Received: 20 November 2020 | Revised: 26 February 2021 | Accepted: 5 March 2021

DOI: 10.1111/vco.12690

ORIGINAL ARTICLE

Veterinary and Comparative Oncology WILEY

Difference in outcome between curative intent vs marginal excision as a first treatment in dogs with oral malignant melanoma and the impact of adjuvant CSPG4-DNA electrovaccination: A retrospective study on 155 cases

Davide Giacobino[1] | Mariateresa Camerino[1] | Federica Riccardo[2] | Federica Cavallo[2] | Lidia Tarone[2] | Marina Martano[3] | Alfredo Dentini[4] | Selina Iussich[1] | Elena Lardone[1] | Paolo Franci[1] | Alberto Valazza[1] | Luca Manassero[1] | Sara Del Magno[5] | Raffaella De Maria[1] | Emanuela Morello[1] | Paolo Buracco[1]

[1]Department of Veterinary Sciences, University of Torino, Torino, Italy

[2]Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Torino, Torino, Italy

[3]Department of Medical Veterinary Science, University of Parma, Italy

[4]Clinica Veterinaria Tyrus, Terni, Italy

[5]Department of Veterinary Medical Science, University of Bologna, Italy

Correspondence
Paolo Buracco, Department of Veterinary Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy.
Email: paolo.buracco@unito.it

Abstract

Canine oral malignant melanoma is locally invasive and highly metastatic. At present, the best option for local control is *en bloc* excision followed by radiation if excision margins are incomplete. Adjuvantly, the role of chemotherapy is dubious while immunotherapy appears encouraging. This retrospective study evaluated 155 dogs with oral malignant melanomas (24 stage I, 54 stage II, 66 stage III and 11 stage IV) managed in a single institution. The aim was to evaluate the differences in median survival time (MST) and disease-free interval (DFI) between dogs which, at presentation, were treated surgically with a curative intent (group 1) vs those marginally excised only (group 2). MST in group 1 was longer than in group 2 (594 vs 458 days), but no significant difference was found (P = .57); a statistical difference was, however, found for DFI (232 vs 183 days, P = .008). In the subpopulation of vaccinated dogs, the impact of adjuvant anti-CSPG4 DNA electrovaccination was then evaluated (curative intent, group 3, vs marginal, group 4); a significant difference for both MST (1333 vs 470 days, respectively, P = .03) and DFI (324 vs 184 days, respectively, P = .008) was found. Progressive disease was significantly more common in dogs undergoing marginal excision than curative intent excision for both the overall population (P = .03) and the vaccinated dogs (P = .02). This study pointed out that, after staging, wide excision together with adjuvant immunotherapy was an effective approach for canine oral malignant melanoma.

KEYWORDS
adjuvant immunotherapy, CSPG4, DNA electrovaccination, dog, oral malignant melanoma, surgery

Paolo Buracco and Emanuela Morello contributed equally to the article.

1 | INTRODUCTION

Malignant melanoma is an aggressive tumour characterized by high local invasiveness and metastatic potential in both humans and dogs. The most frequent location in dogs is the oral cavity, accounting for up to 30-40% of canine oral malignancies.[1-3] The biological behaviour of canine oral malignant melanoma (COMM) can be predicted based on several clinical (site of growth, size and clinical stage)[2-4] as well as on histological and immunohistochemical factors (Ki67 expression, mitotic index, degree of pigmentation and nuclear atypia).[5,6] It has also recently been shown that platelet-derived growth factor receptor expression correlated with prognosis.[7]

It has been reported that COMM invades the bone in 57.0% of cases.[3,8] The metastatic rate is variable ranging from 30.3% to 74.0% at the level of the regional lymph nodes,[3,9] and from 14.0% to 92.0% as distant metastatic spread (lungs and other organs).[3]

Factors such as the site of tumour growth (gum, internal lip/cheek, palate, tongue and tonsil), the size and degree of local soft tissue, and the bone invasion have a heavy impact on the choice of treatment for local tumour control. For local tumour control with a curative intent, a wide surgical excision should be performed. While evidence guiding specific surgical margins is lacking, the authors aim for a minimum of 1.5-2 cm of macroscopically sound tissue all around the COMM, when feasible. The reasons for an excision with no local curative intent may include difficult tumour locations (including the tongue[10] and the tonsil), first excision not performed by a surgeon specialized in veterinary oncological surgery or justified as an excisional biopsy and, finally, marginal excision requested by the owner for palliation because of the signs of foul odour and bleeding from a rapidly tumour growth. Radiotherapy should be considered as adjuvant therapy for those COMMs incompletely excised or, as a primary treatment or in combination with medical treatment, for those cases deemed inoperable or when the owners refuse surgery.[11-18] An alternative to radiotherapy for local tumour control is electrochemotherapy which would be contraindicated when tumour bone erosion is already evident.[19-21]

