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Hypoxia-associated prognostic markers in oral canine melanoma

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Abstract:	Canine oral malignant melanoma (COMM) is the most common neoplasm in the oral cavity characterized by local invasiveness and high metastatic potential. Hypoxia represents a crucial feature of the solid tumor microenvironment promoting cancer progression and drug resistance. Hypoxia-inducible factor-1 α (HIF-1 α) and its downstream effectors vascular endothelial growth factor A (VEGF-A), GLUT1, C-X-C chemokine receptor type 4 (CXCR4) and carbonic anhydrase IX (CAIX) are the main regulators of the adaptive response to low oxygen availability. The prognostic value of these markers was evaluated in 36 COMMs using immunohistochemistry. Additionally, the effects of cobalt chloride-mediated hypoxia were evaluated in one primary COMM cell line. HIF-1 α expression was observed in the nucleus, and this localization correlated with the presence or enhanced expression of HIF-1 α - regulated genes at the protein level. Multivariate analysis revealed that in dogs given chondroitin sulfate proteoglycan-4 (CSPG4) DNA vaccine, COMMs expressing HIF-1 α , VEGF-A, and CXCR4 were associated with a shorter disease-free intervals (DFI) compared to tumors that were negative for these markers ($p=0.03$), suggesting hypoxia can influence immunotherapy response. Western blotting showed that under

	<p>chemically induced hypoxia, COMM cells accumulate HIF-1 α and smaller amounts of CAIX . HIF-1α induction and stabilization triggered by hypoxia was corroborated by immunofluorescence showing its nuclear translocation. These findings reinforce the role of a hypoxic microenvironment in tumor progression and patient outcome in COMM, as previously established in several human and canine cancers. Additionally, hypoxic markers may represent promising prognostic markers, highlighting opportunities for their use in therapeutic strategies for COMMs.</p>

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1 **Hypoxia-associated markers in the prognosis of oral canine melanoma**

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17

18 **ABSTRACT**

19 Canine oral malignant melanoma (COMM) is the most common neoplasm in the oral
20 cavity characterized by local invasiveness and high metastatic potential. Hypoxia
21 represents a crucial feature of the solid tumor microenvironment promoting cancer
22 progression and drug resistance. Hypoxia-inducible factor-1 α (HIF-1 α) and its
23 downstream effectors vascular endothelial growth factor A (VEGF-A), GLUT1, C-X-C
24 chemokine receptor type 4 (CXCR4) and carbonic anhydrase IX (CAIX) are the
25 main regulators of the adaptive response to low oxygen availability. The prognostic
26 value of these markers was evaluated in 36 COMMs using immunohistochemistry.
27 Additionally, the effects of cobalt chloride-mediated hypoxia were evaluated in one
28 primary COMM cell line. HIF-1 α expression was observed in the nucleus, and this
29 localization correlated with the presence or enhanced expression of HIF-1 α -
30 regulated genes at the protein level. Multivariate analysis revealed that in dogs
31 given *chondroitin sulfate proteoglycan-4 (CSPG4)* DNA vaccine, COMMs expressing
32 HIF-1 α , VEGF-A, and CXCR4 were associated with a shorter disease-free intervals
33 (DFI) compared to tumors that were negative for these markers (p=0.03), suggesting
34 hypoxia can influence immunotherapy response. Western blotting showed that under
35 chemically induced hypoxia, COMM cells accumulate HIF-1 α and smaller amounts
36 of CAIX . HIF-1 α induction and stabilization triggered by hypoxia was corroborated by
37 immunofluorescence showing its nuclear translocation. These findings reinforce the
38 role of a hypoxic microenvironment in tumor progression and patient outcome in
39 COMM, as previously established in several human and canine cancers.
40 Additionally, hypoxic markers may represent promising prognostic markers,
41 highlighting opportunities for their use in therapeutic strategies for COMMs.

42

43 **Keywords:** canine, cell culture, cobalt chloride, hypoxia,
44 immunohistochemistry, oral melanoma , prognosis

45

46 Canine oral malignant melanoma (COMM) is the most frequent malignant oral
47 tumor in dogs, accounting for 30-40% of all oral malignancies.⁴ COMM is a solid
48 tumor arising from neoplastic transformation of mucosal melanocytes, and is
49 characterized by aggressive behavior, extensive local invasiveness, and metastatic
50 potential, resulting in poor prognosis.^{3,54}

51 Hypoxia represents one of the most crucial microenvironmental features in
52 solid tumors⁵⁰ and has been associated with invasiveness, angiogenesis,
53 vasculogenic mimicry, and response to therapy in several cancer types, including
54 melanoma.^{10,19} Hypoxia and subsequent acidification of the tumoral
55 microenvironment are reported to promote cancer progression and drug
56 resistance,³⁸ and contribute to immunotolerance of cancer cells, conferring
57 resistance to immunotherapy.^{1,11,15,24,48} Hypoxia-inducible factor-1 α (HIF-1 α) is the
58 primary regulator of the adaptive response to low oxygen availability.^{20,27} Upon
59 stabilization and migration to the nucleus, HIF-1 α acts as transcription factor for
60 several hypoxia-regulating elements. Hypoxia-regulating elements, in turn, induce
61 and modulate various processes, including glycolysis, angiogenesis, cell migration,
62 invasion, metastasis, and chemoresistance.^{11,43,49,50} Vascular endothelial growth
63 factor A (VEGF-A) is a key effector of the hypoxic response, which stimulates
64 angiogenesis that provides nutrients and oxygen to proliferating cancer cells.³⁵
65 Moreover, hypoxia stimulates cell homing and migration via chemokine-mediated
66 stimuli. C-X-C chemokine receptor type 4 (CXCR4) is overexpressed in various

67 human cancers, including melanoma, and primarily contributes to tumor growth,
68 angiogenesis, metastasis, and therapeutic resistance.^{8,31}

69 Cancer cells undergo metabolic reprogramming, exhibiting a highly glycolytic
70 phenotype, which is associated with elevated expression of glucose transporter
71 isoform 1 (GLUT1).¹² The metabolic shift increases lactate production. Lactate is
72 then exported into the extracellular space leading to microenvironment acidification
73 and carbonic anhydrase IX (CAIX) induction.

74 Hypoxic biomarkers have been extensively investigated in human cancers
75 and have been associated with prognosis and therapy resistance.^{18,28} Despite the
76 increasing knowledge on the role of hypoxia in tumors, to date only a few studies are
77 available in dogs.³⁰ Hypoxia has been reported as a frequent condition occurring in
78 COMMs, in which HIF-1 α activation induces the transcription of GLUT1 and CAIX.³⁰
79 A recent study showed that the hypoxia-regulated miRNAs, miR-210 and miR-301,
80 are differentially expressed in primary and metastatic canine melanoma cell lines,
81 and metastatic cells are more resistant to hypoxia stimuli than primary tumor
82 cells.^{17,51} Additionally, it has been demonstrated, by the expression of HIF-1 α , that
83 COMM tumor cells invading the bone are under hypoxic conditions, and this may
84 explain a poorer efficacy of radiotherapy in dogs with bone lysis.³³

85 Based on these premises, and considering the aggressiveness and therapy
86 resistance of COMM,³⁷ the aim of this study was to unveil the prognostic value of
87 HIF-1 α and the associated hypoxia-response proteins, GLUT 1, CXCR4, CAIX, and
88 VEGF-A, in COMM. Additionally, the study aimed to examine the activation of HIF-1 α
89 and its biological alterations after inducing a hypoxic state in a metastatic COMM cell
90 line.

91

92 **Material and Methods**

93 **Sample collection and clinical data**

94 A retrospective study was performed on 36 COMMs diagnosed at the
95 Department of Veterinary Sciences of the University of Turin in the period between
96 2005 and 2018. The cases were retrieved through a data base search using the
97 following keys words: “canine”, “oral cavity”, and “malignant melanomas”. Pre-
98 operative clinical tumor staging ^{4,54} was assessed by means of total body computed
99 tomography in 24 cases, or skull and three views chest radiographs and abdominal
100 ultrasound in 12 cases. The primary tumor was removed by *en-bloc* excision
101 (mandibulectomy, maxillectomy, or lip-cheek excision followed by reconstruction)
102 with regional (ipsilateral or bilateral) lymphadenectomy.

