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6 **A COMBINATION OF ELECTROCHEMOTHERAPY, GENE**
7 **ELECTROTRANSFER OF PLASMID ENCODING CANINE IL-12**
8 **AND CYTOREDUCTIVE SURGERY IN THE TREATMENT OF**
9 **CANINE ORAL MALIGNANT MELANOMA**

10

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29 **ABSTRACT**

30 The aim of this study was to evaluate the safety and efficacy of the
31 combination of electrochemotherapy (ECT) with bleomycin and gene
32 electrotransfer (GET) of plasmid encoding canine interleukin 12 (IL-12) for
33 the treatment of canine oral malignant melanoma (OMM). Our focus was to
34 determine the effect of the treatment on achieving local tumor control and
35 stimulation of an antitumor immune response. Nine dogs with histologically
36 confirmed OMM stage I to III were included in a prospective, non-
37 randomized study. The dogs were treated with a combination of cytoreductive
38 surgery, ECT and IL-12 GET, which was repeated up to five times, depending
39 on the clinical response to the treatment, evaluated according to the follow-
40 up protocol (7, 14 and 28 days after, the last treatment). One month after
41 treatment, the objective response (OR) rate was 67% (6/9). Median survival
42 time (MST) was 6 months and, even though the disease progressed in 8/9
43 patients at the end of the observation period (2 to 22 months), four animals
44 were euthanized due to tumor-unrelated reasons. In addition, we observed a
45 decline in the percentage of regulatory T cells (T_{reg}) in the peripheral blood in
46 the course of the treatment, which could be attributed to a systemic antitumor
47 response to IL-12 GET. The results of this study suggest that a combination
48 of ECT and IL-12 GET may be beneficial for dogs with OMM, especially
49 when other treatment approaches are not acceptable due to their invasiveness
50 or cost.

51

52 **KEYWORDS:** cytoreductive surgery, dogs, electrochemotherapy,
53 interleukin-12, oral malignant melanoma, regulatory T cells

54

55 **DECLARATION OF INTEREST:** None.

56

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62 INTRODUCTION

63 Oral malignant melanoma (OMM) is the most common malignancy of the
64 oral cavity in older dogs (Ramos-Vara et al., 2000), with a high rate of
65 invasion into surrounding bone and metastasis (Ramos-Vara et al., 2000;
66 Withrow and MacEwen, 2013).

67 Dogs with untreated OMM have a median survival time (MST) of 2 months
68 (Harvey et al., 1981). MST for surgically excised OMM ranges from 3 to 17
69 months, depending on the clinical stage of the tumor (Grosenbaugh et al.,
70 2011; MacEwen et al., 1986; Owen, 1980; Sarowitz et al., 2017). In cases of
71 non-resectable or incompletely excised tumors, alternative and/or adjuvant
72 therapies are warranted, mostly radiotherapy (Proulx et al., 2003) or possibly
73 a combination of radiotherapy and chemotherapy (Cancedda et al., 2016;
74 Freeman et al., 2003). Because of the high metastatic potential of the tumor,
75 there is a need for an efficient systemic treatment; the effect of chemotherapy
76 was evaluated in several studies, but its' use as a sole treatment is not
77 recommended (Boria et al., 2004; Boston et al., 2014; Brockley et al., 2013;
78 Dank et al., 2014; Rassnick et al., 2001; Tuohy et al., 2014). As OMM is
79 considered highly immunogenic, many immunotherapeutic modalities with
80 some effect on increasing the MST have been implemented for its treatment
81 (Bianco et al., 2003; Boston et al., 2014; Dow et al., 1998; Finocchiaro and
82 Glikin, 2012; Grosenbaugh et al., 2011; McLean and Lobetti, 2015; Ottinod
83 et al., 2013; Riccardo et al., 2014; Thamm et al., 2010). One of the main
84 features of malignant cells is immune evasion, which is the result of the
85 secretion of cytokines (e.g., interleukin-10 (IL-10), transforming growth
86 factor- β (TGF- β)) in the tumor microenvironment by tumor cells themselves
87 and regulatory T cells (T_{reg}) (Atherton et al., 2016).

