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Clinical evaluation of HuDo-CSPG4 DNA electroporation as adjuvant treatment for canine oral malignant melanoma: comparison of two vaccination protocols

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ABSTRACT

Canine oral malignant melanoma (OMM) is an aggressive, spontaneously occurring tumor carrying a poor to guarded prognosis and relatively limited therapeutic strategies. In this landscape, chondroitin sulfate proteoglycan (CSPG)4 represents a promising immunotherapeutic target. The objective of this bi-center prospective study was to examine the clinical outcome of OMM-bearing dogs treated with surgery and adjuvant electroporation using a DNA vaccine (HuDo-CSPG4) encoding both human (Hu) and canine (Do) portions of CSPG4 through two different vaccination protocols. Dogs with stage I-III surgically resected CSPG4-positive OMM underwent HuDo-CSPG4 plasmid electroporation starting at the 3rd-4th post-operative week; electrovaccination was repeated after 2 weeks. In protocol 1, electrovaccination was then delivered monthly while in protocol 2, electrovaccination was performed monthly four additional times followed by semestral boosters. The survival rates of HuDo-CSPG4-vaccinated dogs were estimated and compared with a control group treated with surgery alone. Significantly longer overall survival times were observed in HuDo-CSPG4 vaccinated dogs as compared with non-vaccinated controls. Dogs receiving protocol 2 showed similar outcomes to those of dogs undergoing protocol 1, despite fewer vaccinations. The comparable humoral response against CSPG4 resulting from the administration of protocol 1 and 2 appears to have similar clinical relevance, highlighting protocol 2 as the optimal vaccination schedule.

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1. Introduction

Malignant melanoma (MM) is the most common oral malignancy in dogs, accounting for up to 45.5% of oral tumors (Nishiya et al. 2016). In most cases, oral MM (OMM) exhibits aggressive behavior with rapid growth, local invasiveness, and high propensity to metastasize, especially to regional lymph nodes and lungs (Ramos-Vara et al. 2000; Esplin 2008; Liptak 2020; Kim et al. 2021). The current recommended treatment is wide surgical excision of the primary tumor, which results in variable survival times, with only up to 29-30% of dogs surviving beyond one year (Boston et al. 2014). Radiotherapy can be used as primary treatment when aggressive surgery is not feasible (due to inoperability or owner's decision) or as an adjunct to surgery, inducing an overall response rate of 75-85% (Cancedda et al. 2016; Baja et al. 2022). Nevertheless, despite local tumor control, many patients succumb to metastases (Proulx et al. 2003; Boria et al. 2004; Kawabe et al. 2015). To control the metastatic spread, chemotherapy (mostly with platinum-based drugs) has been used in combination with surgery and radiotherapy, but no significant survival benefit has been demonstrated (Murphy et al. 2005; Dank et al. 2014). Limited data are available on the use of metronomic chemotherapy in OMM, but a recent study involving several oral tumors suggests some benefit in terms of palliation. Current evidence indicates that the mechanism of action of metronomic chemotherapy, known as low-dose continuous chemotherapy, is achieved by inhibiting tumor angiogenesis, positively stimulating the anti-tumor immune response through immunomodulatory effects, and possibly inducing a state of tumor dormancy (Mutsaers 2009; Milevoj et al. 2022).

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Given the high immunogenicity of MM (Modiano et al. 1999), immunotherapy is attractive when it becomes part of a multimodal therapeutic approach. Different immunotherapeutic strategies have been applied, ranging from vaccines (allogeneic, autologous, xenogeneic) to gene therapy (interleukins, bacterial superantigens), monoclonal antibodies, non-specific immunotherapy based on the use of bacteria, and lymphokine-activated killer cell therapy (Finocchiaro and Glikin 2012; Maekawa et al. 2017; Almela and Anson 2019; Tarone et al. 2019; 2022).

DNA-based vaccination strategies represent a safe and promising tool to activate a specific and long-term immune response against cancer cells. In this perspective, chondroitin sulfate proteoglycan 4 (CSPG4) has gained value as an immunotherapeutic target (Mayayo et al. 2011; Rolih et al. 2017; Yang et al. 2019; Kurokawa and Imai 2024). CSPG4 is a class I oncoantigen (Iezzi et al. 2012) involved in several oncogenic pathways that sustain tumor progression and metastasization, with a scarce expression in healthy tissues. Previous studies have demonstrated that xenogeneic DNA vaccination in association with *in vivo* electroporation (electrovaccination) against CSPG4 was safe, immunogenic, and able to prolong survival in dogs affected by OMM. In particular, dogs were vaccinated with a plasmid encoding human CSPG4 (Hu-CSPG4) to break immune tolerance; the xenogeneic Hu-CSPG4 DNA vaccine was able to induce a consistent humoral response detected in post-vaccination sera, resulting in an improved outcome (Riccardo et al. 2014; Piras et al. 2017). Nevertheless, the affinity of the induced antibodies for the canine CSPG4 (Do-CSPG4) was sub-optimal and many dogs eventually died because of progressive disease; also, a solid cellular response was not detected (Riccardo et al. 2014; Piras et al. 2017). As a further clinical trial to overcome host unresponsiveness, a hybrid DNA vaccine encoding a chimeric CSPG4 molecule derived partly from the human and partly from the canine sequence, called HuDo-CSPG4, has been generated. It was hypothesized that electrovaccination with a hybrid plasmid encoding a chimeric protein containing both xenogeneic (human) and homologous (canine) domains could be effective in breaking host immune tolerance, thus inducing a high affinity immune response against the Do-CSPG4. Indeed, adjuvant HuDo-CSPG4 vaccination led to the development of a high humoral response against both Hu- and Do-CSPG4, with antibody levels significantly associated with survival (Riccardo et al. 2022). In this veterinary study, the vaccination protocol included an initial immunization 3-4 weeks after surgery, followed by a booster dose after 2 weeks, and then monthly immunizations with HuDo-CSPG4 for up to 2 years ((Riccardo et al. 2022); monthly protocol, protocol 1). Despite the positive results obtained, this protocol presents challenges related to the need for monthly general anaesthesia in patients. Besides, preclinical and clinical studies have suggested that long-term repeated vaccination could induce T-cell retention at the injection site, as well as T-cell exhaustion and dysfunction, potentially leading to resistance mechanisms to vaccination (Salerno et al. 2013; Benitez Fuentes et al. 2022). To overcome these potential limitations, we designed a new veterinary trial with a 6-vaccination protocol, followed by boosters every 6 months for up to 2 years (protocol 2), to reduce the number of vaccinations and the potential associated risk of immune-resistance. It was also thought that a positive response to HuDo-CSPG4 electrovaccination in dogs with OMM could have important translational implications for human patients, which could be treated following the same principle.

The aim of this prospective bicentric study was to assess survival time in client-owned dogs with stage I-III CSPG4 positive OMM locally controlled by means of *en-bloc* surgical excision, followed by adjuvant intramuscular electrovaccination with HuDo-CSPG4 DNA vaccine (HuDo-CSPG4 group). These data were compared to a historical control group (Ctrl group) of dogs that achieved local tumor control through surgery alone. The two schedules of HuDo-CSPG4 administration, including the monthly vaccination protocol (protocol 1, P1) and the 6-vaccination protocol (protocol 2, P2), were compared in terms of clinical and immunological response. Finally, based on the data collected, an update of the percentage of CSPG4 expression in OMM was provided.

2. Material and methods

2.1. Patients' enrolment

Dogs adjuvantly electrovaccinated with the HuDo-CSPG4 DNA vaccine were prospectively enrolled at the Veterinary Teaching Hospital of Grugliasco (Turin, Italy) and Tyrus Veterinary Clinic of Terni (Italy) from February 1st, 2015, to March 31st, 2023. Dogs were treated according to the Good Clinical Practice guidelines for animal clinical studies; a written consent form was signed by the owners before dogs' enrolment in the study. Both the Ethics Committee of the University of Turin and the Italian Ministry of Health had approved the trials (0004230-20/02/2018-DGSAF-MDS-P and 0015537-28/06/2017-DGSAF-MDS-P). Pre-treatment work up consisted of thorough physical examination, blood count, serum biochemistry and urinalysis. Complete tumor staging included pre-operative total body computed tomography (CT); alternatively, according to the owners' decision, skull, three views chest radiographs and abdominal ultrasound were performed. For most of the cases a preliminary diagnosis was obtained by incisional biopsy of the primary tumor, in few cases by cytology. Fine needle aspirate of draining lymph nodes was available in some cases. Definitive staging was achieved by regional lymphadenectomy at the time of the primary OMM *en bloc* resection followed by histologic evaluation. Patients were staged according to the World Health Organisation tumor, node, metastases (TNM) guidelines (Owen 1980).