Distant metastasis from COMM, more than local recurrence, represents the definitive cause of death in the majority of dogs; therefore, there is the absolute need, once local control has been reached, to adopt adjuvant systemic treatments in an attempt to delay the metastatic spread. Standard chemotherapy, based mainly on the use of carboplatin, has failed to show real efficacy when used in an adjuvant setting since survival did not appear to be prolonged significantly when compared with local tumour control only.[13-15,22-24] For all these reasons, and also thanks to the immunogenic features of melanoma, several studies dealing with immunotherapy have been carried out. Melanoma-associated antigens have been identified (eg, Tyrosinase and Chondroitin sulphate proteoglycan 4 [CSPG4]) and utilized in producing vaccines capable of evoking an immune response against COMM.[25-32] In particular, the authors' attention was focused on CSPG4, a cellular membrane antigen, characterized by restricted distribution in normal healthy tissues and high expression on neoplastic cells in both human and canine MM. It coordinates several intracellular pathways regulating different cell functions (ie, proliferation, migration and survival), thus being involved in tumourigenesis at multiple levels.[33-36] In addition, CSPG4 has also been shown to be overexpressed in melanoma cancer stem cells and associated with poorer patient prognosis.[29,37] All these features make CSPG4 an ideal antigen to be safely and effectively targeted.

The aim of this retrospective study was to evaluate a) the difference in terms of prognosis (disease-free interval [DFI] and survival time) between COMMs which, immediately after the first presentation, were treated surgically with a curative intent excision vs those marginally excised only, b) the potential relationship between the type of surgery performed and progressive disease, and c) the impact of adjuvant anti-CSPG4 DNA electrovaccination on both survival and DFI in the two groups of dogs.

2 | MATERIALS AND METHODS

2.1 | Patient enrolment

Client-owned dogs affected by COMMs which were presented at the Veterinary Teaching Hospital of Grugliasco (Turin, Italy) of the University of Turin since January 1, 2000 with a minimum follow-up of 1 year as of 30 September 2020 were retrospectively considered for this study. The dogs were presented for surgical treatment, or for anti-CSPG4 DNA electrovaccination or both after having already been operated elsewhere. Written consent was obtained from the owners for the anaesthetic, diagnostic, histological, and surgical procedures before staging. Regarding adjuvant anti-CSPG4 DNA electrovaccination, other prospective studies have been carried out since 2009[27,28] and still others are in progress. The dogs were treated according to the Good Clinical Practice guidelines for animal clinical studies. Both the Ethics Committee of the University of Turin and the Italian Ministry of Health approved the trials (0004230-20/02/2018-DGSAF-MDS-P and 0015537-28/06/2017-DGSAF-MDS-P); specific written consent for entry into the study was obtained from all the owners of the dogs vaccinated.

2.2 | Tumour staging and treatment

Dogs were included if they had undergone a surgical excision of the primary tumour with or without regional lymphadenectomy and if histology confirmed a diagnosis of COMM. The data collected included sex, age, weight and breed as well as tumour localisation and size, and tumour-node-metastasis (TNM) classification based on Owen LN.[38] For both the staging and the evaluation of the general health condition, all dogs underwent a complete clinical examination, a complete blood examination (complete blood count and biochemistry) and a more specific cardiological evaluation (echocardiography or electrocardiogram or both) when indicated; imaging included X-rays of the thorax (three views) and abdominal ultrasound or total body computed tomography (CT). Dogs were excluded from the study if distant metastasis were detected before surgery. Dogs with concurrent

diseases capable of negatively influencing a minimum follow-up of 1 year (mild to severe renal, hepatic or cardiac diseases or other tumours) were also excluded. In addition, all the adjuvant treatments adopted were recorded: metronomic therapy (piroxicam + cyclophosphamide + thalidomide), standard chemotherapy (cisplatin or carboplatin), radiotherapy (hypofractionated protocol) and anti-CSPG4 DNA electrovaccination.[27-29]

The surgical procedure used to resect COMM, excising only the macroscopic tumour without any attempt to include any or just a few millimetres of macroscopically normal surrounding tissue, was considered to be a marginal excision. If the excision was considered to have a curative intent (for local tumour control only), an attempt was made to include at least 1.5 cm up to 2 cm of macroscopically normal bone, soft tissues or both (depending on the tumour location), independent of the result of the histological evaluation of the inked excision margins (infiltrated or non-infiltrated margins at histology). Moreover, the authors. decided to consider as curative an *en bloc* resection performed immediately after a cytological evaluation or within 1 month from a previous incisional or marginal excisional biopsy.