88 A significant increase in the percentage of T_{reg} was found in the peripheral
89 blood of dogs with OMM compared to healthy dogs (Atherton et al., 2016;
90 Tominaga et al., 2010), and this increase correlated with the clinical stage of
91 the tumor (Horiuchi et al., 2010).

92 One of the emerging treatments in veterinary oncology is
93 electrochemotherapy (ECT) (Impellizeri et al., 2016; Kodre et al., 2009;
94 Maglietti et al., 2017; Spugnini et al., 2013; Tamzali et al., 2012; Tozon et al.,
95 2016, 2014) which disrupts the tumor cells locally, but has yet no proven
96 effect on distant metastases when used as a sole treatment (Heppt et al., 2016;
97 Sersa et al., 2015).

98 Gene electrotransfer of plasmid encoding interleukin 12 (IL-12 GET) is an
99 efficient method for tumor control (Cemazar et al., 2016; Cutrera et al., 2015;
100 Reed et al., 2010), especially when the plasmid is injected intratumorally
101 (Pavlin et al. 2009). IL-12 activates primarily NK cells and T lymphocytes.
102 Moreover, IL-12 increases the secretion of IFN- γ from activated T cells,
103 which causes an increase in the production of IL-12 from antigen presenting
104 cells (Colombo and Trinchieri, 2002). ECT combined with IL-12 GET was
105 already used in two dogs with OMM in which a stable disease (Cutrera et al.,
106 2015) or momentarily reduced tumor volume was obtained (Reed et al.,
107 2010). However, an evaluation of this approach in a larger cohort of dogs with
108 OMM has not been performed yet. Therefore, the aim of this study was to
109 evaluate the safety and efficacy of the combination of ECT and IL-12 GET in
110 dogs with OMM. For this purpose, we analyzed the tumor response, local and
111 systemic toxicity and immune response by measuring lymphocyte
112 subpopulations in the peripheral blood.

113

114 **Materials and methods**

115 *Animals and tumors*

116 Nine dogs with histologically confirmed OMM between March 2016 and
117 April 2018 were included in this study (Table 1). Animals were included
118 when the owners declined standard treatment (surgery and/or radiotherapy)
119 and gave a written consent for participation in the clinical study. All animals
120 were in good general health and had basic hematology and biochemistry

121 profiles within the reference limits. Before the treatment, staging was
 122 performed according to the WHO staging criteria (Owen, 1980), along with a
 123 physical examination, basic bloodwork, an examination of three-view
 124 thoracic radiographs (AXIOM Iconos R100, Siemens healthcare, Erlangen,
 125 Germany) and abdominal ultrasonography (Logiq S7 Pro, GE Healthcare,
 126 Solingen, Germany). Fine-needle aspiration biopsy with subsequent
 127 cytological evaluation was performed in four patients with enlarged
 128 mandibular lymph nodes. Basic bloodwork consisted of a complete blood
 129 count with a differential white blood cell count, which was performed using
 130 an automated laser hematology analyzer with species-specific software
 131 (Advia 120, Siemens, Munich, Germany). The automated chemistry analyzer
 132 RX-Daytona (Randox, Crumlin, UK) was used to measure the following
 133 biochemical parameters: blood urea nitrogen (BUN), creatinine, alkaline
 134 phosphatase (ALP) and alanine aminotransferase (ALT). Incisional or
 135 excisional biopsies collected for histopathology were fixed in 10% buffered
 136 formalin, embedded in paraffin, sectioned at 4 μ m thickness and stained with
 137 hematoxylin and eosin. At the histopathological examination all OMM
 138 samples were specifically assessed for the following characteristics: level of
 139 infiltration/invasion, nuclear atypia, mitotic index, the presence of lymphatic
 140 invasion, the presence of necrosis and lymphocytic/mononuclear infiltrates,
 141 according to malignancy criteria for melanocytic neoplasms (Millanta et al.,
 142 2002). Completeness of the excision was assessed in all excisional biopsy
 143 samples. The results of the histopathological examination are presented in
 144 Table 2.