103 Dogs were clinically staged based on tumor size, regional lymph node
104 involvement, and metastasis (TNM) according to Bergman et al 2007.^{3,4} Sixteen out
105 of the 36 COMMs with a chondroitin sulfate proteoglycan-4 (CSPG4)
106 immunohistochemical score $\geq 3/8$ received CSPG4 DNA electro-vaccination
107 according to the Good Clinical Practice guidelines for animal clinical studies.^{29,40,45,59}
108 The remaining 20 cases were treated with surgery alone. Both the Ethics Committee
109 of the University of Turin and the Italian Ministry of Health approved the trial
110 (0004230-20/02/2018-DGSAF-MDS-P).

111 Follow-up for the non-vaccinated dogs consisted of clinical evaluation and
112 thoracic radiographs performed every 3 months during the first year and then every 6
113 months for a minimum of 2 years. Dogs receiving CSPG4 DNA electro-vaccination
114 were clinically evaluated monthly with thoracic computed tomography .

115 **Histological and Immunohistochemical analysis**

116 Formalin fixed and paraffin-embedded histologic sections of the tumors were
117 stained with hematoxylin and eosin. Diagnoses were performed independently by
118 three observers (LM, SI, CG). In case of disagreements, a consensus was reached
119 using a multi-headed microscope.

120 Specimens were classified according to the World Health Organization (WHO)
121 guidelines⁵⁶ and evaluated for prognostic parameters in canine melanocytic
122 neoplasia according to the current literature. Briefly, nuclear atypia was assessed
123 according to what has been described by Spangler and Kass.⁵⁸ A threshold value of
124 30% atypical nuclei was considered. Mitotic count was obtained by counting the
125 number of mitoses in 2.37 mm², considering the regions of highest mitotic activity
126 and avoiding areas of ulceration, necrosis, and inflammation. The mitotic count cut-
127 off value was ≥ 4 according to Smedley et al.⁵⁶ Pigmentation was assessed
128 subjectively as described by Smedley and colleagues⁵⁶ evaluating all available
129 sections and dividing cases into those with $\geq 50\%$ and those with $< 50\%$ pigmented
130 cells. Ki67 was determined as the average number of positive neoplastic cell nuclei
131 per 1 cm² optical grid reticle at 400x magnification/40x objective (5 grid areas
132 counted) in the highest labelling area with a cut- off value of 19.5.

133 Immunohistochemistry was performed on 4 μm thick paraffin sections. After blocking
134 peroxidase activity (0.3% hydrogen peroxide in deionized water for 30 min) and heat-
135 induced antigen retrieval (30 min with citrate buffer at 98°C, pH 6.0), sections were
136 incubated with primary antibodies Ki-67, HIF-1 α , CAIX, CXCR4, GLUT-1, and VEGF-

137 A overnight at 4°C (Table 1); the detection was performed using the Vectastain
138 Universal Quick Kit (Vector Laboratories). Immunolabelled slides were randomized
139 and masked for blinded examination, which was performed by three independent
140 pathologists (SI, ML, LA). In case of disagreements, a consensus was reached using
141 a multi-headed microscope.

142 Antibodies specificity for HIF-1 α , CXCR4, VEGFA, GLUT1, and CAIX was
143 assessed on positive and negative tissues (canine mammary carcinomas, renal
144 carcinoma, skin, and testis) as shown in Supplemental Figure S1.

145 Immunohistochemical evaluation was performed by a semi- quantitative method
146 evaluating both the intensity and the percentage of positive cells using scoring
147 systems previously published (Supplemental Table S2).^{25,29,30,39,44,54}

148 **Cell line and CoCl₂ treatment**

149 A canine malignant melanoma cell line (OLGA), previously established by the
150 authors,⁴⁶ was cultured in Dulbecco's modified Eagle's medium supplemented with
151 10% fetal bovine serum, 1% glutamine, 100 μ g/mL penicillin, and 100 μ g/mL
152 streptomycin at 37°C and 5% CO₂. The OLGA cell line was cultured in chemically
153 induced hypoxia for 12, 24, and 48 hours using a concentration of 200 μ M cobalt
154 chloride (CoCl₂; Sigma Aldrich, 15862-1ML-F).⁴²

155 **Western blot analysis**

156 Proteins from the OLGA cell line, treated with 200 μ M of CoCl₂ for 12, 24, and
157 48 hours or untreated (DMSO alone), were extracted in lysis buffer (1% Triton X-100,
158 10% glycerol, 50mM Tris, 150mM sodium chloride, 2mM EDTA, pH 8.0, and 2mM
159 magnesium chloride) containing Protease Inhibitor Cocktail (P8340 Sigma). Twenty
160 micrograms of total proteins were separated on a 10% SDS-PAGE gel and

161 transferred onto a 0.2 μm pore-size nitrocellulose membrane (Cytiva; Thermo Fisher
162 Scientific). After washing, membranes were incubated in Tris-buffered saline with
163 10% bovine serum albumin at room temperature for 1 hour and then incubated
164 overnight at 4°C with HIF1- α and CAIX antibodies; α -tubulin was used as a
165 housekeeping protein (Supplemental Table S1). Horseradish peroxidase -
166 conjugated secondary antibodies were diluted at 1:15 000. The chemiluminescence
167 substrate (Clarity ECL Substrate; BIO-RAD Laboratories) was used to produce the
168 light signal, acquired with CL-XPosure films (Thermo Fisher Scientific Inc).

169 **Immunofluorescence**

170 To investigate the effects of CoCl_2 on HIF1- α nuclear translocation and CAIX
171 expression, immunofluorescence against HIF1- α and CAIX was performed on OLGA
172 cells exposed to the highest CoCl_2 concentration (200 μM) for 12, 24, and 48 hours.
173 Briefly, 2×10^4 cells were plated in eight-well chamber slides (Lab-Tek II Chamber
174 Slide System; Nalge Nunc International) until 70% confluence. After treatment, cells
175 were fixed with methanol:acetone (1:1 proportion) for 30 seconds. After washing
176 three times with Tris-HCl (0.1M, pH 7.6), cells were blocked with 10% normal goat
177 serum for 1 hour at room temperature and then incubated overnight at 4°C with
178 antibodies to HIF-1 α (1:100) and CAIX (1:200). After washing with Tris-HCl, cells
179 were incubated with a fluorescent secondary Alexa488-conjugated goat anti-rabbit
180 IgG antibody (1:500 dilution, ThermoFisher) and anti-mouse IgG (1:500) for 1 hour
181 in the dark. Subsequently, cell nuclei were stained with DAPI (0.5 $\mu\text{g}/\text{ml}$ in Tris-HCl,
182 Sigma–Aldrich, USA) for 10 minutes, washed three times with Tris-HCl, and then the
183 sections were covered with mounting medium (PermaFluor, Thermo Scientific) and
184 kept overnight in the dark. Fields were randomly selected by microscope TCS SP8
185 (Leica Microsystems CMS GmbH Mannheim, Germany) and z-stacks of 10 nm were

186 acquired. Z-stacks were then processed directly with the Leica LAS-X software to
187 produce maximum projection images of each field. To detect co-localization of
188 different fluorochromes, fluorescent signals in different channels were merged to
189 produce multi-color images. Image acquisitions were performed with a resolution of
190 1024 x 1024 pixels with a 200 Hz sampling frequency.

