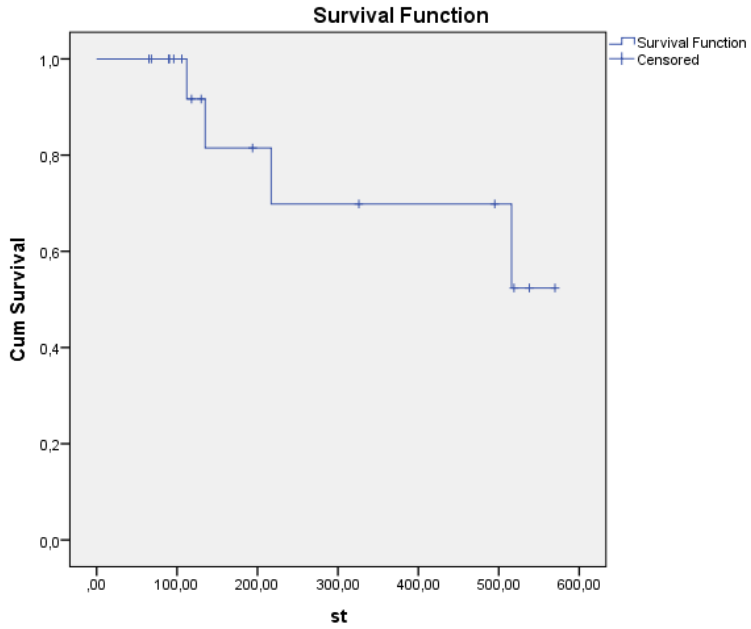


Figure 4: Kaplan-Meier RFI plot for dogs with CMM treated with the melanoma vaccine. The estimated mean RFI was 468 days.

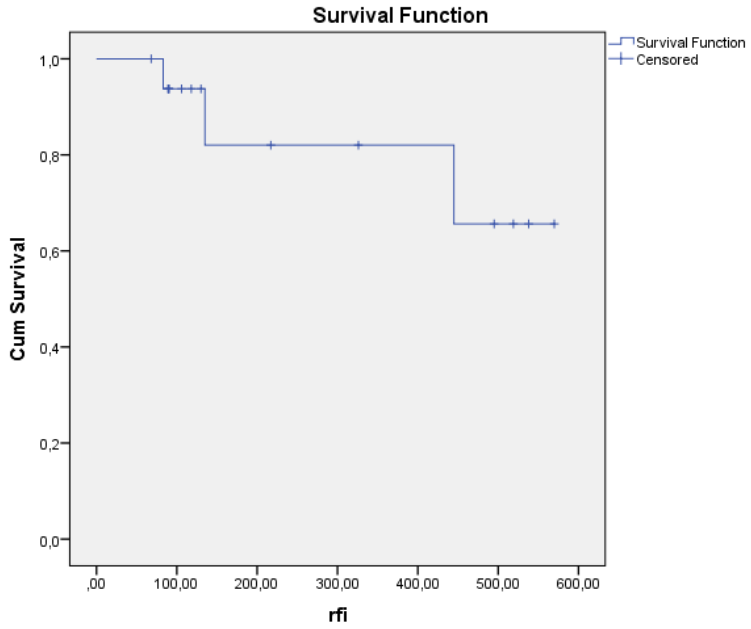


Figure 5: Kaplan-Meier MFI plot for dogs with CMM treated with the melanoma vaccine. The estimated mean MFI was 268 days.

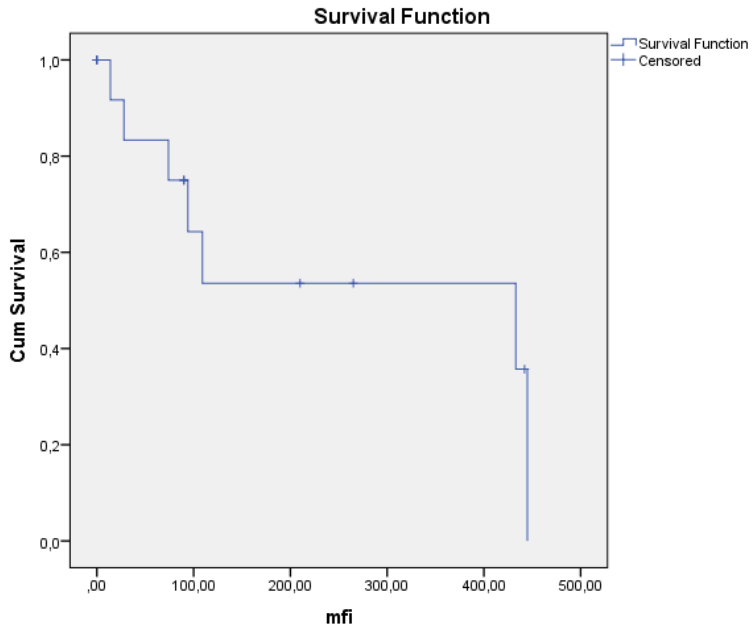
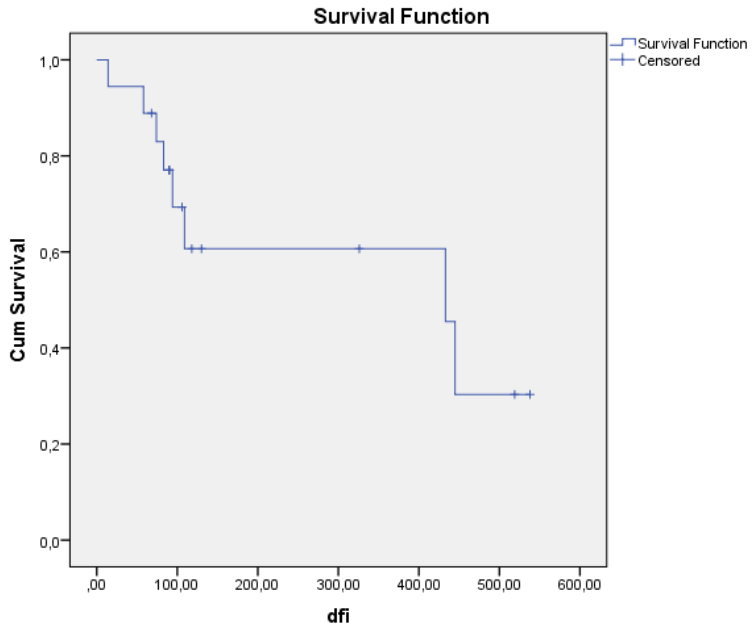


Figure 6: Kaplan-Meier DFI plot for dogs with CMM treated with the melanoma vaccine. The estimated mean DFI was 326 days.