145 The tumor location was determined as caudal when the tumor was caudal to
 146 the third premolar tooth, or rostral when it was rostral to the third premolar
 147 tooth (Verstraete and Lommer, 2012). Furthermore, tumors were described as
 148 either mucosal or gingival (if arising from the dentate jaws).

149 Each nodule was measured in three perpendicular directions (a, b, and c) and
150 the tumor volume calculated using the following formula: $V = a \times b \times c \times$
151 $\pi \div 6$.

152

153 *Plasmid isolation*

154 The pCMVcaIL-12 plasmid, encoding canine IL-12, was constructed in our
155 previous study and thoroughly tested in *in vitro* and *in vivo* conditions
156 (Lamprecht et al., 2015; Lamprecht Tratar et al., 2018). The plasmid was
157 isolated using the Qiagen Endo-Free kit (Qiagen, Hilden, Germany)
158 according to the manufacturer's instructions and was diluted to a
159 concentration of 1 mg/ml in endotoxin-free water (Qiagen). Purified plasmid
160 DNA was subjected to quality control and quantity determinations, which was
161 performed by agarose gel electrophoresis and spectrophotometry.

162

163 *Treatment protocol*

164 Treatment was performed while the patients were under general anesthesia.
165 The dogs were premedicated with an intravenous administration of
166 midazolam (Midazolam Torrex, Torrex Pharma GesmbH, Vienna, Austria;
167 0.2 mg/kg), and general anesthesia was induced using propofol (Diprivan,
168 Zeneca, Grangemouth, United Kingdom; 3-6 mg/kg) and maintained with
169 isoflurane (Isoflurin, Vetpharma Animal Health, Barcelona, Spain) mixed
170 within 100 % oxygen. During anesthesia, the animals received Hartmann's
171 solution (B. Braun Melsungen AG, Melsungen, Germany) at a rate of 5
172 ml/kg/h. Dental charting, dental radiographs and periodontal treatment were
173 performed, but any other oral/dental treatments (apart from tumor-related
174 surgery) were postponed pending a response to the tumor-related treatment.
175 First, patients underwent intracapsular excision of the tumor ("cytoreductive
176 surgery"). After surgery, ECT was performed using an intravenous
177 application of bleomycin (Blenoxane, Bristol-Myers, NY, USA). Bleomycin

178 was dissolved at a concentration of 3 mg/ml and administered intravenously
179 at a dose of 0.3 mg/kg. Ten minutes after the intravenous injection of
180 bleomycin, eight electric pulses were delivered (each of a 100 μ s duration, an
181 amplitude to electrode distance ratio of 1300 V/cm and a frequency of
182 repetition of 5 kHz) with an electric pulse generator, Cliniporator™ (IGEA
183 s.r.l., Carpi, Italy), using two parallel stainless-steel noninvasive plate
184 electrodes 6 mm apart. Electric pulses were first delivered to the margins of
185 the surgical area in order to reduce the blood flow and then continued in
186 concentric circles to the center of the area. Noninvasive electrodes were used,
187 because the tumor was surgically removed and only the surface of the surgical
188 areas was treated.

189

190 Immediately after the first phase of the treatment, the second phase of the
191 treatment procedure, IL-12 GET, was performed. The plasmid encoding
192 canine IL-12 was injected submucosally in two sites close to the tumor in the
193 direction closest to the draining lymph nodes. Based on our previous
194 experience, the dose per patient used was 2 mg (Cemazar et al., 2017).
195 Immediately after the injection of the plasmid, electric pulses were delivered
196 using the aforementioned electric pulse generator and a multi-electrode array
197 (MEA, Iskra Medical, Podnart, Slovenia. MEA electrodes proved highly
198 efficient with minimal or no damage to skin in mice in preclinical studies
199 (Kos et al., 2017; Fig.1). The electrode consists of seven spring-loaded pins
200 arranged in a hexagonal mesh and spaced 3.5 mm apart. During the treatment,
201 a total of 24 electric pulses (two electric pulses between each electrode pair,
202 60 V, duration of 150 ms, frequency of 4 Hz) were delivered.