191

192 **Statistical analyses**

193 Fisher's exact test or the chi-squared test was used to test possible
194 associations among immunohistochemical marker expression and clinical and
195 histopathological data. Disease-free interval (DFI) was calculated as the time
196 between surgery and the first detection of metastases and/or local recurrence, while
197 overall survival (OS) was defined as the period from the day of surgery to the
198 patient's death due to a cancer-related cause. The survival functions of the DFI and
199 OS were estimated with the Kaplan-Meier method, and the comparison of survival
200 function was done by means of the log-rank test. Dogs that died from unrelated
201 causes, were lost to follow-up, or were still alive at the end of the study were right-
202 censored. Univariate and multivariate Cox models were fitted to verify the effects of
203 hypoxia biomarkers on DFI or OS. In multivariate analysis, we considered the
204 following parameters: HIF-1 α , CXCR4, VEGFA, GLUT1, and CAIX
205 immunohistochemical expression. Statistical significance was set at a 0.05 level.
206 Because the clinical samples belonging to stage I were censored, this stage was
207 omitted. GraphPad Prism 8 (GraphPad Software, San Diego, California) and R
208 statistical software (R Core Team, 2018) were used for statistical analysis.

209

210 RESULTS

211 *Patient and tumor characteristics*

212 A total of 36 dogs with COMM met the inclusion criteria. Clinical and follow-up
213 data, and histopathological and immunohistochemical diagnostic features are
214 provided in Tables 1 and 2, respectively. Survival analysis (Fig.1) showed that dogs
215 with clinical stage IV had a worse prognosis compared to dogs with clinical stages II
216 and III ($p=0.002$). Similarly, vaccinated dogs had a significantly higher OS time
217 compared to non-vaccinated dogs ($p<0.001$) (Fig. 2). Although not significant, we
218 found that COMMs with a total absence of melanin or less than 25% pigmented
219 neoplastic cells were associated with a lower DFI when compared to tumors with >
220 50% pigmented neoplastic cells (median DFI: days 180 vs 210 days, respectively, p
221 = 0.06). The OS was not significantly associated with the amount of pigmentation
222 (median OS: 235 days vs 778 days, $p = 0.08$), and none of the other
223 histopathological parameters showed prognostic significance.

224 *Overexpression of hypoxic markers negatively affects prognosis in dogs* 225 *receiving immunotherapy.*

226 The immunohistochemical scores for HIF-1 α , CAIX, GLUT-1, CXCR4, and
227 VEGF-A are summarized in Table 3. Representative images are shown in Figure 3.
228 The CAIX score was associated with the HIF-1 α score ($p = 0.001$). Additionally, we
229 found a statistical association between CXCR4 and Ki-67 index ($p= 0.046$) (Table 4).
230 Multivariate analysis revealed that dogs receiving immunotherapy and
231 overexpressing HIF-1 α , VEGF-A, and CXCR4 had a significantly shorter DFI
232 (median = 111 days) compared to dogs with the same treatment that were negative

233 for all three markers or only positive for 1 or 2 markers (median = 204 days; p =
234 0.03) (Fig. 4), while no significant differences were observed for OS.

235 Multivariate analysis in non-vaccinated patients revealed no statistically
236 significant differences in OS or DFI between dogs with concomitant overexpression
237 of HIF-1 α , VEGF-A, and CXCR4 and dogs negative for the markers or expressing
238 only one or two markers.

239 ***CoCl₂ treatment induces HIF-1 α accumulation and nuclear translocation***

240 Distinct bands corresponding to HIF-1 α (120 kDa) and CAIX (58 kDa)
241 proteins were more pronounced in the Olga cell line treated with CoCl₂ at 12 and 24
242 hours (Fig. 5). In contrast, the expression of these proteins was either negative (HIF-
243 1 α) or very low (CAIX) in untreated cells (Fig. 5).

244 A mild increase of CAIX protein was also present in chemically-induced
245 hypoxic conditions after 24 hours. Under normoxic conditions, HIF-1 α was primarily
246 localized to the cytoplasm, with nuclear localization becoming evident only after
247 CoCl₂ treatment (Fig. 6). In contrast, chemically induced hypoxia did not yield
248 noticeable effects on CAIX protein.

249

250 DISCUSSION

251 COMM is the most commonly diagnosed malignant tumor occurring in the oral
252 cavity of dogs,^{16,54} and it has a high and rapid metastatic rate, resulting in a poor
253 prognosis.³ Recently, a consensus working group was founded to identify potential
254 prognostic markers in this neoplasm and to give accurate recommendations for the
255 diagnosis and histopathologic prognostication of canine melanocytic tumors.⁵⁵
256 Although nuclear atypia, mitotic count, and Ki-67 index are considered the most
257 prognostic factors for COMM, the identification of new markers may improve the ability
258 to prognosticate these neoplasms, as well as aiding in the selection of specific
259 therapies.

260 In this study, we observed that patients with advanced clinical stages (III and
261 IV) had a shorter OS compared to stage II patients, which is consistent with existing
262 literature.³ Furthermore, our data confirmed that *CSPG4* vaccination significantly
263 increased the overall survival of COMM patients.^{40,46}

264 Interestingly, smaller amounts of melanin in COMMs seem to be weakly
265 associated with shorter DFI, suggesting a close association between loss of melanin
266 pigment and tumor dedifferentiation, which may be associated with a more aggressive
267 behavior. This finding doesn't represent a novelty because in 2011, Smedley et al.
268 suggested that a lower level of pigmentation (<50% of pigmented cells) is an
269 unfavorable factor.⁵⁴ In human cutaneous melanoma, decreased pigmentation has
270 also been linked to an aggressive phenotype with implications for prognosis and
271 response to therapy.^{6,53}

272 As previously reported in a number of canine tumor histotypes, a correlation
273 between CAIX and HIF-1 α positivity has been observed, suggesting hypoxia may

274 trigger microenvironment acidosis with metabolic changes in cancer cells growth.³⁶
275 Our findings reinforce the close interaction between these molecules and highlight the
276 strong HIF-1 α -dependent regulation of CAIX as an adaptation of COMM cells to
277 extracellular acidosis.⁶¹ This adaptation may enhance the tumor's ability to survive and
278 grow under adverse conditions.

279 We found that CXCR4 expression was associated with an increased Ki-67
280 index, which is consistent with previous reports in human renal carcinomas⁹ and
281 multicentric lymphoma.⁶³ However, in human cutaneous melanomas, these markers
282 have not been significantly associated.^{31,32,60}

283 Univariate data analysis for the hypoxic markers did not show any statistical
284 significance for DFI or OS. However, these markers are interconnected in the hypoxic
285 cellular pathway signaling. Hence, we investigated the prognostic value of their co-
286 expression. Multivariate data analysis revealed that concurrent expression of HIF-1 α ,
287 VEGF-A and CXCR4 in COMMs treated with a *CSPG4* vaccine is associated with a
288 lower DFI compared to COMMs negative for these markers. This suggests a lower
289 efficacy of *CSPG4* vaccination in tumors displaying hypoxic features. This finding is in
290 line with the literature, in which hypoxia is known to induce immune-resistance and
291 negatively interferes with immune surveillance of tumors, as well as adoptive
292 immunotherapy.^{2,24,62} Likewise, co-expression of hypoxic markers and hypoxia-related
293 signatures have been documented in several human cancer as predictive of a poor
294 outcome.^{7,26,64} This co-expression triggers the activation of HIF-1 α -dependent
295 pathways, which can lead to tumor growth, angiogenesis, and metastasis.⁸ Regarding
296 the relationship between hypoxic microenvironments and *CSPG4* expression, it's very
297 interesting to underscore that in human melanomas, *CSPG4* is regulated by hypoxia
298 in vitro and its expression confers resistance to immunotherapy.^{23,41} On the basis of

299 the preliminary data obtained by the authors, we can hypothesize that in dogs, CSPG4
300 is regulated by hypoxia, strengthening COMM as a good model for comparative
301 oncology.³⁴

302 To gain insight into the mechanisms of hypoxia in COMM, the OLGA cell line
303 was treated with CoCl₂ to mimic a hypoxic stimulus.¹³ We found that *in vitro* hypoxia
304 induction caused an accumulation of HIF-1 α protein after treatment. HIF-1 α induction
305 and stabilization triggered by hypoxia was further corroborated by
306 immunofluorescence, in which nuclear translocation under CoCl₂ treatment to avoid
307 proteasomal degradation was demonstrated.⁵⁰ In the nucleus, HIF-1 α is known to
308 directly coordinate the transcription of hypoxia-regulating elements.⁴⁹ Consistent with
309 the immunohistochemical results, CAIX protein levels were also mildly increased
310 under chemically induced hypoxia. Its accumulation was delayed as compared to HIF-
311 1 α , thus supporting the direct role of HIF-1 α in the upregulation of this protein.²¹

312 In conclusion, our results reinforce the crucial role of a hypoxic
313 microenvironment and acidification in tumor aggressiveness and outcome in COMM,
314 as extensively established in other canine^{14,57} and human cancers.⁵ It becomes critical
315 to develop novel therapeutic strategies considering the hypoxic status of the tumors,
316 and future studies should address the *in vitro* effects of hypoxia in COMM cells and
317 investigate the inhibition of hypoxia-related signaling pathways as potential
318 therapeutic targets. Notably, as shown in this study, it is crucial to consider the
319 interdependent actions of the molecular mechanisms triggered by HIF-1 α and its
320 transcriptional cascade.