Inclusion criteria required a definitive histological and immunohistochemical diagnosis of stage I-III CSPG4^{+} OMM, with local control achieved through surgery alone, and a CSPG4 score of ≥3/8 on the OMM tissue. Dogs were excluded if they had macroscopic local disease making surgery unfeasible, uncertain histological or immunohistochemical confirmation of melanocytic tumor origin, or inconsistent follow-up. Additional exclusions were applied to cases with metastases beyond the cervical lymph nodes, distant metastases at diagnosis, severe systemic conditions (e.g., cardiopathies, significant liver or renal disease). Dogs requiring post-surgical radiotherapy to achieve local tumor control were also excluded.

Enrolled dogs received adjuvant electrovaccination with HuDo-CSPG4 plasmid, with a minimum follow up of one year. For each dog, the following data were collected: signalment, age, sex, neuter status, breed, weight, TNM stage, CSPG4 score, type of surgery, margin status, and type of regional lymphadenectomy.

For the evaluation of surgical excision margins, OMM samples were stained with a specific dye (Tissue Marking Dye [TMD], Triangle Biomedical Sciences, Durham, NC, USA), then fixed in 10% formalin (Ramos-Vara and Borst; 2017). Surgical margins were considered complete if the narrowest histologic margin was >2 mm. The following information was retrieved from the histopathology reports: Ki67 expression (polyclonal Ki67 antibody A-047; DAKO; cut-off of 19.5), mitotic count (MC) (<4/10 high-power fields [hpf] or ≥4/10 hpf), nuclear atypia (% atypical nuclei in 200 cells counted, < or ≥30%), surgical margins and CSPG4 immunohistochemical score (Policlonal Anti-CSPG4 SAB4301658–Sigma Aldrich fi) (Bergin et al. 2011; Kamstock et al. 2011; Mayayo et al. 2011; Smedley et al. 2011; Smedley, Sebastian, et al. 2022; Smedley et al. 2022). The vaccination protocol type, number of vaccine doses, adverse effects and any treatments other than the vaccine were also recorded.

2.2. In vivo electrovaccination

Dogs included in the HuDo-CSPG4 group received the HuDo-CSPG4 plasmid encoding for a chimeric CSPG4 protein which includes at the N-terminal portion the domain 1 and part of the domain 2 of the Hu-CSPG4 protein, and part of domain 2 and the full domain 3 of the Do-CSPG4 protein at the C-terminal. Plasmids were generated as previously described (Riccardo et al. 2014; 2022).

The HuDo-CSPG4 vaccine was administered according to two vaccination schedules. In P1, vaccination started at the 3rd-4th post-operative week and was repeated after 2 weeks, and then monthly until 2 years post-surgery. Patients surviving over 2 years were then boosted every 6 months. In P2, vaccination started at the 3rd-4th week post-operative, it was repeated after 2 weeks and then four times monthly. Then a semestral booster was administered (Figure 1). For both protocols at each vaccination, clinical examination, blood work and restaging with CT of the thorax or three-view chest radiographs were performed. Blood samples for sera were also collected, aliquoted and cryopreserved at −80° C until use. Dogs undergoing P2 were restaged every three months by performing CT scan of the thorax. If distant metastatic disease was detected, metronomic chemotherapy was initiated using a combination of cyclophosphamide (alternatively chlorambucil), piroxicam and thalidomide.

During electrovaccination, dogs underwent a short general anaesthesia, then the vaccine (500 µg HuDo-CSPG4 plasmid in 200 µL of 0.03% NaCl solution) was injected into the muscles of the caudal thighs. Two minutes after the plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 ms, frequency 3 HZ; 1 s pause; 8 low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were delivered within the injection site using the CLINIPORATOR (Igea, Carpi, Italy). Patients recovered quickly from anaesthesia and were discharged within 40-50 minutes. Patients were monitored for acute, late local, or systemic side effects. Toxicities related to the vaccine

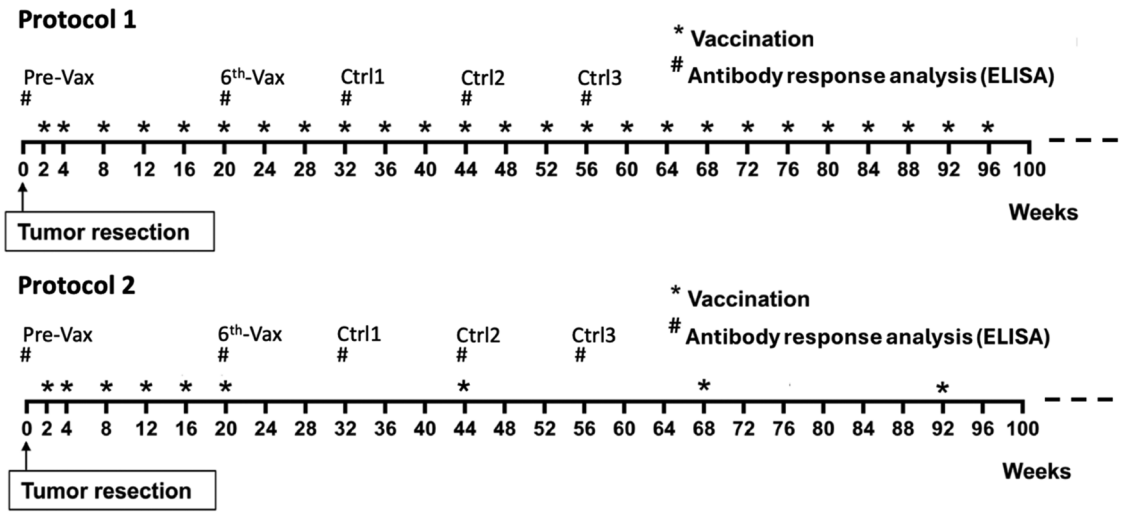

Figure 1. Immunization protocol 1 and protocol 2.

administration were graded based on the Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE v2) (LeBlanc et al. 2021).

2.3. Follow-up data

Follow-up information was gathered from medical records obtained during assessments at the Veterinary Teaching Hospital of Grugliasco and Tyrus Veterinary Clinic of Terni or by phone conversations with referring veterinarians and dog owners. During the follow-up period, data were collected to monitor disease progression and assess treatment efficacy. Clinical assessments included regular evaluations of tumor size, in case of local recurrence, and the appearance of new metastatic lesions. These evaluations were supported by diagnostic imaging (CT scan, thoracic radiographs and abdominal ultrasound). In addition, blood samples were periodically collected to measure antibody titers against CSPG4, allowing for immune response monitoring . The general health status of each dog was assessed, including parameters such as body weight, appetite, and overall behavior, to detect systemic symptoms or adverse effects related to the vaccination or other treatments.

2.4. ELISA

Sera collected from vaccinated dogs before the first (Pre-Vax) and after the sixth (Post-Vax) vaccination and during the follow-up period (every three months for both P1 and P2) were used for ELISA tests (Figure 1) performed as previously described (Riccardo et al. 2022; Tarone et al. 2023). Briefly, thawed sera (diluted 1:100) were incubated in 96-well plates previously coated with different recombinant domains (50 ng/well; GenScript) of the human CSPG4 (Hu-D1) and the canine CSPG4 (Do-D2 + D3) protein. Horseradish peroxidase-conjugated anti-dog IgG (1:10000; from R&D system) was used to detect antibodies bound to the plate. Plates were washed and chromogenic 3,3′,5,5′-tetrameth-ylbenzidine (Sigma-Aldrich) substrate was added. The reaction was stopped by the addition of 2 N hydrochloric acid, and absorbance was measured at 450 nm (absolute antibody titer) on a 680XR microplate reader (Bio-Rad). Vaccinated dogs were considered responders when the fold change between the O.D. at 450 nm of the Post-Vax/Pre-Vax sera was >1.1.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 10.3.1 for Windows, GraphPad Software, San Diego, California, www.graphpad.com), with statistical significance set at a $p < 0.05$.

Mann-Whitney, Kruskal-Wallis, and Fisher tests were used to assess statistical differences among different groups regarding age, weight, stage, MC, Ki67 and CSPG4 expression.Data were summarized using descriptive statistics, and reported as mean, median and range. Survival time was estimated using the Kaplan-Meier method. The median survival times (MST) was defined as the interval from the day of surgery to the date of death or euthanasia, specifying whether it was or not OMM related, while the disease-free interval (DFI) represented the time from surgery to local recurrence and/or metastasis development. Kaplan-Meier survival analysis was used to investigate the effect of stage (I-III) in the vaccinated group and the impact of the two vaccination protocols and of metronomic chemotherapy on survival time. Animals alive at the end of the study or lost to follow-up were censored at the last known date of survival.