For the above parameters also the differences between I+II stage patients (TMN Recode 0) and III+IV stage patients (TMN Recode 1) were calculated (Figure 7 to 10). For the stage I+II patients the estimated mean MFI was 350 days, whereas for stage III+IV patients this was 233 days. The estimated mean DFI for stage I+II patients was 403 days, and for stage III+IV dogs this was 262 days. The mean ST for stage I+II patients could not be estimated because all dogs in this group were still alive at the point of calculation. Also the RFI for stage I+II patients could not be estimated, because no patients in this group developed recurrence of the primary tumor during this study.

Figure 7: Kaplan-Meier ST plot for dogs with CMM treated with the melanoma vaccine. The dogs were now separated into two groups: 0,00 (stage I+II) and 1,00 (stage III+IV).

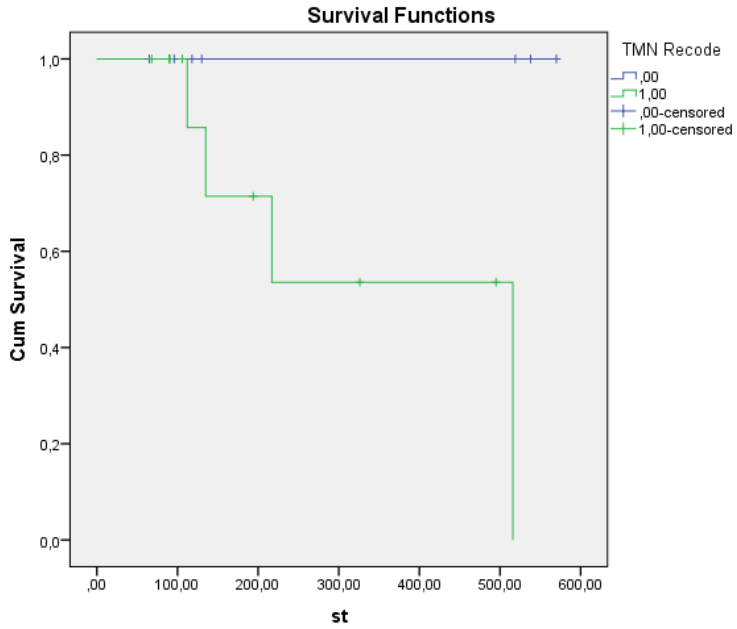


Figure 8: Kaplan-Meier RFI plot for dogs with CMM treated with the melanoma vaccine. The dogs were now separated into two groups: 0,00 (stage I+II) and 1,00 (stage III+IV).

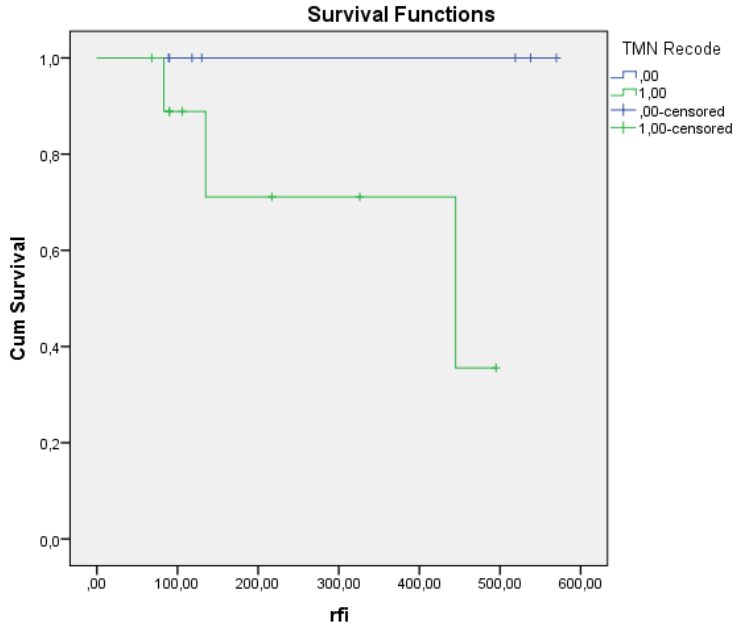


Figure 9: Kaplan-Meier MFI plot for dogs with CMM treated with the melanoma vaccine. The dogs were now separated into two groups: 0,00 (stage I+II) and 1,00 (stage III+IV). The estimated mean MFI for group 0,00 was 350 days, the estimated MFI for group 1,00 was 233 days.