The histological data included the evaluation of the excision margins (not infiltrated, infiltrated or unknown), the mitotic index (MI) (<4/10 high power fields [HPF] or ≥4/10 HPF) and Ki67 expression (<19.5% or ≥19.5%).[5,6] All the excised regional lymph nodes were sent to the histology laboratory for evaluation; if not excised, the mandibular lymph nodes were aspirated and cytologically evaluated.

The entire population of dogs was divided and evaluated based on the type of surgery performed (group 1, curative surgery; group 2, marginal excision). Subsequently, only vaccinated dogs were considered and were divided according to the type of surgery performed (group 3, curative surgery plus electrovaccination; group 4, marginal surgery plus electrovaccination). The DNA electrovaccination procedure was performed only in dogs with COMMs which were characterized by a CSPG4 immunohistochemical expression ≥3/8[37] which was selected as a cut-off value for inclusion in the immunization group. Dogs, under brief general anaesthesia, were vaccinated with plasmids coding for the CSPG4 antigen. The vaccination was started 1 to 3 weeks after surgery and was repeated after 2 weeks and then monthly for a minimum of 6 and a maximum of 24 immunizations. The CSPG4-coding plasmids (500 μg in 200 μL of 0.03% NaCl) were injected into the muscles of the caudal thigh and, 2 minutes later, nine electric pulses (1 high voltage, amplitude 450 V, length 50 milliseconds, frequency 3 HZ; 1 second pause; eight low-voltage amplitude 110 V, length 20 milliseconds, pause 300 milliseconds) were applied to the injection site using the CLINIPORATOR (Igea), an instrument already approved for veterinary application. The dogs were monitored for acute, late local or systemic side effects.[27-29]

2.3 | Patient monitoring

Vaccinated dogs received a monthly re-examination during which a clinical examination, blood examinations and a total body CT scan were performed. Dogs that were not vaccinated had re-examination every 3 months in the first year and every 6 months in the second year. At each of these re-examinations, clinical examination, blood examinations, chest radiographs and abdominal ultrasound were performed.

2.4 | Statistical analysis

The analyses were carried out using GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, San Diego, California, www.graphpad.com), with statistical significance set at a $P < .05$. The data were summarized using descriptive statistics, and were indicated as mean, median and range. Distribution was checked graphically using the Shapiro-Wilk Test; the Wilcoxon Rank Sum Test was then utilized to evaluate the possible differences within the groups for age, weight, Ki67 expression, MI and clinical tumour stage. The median DFI and survival time (MST) were evaluated using the Kaplan-Meier method; the log-rank test was used to calculate the DFI and MST of the dogs in the two treatment groups (curative intent—group 1—vs marginal excision—group 2), and of the subpopulation of dogs which received the anti-CSPG4 DNA vaccination and curative intent surgery (group 3) vs vaccination and marginal surgery (group 4). The DFI was calculated from the day of surgery to the first tumour recurrence or metastasis and ST as the period from the day of surgery to the patient's death. Dogs which died from non-COMM-related causes, those lost to follow-up and those still alive at the end of the study were censored. Finally, the Fisher's exact test was used to test a potential association between treatment types and the probability of local recurrence and/or metastasis.

3 | RESULTS

3.1 | Demographics

One-hundred and fifty-five client-owned dogs were enrolled, of which 61 were female (46 spayed and 15 intact) and 94 were male (26 castrated and 68 intact). The mean and the median ages at presentation were 11.3 and 12.0 years, respectively (range 4-17 years); the mean and the median weights were 22.0 and 21.0 Kg, respectively (range 2-55 kg; Table 1). Approximately one third of the dogs were mixed breeds (53/155, 34.2%) while the remaining dogs (102/155, 65.8%) belonged to 36 different pure breeds. In particular, there were Cocker Spaniels (13 dogs), Golden Retrievers (12 dogs), German Shepherds (8 dogs), 5 each of Dachshunds and Yorkshire Terriers, 4 each of Beagles, Dwarf Schnauzers, Labrador Retrievers and Pinschers, 3 each of Giant Schnauzers, Pekingese, Rottweilers and English Setters, with the remaining dogs belonging to 23 different breeds.