Two-tailed paired Student's t-tests were used to perform statistical analysis for the ELISA data.

3. Results

3.1. CSPG4 expression in OMM

Considering the role of CSPG4 as a clinically relevant immunotherapeutic target for canine tumors (Mayayo et al. 2011; Riccardo et al. 2014; Piras et al. 2017; Riccardo et al. 2019; Camerino et al. 2021; Giacobino et al. 2021; Tarone et al. 2023), 284 OMM samples collected from 2011 to 2023 at the Diagnostic Laboratory of the Department of Animal Pathology, University of Turin, were analysed to update its frequency of expression. Histological and immunohistochemical evaluations were carried out as previously reported (Mayayo et al. 2011). Briefly, a modified semi-quantitative scoring system was adopted to evaluate membrane staining in 10 randomly selected high-power fields (400x) within the tumor. A score representing the estimated proportion of positively stained tumor cells was assigned as follows: 0 (none); 1 (<1/100); 2 (1/100–1/10); 3 (1/10–1/3); 4 (1/3–2/3); and 5 (>2/3). An intensity score was also assigned to represent the estimated average staining intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The proportion and intensity scores were then summed to obtain a total score ranging from 0 to 8. Samples with a total score greater than or equal to 3 were considered positive (Figure 2) (Mayayo et al. 2011). Of the 284 OMM samples, 253 (89.1%) had a CSPG4 score ≥3, confirming a higher positivity rate compared to previous reports (Mayayo et al. 2011).

3.2. Patient characteristics, groups, and protocols

Of the aforementioned population, 80 dogs fulfilling the inclusion criteria for this study were prospectively enrolled and received adjuvant HuDo-CSPG4 vaccination after achieving local disease control through surgery (HuDo-CSPG4 group).

Of these cases, 42 were males (12 castrated, 30 intact) and 38 females (36 spayed, 2 intact). Their ages ranged from 7.5 to 14 years (mean and

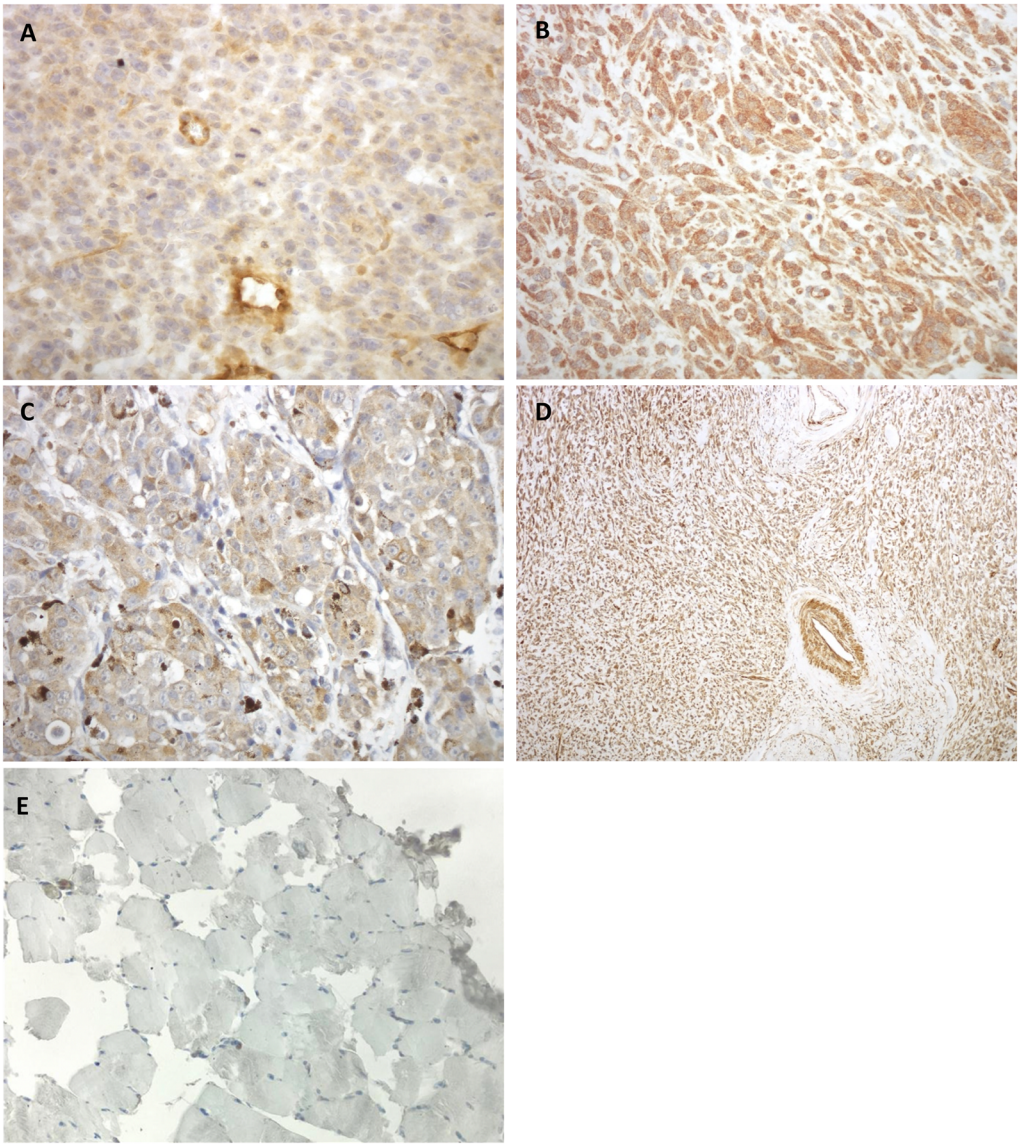

Figure 2. Immunohistochemical staining of canine OMM with an anti-CSPG4 antibody. (A) Moderate expression within tumor cells (score = 5/8). (B-D) high expression of CSPG4 in scattered neoplastic cells (score 8/8, B; score 7/8, C and D). Melanin (stained in brown) is clearly distinguishable (C) from the immunohistochemical reaction. Skeletal muscle used as negative antibody tissue control (E).

Table 1. Patients' characteristics, groups, and protocols.

Groups	n. of dogs	Males (Castrated/ Intact)	Females (Spayed/ Intact)	Age Range (Years)	Weight Range (kg)	Vaccination Protocol
HuDo-CSPG4 (Vaccinated)	80	42 (12/30)	38 (36/2)	7.5-14 (Mean: 11, Median: 11)	4-45 (Mean: 21.2, Median: 20)	P1 = 18 dogs P2 = 62 dogs
Control (Surgery Alone)	39	27 (6/21)	12 (9/3)	4-16 (Mean: 11.5, Median: 12)	5-55 (Mean: 22.2, Median: 16)	No vaccination

median 11 years), and their weight from 4 to 45 kg (mean 21.2 kg, median 20 kg). The represented breeds included 27 mongrels, 4 Pinschers, 7 Golden Retrievers, 5 Labrador Retrievers, 3 German Shepherds, 5 English Cocker Spaniels, 2 of each of Setters, English bulldogs, Dachshunds, Pekingese, Jack Russel Terriers, Poodles, Shar Peis, Miniature Schnauzers and one each of Akita-Inu, Dogue de Bordeaux, Fox Terrier, Malinois, Pug, Hovawart, Amstaff, Australian Sheperd, Alaskan Malamute, Rottweiler, Shih Tzu, Yorkshire and Beagle (Table 1).

Two vaccination protocols were used; both included adjuvant HuDo-CSPG4 electrovaccination starting 3-4 weeks after surgery, followed by a second dose 2 weeks later. Eighteen dogs continued the treatment with monthly vaccinations for up to 2 years (P1), while the remaining 62 dogs received only four additional monthly immunizations, for a total of six electrovaccinations (P2) and then were vaccinated every six months (Table 1, Figure 1).

Thirty-nine dogs were retrospectively included in the control group and were treated with surgery alone (Table 1). Among them, there were 27 males (6 castrated, 21 intact) and 12 females (9 spayed, 3 intact), with ages ranging from 4 to 16 years (mean 11.5 years, median 12 years). The mean weight

was 22.2 kg, with a median of 16 kg (range 5-55 kg). The represented breeds included 16 mongrels, 2 Golden Retrievers, 3 Labrador Retrievers, 4 English Cocker Spaniels, 2 Dachshunds, and one of Alaskan Malamute, Poodle, Dogue de Bordeaux, Pomeranian, Beagle, Dobermann, Breton Spaniel, Rottweiler, Springer Spaniel, Segugio Italiano, German Sheperd and Yorkshire Terrier.