203 At the end of the procedure, all patients received a single intravenous
204 application of carprofen (Rimadyl, Pfizer, NY, USA; 4 mg/kg). Patients were
205 discharged from the hospital when they were fully recovered from anesthesia.
206 All patients received chlorhexidine-based oral gel (Stomodine gel, ICF,
207 Milan, Italy). In patients with a heavy tumor burden for which extensive

208 necrosis was expected, and in those that developed leukocytosis with
209 neutrophilia after treatment, a course of antibiotic treatment was introduced
210 (amoxicillin with clavulanic acid; Clavaseptin, Vetoquinol, Lure Cedex,
211 France; 20 mg/kg/12 h po) until the improvement of clinical signs and/or
212 laboratory parameters was achieved.

213

214 *Evaluation of treatment outcome and sample collections*

215 Local and systemic treatment outcomes and possible side effects were
216 evaluated at regular follow-up examinations at 7, 14 and 28 days and 2
217 months, 6 months and one year after treatment and then annually until the end
218 of the observation period (20 April 2018). At each time point, a clinical
219 examination of the patient, including measurements of the tumor and three-
220 view thoracic radiographs were performed. Furthermore, before treatment
221 and at each follow-up examination, blood samples were collected for
222 complete blood count and flow cytometry.

223 The local response to treatment was evaluated by measurements of the tumor
224 size. The response to the treatment was determined 4 weeks after the last
225 treatment and at the end of the observation period, and it was determined as a
226 complete response (CR), partial response (PR), stable disease (SD) or
227 progressive disease (PD) according to the Response Evaluation Criteria in
228 Solid Tumors (RECIST) (Nguyen et al., 2015). Possible side effects were
229 determined at each follow-up examination according to the Veterinary
230 Cooperative Oncology Group toxicity scale (VCOG–CTCAE) (VCOG–
231 CTCAE, 2016).

232

233 *Isolation of peripheral blood mononuclear cells (PBMC)*

234 Blood samples were examined for the presence of different subpopulations of
235 lymphocytes. Blood was drawn on the day of the treatment and 7 days and 4
236 weeks later. Peripheral blood mononuclear cells (PBMC) were isolated using
237 Ficoll-Paque™ Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

238 and Septmate (STEMCELL Technologies Inc., Vancouver, BC, Canada)
239 tubes. Two ml EDTA blood was diluted with 2 ml phosphate-buffered saline
240 (PBS) solution. The diluted blood was gently pipetted into the Septmate
241 (STEMCELL Technologies Inc) tubes, in which 4 ml Ficoll Paque™ Plus
242 (GE Healthcare Bio-Sciences AB) was pipetted beforehand. The samples
243 were centrifuged for 10 minutes at 1200 rpm (Heraeus MULTIFUGE 1S-R
244 Centrifuge, Thermo Fisher Scientific, Massachusetts, USA). The top layer
245 consisting of PBMC was poured into a new tube and mixed with the same
246 amount of PBS. The samples were centrifuged for 5 minutes at 1500 rpm
247 (Heraeus MULTIFUGE 1S-R centrifuge). The sediment containing the
248 PBMC was mixed with 1 ml serum-free freezing media (Biological
249 Industries, Beit HaEmek, Israel) and stored in cryovials at -80°C. For the flow
250 cytometry analysis, the cryovials containing PBMC were defrosted in a 37°C
251 bath. The content of the cryovials were mixed with 1 ml cold RPMI media
252 and centrifuged for 5 minutes at 3000 rpm (Heraeus MULTIFUGE 1S-R
253 centrifuge). The sediment was mixed with 12 ml RPMI media and put in the
254 Petri dish for 2 hours at 37°C in order to restore the viability of the cells. After
255 2 hours, the media containing the PBMC was centrifuged for 5 minutes at
256 1500 rpm (Heraeus MULTIFUGE 1S-R centrifuge). The PBMC of all nine
257 patients were defrosted, but the PBMC for one patient (patient 8) were not
258 used for the detection of cells with flow cytometry due to the poor viability
259 of the cells in that patient.