321

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LEGEND OF THE FIGURES

476

Figure 1

478 Kaplan-Meier survival curves for stage II, III, and IV canine oral malignant
479 melanomas. (a) Disease-free interval (DFI). (b) Overall survival (OS) . Stage II
480 (median DFI = 187 days, median OS = 397 days); Stage III (median DFI = 209 days,
481 median OS = 260 days); Stage IV (median DFI = 36 days, median OS= 76). DFI: p =
482 0.06 , OS: p = 0.002

483

Figure 2

485 Kaplan-Meier survival curves of dogs with canine oral malignant melanomas treated
486 with or without *chondroitin sulfate proteoglycan-4 (CSPG4)* DNA vaccination. (a)
487 Disease-free interval (DFI). Vaccinated median DFI = 205 days, not vaccinated
488 median DFI = 187 days ($p > 0.05$). (b) Overall survival (OS). Vaccinated median OS
489 = 972 days, not vaccinated median OS = 215 days ($p < 0.001$).

490

Figure 3

492 Immunohistochemistry (IHC) for hypoxia-associated factors in canine oral
493 malignant melanomas, dog. (a) Diffuse moderate cytoplasmic immunolabeling for
494 vascular endothelial growth factor A (VEGFA) in amelanotic melanoma. VEGFA IHC.
495 (b) Diffuse strong cytoplasmic and membrane immunolabeling for C-X-C chemokine
496 receptor type 4 (CXCR4) in spindle amelanotic melanoma. CXCR4 IHC. (c)
497 Multifocal nuclear immunolabeling for hypoxia-inducible factor-1 α (HIF-1 α) in poorly

498 melanotic melanoma. HIF-1 α IHC. (d) Multifocal moderate cytoplasmic
499 immunolabeling for glucose transporter 1 (GLUT1) in poorly melanotic melanoma.
500 GLUT1 IHC. (e) Diffuse weak cytoplasmic immunolabeling for carbonic anhydrase IX
501 (CAIX) in poorly melanotic melanoma. CAIX IHC. (f) Negative reagent control.
502 diaminobenzidine chromogen hematoxylin counterstain.

503 **Figure 4**

504 Kaplan-Meier survival curves of dogs with canine oral malignant melanomas treated
505 with chondroitin sulfate proteoglycan-4 DNA vaccination that either co-express
506 hypoxia-inducible factor-1 α (HIF-1 α), C-X-C chemokine receptor type 4 (CXCR4),
507 and vascular endothelial growth factor A (VEGFA) or lack the expression of at least
508 one of protein (others). (a) Disease-free interval (DFI). Patients overexpressing HIF-
509 1 α , CXCR4, and VEGFA had significant shorter DFIs ($p < 0.05$) compared to dogs
510 that were negative for all three markers, as well as those positive to only 1 or 2
511 markers (others).
512 (b) Overall survival (OS). There were no statistically significant differences in OS (p
513 > 0.05).

514 **Figure 5**

515 Western blot analysis of Olga cells that were untreated (Ctrl) or cells that were
516 treated for 12, 24, or 48 hours with cobalt chloride (CoCl₂) to induce hypoxic
517 conditions. HIF-1 α , hypoxia-inducible factor-1 α ; CAIX, carbonic anhydrase IX .

518 **Figure 6**

519 Representative immunofluorescence of primary canine oral malignant melanoma
520 OLGA cell line, either (a-d) untreated or (e-h) treated with 200 μ m cobalt chloride
521 (CoCl₂) for 24 hours. (a, e) DAPI nuclear staining, blue (b, f) hypoxia-inducible

522 factor-1 α (HIF1- α), green. (c, g) carbonic anhydrase IX (CAIX), red. (d, h) merged
523 fluorescent signals of DAPI, HIF1- α , and CAIX.

524

525

526

527

Authors' Contributions

528

529 RDM, CG, designed and performed the experimental design. SI, LM, performed
530 histological diagnosis; LM, PM, KV performed IHC evaluations; EM, DG, MO
531 enrolled and surgically treated COMMs

532 EM* performed statistical analysis.

533 PA EM** performed in vitro assay, western blot and immunofluorescence.

534 The manuscript was written by RDM, CG, LM with contribution of LA.

535

536

Table 1.

Clinical features of dogs included in the study

Clinical features		Values	
Age (years)	Mean	11.4	
	Median	12	
	Range	6-16	
Gender, n (%)	Female	14 (38.9)	
	Male	22 (61.1)	
Breed, n (%)	Crossbreed	15 (41.7)	
	Golden retriever	3 (8.3)	
	Yorkshire terrier	3 (8.3)	
	Cocker spaniel	3 (8.3)	
	Schnauzer	2 (5.6)	
	Others breeds	10 (27.8)	
Weight (kg) n (%)	Mean	19	
	Median	14	
	Range	2-43	
Location, n (%)	Mandible	16 (44.4)	
	Cheek	8 (22.2)	
	Maxilla	6 (16.7)	
	Lip	3 (8.3)	
	Gingiva	1 (2.8)	
	Soft palate	1 (2.8)	
	Tongue	1 (2.8)	
Lymphadenectomy, n (%)	Unilateral	28 (77.8)	
	Bilateral	8 (22.2)	
Histological evaluation of surgical margins, n (%) ^a	Non infiltrated	16 (64)	
	Infiltrated	9 (36)	
Local recurrence n (%)	No	17 (47.2)	
	Yes	19 (52.8)	
Pulmonary metastasis n (%)	no	18 (50)	
	yes	18 (50)	
Clinical stage n (%)	I	3 (8.3)	
	II	19 (52.8)	
	III	10 (27.8)	
	IV	4 (11.1)	
Follow-up (days)	DFI	Mean	292
		Median	197
		Range	21-1629
	OS	Mean	506
		Median	283
		Range	41-1951
CSPG4 positivity n (%)		31 (86)	

^aExcision margins data are not available for the remaining samples
Abbreviations: DFI, disease-free interval; OS, overall survival.

Table 2

Histopathological and immunohistochemical diagnostic features of Canine Oral Malignant Melanomas

	Histological findings	<i>n</i> (%)
Histotype	Epithelioid	11 (30.5)
	Fusiform	10 (27.8)
	Mixed	15 (41.7)
Mitotic count in 2.37 mm ²	< 4/10	6 (16.7)
	≥4/10	30 (83.3)
Pigmentation	< 50% of cells	34 (94.4)
	≥ 50% of cells	2 (5.6)
Nuclear atypia	< 30%	8 (22.2)
	> 30%	28 (77.8)
Ki67 index ^a	< 19.5	5 (14.7)
	≥ 19.5	29 (85.3)

^aImmunohistochemistry not available in the remaining samples

Table 3.