3.2 | Tumour location, clinical staging and adjuvant treatment

The COMM was localized at the level of the gum of the lower arcade in 56 dogs (36.1%), the gum of the upper arcade in 36 dogs (23.2%),

TABLE 1 Clinical characteristics of the dogs enrolled in the study

		Overall population (155)	Curative intent surgery (109)	Marginal surgery (46)
Age (Years)	Mean	11.3	11.6	10.7
	Median	12	12	11
	Range	4-17	4-17	5-14.5
Weight (kg)	Mean	22	21.3	23.7
	Median	21	19	24
	Range	2-55	2-52	3-55
Sex (%)	Female intact	15 (9.7%)	9 (8.3%)	6 (13%)
	Spayed	46 (29.7%)	31 (28.4%)	15 (32.6%)
	Male intact	68 (43.8%)	49 (45%)	19 (41.4%)
	Neutered	26 (16.8%)	20 (18.3%)	6 (13%)
Localisation (%)	Mandible	56 (36.1%)	40 (36.7%)	16 (34.8%)
	Maxilla	36 (23.2%)	26 (23.9%)	10 (21.8%)
	Cheek	22 (14.2%)	19 (17.4%)	3 (6.5%)
	Lip	21 (13.5%)	18 (16.5%)	3 (6.5%)
	Tongue	10 (6.5%)	0 (0%)	10 (21.8%)
	Palate	8 (5.2%)	6 (5.5%)	2 (4.3%)
	Tonsil	2 (1.3%)	0 (0%)	2 (4.3%)
Clinical stage (%)	Stage I	24 (15.5%)	19 (17.4%)	5 (10.9%)
	Stage II	54 (34.8%)	32 (29.4%)	22 (47.8%)
	Stage III	66 (42.6%)	50 (45.9%)	16 (34.8%)
	Stage IV	11 (7.1%)	8 (7.3%)	3 (6.5%)

the mucosa of the cheek in 22 dogs (14.2%), the mucosa of the lip in 21 dogs (13.5%), the tongue in 10 dogs (6.5%), the palatine mucosa in 8 dogs (5.2%), and the tonsil in 2 dogs (1.3%) (Table 1).

Curative intent surgery consisted of 45 mandibulectomies, 27 maxillectomies and 37 cheek/lip *en bloc* excisions with or without mucosal reconstruction or skin flap reconstructions or a combination of both.

Mandibular lymphadenectomies were performed in 135 dogs, in 42 cases bilaterally (in three, the medial retropharyngeal lymph nodes were also removed) while in 93 dogs only the ipsilateral mandibular lymph nodes were removed. In 20 dogs, the lymph nodes were evaluated using fine needle aspiration and cytological examination only. The overall metastatic rate at the level of the regional lymph nodes was 41.9% (65/155). In particular, 63/135 (46.7%) of the nodes surgically excised were histologically metastatic (20/42 [47.6%] bilaterally removed and 43/93 [46.2%] ipsilaterally removed); of the 20 cases in which only a cytological evaluation was performed, only 2 (10%) were metastatic. This allowed establishing the definitive postoperative tumour stage (parameter N of the TNM system).[38]

Clinical staging identified 24 stage I (15.5%), 54 stage II (34.8%), 66 stage III (42.6%) and 11 stage IV (7.1%) COMMs (bilateral metastasis at the level of the regional lymph nodes only); stage IV COMMs with distant metastasis were excluded (Table 1).

Data regarding adjuvant treatment are summarized in Table 2. Eighty-two dogs underwent adjuvant DNA electrovaccination against CSPG4, 10 dogs received adjuvant radiotherapy, 40 dogs had metronomic treatment based on piroxicam, cyclophosphamide and thalidomide, eight received standard chemotherapy (cisplatin in four dogs and carboplatin in other four dogs) while electrochemotherapy was used in five dogs (with bleomycin intravenous injection; Table 2).

3.3 | Histological evaluation and immunohistochemical characterization of COMMs

Histology of the excision margins identified 68 (43.9%) COMMs with non-infiltrated margins (67 curative intent and 1 marginal) and 43 (27.7%) COMMs with infiltrated margins (11 curative intent and 32 marginal); the excision margin status was unknown in 44 (28.4%) COMMs (31 curative intent and 11 marginal; Table 3).

The mitotic index was ≥4/10 HPF in 102 COMMs, <4/10 HPF in 23 COMMs and was not available in 30 COMMs. The Ki67 expression was ≥19.5% in 83 COMMs, <19.5% in 29 cases and not available in 43 COMMs (Table 3). Immunohistochemistry for CSPG4 was <3 in 40 COMMs (26%); the dogs bearing this COMM were only operated and not vaccinated.