260

261 *Flow cytometry*

262 To define the immunophenotype, the cell suspension was divided into three
263 tubes to gate different lymphocyte subpopulations. The first tube was used
264 to detect leukocytes (CD45+), the second to determine the percentage of B
265 lymphocytes (CD21+) and T lymphocytes (CD3+), and the last tube to
266 determine the percentage of helper (CD4+), cytotoxic (CD8+) and
267 regulatory T cells (CD4+CD25+Foxp3+). The cells were stained with

268 appropriate amounts (5 or 10 μ l) of antibodies. The panel of antibodies used
 269 for surface staining included CD3 FITC (clone CA17.2A12) and CD21 PE
 270 (clone CA2.1D6) from Bio-Rad (California, USA) and CD45 eFluor® 450
 271 (clone YKIX716.13), CD4 PE-Cy7 (clone YKIX302.9), CD8a eFluor 450
 272 (clone YCATE55.9) and CD25 (clone P4A10) from eBioscience (MA,
 273 USA). After incubation in the dark for 20 minutes with surface antibodies,
 274 the cells were washed twice in PBS at 1500 rpm for 5 minutes (Hettich,
 275 Universal 32 centrifuge, Kirchleugern, Germany). The supernatant was
 276 discarded, and 300 μ l cell fix (BD Biosciences, CA, USA) was added.
 277 To detect the intracellular expression of canine Foxp3, the cells were fixed,
 278 permeabilized (Foxp3/Transcription Factor Staining Buffer Set Kit,
 279 eBioscience) and stained with the FOXP3 APC antibody (clone FJK-16s,
 280 eBioscience) according to the manufacturer's instructions. Briefly, after
 281 surface staining, the cells were fixed in 1 ml freshly prepared
 282 fixation/permeabilization working solution for 90 minutes. Thereafter, the
 283 cells were washed twice with permeabilization buffer. FOXP3 APC
 284 antibody and 100 μ l permeabilization buffer were added, incubated in the
 285 dark for 45 min, and then washed twice with 2 ml permeabilization buffer
 286 (1500 rpm, 5 minutes Hettich, Universal 32 centrifuge). After incubation,
 287 1.5 ml PBS was added to the sample, and the sample was mixed and
 288 centrifuged at 1500 rpm for 5 minutes (Hettich, Universal 32 centrifuge).
 289 The supernatant was discarded, and 200 μ l PBS was added.
 290 The samples were acquired using a ten-color flow cytometer, FACSCanto
 291 (BD Biosciences). During the sample acquisition, whole samples were
 292 measured. The measurement results were analyzed using BD FACSDiva
 293 software (v.8.0.1, BD Biosciences). For the results analysis, combined side-
 294 scatter (SSC) and the intensity of the CD45 surface marker gating was used
 295 for leukocyte gating (Fig. 2a). For the results analysis of the percentage of
 296 CD21 (B lymphocyte) and CD3 (T lymphocyte) (Fig. 2b), combined side-
 297 scatter (SSC) and surface marker (CD21/CD3) gating was used. We

determined FoxP3+ cells from the percentage of FoxP3 cells within the total (Fig. 2c) CD4 (helper T cell) lymphocyte population (FoxP3+, CD3+, CD4+) (Fig. 2d) and within the CD25+ and CD4+ lymphocyte population (Fig. 2e).

Data analysis

All data were tested for normality of distribution with the Shapiro-Wilk test. When data were normally distributed, the differences between the experimental groups were statistically evaluated by one-way analysis of variance (one-way ANOVA) followed by a Holm-Sidak test for multiple comparisons. When the normality test failed, Kruskal-Wallis one-way analysis of variance on ranks was used to compare the medians of our data. Non-normally distributed data were presented as the median and interquartile range (IQR), the range between the 1st (Q1) and 3rd (Q3) quartiles. A P value of less than 0.05 was considered to be statistically significant. MST was calculated with Kaplan-Meier survival analysis, and dogs that were euthanized due to tumor-unrelated reasons were censored for analysis. SigmaPlot software (Systat Software, San Jose, CA, USA) was used for graphical representations and for the statistical analysis.