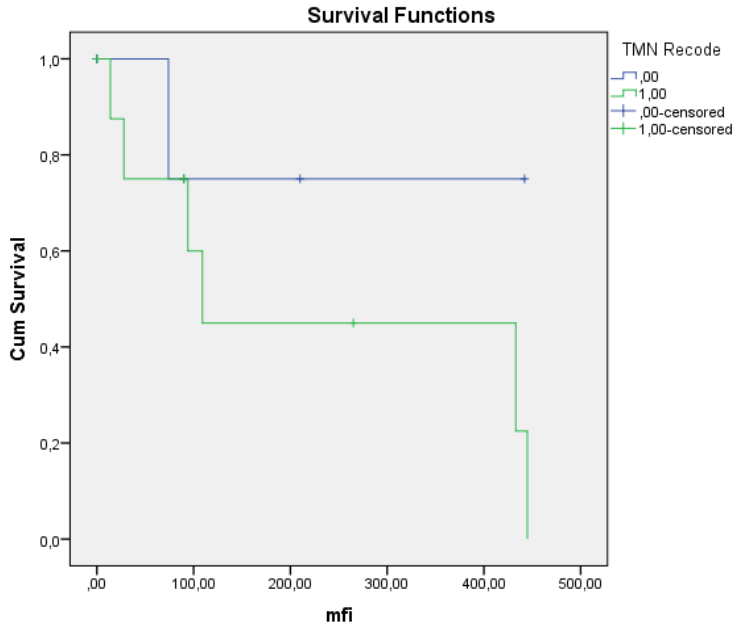
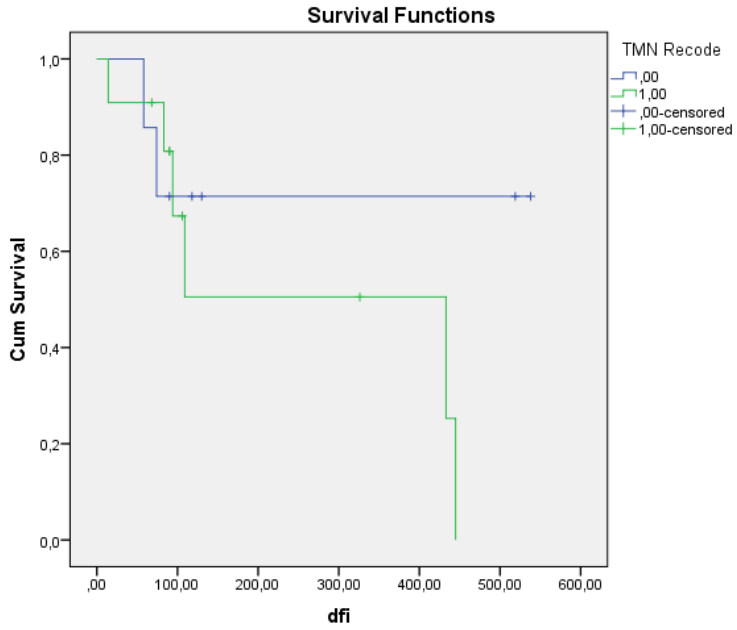


Figure 10: Kaplan-Meier DFI plot for dogs with CMM treated with the melanoma vaccine. The dogs were now separated into two groups: 0,00 (stage I+II) and 1,00 (stage III+IV). The estimated mean DFI for group 0,00 was 403 days, the estimated DFI for group 1,00 was 262 days.



Discussion and conclusions

The aim of the study was to investigate the effects of the Oncept ® DNA vaccine on survival time and quality of life, when administered in addition to conventional therapy to dogs with CMM. The hypothesis was that administration of this DNA vaccine to dogs with CMM, in addition to conventional therapy, would increase both their survival time and quality of life compared to conventional therapy alone.

The mean ST of all 19 dogs was 437 days from the start of the vaccine treatment. No median ST could be calculated at this point of study, because the 0.5 cumulative survival was not yet reached (12 of 19 dogs were still alive). Previous studies described median STs of 2 to 14 months during conventional therapy, dependent of the stage of disease. Although a comparison to these data is difficult to make, it is certain that the median ST of this group of dogs (when reached) will be longer than 437 days. The survival time of dogs with CMM after conventional therapy and Oncept® treatment is therefore longer than the survival time of dogs with CMM after conventional therapy alone.

The stage I and II dogs had significantly higher STs than the stage III and IV dogs (P=0.042). In this study all stage I and stage II dogs (8) were still alive when the data were calculated in September 2012 (the dogs died of another cause than melanoma were censored). Four of the 11 stage III and IV dogs died of melanoma disease.

As for the quality of life, the data RFI, MFI and DFI could represent that. It is assumable that a greater interval free of primary tumor recurrence, metastases or clinical symptoms reflects a better quality of life. The mean RFI of all dogs was 468 days, the mean MFI was 268 days and the mean DFI was 326 days. Stage I+II dogs had longer intervals than stage III+IV dogs, however, these differences were not significant (P=0.096 for RFI, P=0.365 for MFI, P=0.240 for DFI). As these data were not used in previous studies, it is uncertain whether these intervals are longer than with conventional therapy alone. However, especially the long mean RFI (which is longer than the median ST of 2-14 months in conventional therapy) is an indication for the health and well-being of the patients.

A point of discussion is the relatively short mean MFI of 268 days compared to the RFI and DFI. The Oncept® vaccine was developed because of a lack in response of metastases to conventional therapy such as chemotherapy. Nevertheless, a total of 7 dogs developed (new) metastases during the DNA vaccine treatment. Of these dogs, 1 was stage II, 3 were stage III and 3 were stage IV. The location, number and size of metastases differed among them. As no stage I dogs and only one stage II dog developed metastases after vaccination, it seems that the Oncept ® DNA vaccine can help prevent metastases when they are absent in the onset of the treatment. However, in 3 of 7 stage III dogs and 3 of 4 stage IV dogs new metastases developed during vaccination treatment.

The staging scheme of the WHO has been used in several CMM studies, however, according to Bergman et al (2008), this staging scheme has some limitations. Firstly, the size of the tumor, is not standardized to the size of the patient. A tumor in a Chihuahua, 1.5 cm in size is staged as a stage I, but so is a tumor of this size in a Rottweiler. Secondly, the histologic appearance is not included as a prognostic factor in this scheme. According to several investigators, the following prognostic factors should be included: lesser degree of extirpation

and incomplete surgical margins, location (caudal mandibular or rostral maxillar tumors have a more negative prognosis), tumor mitotic index >3, and bone invasion or lysis.[19]
According to these opinions further investigations using a different staging scheme is recommended.

As the median is still not reached after 1.5 years, the Oncept® vaccine seems to positively influence the survival times of CMM patients. However, these 19 dogs were not simultaneously included in this study. Some of the patients were only just included, a couple months or even weeks before calculating the data. Others were included from the onset and are still alive and free of recurrence, metastases and disease. To make definite conclusions about the effectiveness of the vaccine, the median should be reached so that a median ST can be calculated.