3.4 | Follow-up and statistical data

One-hundred and nine dogs (70.3%) experienced progressive disease, of which 41 (37.6%) had a local recurrence only, 23 (21.1%) both a

TABLE 2 Adjuvant therapy used in the dogs enrolled in the study

	Overall population (155)	Curative intent surgery (109)	Marginal surgery (46)
Anti-CSPG4 electrovaccination	82 (52.9%)	51 (46.8%)	31 (67.4%)
Metronomic chemotherapy	40 (25.8%)	22 (20.2%)	18 (39.1%)
EV chemotherapy	8 (5.2%)	7 (6.4%)	1 (2.2%)
Radiation therapy	10 (6.5%)	5 (4.6%)	5 (10.9%)
Electrochemotherapy	5 (3.2%)	3 (2.7%)	2 (4.4%)

TABLE 3 Histological and immunohistochemical parameters of canine oral malignant melanoma present in the study

		Overall population (155)	Curative Intent Surgery (109)	Marginal Surgery (46)
Margins	Not infiltrated	68 (43.9%)	67 (61.5%)	1 (2.2%)
	Infiltrated	43 (27.7%)	11 (10.1%)	32 (69.6%)
	Unknown	44 (28.4%)	31 (28.4%)	13 (28.2%)
Mitotic index (MI)	≥4/10 HPF	102 (65.8%)	64 (58.7%)	38 (82.6%)
	<4/10 HPF	23 (14.8%)	17 (15.6%)	6 (13.0%)
	Unknown	30 (19.4%)	28 (25.7%)	2 (4.4%)
Ki67	≥19,5	83 (53.6%)	54 (49.5%)	29 (63.0%)
	<19,5	29 (18.7%)	16 (14.7%)	13 (28.3%)
	Unknown	43 (27.7%)	39 (35.8%)	4 (8.7%)

TABLE 4 Follow-up of the dogs enrolled in the study

		Local recurrence	Distant metastasis (lung)	Both metastasis and local recurrence
Overall population (155)	Alive (14)	1 (7.1%)	3 (21.4%)	0 (0.0%)
	COMM-related Death (85)	27 (31.8%)	36 (42.3%)	22 (25.9%)
	Unrelated death (50)	11 (22.0%)	6 (12.0%)	1 (2.0%)
	Lost to follow-up (6)	2 (33.3%)	0 (0.0%)	0 (0.0%)
Curative intent surgery (109)	Alive (12)	1 (8.3%)	2 (16.7%)	0 (0.0%)
	COMM-related death (57)	22 (38.6%)	25 (43.9%)	10 (17.5%)
	Unrelated death (36)	7 (19.4%)	2 (5.6%)	1 (2.8%)
	Lost to follow-up (4)	1 (25%)	0 (0.0%)	0 (0.0%)
Marginal surgery (46)	Alive (2)	0 (0.0%)	1 (50.0%)	0 (0.0%)
	COMM-related death (28)	5 (17.8%)	11 (39.3%)	12 (42.9%)
	Unrelated death (14)	4 (28.6%)	4 (28.6%)	0 (0.0%)
	Lost to follow-up (2)	1 (50.0%)	0 (0.0%)	0 (0.0%)

local recurrence and distant metastasis, and 45 (41.3%) distant metastases only (Table 4). At the end of the study, 14 (9.0%) dogs were still alive (range 386-2632 days) while 135 (87.1%) had died, 85 (62.9%) of which from COMM-related causes, and 6 (3.9%) were lost to follow-up.

When the dogs were divided based on the type of surgery, 109 dogs had undergone a curative intent (group 1, 70.3%) and 46 dogs a marginal (group 2, 29.7%) excision. The MST in groups 1 and 2 was 594 (range 46-2632 days) and 458 days (range 149-1063 days), respectively; no statistical difference was found (P = .57, Figure 1). The DFI in groups 1 and 2 was 232 (range 22-2632 days) and 183 days (range 13-1049 days), respectively; a statistical difference was found (P = .008, Figure 2).

Since many dogs underwent adjuvant anti-CSPG4 DNA electrovaccination, they were additionally subdivided with the aim of verifying how much the immunological treatment had impacted the outcomes. Eighty-two dogs (52.9%) underwent anti-CSPG4 DNA electrovaccination, of which 51 had curative intent surgery (group 3, 62.2%) and 31 a marginal resection (group 4, 37.8%). The MST in groups 3 and 4 was 1333 days (range 78-2632 days) and 470 days (range 187-1063 days), respectively; a statistical difference was found (P = .03, Figure 3). The DFI in Groups 3 and 4 was 324 (range

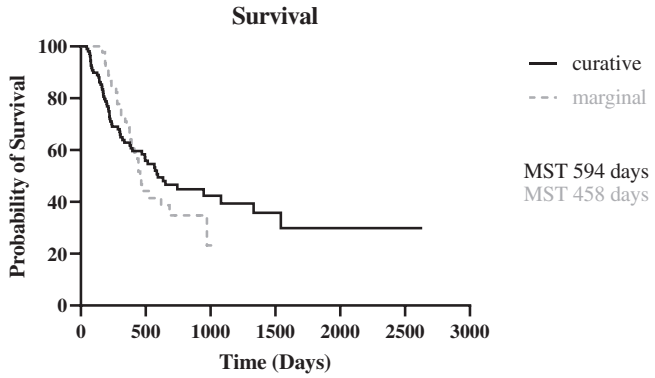

FIGURE 1 Groups 1 and 2. Median survival time (MST) for curative intent vs marginal surgery