Results

Treatment results

Nine dogs with histologically confirmed OMM, with tumor stages ranging from I to III, were enrolled in our study. In four patients (patients 2, 5, 4 and 7) with enlarged mandibular nodes, fine-needle lymph node aspiration biopsy was performed, and there was no evidence of metastatic disease in any of the sampled nodes. The decision about the repetition of treatment was discussed continuously, depending on the response to treatment. One month after the last treatment, the objective response (OR) rate was 67 % (6/9). At the end of

the observation period (2 to 22 months, median: 6 months), PD occurred in all but one of the patients. The achieved MST was 6 months, and four patients were euthanized due to tumor-unrelated reasons and were censored for the statistical analysis (Fig. 3). The stage, location and recurrence of the tumors did not influence the MST. A summary of the treatment results is presented in Table 3. Importantly, in patient 4, after the fourth (last) treatment (week 8), radiographic evidence of new mandibular bone formation/mineralization was observed, which was consistent with the complete clinical response to the treatment. Thereafter, at 7 months posttreatment, the dog developed multiple lung metastases and was euthanized 10 months after initial presentation due to tumor-unrelated causes (development of a histologically different cutaneous tumor) (Fig. 4).

To evaluate the possible systemic effects of the treatment, the Veterinary Cooperative Oncology Group toxicity scale (VCOG-CTCAE) was followed at each post-treatment examination. No major side effects of the treatment were noted, and the treatment was well tolerated by all patients. Muscle contractions were observed at the time of application of electric pulses but disappeared at the end of pulse delivery. The intravenous application of bleomycin did not result in any local or systemic toxicity. The only local side effect of treatment was necrosis of the tumor, and in four patients (patients 3, 4, 5 and 8), transient leukocytosis with neutrophilia occurred, which was resolved with a short course of antibiotic treatment. After finishing treatment with ECT and GET, none of the patients suffered from a local or systemic infection.

Flow cytometry

There was no difference in the percentages of helper (CD4+) and cytotoxic T (CD8+) cells before and after the treatment, but there was a difference in percentages of T_{reg}. The results showed that the percentage of T_{reg} declined by the end of the observation period. At the time of treatment, the median

percentage of T_{reg} was 1.2 % (Q1: 1.1 %, Q3: 2.8 %). This number decreased to 1.0 % after 4 weeks (Q1: 0.55 %, Q3: 2.13 %). At the end of the observation period, the percentage of T_{reg} was 0.3 % (Q1: 0.3 %, Q3: 0.58 %). The results showed a statistically significant difference in percentages of T_{reg} before and after the treatment (Fig. 5).

Discussion

The results of our study demonstrate that the combination of ECT and IL-12 GET is a safe and effective treatment for local tumor control in dogs with OMM. Furthermore, we proved that the percentage of T_{reg} in the peripheral blood declined over the course of the treatment, which could be attributable to a systemic antitumor immune response.

There were three major limitations of this study. The first is the small sample size, which makes it impossible to draw clear conclusions about the survival times and significant differences between patients with different stages of OMM. The second limitation is that fine-needle aspiration biopsies were not performed in all of the patients but rather just in those with enlarged lymph nodes, while it has been described that lymph node size is not always a predictive factor for metastasis (Williams and Packer, 2003). However, the correlation between cytology and histopathology for staging of lymph nodes in dogs with oral melanocytic neoplasms was recently found to be poor (Grimes et al., 2017). The third limitation is the unavailability of advanced diagnostic techniques (i.e., CT, MRI scanning, etc.) at the time of staging of the patients. The latter two limitations may have caused an underestimation of the clinical stages of the patients.