Immunohistochemical scoring of hypoxia-related markers

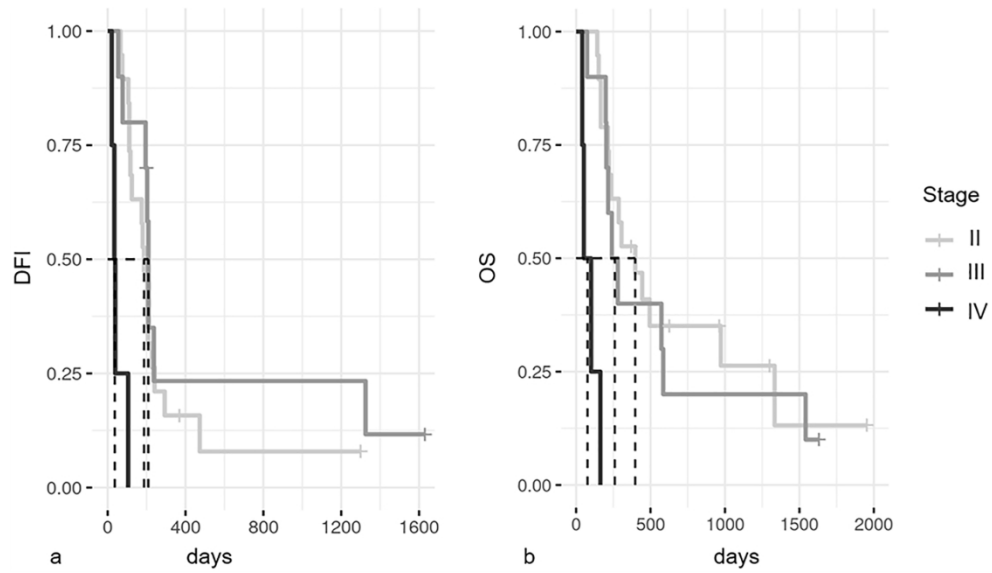
Marker	IHC Labelling	n (%)	Total
HIF-1 α	Negative	20 (55.6)	36
	Positive	16 (44.4)	
CAIX	Score 0	9 (29)	31 ^a
	Score 1	7 (22.6)	
	Score 2	15 (48.4)	
GLUT-1	Score 0	1 (2.8)	36
	Score 1	12 (33.3)	
	Score 2	23 (63.9)	
CXCR4	Negative	17 (42.2)	36
	Positive	19 (47.2)	
VEGF-A	Score 0	3 (8.3)	36
	Score 1	27 (75)	
	Score 2	6 (16.7)	

^aImmunohistochemical scoring not assessable in the remaining samples

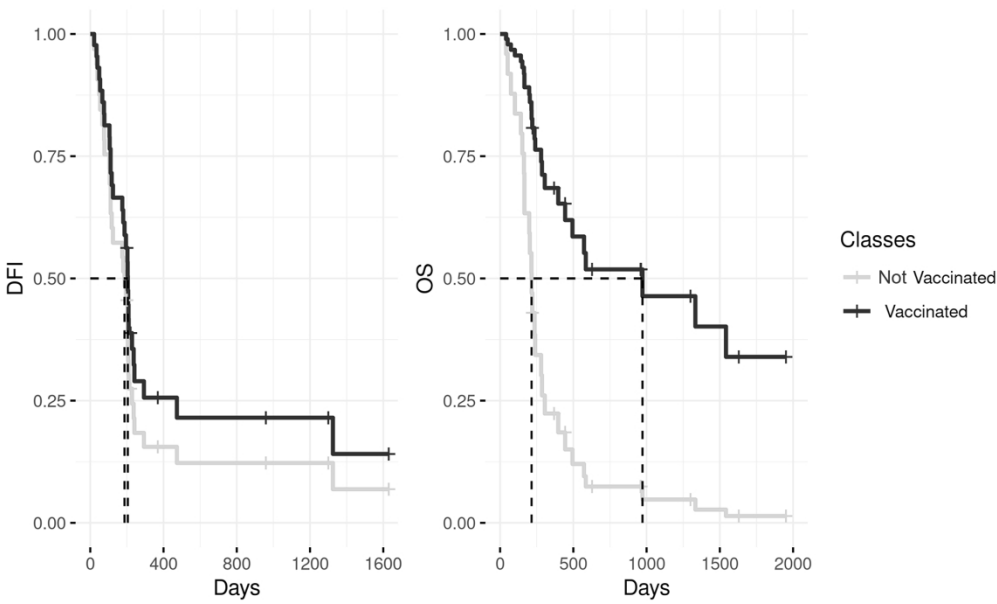
Table 4
Contingency tables of significantly associated hypoxia immunohistochemical markers

		HIF-1α		Total n (%)
		Negative	Positive	
CAIX	0-30%	14	2	16 (51.6)
	>30%	4	11	15 (48.4)
	Total n (%)	18 (58.1)	13 (41.9)	
p-value		0.001		

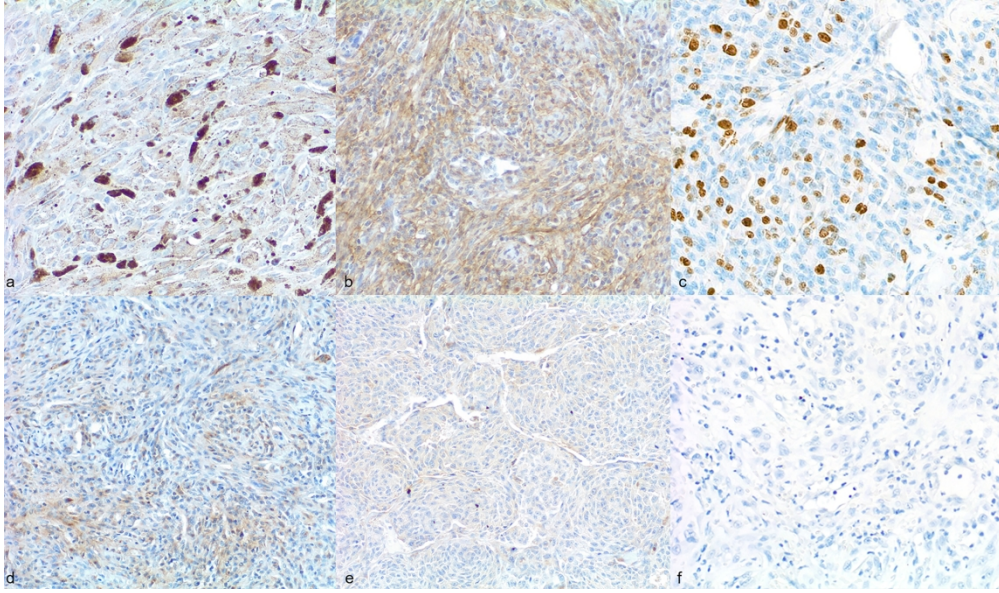
		Ki67		Total n (%)
		<19.5	\geq19.5	
CXCR4	Negative	0	16	16 (47.1)
	Positive	5	13	18 (52.9)
	Total n (%)	5 (14.7)	29 (85.3)	
p-value		0.046		



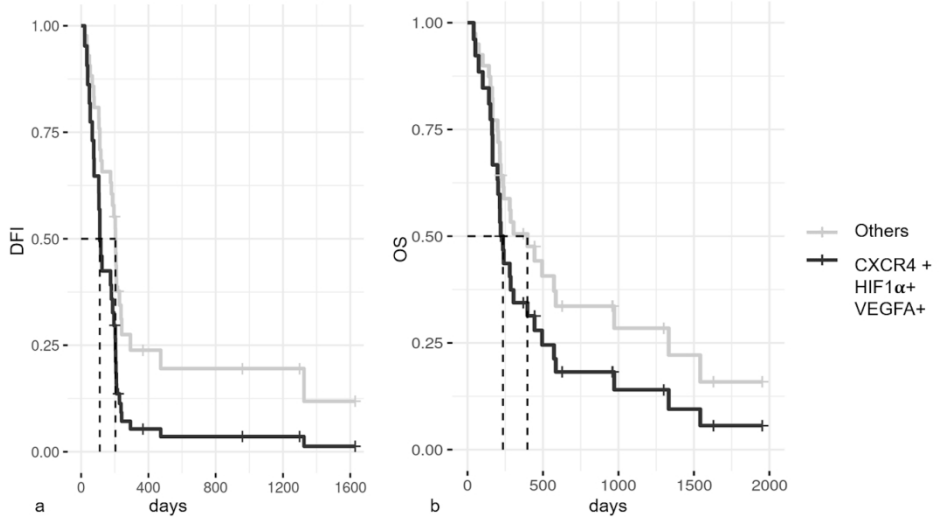
180x101mm (300 x 300 DPI)