Literature study: The possible effects of the human tyrosinase vaccine on canine fetuses when administered during gravidity

Introduction

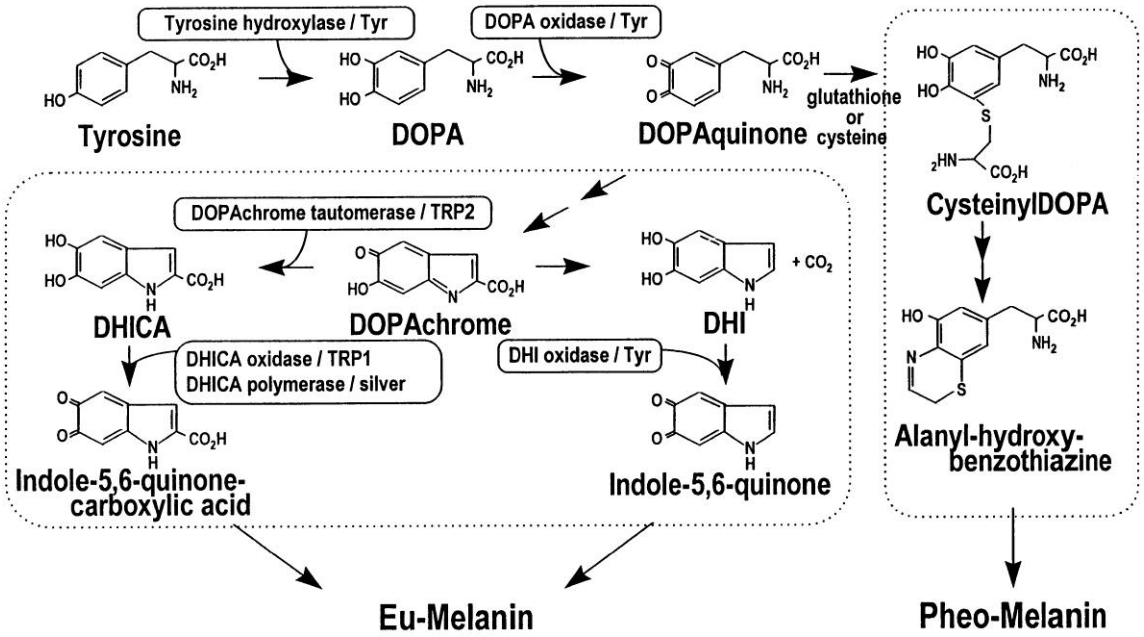
Many vaccinations are administered to animals these days, with various purposes. Some of these purposes are: to prevent disease in an individual, to decrease the risk of infection in herds and to reduce the severity of symptoms. Also, it provides the fetuses with maternal immunity when it is administered to the mother during pregnancy. One can wonder whether the Oncept ® vaccine containing human DNA encoding tyrosinase can have effects on fetuses when administered to pregnant dogs. Can this procedure, for instance, result in albinism or vitiligo in the offspring? Unfortunately, this question cannot be answered easily, as it is unknown. No studies have been done to examine this subject, but such investigation would undoubtedly unleash an ethical discussion. One can question the clinical importance of the hypothetical answers of the above question, because most dogs with CMM are not reproductive anymore (as the mean age to develop CMM is around 9 years). However, the use of this DNA vaccine in dogs is considered to be representative of the use of xenogeneic DNA vaccines in humans. Though pregnancy and placentation is different in humans compared to dogs, it is interesting to consider the possible effects of this vaccination on fetuses.

Melanogenesis

Melanin exists in two different forms: eumelanin and pheomelanin. Eumelanin contains mainly tyrosine causing black or brown coloration of the skin and hairs, whereas pheomelanin contains various amounts of cysteine in addition to tyrosine causing reddish brown or yellowish coloration. Both forms of melanin originate from a common metabolic pathway, in which dopaquinone is the key intermediate (Figure 11). The direction in which the melanin synthesis ends is possibly dependent of the amount of tyrosinase. High amounts of tyrosinase should cause eumelanin synthesis whereas low amounts will lead to the production of pheomelanin. Also, as pheomelanin contains a high amount of sulfur, the pathway will end in the production of this type of melanin when sulfhydryl groups are present.
The production of pigment (melanogenesis) is mainly determined by the expression of several genes and hormones, such as the Melanocyte Stimulating Hormone (MSH). However, the influence of this hormone on the physiology and pathology of melanogenesis is still unknown.

The melanin synthesis occurs in melanocytes, which are found at different locations including the basal layer of the epidermis, the outer root sheath and hair matrix of hair follicles, the ducts of sebaceous sweat glands and the superficial dermis. Two types of melanocytes can be found; the epidermal type (which provide the skin of melanin) and the follicular type (which provide the hairs of melanin). Because melanocytes do not stain in H&E, they appear as clear cells under the microscope. Both melanocyte types have long dendrites that weave among keratinocytes in the basal cell layer.[2]

Figure 11: The synthesis of eumelanin or pheomelanin from tyrosine requires the enzyme tyrosine hydroxylase, also called tyrosinase.[25]



Melanocytes express several cell surface receptors, which enable them to communicate with other cell types including keratinocytes, Langerhans' cells, fibroblasts, lymphocytes and macrophages. Because of these receptors, melanocytes can respond to growth factors (e.g. β fibroblast growth factor), hormones, interferons, interleukins, eicosanoids, retinoic acid, vitamin D_3 and a host of other cytokines. Apart from the expression of receptors, melanocytes are able to produce some of these themselves, for instance several cytokines. Therefore, melanocytes can participate in inflammatory and immunologic reactions. However, many of the precursors and intermediates in the melanin biosynthetic pathway are cytotoxic.

Melanogenesis can only occur in an organelle in melanocytes called the melanosome. Dermal melanocytes eject melanosomes into keratinocytes via migration along the dendrites, a transfer process that is biologically unique and is called cytocrinia.

The coloration of the skin is mainly dependent of the amount, the size, the type and the distribution of the melanosomes to the epidermis. Most of the pigment found in the skin is present in the basal layer of the epidermis, however, in dark skinned animals the whole epidermis or even the superficial dermis can be involved.