No statistical differences were found regarding the distribution of sex, age, and weight between the HuDo-CSPG4 vaccinated group and the control group.

3.3. Clinical staging and histology

Clinical staging was performed by total body CT in all dogs of HuDo-CSPG4 group (100%) and 28 out of 39 (71.8%) of the control group, with the remaining dogs being staged with thoracic radiographs and abdominal ultrasound.

In the HuDo-CSPG4 group there were 15 (18.8%) OMM stage I, 34 (42.5%) stage II and 31 (38.7%) stage III; in the control group, 9 (23%) OMM stage I, 10 (25.7%) stage II and 20 (51.3%) stage III. Twenty-one (26.2%) dogs out of 80 of the HuDo-CSPG4 group and 14 (35.9%) out 39 of the control group were identified with metastatic lymph nodes.

Histopathological data for MC, nuclear atypia, immunohistochemical expression for Ki67, margin status, and score for CSPG4 were evaluated and are summarized in Tables 2 and 3. Specifically, in the HuDo-CSPG4 group MC was ≥4/10 hpf in 72 (90%) OMM (mean 18, median 14, range 0-55), in the control group MC was ≥4/10 hpf in 35 (89.7%) dogs (mean 20.3, median 15.5, range 1-58). Nuclear atypia was ≥30% in 54 (67.5%) OMM of the HuDo-CSPG4 group and in 26 (66.7%) of the control group.

In the HuDo-CSPG4 group, Ki67 expression was ≥19.5 in 50 (62.5%) cases (mean 37, median 38, range 3-83); in 3 cases this data was not available. In the control group Ki67 was ≥19.5 in 27 (69.2%) cases (mean 38, median 26.4, range 3-80), while in 5 dogs this data was not detectable.

Margin status analysis was available for 74 out 80 (92.5%) dogs in the HuDo-CSPG4 group and for 36 out of 39 (92.3%) dogs in the control group. This information was not retrievable for the remaining dogs of both groups.

No statistical differences were observed between the two groups regarding MC, nuclear atypia, margin status, stage, Ki67 and CSPG4 expression (Table 2).

Table 2. Histological and immunohistochemical parameters of OMMs in each group.

Parameters	Threshold	Group HuDo-CSPG4 (= 80)	Group ctrl (= 39)	P-value
Nuclear atypia	<30 %	26 (32.5 %)[a]	13 (33.3 %)[a]	0.43
	≥30 %	54 (67.5 %)[a]	26 (66.7 %)[a]	
Mitotic count (MC)	<4/10 hpf	8 (10.0 %)[a]	4 (10.3 %)[a]	0.36
	≥4/10 hpf	72 (90.0 %)[a]	35 (89.7 %)[a]	
Ki67	<19.5%	27 (33.8 %)[a]	7 (17.9 %)[a]	0.56
	≥19.5%	50 (62.5 %)[a]	27 (69.2 %)[a]	
	unknown	3 (3.7 %)[a]	5 (12.8 %)[a]	
Margin status	clear	57 (71.2 %)[a]	32 (82.0 %)[a]	0.29
	infiltrated	17 (21.3 %)[a]	4 (10.3 %)[a]	
	unknown	6 (7.5 %)[a]	3 (7.7 %)[a]	
Stage	I-III	15 (18.8 %) I 34 (42.5 %) II 31 (38.7 %) III	9 (23 %) I 10 (25.7 %) II 20 (51.3 %) III	0.72
CSPG4	≥ 3/8	80 (100%)	39 (100%)	0.38

[a]% in brackets.

Table 3. Immunohistochemical CSPG4 score of OMM from dogs included in the study.

CSPG4 score	Group HuDo-CSPG4 (= 80)	Group ctrl (= 39)
3/8	4 (5.0 %)[a]	4 (10.3 %)[a]
4/8	13 (16.2 %)[a]	6 (15.4 %)[a]
5/8	11 (13.8 %)[a]	9 (23.0 %)[a]
6/8	22 (27.6 %)[a]	6 (15.4 %)[a]
7/8	21 (26.2 %)[a]	10 (25.6 %)[a]
8/8	9 (11.2 %)[a]	4 (10.3 %)[a]

[a]% in brackets.

3.4. Treatments

Table 4 summarizes the different treatments received by both HuDo-CSPG4 vaccinated and control groups, including surgery, mandibular and medial retropharyngeal lymphadenectomy (bilateral or ipsilateral), and metronomic chemotherapy. Surgical procedures included maxillectomy, mandibulectomy, and/or *en bloc* excision of the lip and cheek. Among the 80 dogs in HuDo-CSPG4 group, 16 (20%) had undergone a previous excisional biopsy performed by referring veterinarians, followed by revision surgery in 7 cases, before starting immunotherapy. For the remaining 9 patients, surgical revision was not performed as no macroscopic disease was detected at clinical examination. In the control group, 4 out of 39 (10.3%) dogs underwent excisional biopsy followed by revision surgery.

In the HuDo-CSPG4 group the mean number of electrovaccinations for dogs undergoing P1 and P2 were 13 (range 6-26) and 7 (range 4-10), respectively. Both P1 and P2 protocols were well tolerated. A few dogs exhibited a transient (lasting no longer than one day) mild limping in the hind limb used for electrovaccination, which did not require any medical treatment.

Table 4. Type of treatments administered to dogs included in the study.

Type of treatment	Group HuDo-CSPG4 (= 80)	Control Group (= 39)
Surgery	80 (100 %)[a]	39 (100 %)[a]
Maxillectomy	16 (20.0 %)	21 (53.8 %)
Mandibulectomy	25 (31.2 %)	8 (20.5 %)
En bloc excision lip/cheek	30 (57.3 %)	11 (28.2 %)
Excisional biopsy	16 (20.0 %)	4 (10.3 %)
Revision surgery	7 (8.7 %)	4 (10.3 %)
Lymphadenectomy	67 (83.8 %)[a]	38 (97.4 %)[a]
Bilateral mandibular	11 (16.4 %)	17 (44.7 %)
Ipsilateral mandibular	38 (56.7 %)	21 (55.3 %)
Bilateral mandibular and rethropharyngeal	18 (26.9 %)	0 (0.0 %)
Metronomic chemotherapy	23 (28.7 %)[a]	0 (0.0 %)

[a]% in brackets.

3.5. Clinical outcome and response to anti-CSPG4 electrovaccination

Table 5 summarizes the survival rate and the MST for both the HuDo-CSPG4 and control groups. In HuDo-CSPG4-vaccinated dogs, the survival rates at 6, 12, 18, and 24 months were 97.5% (78/80), 75.3% (55/73), 46.9% (31/66), and 37.7% (23/61), respectively. As of March 31st, 2024, 15 dogs (18.7%) were still alive, while 65 (81.3%) had died; among the latter, 43 (66.2%) due to OMM and 20 (30.8%) for unrelated causes. Two dogs (3%) were lost to follow up at 512 and 879 days.

In the control group, the survival rates at 6, 12, 18, and 24 months were 71.8% (28/39), 48.6% (18/37), 34.3% (12/35), and 27.3% (9/33), respectively. By the end of the study, all patients have deceased. Twenty-seven dogs (69.2%) succumbed to the tumor, 10 (25.6%) to unrelated causes, and 2 (5.2%) were lost to follow up at 715 and 1371 days.

The MST for the HuDo-CSPG4 and control groups was 598 days (range 171-2252 days) and 363 days (31-2387 days), respectively. Dogs in the HuDo-CSPG4 group lived significantly longer than those in the control group (Figure 3, $p = 0.01$), confirming the efficacy of adjuvant anti-CSPG4 vaccination.

Within the HuDo-CSPG4 group, dogs undergoing P1 or P2 showed similar MST of 619 days (range 238-1504 days) and 598 days (range 171-2252 days) days, respectively, with no statistical difference (Figure 4A, $p = 0.99$). Considering DFI, no statistical differences were found between patients included in the two different HuDo-CSPG4 immunization protocols, resulting in 193 days (range 13-1504 days) and 180 days (34-2203 days) for P1 and P2, respectively (Figure 4B, $p = 0.18$). However, a trend toward an improved DFI was observed in the group of HuDo-CSPG4 vaccinated dogs included in P2. When comparing the survival of dogs receiving P1 (MST 619 days) and P2 (MST 598 days) individually with the control group (MST 363 days), both vaccinated groups displayed longer survival (Figure 5A, $p = 0.99$, Figure 5B, $p = 0.01$).