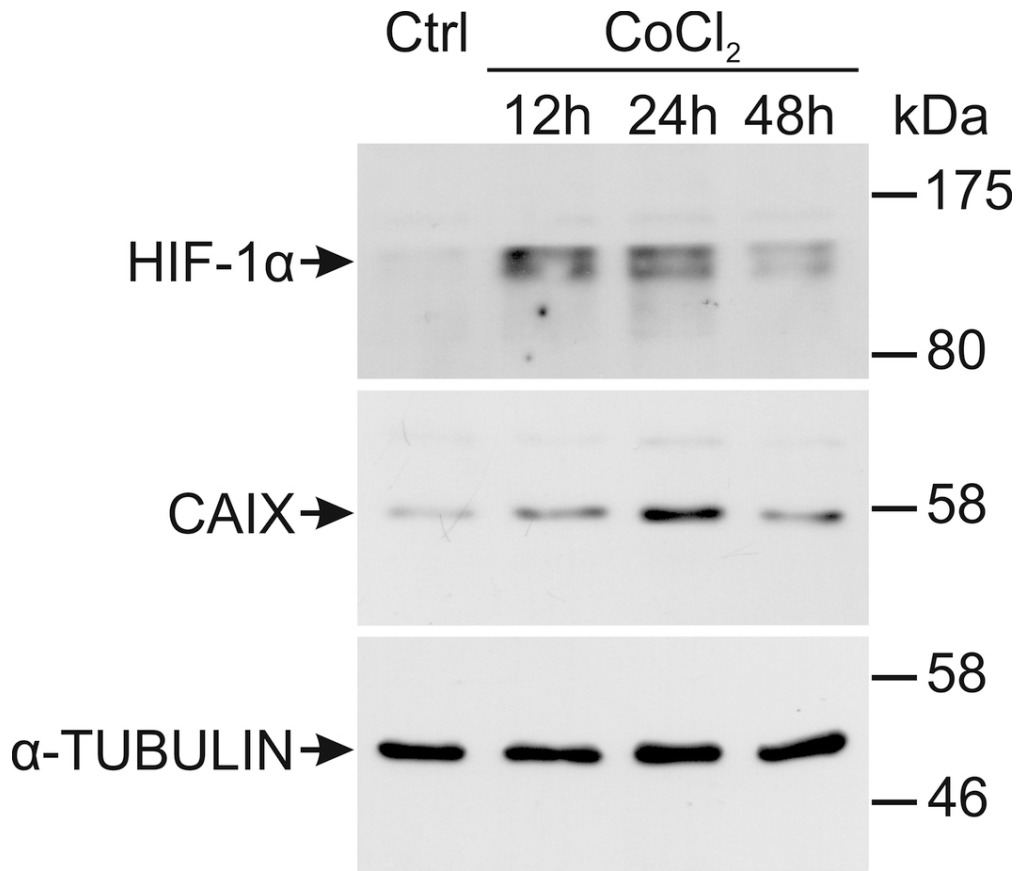
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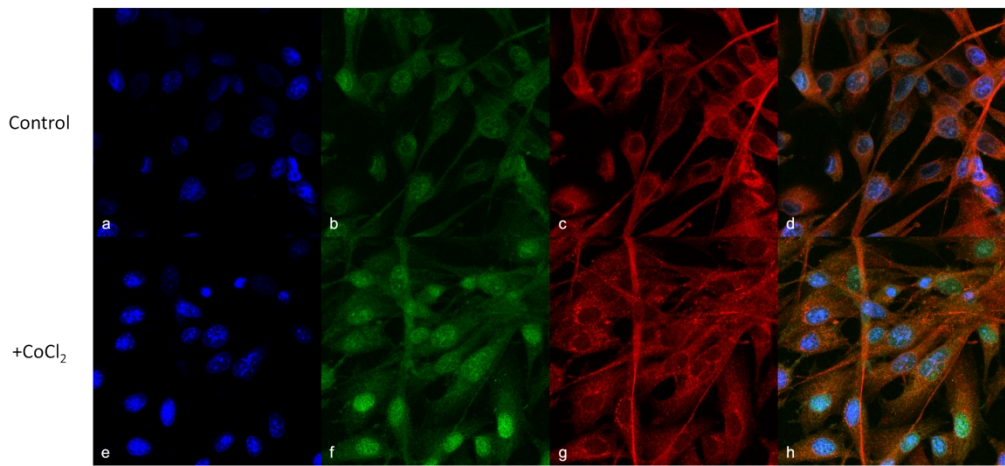
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90x77mm (300 x 300 DPI)



180x82mm (300 x 300 DPI)

Supplementary Table 1.

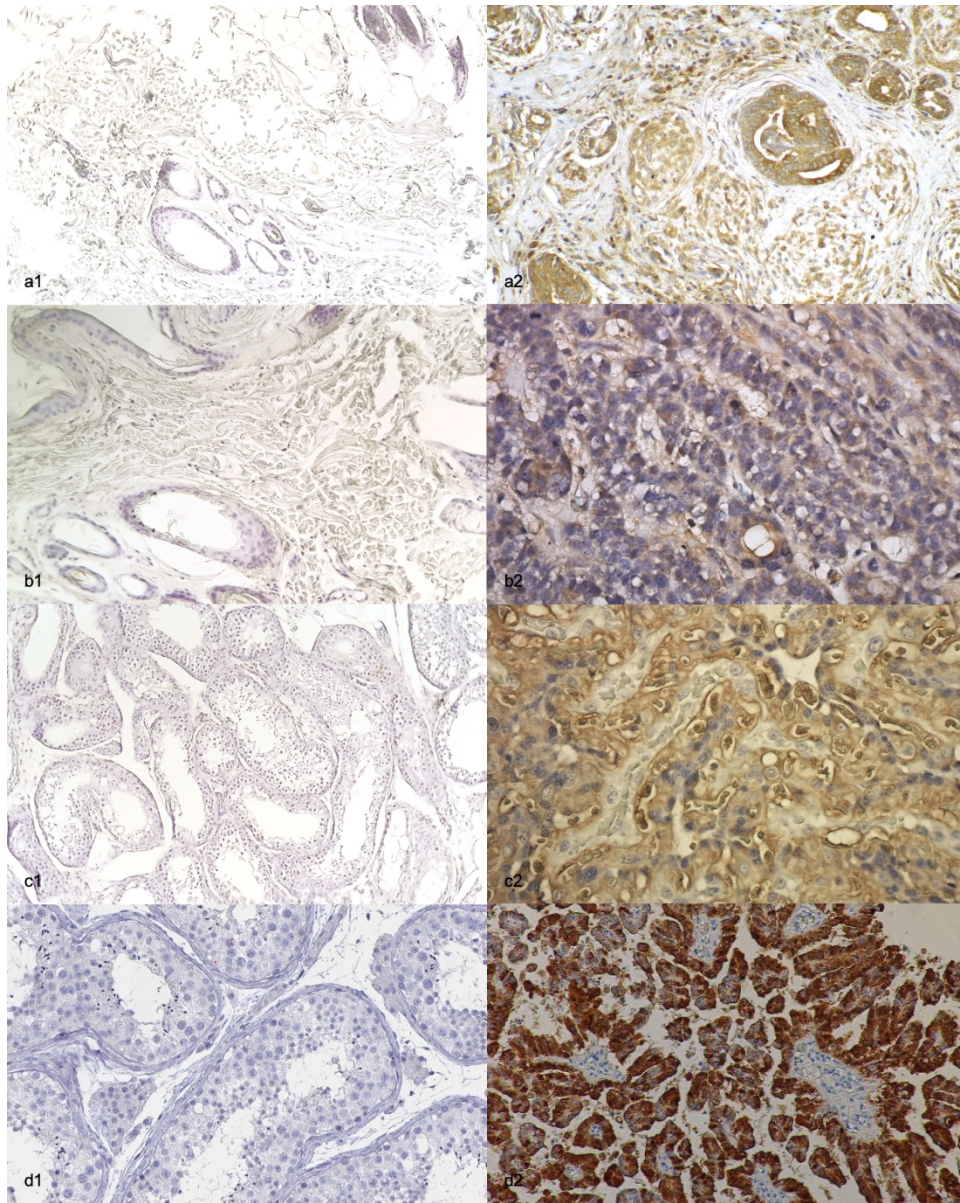
Primary antibodies used in this study.