Because of the malignant biologic behavior of the tumor and high rate of metastasis, OMM remains one of the most challenging tumors for veterinary oncologists. The most successful treatment method is radical excision of the tumor, which frequently requires major maxillofacial surgical procedures,

possibly resulting in impaired function and cosmesis (Arzi et al., 2015; Sarowitz et al., 2017). OMMs are considered highly immunogenic; therefore, many immunotherapeutic modalities for its treatment have been implemented. Several different agents, e.g., tumor necrosis factor- α (TNF- α), interleukin-2 (IL-2), *Corynebacterium parvum*, granulocyte-macrophage colony-stimulating factor (GM-CSF), chondroitin sulfate proteoglycan-4 (CSPG4), liposome-encapsulated muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) and suicide gene therapy combined with a vaccine composed of tumor cells extracts and xenogeneic cells producing human IL-2 and GM-CSF, were proven to induce an antitumoral immune response. The achieved MST was from 6 to 23 months, depending on the clinical stage of the disease (Bianco et al., 2003; Dow et al., 1998; Macewen et al., 1999; MacEwen et al., 1986; Piras et al., 2017; Thamm et al., 2010), and not reached in one study (Finocchiaro and Glikin, 2012). A xenogenic human DNA-encoding tyrosinase protein vaccine (Oncept™, Merial®, Duluth, GA, U.S.A.) was developed for the adjuvant treatment of stage II to IV OMMs (Grosenbaugh et al., 2011). The vaccine prolonged the survival in two studies (MST of 29 months and not reached in another study) (Grosenbaugh et al., 2011; McLean and Lobetti, 2015), but did not prolong the survival time compared to dogs treated with surgery and/or radiotherapy in other studies (Boston et al., 2014; Ottinod et al., 2013). ECT combined with IL-12 GET already proved to be a safe and efficient method for treating tumors. A pronounced and long-term antitumor effect was observed in mast cell tumors with 72 % CR (median observation time: 40 months) (Cemazar et al., 2016), while on the histological level, an effective induction of a cellular response against neoplastic cells, characterized mainly by the recruitment of T-lymphocytes and macrophages and a fibrotic proliferation with reduction of microvessels, was seen (Salvadori et al., 2017). The same treatment was also already used in selected oral tumors, such as acanthomatous ameloblastoma and squamous cell carcinoma, in which ORs of 70 % and up to 100 % were

416 achieved (Cutrera et al., 2015, 2008; Reed et al., 2010). However, these
417 tumors are generally less locally aggressive and have a lower metastatic
418 potential and, thus, carry a better prognosis than OMM (Withrow and
419 MacEwen, 2013). The combination of ECT and IL-12 GET was attempted
420 for the treatment of two OMMs where SD (Cutrera et al., 2015) or PR (Reed
421 et al., 2010) was achieved. In our study, we achieved a 44 % CR at four weeks
422 after the treatment; however, in eight animals, the disease progressed at the
423 end of the observation period. The achieved MST was 6 months, and in
424 contrast with previously reported data, the clinical stage of the tumor did not
425 influence the MST in our study. Interestingly, the three patients with stage III
426 tumors all survived from 5 months to 12 months, and two of them were
427 euthanized due to tumor-unrelated reasons. These achieved survival times are
428 longer than the reported survival times for stage III tumors treated with radical
429 surgical excision (MST: 3 months) (Withrow and MacEwen, 2013). We
430 believe this finding might be attributable to the systemic effects of IL-12
431 GET, causing a delay in metastatic spread and boosting the antitumoral
432 effects of the immune system. The histopathological characteristics of the
433 tumors did not influence the MST and, therefore, are not presented in detail.

434 One of the main advantages of the combination of ECT and IL-12 GET is
435 minimal trauma and therefore better immediate post-treatment function and
436 cosmetic appearance when compared to major surgical procedures that are
437 commonly indicated for the treatment of OMM. The treatment described here
438 can also be performed on an outpatient basis and is repeated less frequently
439 compared to hypofractionated radiation therapy currently recommended for
440 OMM (Withrow and MacEwen, 2013). However, even though we managed
441 to increase the overall survival of the animals and achieved good local control
442 of the disease, we were unable to prevent the metastatic spread of the disease.
443 Therefore, improvements of the treatment protocols and possible markers for
444 the treatment planning are crucial for obtaining even better results of the
445 treatment.