Evaluating the MST according to the clinical stage in the HuDo-CSPG4 group, dogs with stage I OMM experienced longer survival (median not reached, range 183-2203 days) compared with stage II (619 days, range 187-2252 days) and stage III (390 days, range 171-2004 days), and vaccinated dogs in stage II OMM survived longer compared to those with stage III; however, statistical significance was detected only between stage I and stage III ($p = 0.01$). In the control group, dogs with stage I OMM had longer survival (median not reached, range 95-2387 days) compared with dogs in stage II (427 days, range 54-1081 days) and stage III (240 days, range 54-1325 days); dogs with stage II OMM survived longer compared to those with stage III.

In the HuDo-CSPG4 group, 23 out of 80 dogs (28.7%) received metronomic chemotherapy during the vaccination protocol upon the onset of distant metastatic disease. The introduction of the metronomic therapy did not influence the disease-free period, but may have impacted the survival of these dogs. This group was compared to 26 dogs (32.5%) within the same HuDo-CSPG4 group who also developed distant progressive disease but did not receive metronomic chemotherapy, primarily due to owner choice or concerns about potential side effects. The MST for dogs treated with metronomic chemotherapy was 375 days (range 171-1639 days), compared to 529 days (range 172-2252 days) for those not receiving this therapy. This difference was not statistically significant (Log rank test, $p = 0.18$). Among the 23 dogs treated with metronomic chemotherapy, 9 (39.1%) received P1, while the remaining 14 (60.9%) were treated with P2. In the group of 26 dogs not receiving metronomic therapy, 5 (19.2%) underwent P1 and 21 (80.8%) underwent P2. Statistical analysis for these two sub-groups (P1 vs P2) was not conducted due to the very small sample sizes.

3.6. Immunogenic response to HuDo-CSPG4 vaccination

Sera from HuDo-CSPG4 vaccinated dogs were collected before starting the vaccination protocols (Pre-Vax) and after each immunization (Post-Vax). Patients enrolled in either P1 or P2 received 6 consecutive vaccinations following the surgical removal of the primary tumor; after this, the two protocols diverged. Antibody titers against CSPG4 were assessed after the 6th vaccination (6th-Vax) and at 3 (Ctrl1) and 6 (Ctrl2) months later (Figure 1). When possible, additional testing was performed with sera collected 9 months (Ctrl3) after the 6th vaccination. Nineteen dogs, whose sera corresponding to at least Ctrl2 were available, were included in the analysis. Of these, 7 dogs received P1 and 12 received P2. The HuDo-CSPG4 plasmid encodes a chimeric CSPG4 protein comprising the first domain (D1) of human CSPG4 and part of the second (D2) and the entire third (D3) domains of canine CSPG4. ELISA was performed using recombinant Hu-D1 and canine D2 and D3 (Do-D2 + D3) CSPG4 domains. After the 6th vaccination, a significant increase in anti-CSPG4 absolute titers (measured as O.D.) was observed between Pre-Vax and Post-Vax sera (Figure 6A and B). Specifically, 9 out of 19 patients (47%) developed antibodies against Hu-D1, and 10 out of 19 (53%) developed

Table 5. MST and percentages of survival in the HuDo-CSPG4 and control groups.

		Survival rates (%)			
	MST (days)	6 months	12 months	18 months	24 months
HuDo-CSPG4 group	598 (range 171-2252)	97.5 (78/80)	75.3 (55/73)	46.9 (31/66)	37.7 (23/61)
Ctrl group	363 (range 31-2387)	71.8 (28/39)	48.6 (18/37)	34.3 (12/35)	27.3 (9/33)

antibodies against Do-D2 + D3 after the 6th vaccination cycle. Overall, anti-CSPG4 titers remained high or increased when sera were tested at subsequent time points (Figure 1). By the final time point (Ctrl3), 15 out of 19 patients (79%) exhibited a response to the Hu-D1 and 14 out of 19 (74%) responded to Do-D2 + D3. When comparing antibody levels against Hu-D1 or Do-D2 + D3 between the two groups (P1 or P2), no differences were observed in sera collected after surgery (Pre-Vax), after the 6-vaccination cycle (6th-Vax), or during the follow-up period. These findings suggest that the interruption of vaccination in P2 did not affect anti-CSPG4 antibody titers in canine sera (Figure 6C and D). In the analyzed population, the anti-CSPG4 absolute titer was not correlated with age, weight or the CSPG4 score of the tumor (Supplementary Figure S1).

Since Pre-Vax sera indicated a spontaneous response against Do-CSPG4 (Figure 6B and D), we also analyzed the vaccine-induced response by considering the Post-Vax/Pre-Vax ratio (fold change). This analysis confirmed a significant increase in anti-CSPG4 antibodies in vaccinated dogs (Figure 6A and B), independent of age, weight, or CSPG4 score (Supplementary Figure S1).

A significantly higher anti-CSPG4 antibody titer against Do-D2 + D3 was observed in stage II vaccinated dogs compared to stage III dogs. However, when considering the fold change, no significant differences were found between these stages. This suggests that the HuDo-CSPG4 vaccine can boost the spontaneous anti-CSPG4 response similarly in both stages (Supplementary Figure S1), supporting the rationale for vaccinating stage III OMM-affected dogs and potentially dogs in more advanced stages.

Importantly, the anti-canine CSPG4 immune response appears to have clinical significance in the immunized population. Specifically, dogs classified as responders, i.e. those achieving an anti-CSPG4 fold change >1.1 after the 6th vaccination (Figure 7A) or at any subsequent time points during treatment (Figure 7B), showed a positive trend toward prolonged overall survival compared to non-responders. Among the entire vaccinated population, dogs with higher anti-CSPG4 antibody levels in 6th-Vax sera exhibited prolonged, though not statistically significant, overall survival compared to those with lower levels (Figure 7C). This apparent survival benefit became more pronounced and significant when comparing responders with the higher anti-CSPG4 absolute titers to those with lower titers, using the median of the O.D. in 6th-Vax sera as a cut-off (Figure 7D).

Considering the clinical risk of recurrences in the oral mucosa despite the local tumor control, we also evaluated the fold change of IgA against the Do-D2 + D3. This analysis identified 6 responders (31.6%) among the 19 dogs analyzed, who demonstrated clearly prolonged disease-free survival compared to non-responders (median DFI: 241 days vs. 180 days, respectively; Supplementary Figure S2).

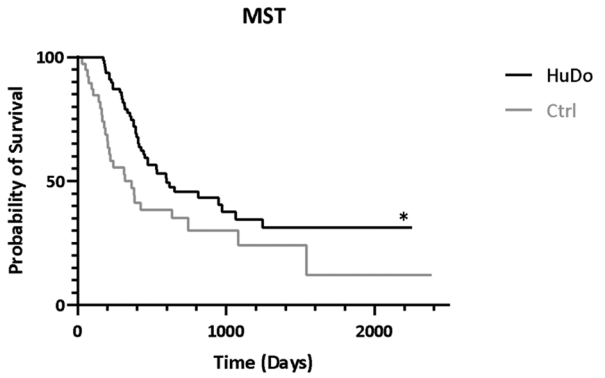

Figure 3. Kaplan-Meier curves comparing the MST of HuDo-CSPG4 (black line) and control (grey line) groups. Log-rank test, *$p = 0.01$.

4. Discussion

The main therapy for canine OMM remains surgery, typically involving resection of the primary tumor and regional lymphadenectomy. This is often complemented by adjuvant radiotherapy. In specific cases, radiotherapy may be employed as a sole treatment for controlling macroscopic disease (Boston et al. 2014; Tuohy et al. 2014; Cancedda et al. 2016; Grimes et al. 2019).

Immunotherapy, which targets key molecules involved in melanoma progression, is emerging as a promising component of a multimodal therapeutic approach.

Immunotherapeutic strategies in veterinary medicine are increasingly being explored, with numerous

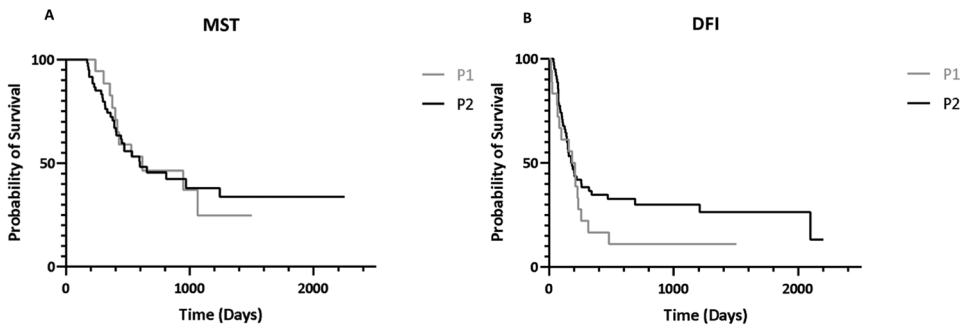

Figure 4. Kaplan-Meier curves comparing the MST (A) and DFI (B) of dogs undergoing protocol I (P1, grey line) and protocol 2 (P2, black line). Log Rank test, MST, $p = 0.99$; DFI, $p = 0.18$.