Melanosomes originate from the Golgi apparatus, where the enzyme tyrosinase is formed. This enzyme catalizes the conversion of tyrosine to dopa, which is a rate-limiting step in melanin production. Tyrosinase is a copper-containing enzyme and is exclusively found in melanocytes. It is an unusual enzyme for it has three distinct catalytic activities. The tyrosine hydroxylase activity – the one that converts tyrosine to dopa – is the most critical in the melanogenesis. Tyrosinase also has the capability to use dopa or 5,6-dihydroxyindole (DHI) as substrates for oxidase activities. Other enzymes that are involved in melanogenesis are the dopachrome tautomerase (DCT) and DHICA oxidase (TRP1) (Figure 11)[2]. The canine tyrosinase exists of 530 amino acids and is a membrane bound glycoprotein.[28]

Melanogenesis in the fetus

Skin and hair melanocytes derive from neural crest cells. During embryogenesis neural crest cells migrate into the dermis and differentiate into melanoblasts.[8] After this migration, which occurs in mice at 12 days of gestation, they penetrate the basement membrane and enter the epidermal ectoderm. These cells, which will become melanocytes, produce the melanin-containing melanosomes. Once the melanoblasts arrive in the skin, they become integrated into the developing hair follicles. It is not until around birth, that the melanocytes secrete melanin granules into the hair as it grows.[9]

Whereas melanocytes of the skin and hair follicles derive from the neural crest, the pigmented epithelial cells of the retina derive from the optic cup. Tyrosinase is produced in both cell types after expression of the tyrosinase gene TYR. In mice TYR is expressed in the pigment epithelium of the retina as early as day 10.5 of gestation and in the hair follicle it is expressed from day 16.5 onwards. Also, from day 16.5, melanocytes expressing tyrosinase mRNA were found in both dermis and epidermis.[9]

The exact moment of the TYR expression in the hair follicle of the canine fetus is still uncertain. The length of gestation in mice is 19-21 days, whereas it is 58-68 days in dogs. Reasoned from the analogy between different species, the TYR gene expression would then occur around day 50 in the gestation of the dog.[25,27]

The expression of TYR is cell type specific, temporal and regulated by several transcription factors.[9] One of these is the Microphthalmia-associated Transcription Factor (*Mitf*), which coordinates the differentiation of melanocyte stem cells into melanocytes by activation of associated genes. The gene, which expresses *Mitf*, is activated by the proteins *Sox10* and *Pax3*. Especially *Sox10* is important for the expression of the enzyme-coding genes in the melanin pathway, while *Pax3* competes with *Mitf* for the enhance sites of the genes for melanin production. While the melanocytes are in their stem cell niche, their differentiation is inhibited. Therefore, the genes for melanin production remain unexpressed until the melanocytes leave their niche. Once the stem cells are outside of the niche *Pax3* is removed from the cells, allowing *Mitf* to bind. When *Sox10* is also bound, the enzyme-coding genes can be transcribed and melanin production can occur.[8]

Albinism

As pigmentation of skin, hair and eye depends on melanin being present in these tissues, deficiency of melanin-production results in depigmentation.[8] Because albino patients are not able to produce melanin properly, their hair, skin and mucous membranes remain amelanotic.[2]

Albinism disease appears to be rare in domestic animals, and must be differentiated from other forms of white spotting or dilution (such as piebaldism, extreme white or vitiligo). Pigmentary abnormalities may be acquired or hereditary. Albinism is a hereditary condition, transmitted as an autosomal recessive trait. In true albinism patients have pale irises (in humans pink or red, in dogs blue), visual defects and increased risk of solar radiation-induced neoplasms of the skin.[30] In patients with albinism the amount and structure of melanocytes appear to be normal, but these cells lack tyrosinase causing the failure of melanogenesis.[2,12]

The two main mechanisms in which pigmentation of the skin is deficient, are either the absence or relatively inactivity of melanocytes. Decrease or elimination of pigment by either of these basic mechanisms can be regional, or can involve the entire animal. When animals regionally or systemically lack melanocytes, white spotting or dilution mechanisms can occur. This lack of pigment can originate from the embryonic stage, where failures can occur on several levels. For example, the melanoblasts may fail in differentiation to melanocytes, in migration to the skin or may die after migration has occurred.[24]

Genetic mutations causing albinism

In human, mutations in seven different genes have been found to be responsible for different types of albinism. These include the tyrosinase gene (TYR), the P gene, the tyrosinase-related protein-1 gene (TYRP1), the HPS1 gene, the beta-3A-adaptin gene (ADTB3A), the CHS1 gene, and the OA1 gene. There may be more genes involved, for there are also albino patients without any mutation in each of the genes described above.[12]
In this study only the consequences of TYR mutations in will be discussed, because the DNA vaccine only targets cells, which present tyrosinase on their surfaces. Mutations in this gene, which encodes the tyrosinase protein, are associated with Oculocutaneous Albinism type 1 (OCA1) in humans.

Durham was the first to describe tyrosinase activity in mammalian skin in 1904, and later he also reported a lack of tyrosinase activity in the skins of albino animals. However, Sir Archibald Garrod was the first to make a hypothesized association between albinism in man and a lack of enzyme activity in his 'Inborn Errors of Metabolism' (1958). Then, it was still unclear whether the lack of tyrosinase activity was due to a mutation in the gene encoding tyrosinase or to the absence of melanocytes in the skin. Many decades later, melanocytes were identified in the skin of both mouse and human albino patients, but appeared to have no tyrosinase activity.

The mouse and human TYR genes were isolated in 1987 and the first mutation associated with human OCA was reported in 1989. At present, over 90 mutations of the TYR gene, associated with albinism, have been described. These mutations include missense, nonsense, frameshift, and splice site mutations, and a deletion of the entire coding sequence.[12]
The canine TYR locus maps to chromosome 21. Polymerase chain reaction amplified an 819 bp fragment. Chromosome painting studies indicated also that canine chromosome 21 was homologous with human chromosome 11q21, where TYR already had been mapped.[28]

As discussed before, expression of the tyrosinase gene is controlled by several transcription factors, such as *Mitf*. It is unknown whether the DNA vaccine Oncept ®, which is used in this study in dogs with melanoma, has influence on the expression of the TYR gene. If that would be the case, the vaccination should have effect on one or more transcription factors, or should affect the transcription or translation into the tyrosinase protein.