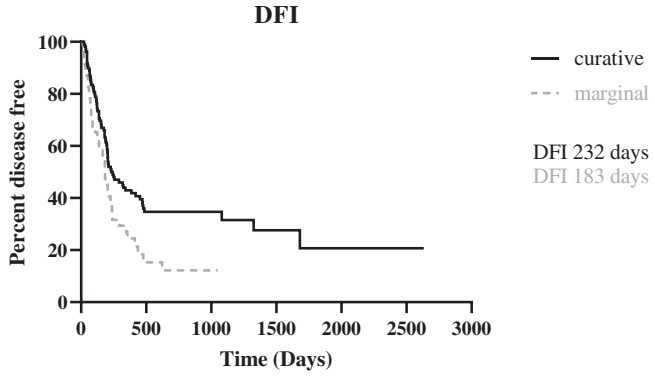

FIGURE 2 Groups 1 and 2. Disease-free interval (DFI) for curative intent vs marginal surgery

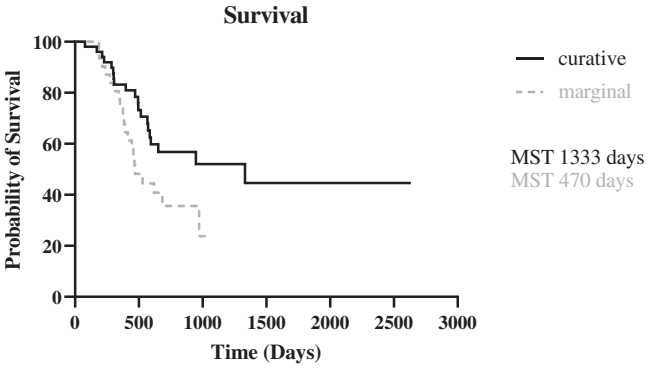

FIGURE 3 Groups 3 and 4. Median survival time (MST) for curative intent vs marginal surgery in vaccinated dogs

37-2632 days) and 184 days (range 13-1049 days), respectively, with a significant difference between the two groups (P = .008, Figure 4).

The survival and disease-free rates at 6, 12, 18 and 24-months are reported in Table 5.

No association between the type of surgical excision performed and the occurrence of local recurrence, either in the entire canine population (P = .48) or in the dogs receiving the anti-CSPG4 DNA

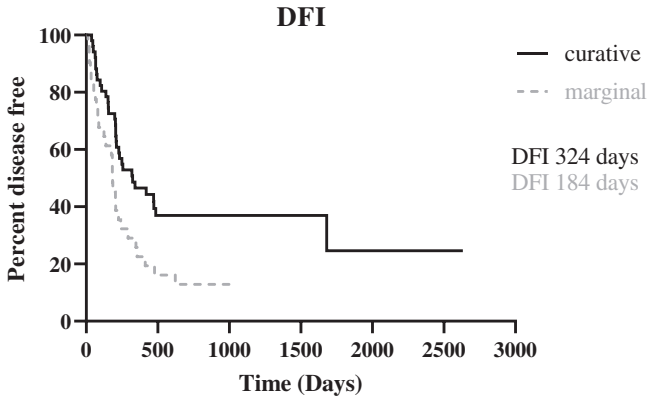

FIGURE 4 Groups 3 and 4. Disease-free interval (DFI) for curative intent vs marginal surgery in vaccinated dogs

electrovaccination (P = .49) was demonstrated. No association was also found when the histology of the excision margins was compared with the outcome (P = .18). Instead, a significant association was found when the comparison was between the type of surgical excision (curative intent vs marginal) and progressive disease (local recurrence, metastasis only or recurrence plus distant metastasis) in both the overall population (P = .03) and in vaccinated dogs (P = .02). Finally, a significant association was found in the vaccinated dogs between the status of the histological excision margins and local recurrence (P = .04; Table 6).

4 | DISCUSSION

This retrospective study describes 155 dogs with COMMs treated with surgery, of which 82 dogs also received adjuvant anti-CSPG4 DNA electrovaccination. Compared with the existing literature, no relevant difference regarding age, breed, sex, weight and site of growth of the COMM was found in this population of dogs or among the groups.[1-3,39]

The dogs included in the study were first divided into two groups, the first including dogs which had undergone curative intent surgery, such as mandibulectomy, maxillectomy or lip/cheek *en bloc* resection, followed, if required, by plastic reconstruction while the second group included those dogs which had undergone a marginal excision only, with no locally curative intent. Primary COMMs of the tongue and tonsils were also included in the second group. The goal of this division was to look for a potential statistical difference in outcome between these two groups of dogs.