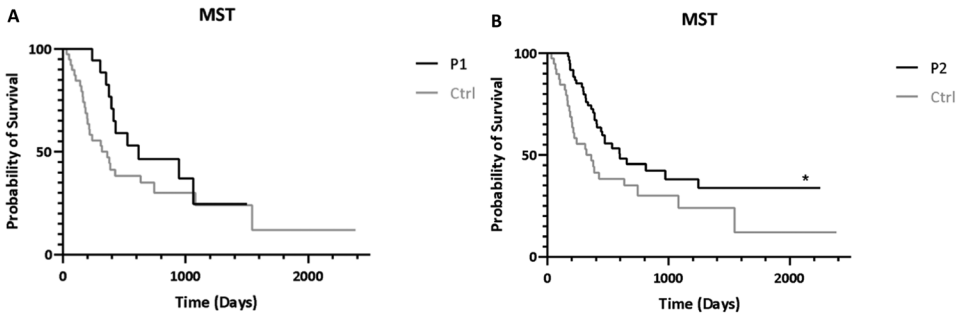

Figure 5. (A) Kaplan-Meier curves comparing the MST of dogs undergoing protocol I (P1, black line) and control group (ctrl, grey line). Log Rank test, $p = 0.99$. (B) Kaplan-meier curves comparing the MST of dogs undergoing protocol II (P2, black line) and control group (ctrl, grey line). Log Rank test, $*p = 0.001$.

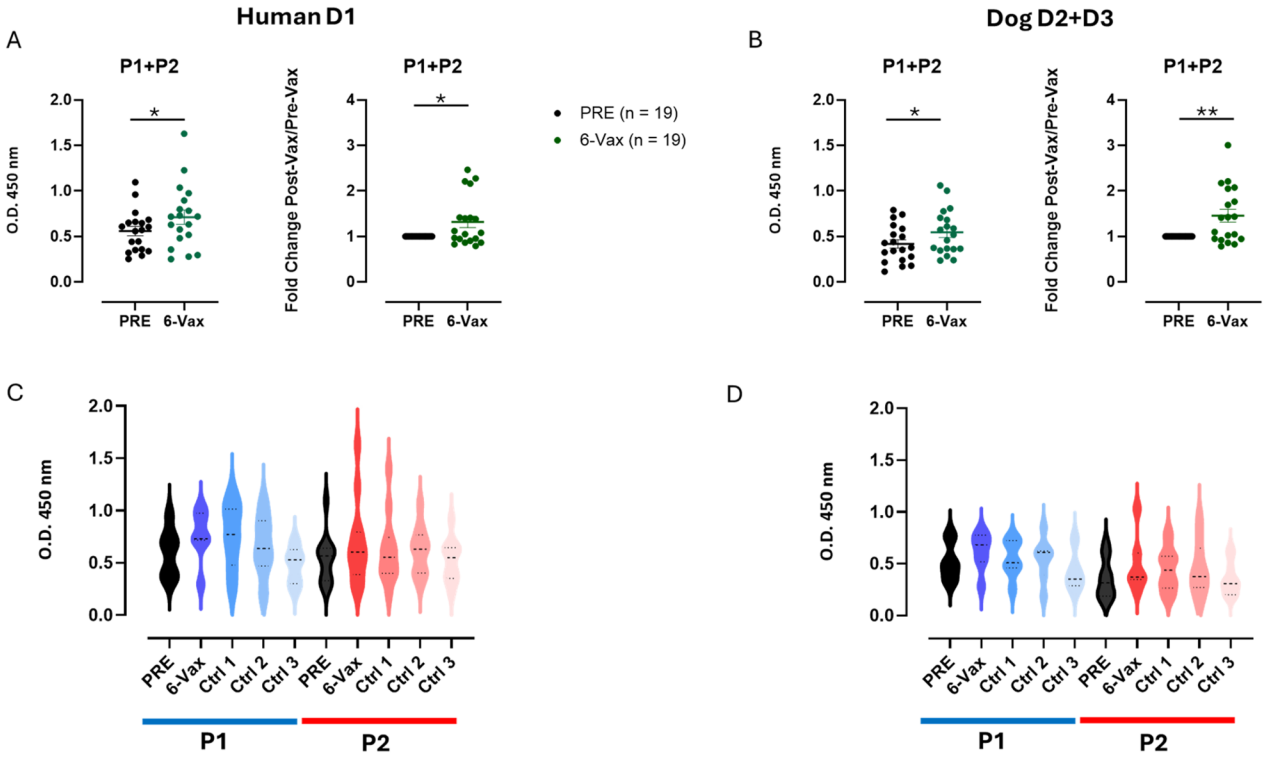

Figure 6. HuDo-CSPG4 electroporation induces a specific cspg4 antibody response in OMM-affected dogs included in both vaccination protocols. Analysis, by means of ELISA assay, of the presence of IgG against the human D1 (a and C) and canine D2 + D3 (B and D) domains of the CSPG4 protein in the sera of dogs collected before the first (Pre-vax), after the 6th (6-vax) vaccination (a and B) and at three-month intervals thereafter (ctrl 1,2,3; C and D). Results express the optical density (O.D.) at the absorbance measured at 450 nm or the fold change between the O.D. measured in the post-6th vax/Pre-vax. Paired student's t-test: $*p < 0{,}0238$; $**p = 0.0046$.

trials conducted not only in the melanoma setting but also across various tumor types. These strategies range from autologous and allogeneic vaccines to DNA- and peptide-based immunization approaches (Tarone et al. 2019; Bryan and Maitz 2024; Ruzzi et al. 2025). In this context, several melanoma-associated antigens have been investigated as vaccination targets, including disialogangliosides GD2 and GD3 (Milner et al. 2006; Albertini et al. 2024), tyrosinase (Bergman et al. 2003; Grosenbaugh et al. 2011), and gp100 (Alexander et al. 2006). Among these advancements, ONCEPT (Merial) became the first FDA-approved DNA vaccine for anti-tumor therapy. Designed to treat dogs with locally controlled melanomas, it encodes human tyrosinase. Studies suggest that ONCEPT increases survival times in treated dogs compared to unvaccinated controls, with no reported adverse events. Despite some criticism regarding its effectiveness, ONCEPT's approval marked a significant milestone in canine melanoma treatment, fostering hope for the broader application of anti-cancer DNA vaccines (Bergman et al. 2006; Ottnod et al. 2013; Verganti et al. 2017; Pellin 2022). Building on this momentum, our DNA-based vaccination strategy holds the potential to give a significant impact in this field due to several advantages. CSPG4, with its restricted distribution in normal tissue and high expression in melanoma cells, appears to be an ideal target for anti-tumor vaccination (Cavallo et al. 2007; Rolih et al. 2017; Tarone et al. 2019; Kurokawa and