Maternal cellular immune response

Could the Oncept ® vaccination cause lack of pigmentation in the fetus and neonate because of the immune response of the mother against tyrosinase-presenting cells? To give an answer to this question, two types of immune response are described below; the cellular immune response and the humoral immune response.

Once introduced in the muscle of canine patients the DNA sequence is transcribed and translated into the human tyrosinase protein. Cells of the immune system (the antigen-presenting cells) are able to present the tyrosinase antigen on their surfaces.[17]
In the adaptive immune response, antigen recognition occurs by either the immunoglobulins - that serve as antigen receptors of B cells - or the antigen-specific receptors of T cells. The Major Histocompatibility Complex (MHC) molecules are evident in this process. These molecules, which are specialized host-cell glycoproteins, present (foreign) peptides on the cell surface. Once a protein is incorporated in a cell, the protein antigens are degraded into peptides. These peptides can be generated either in intracellular vesicles or in the cytosol, and are then carried to the cell surface where they bind to MHC molecules.[5]

Two classes of MHC molecules exist – MHC class I and MHC class II – which bind respectively peptides from the cytosol and peptides from the vesicles. The two classes of MHC proteins differ in both structure and expression pattern. Therefore, different functional sets of T cells interact with them. Peptides bound to MHC class I molecules mostly derive from pathogens (commonly viruses) and are recognized by CD-8 cytotoxic T cells which are specialized to kill these cells. As viruses can infect all cells containing a nucleus, almost every cell type in an individual is able to express MHC class I molecules.[5]

Only certain cells can present peptides bound to MHC II molecules on their surfaces. These cells are recognized by CD-4 T cells. The cells which express MHC II molecules are only those that participate in immune responses, such as B lymphocytes, dendritic cells and macrophages. Once the antigen on the cell surface is recognized, the CD-4 T cells activate other effector cells of the immune system. This results in the production of antibodies (when the antigen is presented on B cells) or the destruction of pathogens in the vesicles of the cell (when the antigen is presented on macrophages).[5]

The presentation of the tyrosinase antigen in malignant melanoma cells differ from that in normal melanocytes. During malignant transformation the expression of MHC class II molecules is upregulated.[20] Whereas the normal melanocytes only express MHC class I molecules on their surface, malignant melanoma cells express both MHC class I and MHC class II molecules.[11,20] The expression of MHC molecules in a cell is regulated by cytokines such as interferons. Particularly interferon-γ plays an important role in this process, as it is able to induce the expression of MHC II class molecules in cells that do not normally express them. The immune system is activated by this phenomenon and reacts with both cellular and humoral immune responses against these cells.[5]

After vaccination, the human tyrosinase antigen is bound to MHC class II molecules on the surface of antigen-presenting cells, mainly dendritic cells. The plasmid that is introduced in the dog contains immunostimulary DNA sequences, which enhance the immune response even more.[16] Grosenbaugh et al (2011) found that the immune system selectively targets the cells presenting the human tyrosinase and the malignant melanoma cells above normal

melonocytes. Furthermore, this study showed antigen specific interferon-γ T cell response in dogs as a result of vaccination with a DNA sequence encoding human tyrosinase.[20]
According to Liao et al (2007) the differences in protein sequences between human and canine tyrosinase may provide higher affinity for MHC class II proteins, which then activate CD-4 T cells with intermediate- or low-affinity receptors for the normally poorly immunogenic allogeneic tyrosinase.[18]

Concluding, the immune system selectively targets the cells presenting the human tyrosinase and the malignant melanoma cells presenting the canine tyrosinase. The cellular immune response against these cells probably do not affect benign melanocytes or other normal tissue cells as they do not express MHC II molecules. However, Đorđić et al (2012) describes immune reactions in non-vaccinated humans with melanoma, which can cause vitiligo as a side effect of melanoma regression.[23] This kind of hypopigmentation, which is associated with melanoma and probably is caused by the humoral immune response, is discussed later.

Fetal tolerance to maternal T cells

As a fetus carries other (paternal) MHC and minor histocompatibility proteins than the mother, the fetus could by recognized as 'a foreign body' by the maternal immune system. However, the fetus is tolerated by two main mechanisms: the nonimmunogenic tissue barrier (the placenta), which protects the fetus, and the local immunosuppressive response, which is induced by the presence of the fetus.

The placenta, which is a fetus-derived tissue, seems to sequester the fetus from the maternal T-cells. One way in which this is established is by the trophoblast, which is the outer layer of the placenta. The trophoblast does not express classical MHC class I and MHC class II antigens, and therefore the maternal T cells cannot recognize and attack this fetal tissue. On the other hand, tissues that lack MHC class I expression are likely to be attacked by NK cells.

The fetus is also sequestered from the maternal T cells by an active mechanism of nutrient depletion. The catabolic enzyme indoleamine 2,3-dioxygenase (IDO), which is expressed at a high level by cells at the maternal-fetal interface, depletes tryptophan at this site. Tryptophan is an essential amino acid and T cells starved of tryptophan show reduced responsiveness. Some experiments have been done in pregnant mice to investigate the effects of inhibition of this enzyme. The usage of the inhibitor 1-methyltryptophan caused rapid rejection of allogeneic but not syngeneic fetuses. Maternal T cells, thus, seem to autoreact to paternal MHC molecules of the fetus, but might be inhibited to do so by tryptophan depletion.