The evaluation of these two groups did not reveal any significant difference in terms of Ki67 expression, MI and clinical stage, thus confirming a uniform distribution of the prognostic indicators reported.[5,6] The MST in group 1 was longer than in group 2; however, no significant difference was found (P = .57). Instead, significance was demonstrated when the DFI of these two groups was evaluated (P = .008). It should be noted that the MST recorded in both groups 1 and 2 was superior to that reported in the study of Boston et al. (2014) in which

TABLE 5 Survival in months and DFI rate

		Overall population (155)		Vaccinated dogs (82)	
	Months	Curative intent surgery (109)	Marginal surgery (46)	Curative intent surgery (51)	Marginal surgery (31)
Survival rate	≥6	78.9%	95.6%	94.1%	100%
	≥12	51.1%	65.2%	72.5%	74.2%
	≥18	39.8%	35.3%	54%	40%
	≥24	28.1%	20%	35.5%	23.3%
DFI rate	≥6	65.1%	52.2%	72.5%	58%
	≥12	37.6%	21.7%	43.1%	22.6%
	≥18	25.9%	11.1%	30%	16.6%
	≥24	22.3%	6.7%	24.4%	10%

TABLE 6 Fisher's Exact test

	Curative intent surgery	Marginal surgery	*P* value	Clear margins	Infiltrated margins	*P* value
Overall population (155)						
Local recurrence	43 (39.4%)	21 (45.7%)	.48	28 (41.2%)	21 (48.8%)	.44
No recurrence	66 (60.6%)	25 (54.3%)		40 (58.8%)	22 (51.2%)	
Total	109	46		68[a]	43[a]	
Progressive disease	71 (65.1%)	38 (82.6%)	**.03**	47 (69.1%)	35 (81.4%)	.18
Stable disease	38 (34.9%)	8 (17.4%)		21 (30.9%)	8 (18.6%)	
Total	109	46		68[a]	43[a]	
Vaccinated dogs (82)						
Local recurrence	23 (45.1%)	17 (54.8%)	.49	20 (51.3%)	14 (82.4%)	**.04**
No recurrence	28 (54.9%)	14 (45.2%)		19 (48.7%)	3 (17.6%)	
Total	51	31		39[a]	17[a]	
Progressive disease	32 (62.8%)	27 (87.1%)	**.02**	27 (69.2%)	16 (94.1%)	.08
Stable disease	19 (37.6%)	4 (12.9%)		12 (30.8%)	1 (2.9%)	
Total	51	31		39[a]	17[a]	

Note: Associations between type of surgery/histological margins and the probability of local recurrence and/or progressive disease. Bold values are for those that are statistically significant

[a]Unknown margins were not considered.

a large proportion of dogs were only operated on (MST of 352 days), and the use of different adjuvant therapies did not substantially change the outcome (MST of 335 days)[15]; it was however lower when compared with the results of the study of Tuohy et al. (2014) in which the MST was 723 days.[40] In the latter study, however, 74.3% of the COMMs were stages I and II (51.4% and 22.9% respectively), and only 20% were stages III and IV (18.6% and 1.4% respectively) while, in the present study, 50.3% of COMMs were stages I and II and 49.7% were stages III and IV (Table 1). The elevated number of higher stages of COMMs in the present population may explain this difference.[40]

An important variable in this study was that many of the dogs in both group 1 and group 2 also underwent anti-CSPG4 DNA electrovaccination, which had already demonstrated to be able to increase both MST and DFI in COMMs.[27-29] The authors' previous and ongoing studies have demonstrated the high expression and prevalence of the CSPG4 antigen in COMMs.[27-29,36,37] The CSPG4 antigen has several interesting features ie high expression in the cell membranes of neoplastic cells and low expression in the cells of healthy tissues as well as playing a key role in multiple tumourigenic processes.[36] Authors believe that these features make this molecule an ideal target in COMMs; DNA vaccination has been demonstrated to be safe in companion animals and effective in inducing a specific humoral and cellular response which could be long lasting.[25,26,41,42] All these factors prompted the Authors to investigate the anti-CSPG4 DNA vaccination as an adjuvant option in treating COMM patients.[27,28] Both the latter studies and the ongoing clinical trials have revealed the ability of anti-CSPG4 DNA electrovaccination to induce a significant antibody response capable of binding the antigen and likely inducing its down-modulation, impairing its tumourigenic function.[27-29 and unpublished data]

The rate of CSPG4 expression at immunohistochemistry in this series of dogs was not prognostic, as also already previously reported.[27] Additionally, when not vaccinated dogs were compared based on the cut-off value of CSPG4, that is, < 3 vs ≥ 3, there was no

significant difference regarding the MST (280 days vs 320 days [P = .57]).