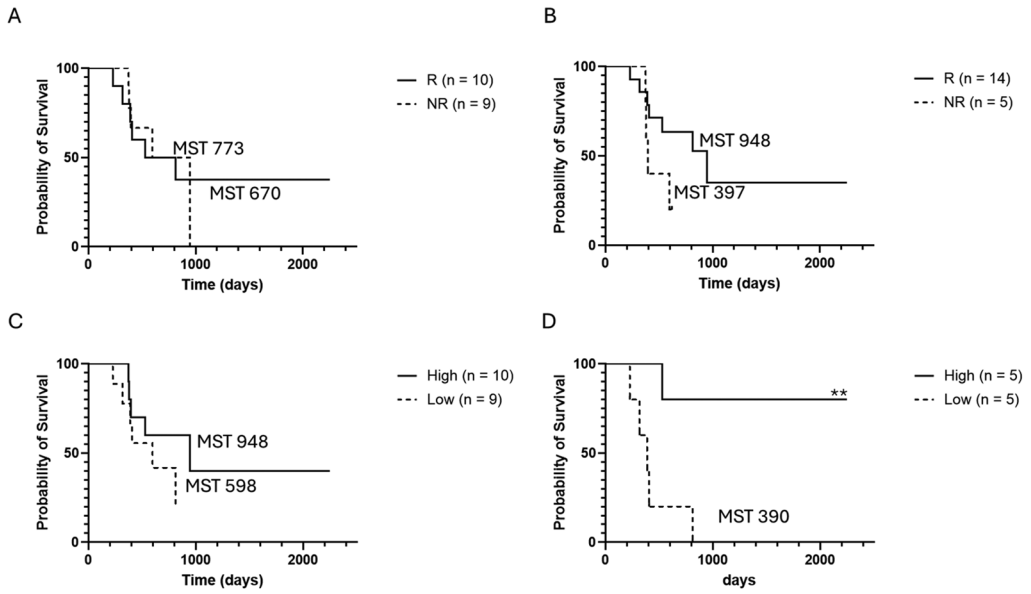

Figure 7. (A and B) Kaplan-Meier curves correlating the overall survival of responder (R, continuous black lines) versus non-responders (NR, dotted black lines) dogs to the anti-CSPG4 vaccination. "responders" are the dogs which developed a specific IgG response against the do–D2 + D3 domains, with a higher level in the Post-Vax sera as compared to Pre-Vax sera (measured by ELISA, fold change Post-Vax/Pre-vax O.D. at 450 nm >1.1), considering the 6th vaccination (A) or any time points during treatment. (C and D) Kaplan-Meier curves correlating the overall survival of the entire population (C) or of the responder dogs only (D), with a high (continuous black lines) or low (dotted black lines) absolute antibody titer, measured in their post-vaccination sera by ELISA, considering as cut-off the median of the absorbance at 450 nm. Log Rank test, $^{**}p = 0.008$.

Imai 2024). Furthermore, CSPG4 is overexpressed on cancer stem cells (CSC), making it an attractive target for eliminating drug-resistant, recurrent, and metastatic CSPG4-expressing CSC (Ruiu et al. 2019; Quaglino et al. 2020; Ruzzi et al. 2025). As a plasma membrane receptor, CSPG4 can be targeted by both T cells and antibodies, addressing the challenge of MHC-I downregulation that cancer cells often use to evade T cell recognition (Cavallo et al. 2007; 2011; Lollini et al. 2011; Iezzi et al. 2012).

Like other DNA vaccines, our strategy must address the challenge of low intrinsic immunogenicity. However, the use of an innovative chimeric plasmid offers a significant advancement in breaking immune tolerance and developing effective anti-cancer vaccines against self-tumor-associated antigens, distinguishing it from previous DNA vaccine strategies (Cavallo et al. 2014; Riccardo et al. 2022; Tarone et al. 2023). Moreover, recent studies in human medicine, targeting antigens such as MART-1, gp100, and NY-ESO-1, have demonstrated that electroporation significantly enhances transfection efficiency and immunogenicity (Rezaei et al. 2021; Ruzzi et al. 2025). Our study aligns with these advances, employing electroporation to maximize the efficacy of the anti-CSPG4 plasmid. This approach underscores the translational potential of this technology, suggesting its applicability to human medicine.

This prospective clinical trial aimed to validate the prolonged survival benefit of adjuvant electrovaccination using a hybrid plasmid (HuDo-CSPG4) encoding a human-dog chimeric CSPG4 protein in dogs with stage I-III OMM. The study also sought to compare two vaccination protocols to evaluate whether reducing the number of vaccinations (as in P2) and, consequently, the number of anaesthetic events, could achieve comparable clinical outcomes.

In a previous veterinary trial testing HuDo-CSPG4 (Riccardo et al. 2022), a monthly vaccination protocol lasting up two years was implemented. Since there were no guidelines regarding the minimum number of immunizations needed to overcome immune tolerance against the CSPG4 and induce an effective anti-cancer immune response, this initial schedule was based on the safety and positive immunological results observed with another plasmid encoding the xenogeneic Hu-CSPG4. This plasmid had been previously tested by the authors for treating canine OMM (Riccardo et al. 2014; Piras et al. 2017). These earlier studies confirmed the safety of long-term, repeated vaccinations against the CSPG4 using the chimeric HuDo-CSPG4 plasmid. Notably, the majority of vaccinated dogs in those studies developed a significant anti-CSPG4 immune response after the fourth immunization, with further increases in vaccine-induced antibodies observed following the fifth and the sixth vaccinations. These findings formed the rationale for exploring the feasibility of a 6-vaccination protocol.

In veterinary oncology, a variety of immunization protocols have been proposed for different antigens and tumor types, ranging from a few to multiple vaccine administrations. Collectively, these studies suggest that a prime-and-boost strategy is essential to elicit an effective anti-cancer immune response (Bergman et al. 2003; Peruzzi et al. 2010; Grosenbaugh et al. 2011; Gavazza et al. 2013; Treggiari et al. 2016; Impellizeri et al. 2018; Doyle et al. 2021; Marconato et al. 2022). Conversely, preclinical and clinical

studies on peptide vaccines have indicated that prolonged and repeated immunizations can result in the accumulation and persistent retention of T cells at the injection site. This phenomenon may lead to dysfunction of these effector cells, potentially limiting vaccine-induced tumor control (Salerno et al. 2013; Hailemichael et al. 2022). Considering these factors, and to mitigate the risk of immune resistance and the potential hazards associated with repeated anesthesia during electroporation, we opted to reduce the number of immunizations. We hypothesized that even with fewer vaccinations under P2, the antibody response against CSPG4 would remain robust and provide clinical benefits comparable to those achieved with the more frequent monthly vaccinations in P1.

Consistent with previous findings (Riccardo et al. 2022), our study confirms that the HuDo-CSPG4 electrovaccination is safe, immunogenic and significantly extends the survival of dogs with OMM. The rationale for using a chimeric DNA vaccine in dogs with OMM is supported by several reasons. Firstly, it is widely recognized that DNA vaccines are easy to produce, stable, and characterized by low manufacturing costs; additionally, they can induce both humoral and cellular immune responses (Fioretti et al. 2010). Secondly, being the hybrid plasmid encoding a chimeric CSPG4 protein partially derived from the human and partially from the dog CSPG4 sequence, the heterologous and autologous domains could, on one hand, break the host immunotolerance to self-CSPG4 and, on the other hand, induce a high affinity immune response that could protect from local recurrence and metastatic disease (Riccardo et al. 2022; Tarone et al. 2023).

Dogs with CSPG4-negative OMM were excluded from this study, as they were unlikely to benefit from CSPG4-targeted immunotherapy and could confound the evaluation of the vaccine's efficacy. Notably, the CSPG4 positivity rate in OMM observed in this study was higher than previously reported. Mayayo et al. (Mayayo et al. 2011) reported a CSPG4 positivity rate of about 60%, in contrast, the present investigation found a CSPG4 positivity rate of 89.1% in OMM samples, suggesting a broader potential benefit from CSPG4 immunization.

The study by Mayayo et al. (Mayayo et al. 2011) is an older investigation that utilized biopsies and post-surgical samples, some of which were sourced from outside our hospital and retrieved from the collection of the Pathology Section of our Department. Notably, none of the dogs in that study had been vaccinated. As our study progressed, we observed that the previously reported percentage of CSPG4 positivity in OMM appeared lower than what we were finding in our own cohort. This observation prompted us to recalculate the CSPG4 positivity rate, which revealed a higher value. This discrepancy could potentially be attributed to several factors, including methodological differences, such as variations in immunohistochemical protocols and antibody specificity. Additionally, differences in patient populations and sample selection may have influenced the results. The samples analyzed in Mayayo et al. (Mayayo et al. 2011) included 65 melanoma samples, 10 of which were subungual, which may exhibit different CSPG4 expression patterns. In contrast, our study focused exclusively on OMM, which might inherently have higher CSPG4 positivity. The positivity rate reported here aligns more closely with the levels observed in human studies (Chauhan et al. 2023; Grossauer et al. 2023; Chen et al. 2024).

From a translational perspective, using this hybrid human-dog vaccine structure could facilitate translation to human clinical settings, given the promising results from veterinary studies. CSPG4 plays a significant role in promoting MM in humans, with its expression linked to phenotypic features crucial for tumor progression (Campoli et al. 2010; Price et al. 2011).

HuDo-CSPG4 vaccinated dogs showed significantly longer survival ($p = 0.01$) compared with the control group (Figure 3, Table 5). While DFI could be a superior endpoint, unaffected by owner decisions, it was not considered appropriate for validating vaccine efficacy when compared with the historical control group, given the differences in the monitoring frequency between the two groups of dogs. Regular re-staging using CT scans allowed a more standardized assessment, showing similar MST and DFI in both P1 and P2 (Figure 4A and B). However, a positive trend in the long-term, according to the Kaplan-Meier curve for DFI, was observed in the P2 dogs, suggesting P2 as the preferred protocol due to both the reduced anesthesia sessions, as well as practical and financial advantages.