Fetal tolerance is probably a multifactorial process, in which also secretion of cytokines plays a role. The uterine epithelium, as well as the trophoblast, secrete several cytokines including TGF-β, IL-4 and IL-10. This combination of cytokines appear to suppress T_H1 responses, whereas other cytokines such as IFN-γ and IL-12 stimulate these responses[5,15]. In experimental animals, in which the latter combination of cytokines was induced or injected, fetal resorption occurred.[5]

The maternal humoral immune response

In dogs, vaccinated with human tyrosinase DNA (huTyr), Bergman et al (2006) and Liao et al (2007) found in 3 of 9 dogs increased concentrations of specific antibodies against huTyr. In two of these three dogs, the antibodies appeared to be also reactive to syngeneic canine tyrosinase.[17,18]

In a humoral response to foreign proteins, the first type of antibody to be produced by B cells is the immunoglobulin isotype IgM. The other isotypes (IgG, IgA and IgE) are smaller than the pentameric structured IgM, and diffuse more easily out of the blood into tissues. Whereas IgA can form dimmers, IgG and IgE are always monomeric. The locations and functions of the isotypes differ. Whereas IgA is the principal isotype in secretions, mainly in the epithelium of the intestinal and respiratory tract and acts locally, IgG is present in the bloodstream and extracellulair fluid and is active in the entire body. IgM is also present in the blood and (to a smaller amount) in the lymph, but is especially found in the peritoneal cavity and pleural spaces. Also, IgE can be found in the blood and extracellular fluid, but at very low levels, for it is mostly bound to mast cells. These mast cells are located just beneath the skin and mucosa and along blood vessels in connective tissue.[5]

The immunoglobulin isotypes have several functions, including neutralization or opsonization of pathogens, sensitization for killing by NK cells, sensitization of mast cells of the activation of the complement system. The IgM isotype especially activates the complement system, along with the IgG3 isotype. The sensitization of mast cells, naturally, is a specialty of the IgE isotype. Neutralization and opsonization can be established when IgM, IgA and several types of IgG are present, while sensitization of killing by NK cells only occurs when IgG1 or IgG3 is present.[5]

Several investigators have found different antibody responses in different kind of patients. Antibodies against tyrosinase can be found, but also other types of antibodies, for example against melan-A. The isotype which is measured in several studies is the IgG isotype.[10,14] Merimsky et al (1998) compared the different IgG levels against tyrosinase in the sera of human melanoma patients having either metastatic disease or no evidence of disease, in patients with melanoma and melanoma-associated hypopigmentation (MAH), in patients with vitiligo, and in healthy individuals. In this study, the levels of anti-tyrosinase IgG appeared to be significantly higher ($p = 0.03$) in sera of patients with metastatic disease, compared to that in healthy controls. Also a higher level of anti-tyrosinase IgG was found in sera of patients with metastatic disease compared to that in patients with no evidence of disease. This difference, however, was not statistically significant.[10]

In human melanoma patients several isotypes of immunoglobulines against both melanin and tyrosinase have been found by Đorđić et al (2012). In this study the isotypes IgM, IgA and IgG were investigated in patients with melanoma, in patients with vitiligo and in healthy controls when exposed to mushroom tyrosinase and synthetic melanin. The results showed significantly lower levels of anti-tyrosinase IgM in both melanoma and vitiligo patients ($p < 0.0000004$ and $p < 0.04$ respectively) compared to those in healthy people. Beside, this study described the levels of IgM in melanoma patients to be significantly lower compared to that found in vitiligo patients ($p < 0.05$). When looked at the other immunoglobulins (i.e. IgA and IgG), no significant difference between levels of IgA and IgG anti-tyrosinase autoantibodies were found in patients with melanoma or vitiligo compared to controls. [23]

These results seem contradictory to what Merimsky et al (1998) found. However, Merimsky describes differences in IgG levels, whereas Dordic found significant differences in IgM levels. Merimsky gives a possible explanation for the lower levels of IgM in melanoma patients: absorption of antibodies by melanoma cells results in a reduced free fraction of antibodies in the serum.[10]

Vitiligo and Melanoma-associated Hypopigmentation

Vitiligo, a type of hypopigmentation, is a hereditary disease found in many species.[30] The patches of discoloration in patients in vitiligo is thought to be caused by high levels of circulating antibodies against melanocytes. This type of hypopigmentation can also be observed in patients with melanoma, and this type is therefore called Melanoma-associated Hypopigmentation (MAH). In MAH the clinical signs are probably also caused by B cell induced destruction of melanocytes, however, the pathophysiology of both diseases differ. As MAH is related to the presence of a melanoma in a patient, vitiligo is considered to be an autoimmune disorder and may be associated with several autoimmune disorders (e.g. hyperthyreoidism, hypothyreoidism, pernicious anemia, alopecia areata, hypo-parathyreoidism, diabetes mellitus).[10]

Merimsky et al (1998) found that in human patients with vitiligo the sera titers of antityrosinase IgG antibodies were higher than those in patients with melanoma (metastatic or without evidence of disease), patients with MAH or healthy individuals. The anti-tyrosinase IgG levels in sera of patients with vitiligo were also found to be significantly ($p < 0.0001$) higher compared to healthy individuals. In patients with MAH the sera titers were not significantly different from those in the group of patients with metastatic disease ($p = 0.8$) and, thus, were also lower than in patients with vitiligo.[10]

The appearance of the high levels in vitiligo patients can be explained by the absorption of antibodies by melanocytes. Once absorbed in melanocytes, the IgG-molecules cause destruction of this cell type, and are then detected by ELISA as free-antibody in the sera.
In patients with MAH the antibodies are also absorbed by malignant melanoma cells, as well as by normal melanocytes. The fraction of antibody that is bound to normal melanocytes (and lyse them) causes an increase of the free-antibody fraction in the serum. However, the antibodies bound to malignant melanoma cells are not detected by ELISA, because they do not contribute to the free-antibody fraction. The more anti-tyrosinase IgG is present in the circulatory system, the more likely it is that the individual shall develop MAH. However, only in approximately 10% of the human melanoma patients MAH does occur.[10]
As in dogs, Bergman et al (2006) found moderate foot-pad vitiligo in one dog, developed during immunotherapy using a vaccine containing murine tyrosinase DNA.[17]

Placental transfer of antibodies

The cellular immune response does not seem to reach the fetus, but what about the humoral immune response? To answer this question the placental transfer of antibodies in the dog is discussed below.