One of the proposed markers for the treatment planning is the percentage of T_{reg} cells in the peripheral blood (Sersa et al., 2015). The mechanism of action of these cells is based on eliciting different suppressive molecules, receptors and cells. Among other roles, the direct suppressive role is based also on the suppression of effector T lymphocytes activity that play a pivotal role in tumor elimination (Chaudhary and Elkord, 2016). The group of Tominaga et al. (2010) showed that the percentage of T_{reg} in the peripheral blood of dogs with OMM was statistically significantly higher than in that of healthy dogs. In our study, the T_{reg} percentages were increased before the treatment and decreased in the following days after the treatment. At the end of the observation period, the percentage of T_{reg} evidently declined. This observation is in agreements with other studies in which the decline of T_{reg} after the immune treatment with IL-12 was observed (Zhao et al., 2012). The decline was also evident in the patients in which the disease progressed; therefore, the percentage of T_{reg} cannot serve as a prognostic marker, but our results demonstrated a negative impact of IL-12 on T_{reg} , cells that are associated with a poor prognosis. One of the reasons the tumors progressed despite the decrease of T_{reg} could be that the tumors used different pathways of immune evasion that still need to be elucidated.

Conclusion

In conclusion, our results suggest that a combination treatment with ECT and IL-12 GET may be beneficial for dogs with OMM, as it prolongs the MST and is well tolerated by the animals. The achieved MST is comparable to the reported MST of animals treated with surgery and/or radiotherapy; therefore, the combined treatment is beneficial, especially in cases that would otherwise require major maxillofacial surgery or where radiotherapy is unavailable or poses a financial restraint to the owner. Additional studies to prove the clinical benefit of the combination treatment with ECT and IL-12 GET are warranted.

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477 The manuscript was edited for language by "American Journal Experts".

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 720

721 **FIGURE LEGENDS**

722

723 **Fig. 1** Non-invasive MEA that was used for IL-12 GET (Kos et al., 2017).

724

725 **Fig. 2** Identification of lymphocyte populations by flow cytometry. a) Canine
726 lymphocytes gating according to CD45 V450 (78 %) intensity and side-
727 scatter characteristics (SSC). b) Identification of T lymphocyte percentages
728 using CD3 FITC intensity and side-scatter characteristics (86 % (63 %)). c)
729 Identification of CD4+ (helper) T lymphocyte population (63 %). d)
730 Identification of FoxP3 cells within CD3+ and CD4+ lymphocyte populations
731 (5,8 %). e) Identification of FoxP3 cells within CD3+, CD4+ and CD25+
732 (regulatory T cells) lymphocyte populations (3,4 %).

733

734

735 **Fig. 3** Survival of dogs (n=9) with oral malignant melanoma treated with a
736 combination of cytoreductive surgery, ECT and IL-12 GET. The results are
737 expressed using a Kaplan-Meier curve. Dots represent censored patients that
738 were euthanized due to tumor-unrelated reasons.

739

740

741 **Fig. 4** Treatment response of a 13-year-old Soft-Coated Wheaten Terrier
742 with stage III OMM (patient 4). After the fourth treatment (week 8), we
743 observed radiographic evidence new mandibular bone
744 formation/mineralization (*), which was consistent with the clinical
745 response to the treatment. The dog had multiple lung metastases at 7 months
746 posttreatment and was euthanized 10 months after initial presentation due to
747 tumor-unrelated causes. Photos and dental radiographs were taken at the
748 first treatment (week 0; a, b), second treatment (week 2; c, d), third

749 treatment (week 4; e, f), fourth treatment (week 8; g, h) and at 9 months
750 after the first treatment (i, j).

751

752 **Fig. 5** Percentage of T_{reg} over the course of treatment. The percentage was
753 measured by flow cytometry on the day of the treatment, at four weeks later
754 and at the end of the observation period. The percentage of T_{reg} was
755 significantly lower at the end of observation period compared to the
756 percentage on the day of treatment. *p<0.05.

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