Antibody	Application	Dilution	Code	Source
CAIX	IHC	1:1000	AB15086	Abcam
CSPG4	IHC	1:40	SAB4200621	Sigma Aldrich
CXCR4	IHC	1:300	NB100-56437	Novus Biologicals
GLUT-1	IHC	1:80	NB120-15309	Novus Biologicals
	IHC	1:100	A300-286A	Bethyl Laboratories
HIF-1 α	WB	1:1,000	610959	Becton and Dickinson Biosciences
	IF	1:100		
Ki67	IHC	1:200	A-047	Dako
VEGF-A	IHC	1:25	SC-65617	Santa Cruz Biotechnology
α -tubulin	WB	1:10,000	T-5168	Sigma Aldrich

Supplementary Table 2.

Scoring system used for immunohistochemical evaluations.

Antibody	Scoring			Reference
CAIX	<i>% of positive cells</i> 0 = < 1% 1 = 1-30% 2 = > 30%			Meier et al. (2016) ³⁰
CSPG4	<i>% of positive cells</i> 0 = none 1 = < 1% 2 = 1-10% 3 = 10-33% 4 = 33-66% 5 = > 66%	<i>Intensity</i> 0 = negative 1 = weak 2 = intermediate 3 = strong	<i>Total score [% + Int]</i> Negative = 0-2 Positive = ≥3	Mayayo et al. (2011) ²⁹
CXCR4	<i>% of positive cells</i> 0 = up to 5% 1 = 10-20% 2 = 21-40% 3 = >40%	<i>Intensity</i> 0 = negative 1 = weak 2 = moderate 3 = strong	<i>Total score [% + Int]</i> Negative = 0-2 Positive = >3	Ren et al. (2016) ⁴⁴
GLUT-1	<i>% of positive cells</i> 0 = none 1 = up to 50% 2 = > 50%	<i>Intensity</i> 0 = negative 1 = weak 2 = strong	<i>Total score [% x Int]</i> Negative = 0 Moderate = 1-2 Strong = 4	Petty et al. (2008) ³⁹
HIF-1 α	Negative = only cytoplasmatic staining Positive = cytoplasmatic and nuclear staining			Noguchi et al. (2022) ³³
Ki67	Negative = none or cytoplasmatic staining Positive = nuclear staining			Smedley et al. ⁵⁵ (2022)
VEGF-A	<i>% of positive cells</i> 0 = 0-10% 1 = 10-30% 2 = 31-50% 3 = 51-75% 4 = >75%	<i>Intensity</i> 0 = negative 1 = yellow 2 = light brown 3 = dark brown	<i>Total score [% x Int]</i> Negative = 0 Moderate = 1-8 Strong = 9-12	Liu et al. (2017) ²⁵



500x624mm (96 x 96 DPI)

Supplementary Figure 1

Validation of immunohistochemistry (IHC) antibodies on canine tissues. (a, b) Hypoxia-inducible factor-1 α (HIF-1 α) IHC. (a) Haired skin, negative tissue control. (b) Mammary carcinoma, positive tissue control. (c-d) C-X-C chemokine receptor type 4 IHC. (c) Haired skin, negative tissue control. (d) Mammary carcinoma, positive tissue control. (e, f) glucose transporter 1 IHC. (e) Testis, negative tissue control. (f) Placenta, positive tissue control. (g, h) Carbonic anhydrase IX. (g) Testis, negative tissue control. (h) Renal cell carcinoma, positive tissue control.

For Peer Review

1 **Hypoxia-associated markers in the prognosis of oral canine melanoma**

2

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17

18 **ABSTRACT**

19 Canine oral malignant melanoma (COMM) is the most common neoplasm in the oral
20 cavity characterized by local invasiveness and high metastatic potential. Hypoxia
21 represents a crucial feature of the solid tumor microenvironment promoting
22 cancer progression and drug resistance. Hypoxia-inducible factor-1 α (HIF-1 α) and its
23 downstream effectors ~~VEGF-A (Vascular~~vascular endothelial growth factor A
24 ~~(VEGF-A)~~, GLUT1~~(Glucose transporter 1)~~, C-X-C chemokine receptor type 4
25 ~~(CXCR4)(C-X-C chemokine receptor type 4)~~, and ~~carbonic anhydrase IX (CAIX)~~
26 ~~(Carbonic anhydrase IX)~~ are the main regulators of the adaptive response to low
27 oxygen availability. ~~In this study, the~~The prognostic value of these markers was
28 evaluated in 36 COMMs using immunohistochemistry. Additionally, the ~~in vitro~~
29 effects of cobalt chloride-mediated hypoxia were evaluated in one primary COMM
30 cell line. HIF-1 α expression was observed in the nucleus, and this ~~subcellular~~
31 localization ~~appeared to correlate~~ with the presence or enhanced expression of
32 HIF-1 α -regulated genes at the protein level. Multivariate ~~data~~ analysis revealed that
33 in dogs given *chondroitin sulfate proteoglycan-4 (CSPG4)* DNA vaccine, COMMs
34 expressing HIF-1 α , VEGF-A, and CXCR4 were associated with a shorter ~~disease-~~
35 ~~free intervals (DFI) (disease free interval)~~ compared to tumors that were negative for
36 these markers ($p=0.03$), suggesting hypoxia can influence immunotherapy response.
37 Western blotting analysis showed that under chemically induced hypoxia, COMM
38 cells ~~line~~ accumulates HIF-1- α and smaller amounts of CAIX ~~proteins~~. HIF-1 α
39 induction and stabilization triggered by hypoxia was ~~further~~ corroborated by
40 immunofluorescence showing its nuclear translocation. These findings reinforce the
41 ~~crucial~~ role of a hypoxic microenvironment in tumor progression and patient outcome
42 in COMM, as previously established in several human and canine cancers.

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43 Additionally, ~~the~~ hypoxic markers may represent promising prognostic markers,
 44 highlighting opportunities for their use in therapeutic strategies for COMMs.

45

46 **Keywords:** canine, [cell culture](#), cobalt chloride, hypoxia, [in vitro](#)
 47 [assayimmunohistochemistry](#), oral melanomas, prognosis

48

49 Canine oral malignant melanoma (COMM) is the most frequent malignant oral
 50 tumor in dogs, accounting for 30-40% of all oral malignancies.⁴ COMM is a solid
 51 tumor arising from neoplastic transformation of mucosal melanocytes, and is
 52 characterized by aggressive behavior, extensive local invasiveness, and metastatic
 53 potential, resulting in poor prognosis.^{3,54}

54 **Hypoxia** represents one of the most crucial microenvironmental features in
 55 solid tumors⁵⁰ and has been associated with invasiveness, angiogenesis,
 56 vasculogenic mimicry, and response to therapy in several cancer types, including
 57 melanoma.^{10,19} Hypoxia and subsequent acidification of the tumoral
 58 microenvironment are reported to promote cancer progression, ~~and~~ drug
 59 resistance,³⁸ and contribute to immunotolerance of cancer cells, conferring
 60 resistance to immunotherapy.^{1,11,15,24,48} Hypoxia-inducible factor-1 α (HIF-1 α) is the
 61 primary regulator of the adaptive response to low oxygen availability.^{20,27} Upon
 62 stabilization and migration to the nucleus, HIF-1 α acts as transcription factor for
 63 several hypoxia-regulating elements (~~HREs~~). ~~HREs~~ **Hypoxia-regulating elements**, in
 64 turn, induce and modulate various processes, including glycolysis, angiogenesis, cell
 65 migration, invasion, metastasis, and chemoresistance.^{11,43,49,50} ~~Vascular Endothelial~~
 66 ~~endothelial Growth-growth Factor factor~~ **A** (VEGF-A) is a key effector of the hypoxic
 67 response, which stimulates angiogenesis ~~and that~~ provides nutrients and oxygen to