In the dog, placentation is initiated around days 17 to 18 of fetal development.[8] The dog has, as all carnivores have, an endotheliochorial placenta. In this placenta type there is a complete

erosion of the endometrial epithelium and underlying interstitium. Thus, maternal capillaries are directly exposed to epithelial cells of the chorion. Therefore, this placenta type has more connection to the fetal tissue than for instance the epitheliochorial placenta, in which the endometrial epithelium is present and completely intact.[4]

The placenta of the dog is also called a zonary placenta, as it consists of two zones. The labyrinthine zone is in the middle and is flanked on both sides by a marginal haematoma known as the haemophagous zone. Whereas maternal tissue is eroded up to the endothelium in the labyrinthine zone (endotheliochorial placenta), cytotrophoblast cells of the haemophagous zone are in immediate contact with the maternal blood.[4,13]

With the exception of some immunoglobulins, maternal proteins do not cross the placental barrier. Thus, the human tyrosinase protein, circulating in the bloodstream of a pregnant dog when vaccinated, cannot be transferred to the fetus. Some immunoglobulins, however, can be transported from the maternal to the fetal side, but only in a hemochorial placenta (such as in primates and rodents) or an endotheliochorial placenta (such as in carnivores like cats and dogs).[4]

Which type of immunoglobulines passes the placenta barrier is dependent of the species.[5] In lagomorphs and rodents the transfer of both IgG and IgM occur, whereas in humans and dogs only the IgG isotype passes the placenta. This transportation occurs during the last trimester of gestation.[5,13] According to Stoffel et al (2000) the maternal IgG proteins are present in the labyrinthine zone and in all layers of the materno-fetal barrier including the fetal capillaries. In the cytotrophoblast of the haemophagous zone maternal IgG was only localized in phagolysosomes and was not detected within fetal vessels. Cytotrophoblast cells of the haemophagous zone phagocytize maternal erythrocytes to provide the fetus with iron. Along with the erythrocytes, blood plasma containing IgG is present in the phagolysosomes. The immunoglobulins are degraded within the phagolysosomes, along with the other contents. Thus, the transplacental transport of IgG seems to be restricted to the maternal vessels in the labyrinthine zone.[13]

In humans, maternal IgG is transported directly into the bloodstream of the fetus by a specialized transport protein, *FcRn*. This protein has great structural similarity with MHC class I molecules, but binds IgG differently because the peptide-binding groove is occluded. Two molecules of *FcRn* bind to the Fc portion of one IgG-molecule, bearing it across the placenta. In dogs, this transplacental transport route has not yet been described.[5]
As Stoffel et al found no IgG in intercellular spaces, transcellular transport is assumed. The concentration of IgG was found highest in maternal basement membranes which are, like all basement membranes, considerably permeable for proteins. The transfer of IgG does occur in dogs, however, the IgG concentration in canine umbilical cord blood amounts to no more than 2-18% of the IgG concentration in the mother. When the pups are born, the level of maternal antibodies in their blood is relatively low.[13]

Discussion and conclusions

As we have seen in the above, hypopigmentation can occur in many different ways. When the Oncept® vaccine is administered, both cellular and humoral immune responses may cause the destruction of melanocytic cells – malignant or benign. We have seen that these responses seem to target particularly the malignant cells, because of a different way of tyrosinase expression. Benign melanocytic cells do not express MHC II on their surface where malignant melanocytic cells express both MHC I and MHC II on their surface. For this reason, the immune system specifically attacks the malignant cells instead of all cells with melanocytic origin. However, some investigators have found patches of hypopigmentation as a side effect when using the Oncept ® vaccine on dogs with CMM.

The question whether the tyrosinase protein can cross the placenta barrier and cause destruction of melanocytes in the fetus, cannot easily be answered. As discussed above, no proteins other than the IgG immunoglobulin isotype can cross the placenta in the canine species. So, the human tyrosinase, produced after administration of the vaccine to the mother, should not be able to reach the fetus. But what about those anti-tyrosinase antibodies?

As described, a part of the IgG antibodies, which circulate in the bloodstream of the mother, also circulate in the bloodstream of the fetus. However, the amount of [IgG] in the blood of the fetus accounts for only 2-18% of the total amount circulating in the mother's blood. Additionally, the possible effects of these antibodies would only be temporary, as the amount of maternal IgG decreases in the first weeks after parturition while the immune system of the newborn gradually takes over. So, when the pup has no maternal antibodies left in the blood, the possible effects of these antibodies should disappear.

The newborn should still be able to produce its own tyrosinase, for it is unlikely that the Oncept® vaccine causes mutations in the *TYR* gene. As the Oncept® vaccine contains a sequence of human DNA, encoding the *TYR* gene, the expression of this gene in the dog, and furthermore the transcription and translation into tyrosinase, is required. After administration of the vaccine, the immune system recognizes the cells presenting the foreign tyrosinase protein, and attack these cells. It is therefore assumable that the expression, transcription and translation of the *TYR* gene could not be affected by this vaccine. *In this line of thought the conclusion can be made that administration of the Oncept® vaccine does not necessarily result in the birth of albino pups.*

As for the cellular immune response of the mother, the fetus is sequestered from destruction by maternal T cells by both the placenta and the local immunosuppressive response. This local response consists of a combination of several cytokines and the trophoblast, which does not express MHC I or MHC II antigens on their surface. In this line, the conclusion can now be made that maternal T cells, produced in a pregnant dog in response to the human DNA vaccine, probably do not affect her fetuses.

Concluding, the Oncept® vaccine containing xenogeneic DNA encoding human tyrosinase should not have much effect on the melanogenesis in the fetus when administered to a pregnant dog. The produced antibodies against tyrosinase hypothetically may cause some reversible hypopigmentation. However, further investigation on this subject is needed to exclude the possibility that the administration of the vaccine increases the risk of mutations in the canine *TYR* gene and therefore cause irreversible albinism.

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