In order to avoid any potential confounding elements in evaluating the impact of the two different surgical approaches (curative intent vs marginal) on the outcome, only vaccinated dogs receiving a curative intent (group 3) or a marginal (group 4) surgical excision were compared. Analysis showed a significant difference fin both MST (P = .03) and DFI (P = .008), thus emphasizing the role of local surgical control in dogs subsequently treated with anti-CSPG4 electrovaccination in an attempt to also improve systemic control.

This study had some limitations. First of all, albeit this study dealt with COMMs treated in a single institution, it was a retrospective study which spanned a wide period of time during which imaging procedures to detect systemic metastases progressively changed (from three view X-rays of the thorax and abdominal ultrasound to total body CT scan), the latter being the most used in recent years); even the procedures for histological evaluation of the excision margins have been progressively improved. It should be noted that the histological evaluation of the excision margins was not available in 44/155 (28.4%) cases.

The second issue was relative to the evaluation of the regional lymph node status. In the past, this evaluation was based on cytology only or on its/their removal only when enlarged; recently it has been shown that this method does not reliably determine the N parameter of the TNM staging system.[9,43] Currently, lymph node staging is based on their surgical removal and histologic examination, regardless of size and shape; in addition, more accurate imaging techniques, addressed to identifying either the regional or, more specifically, the sentinel lymph nodes, have been implemented.[44-50] According to this, some of the dogs in the present study had undergone cytology only (older cases), others ipsilateral mandibular lymph node excision, others ipsilateral mandibular and medial retropharyngeal lymph node excision, and, more lately, bilateral and medial retropharyngeal lymph node excision. At present, the latter is the procedure which the authors routinely perform for COMM. Finally, the results of the cytological vs histological evaluation of the regional lymph nodes reported herein would indicate that histology appears to be more accurate than cytology in establishing the lymph node status (10% of the lymph nodes examined cytologically vs over 40% in those examined histologically).

The above limitations may have underestimated the tumour clinical stage, especially of the older cases but, on the other hand, this should be considered favourably when compared with the most recent retrospective studies.[15,40]

An important difference between the vaccinated (vaccination is still a clinical trial approved by the Italian Ministry of Health) and not vaccinated dogs is the closer monitoring of the former. As a consequence, local recurrences, systemic metastasis or both may have been diagnosed later in not vaccinated dogs compared with vaccinated dogs. Therefore, the DFI in not vaccinated dogs may have been overestimated. However, this unequal follow-up does not influence the overall survival data.

At present, the authors do not have any specific data to present regarding metronomic therapy. They strongly believe that this would require a clinical trial addressed only to the evaluation of its efficacy. This variable was very difficult to monitor; however, it may be said from an empirical point of view that this treatment was capable, likely together with electrovaccination, of stabilizing the disease in many cases, apparently avoiding the rapid progression of metastasis of COMM. However, additional studies on this treatment are warranted.

It can be concluded that a curative intent surgical approach, when feasible, is advisable in an attempt to prolong both the DFI and survival. The positive association between the type of surgery and the outcome confirmed this aspect; an association was also found when the histological status of the excision margins was considered in the vaccinated dogs; however, this was not the case in the overall population. The latter result should be considered cautiously as the histological evaluation of the excision margins was not available in all cases. Finally, it can also be concluded that adjuvant anti-CSPG4 DNA electrovaccination may additionally improve the outcome. Therefore, in the authors' experience a curative intent surgical procedure plus an adjuvant anti-CSPG4 DNA electrovaccination represented a valid therapeutic approach for COMM when considering MST, DFI, survival time and DFI rates.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with the article.

DATA AVAILABILITY STATEMENT

Encourages Data Sharing

ORCID

Mariateresa Camerino https://orcid.org/0000-0003-2850-6794
Federica Cavallo https://orcid.org/0000-0003-4571-1060
Sara Del Magno https://orcid.org/0000-0002-2900-6989
Raffaella De Maria https://orcid.org/0000-0002-7028-2280
Paolo Buracco https://orcid.org/0000-0002-0181-0887

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How to cite this article: Giacobino D, Camerino M, Riccardo F, et al. Difference in outcome between curative intent vs marginal excision as a first treatment in dogs with oral malignant melanoma and the impact of adjuvant CSPG4-DNA electrovaccination: A retrospective study on 155 cases. *Vet Comp Oncol*. 2021;19:651–660. https://doi.org/10.1111/vco.12690