Significant differences in MST were also evident when evaluating dogs receiving P2 with those of the control group (598 days vs 363 days) (Figure 5B). In support of previous studies (Riccardo et al. 2022), HuDo-CSPG4 electrovaccination demonstrated its effectiveness in inducing a significant increase in the antibody response against the human (Hu-D1) and the canine (Do-D2 and Do-D3) CSPG4 domains included in the chimeric plasmid (Figure 6A and B), notably capable of binding the antigen and inducing its down-modulation, thus impairing the tumorigenic properties of melanoma cells (Piras et al. 2017; Riccardo et al. 2022).

Additionally, in this study we have observed that the level of antibodies remained high and stable for months, even though dogs did not undergo monthly vaccination as in P1 (Figure 6C and D). Considering this prolonged humoral response, these data further support that P2 should be preferred over P1.

No correlation was observed between age, weight, CSPG4 expression in the tumor, and vaccine-induced anti-CSPG4 antibody levels in the vaccinated dogs. However, when comparing disease stages, a significantly higher absolute antibody level was observed in the Post-Vax sera of stage II canine patients. This finding suggests that dogs with more advanced disease (stage III) may respond less effectively to the vaccine, likely due to a more immunosuppressive

environment. Nevertheless, when considering the fold change between Pre-Vax and Post-Vax sera and the MST of stage III vaccinated dogs compared to stage III controls (390 vs. 240 days, respectively; Gehan-Breslow-Wicoxon test, $*p = 0.0178$), the HuDo-CSPG4 vaccination appears to confer a clinical benefit. This supports the rationale for proposing the vaccine as a treatment option for patients with advanced-stage disease. Interestingly, the anti-CSPG4 antibody response appears to have a clinical relevance in the immunized population. Among the analyzed cohort, approximately 52% of vaccinated dogs showed increased antibody levels against the Do-D2 + D3 following the 6th vaccination compared to the Pre-Vax sera (responders). A further increase in vaccine-induced antibodies was detected following subsequent vaccinations, with approximately 73% of vaccinated dogs classified as responders. A trend toward improved overall survival was noted in responder dogs that developed a higher anti-CSPG4 IgG response after vaccination compared to Pre-Vax sera (responder vs. non-responder dogs; Figure 7A and B). Additionally, a significant correlation was observed between higher Post-Vax anti-CSPG4 antibody level and prolonged overall survival (Figure 7C and D).

It is important to note that a spontaneous, low-affinity anti-CSPG4 antibody response was previously detected in melanoma-bearing dogs following local tumor control and prior vaccination (Riccardo et al. 2022). This pre-existing response in Pre-Vax sera may have led to an underestimation of the percentage of responder dogs and, consequently, an underestimation of the clinical correlation. Furthermore, mucosal immunity induction was observed in approximately 31% of 6th-Vax sera, as evidenced by elevated IgA levels as compared to Pre-Vax. In responder dogs, a positive trend towards higher IgA levels and prolonged DFI was noted. However, these results should be interpreted cautiously due to the small sample size and the fact that IgA were measured in the sera rather than saliva, which may be more relevant for assessing protection against oral malignancies.

Despite these promising findings, which highlights the crucial role of the vaccine-induced antibody response in melanoma inhibition, a deeper understanding of the cellular immune response and the immune phenotyping is essential. Future studies should include a more comprehensive analysis of diagnostic biopsies and, where feasible, metastatic lesions to evaluate the expression of checkpoint inhibitors and other relevant markers. Such analyses could establish correlations with treatment outcomes, identify new therapeutic targets, and support the development of combinatorial treatment approaches. Enhanced understanding of these mechanisms will improve patient selection, optimize vaccination outcomes, and inform the design of future trials.

In the HuDo-CSPG4 group, dogs with progressive disease treated with metronomic chemotherapy had shorter survival times (MST 375 days) compared to dogs not receiving metronomic chemotherapy (MST 529 days). Although this difference was not statistically significant, it suggests that the vaccine may prolong survival regardless of metronomic chemotherapy administration. However, the precise impact of metronomic chemotherapy in this study remains unclear due to its combination with electrovaccination and the limited sample size. Similarly, the influence of metronomic chemotherapy on the survival of vaccinated dogs with metastatic disease could not be definitively assessed, as no comparable population existed within the control group. Specifically, no dogs in the historical control group received metronomic treatment, primarily because this therapy was introduced after the control goup's enrollment period. Determining whether metronomic chemotherapy improves outcomes in stage IV OMM, with or without the HuDo-CSPG4 vaccination, should be a priority for future research. This could be achieved through a dedicated veterinary study that, based on refined inclusion and exclusion criteria, enrolls a sufficiently large cohort to yield statistically meaningful conclusions.

Clinical stage based on World Health Organization criteria is a well-recognized prognostic factor in melanoma patients (Bostock 1979; Harvey et al. 1981). Our study suggests that dogs with early stage (stage I) OMM treated with HuDo-CSPG4 have longer survival compared to stage II-III OMM. This has been also confirmed in a previous paper (Tuohy et al. 2014). Considering only stage I OMM, our results and those of Tuohy et al. (Tuohy et al. 2014) are difficult to compare. Stage I OMM in the present series showed a longer survival, but whether this is an effect of the vaccination and/or the low clinical stage cannot be established with certainty, as different treatments were also used in the study by Tuohy et al. (Tuohy et al. 2014). Even though a more pronounced immunogenicity of OMM can be assumed in its early stages, the longer survival recorded here compared to that reported by Tuohy et al. (Tuohy et al. 2014) in stage I OMM does not allow us to definitively conclude that our prolonged survival is solely due to the vaccine. However, authors believe that anti-CSPG4 vaccination should be considered even in stage I OMM when additional negative prognostic factors are present, as some cases can exhibit aggressive behavior despite the small tumor size. Overall, the results support the benefit of adjuvant anti-CSPG4 vaccination in treating canine patients at any disease stage.

Finally, it should also be considered that different lymphadenectomy procedures (bilateral vs ipsilateral; mandibular only or submandibular and retropharyngeal lymph nodes) may have influenced the final definition of N status in TNM staging and thus impacting the final clinical staging. This is due to the progressive changes in the standard of OMM management over time in the field of veterinary oncology.

None of the vaccinated dogs developed local or systemic adverse reactions following vaccine administration, as reported by owners and confirmed by data collected on the day of electrovaccination.

One limitation of this study is the non-randomized enrolment of dogs into the HuDo-CSPG4 or control groups. As is common in veterinary studies,

treatment decisions that deviate from standard care are often influenced by owner compliance, particularly when expenses are not covered by dedicated fundings. Additionally, the comparison between control and vaccinated groups may be biased by the more frequent and intensive monitoring of vaccinated dogs, which could lead to an underestimation of tumor progression in the control group. For this reason, DFI was not considered a reliable parameter for comparing outcomes between HuDo-CSPG4 vaccinated and control dogs; instead, overall survival was used as the primary measure. It is also important to emphasize that the primary objective of this study was to evaluate and compare the safety, immunogenicity, and clinical effectiveness of the two proposed vaccination protocols (P1 vs P2). The study design minimizes bias related to the enrolment of dogs into these two groups, their clinical follow-up, immune monitoring, and other factors relevant to the core objectives. As a secondary objective, the study validated the clinical efficacy of the adjuvant vaccination with the HuDo-CSPG4 plasmid within this cohort of dogs, compared to historical controls. These findings provide additional support for previous results reported in Riccardo et al. (2022). Finally, based on the collected data, P2 should be preferred over P1 as the recommended first-line HuDo-CSPG4 vaccination protocol.

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Author contributions statement

Federica Riccardo, Paolo Buracco and Federica Cavallo conceptualized and designed the project. Mariateresa Camerino and Davide Giacobino managed the clinical cases enrolled in the project. Federica Riccardo and Lidia Tarone performed the laboratory studies; Mariateresa Camerino, Davide Giacobino, Federica Riccardo, Lidia Tarone analyzed the data. Selina Iussich and Lorella Maniscalco performed histopathological analysis; Luca Manassero followed all the imaging studies.

Mariateresa Camerino, Federica Riccardo and Paolo Buracco wrote the manuscript. Mariateresa Camerino, Federica Riccardo, Paolo Buracco and Federica Cavallo revised the manuscript. Paolo Buracco, Alfredo Dentini, Emanuela Morello and Marina Martano supervised the clinical veterinary trial. All authors agree to be accountable for all aspects of the work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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