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68 proliferating cancer cells.³⁵ ~~In addition, cancer~~ Moreover, hypoxia stimulates cell
69 ~~homing and migration via chemokine-mediated stimuli. C-X-C chemokine receptor~~
70 ~~type 4 (CXCR4) is overexpressed in various human cancers, including melanoma,~~
71 ~~and primarily contributes to tumor growth, angiogenesis, metastasis, and therapeutic~~
72 ~~resistance.~~^{8,31}

73
74 Cancer cells undergo metabolic reprogramming, exhibiting a highly glycolytic
75 phenotype, which is associated with elevated expression of glucose transporter
76 isoform 1 (GLUT1).¹² The metabolic shift increases lactate production. ~~The lactate~~
77 Lactate is then exported into the extracellular space leading to microenvironment
78 acidification and carbonic anhydrase IX (CAIX) induction. ~~Moreover, hypoxia~~
79 ~~stimulates cell homing and migration via chemokine-mediated stimuli. C-X-C~~
80 ~~chemokine receptor type 4 (CXCR4) is overexpressed in various human cancers,~~
81 ~~including melanoma, and primarily contributes to tumor growth, angiogenesis,~~
82 ~~metastasis, and therapeutic resistance.~~^{8,34}

83 Hypoxic biomarkers have been extensively investigated in human cancers
84 and have been associated with prognosis and therapy resistance.^{18,28} Despite the
85 increasing knowledge on the role of hypoxia in tumors, to date only a few studies are
86 available in dogs.³⁰ Hypoxia has been reported as a frequent condition occurring in
87 COMMs, in which HIF-1 α activation induces the transcription of GLUT1 and CAIX.³⁰
88 A recent study showed that the hypoxia-regulated miRNAs, miR-210 and miR-301,
89 are differentially expressed in primary and metastatic canine melanoma cell lines,
90 and metastatic cells are more resistant to hypoxia stimuli than primary tumor
91 cells.^{17,51} Additionally, it has been demonstrated, that by the expression of HIF-1 α ,

92 ~~that in~~ COMM tumor cells invading the bone are under hypoxic conditions, and this
93 may explain a poorer efficacy of ~~RT~~ (radiotherapy) in dogs with bone lysis.³³

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94 Based on these premises, and considering the aggressiveness and therapy
95 resistance of COMM,³⁷ the aim of this ~~research-study~~ was to unveil the prognostic
96 value of HIF-1 α and the associated hypoxia-response ~~genes~~proteins, GLUT 1,
97 CXCR4, CAIX, and VEGF-A, in COMM. Additionally, the study aimed to examine the
98 activation of HIF-1 α and its biological alterations after inducing a hypoxic state in a
99 metastatic COMM cell line.

100

101 **Material and Methods**

102 **Sample collection and clinical data**

103 A retrospective study was performed on 36 COMMs diagnosed at the
104 Department of Veterinary Sciences of the University of Turin in the period between
105 2005 and 2018. The cases were retrieved through a data-base ~~research~~ using the
106 following keys words: "canine", "oral cavity", and "malignant melanomas". Pre-
107 operative clinical tumor staging^{4,54} was assessed by means of total body computed
108 tomography (~~CT scan~~) in 24 cases, or skull and three views chest radiographs and
109 abdominal ultrasound in 12 cases. The primary tumor was removed by *en-bloc*
110 excision (mandibulectomy, maxillectomy, or lip-cheek excision followed by
111 reconstruction) with regional (ipsilateral or bilateral) lymphadenectomy.

112 Dogs were clinically staged based- on- tumor size, regional lymph nodes
113 involvement, and metastasis (TNM) according to Bergman et al 2007.^{3,4} Sixteen out
114 of the 36 COMMs -with a chondroitin sulfate proteoglycan-4 (CSPG4)

115 immunohistochemical score $\geq 3/8$ received CSPG4 DNA ~~electro-electro~~-vaccination
116 according to the Good Clinical Practice guidelines for animal clinical studies.^{29,40,45,59}
117 The remaining 20 cases were treated with surgery alone. Both the Ethics Committee
118 of the University of Turin and the Italian Ministry of Health approved the trial
119 (0004230-20/02/2018-DGSAF-MDS-P).

120 Follow-up for the non-vaccinated dogs consisted of clinical evaluation and
121 thoracic radiographs performed every 3 months during the first year and then every 6
122 months for a minimum of 2 years. Dogs receiving CSPG4 DNA ~~electro-electro~~-
123 vaccination were clinically evaluated monthly with thoracic ~~CT-computed~~
124 ~~tomography~~scan.

125 **Histological and Immunohistochemical analysis**

126 Formalin-fixed and paraffin-embedded histologic sections of the tumors were
127 stained with hematoxylin and eosin. ~~Diagnosis-Diagnoses was-were~~ performed
128 independently by three observers (LM, SI, CG). In case of disagreements, a
129 consensus was reached using a multi-headed microscope.

130 Specimens were classified according to the World Health Organization (WHO)
131 guidelines⁵⁶ and evaluated for prognostic parameters in canine melanocytic
132 neoplasia according to the current literature. Briefly, nuclear atypia was assessed
133 according to what has been described by Spangler and Kass.⁵⁸ A ~~t~~threshold value
134 of 30% atypical nuclei ~~evaluating almost 200 cells~~ was considered. ~~MC-(mM~~mitotic
135 count) was obtained by counting the ~~absolute~~ number of mitoses in ~~the area~~
136 2.37 mm^2 , considering the regions of highest mitotic activity and avoiding areas of
137 ulceration, necrosis, and inflammation ~~when counting mitoses~~. The MCmitotic
138 count ~~cut~~-off value was ≥ 4 according to Smedley et al.⁵⁶

139 Pigmentation was assessed subjectively as described by Smedley and colleagues⁵⁶
 140 evaluating all available sections and dividing cases [into those](#) with ≥50% and [those](#)
 141 [with](#) < 50% [of](#) pigmented cells. Ki67 was determined as the average number of
 142 positive neoplastic cell nuclei per [area of a](#) 1 cm² optical grid reticle at 400x
 143 magnification/40x objective (5 grid areas counted) in the highest [labelling](#) area with
 144 a [cut-off](#) value of 19.5.
 145 Immunohistochemistry was performed on 4 [µm](#)-thick paraffin sections. After
 146 blocking peroxidase activity (0.3% [H2O2-hydrogen peroxide](#) in deionized water for
 147 30 min) and heat-induced antigen retrieval (30 min with citrate buffer at 98°C, pH
 148 6.0), sections were incubated with primary antibodies Ki-67, HIF-1α, CAIX, CXCR4,
 149 GLUT-1, and VEGF-A overnight at 4°C ([Table 1](#)); the detection was performed using
 150 the Vectastain Universal Quick Kit® (Vector Laboratories). Immunolabelled slides
 151 were randomized and masked for blinded examination, which was performed by
 152 three independent pathologists (SI, ML, LA). In case of disagreements, a consensus
 153 was reached using a multi-headed microscope.

154 Antibodies specificity for HIF-1α, CXCR4, VEGFA, GLUT1, and CAIX was
 155 assessed on positive and negative tissues (canine mammary carcinomas, renal
 156 carcinoma, skin, and testis) as shown in [supplementary Supplemental Figure S1](#).

157 Immunohistochemical evaluation was performed by a semi-quantitative method
 158 evaluating both the intensity and the percentage of positive cells using scoring
 159 systems previously published ([Supplementary Supplemental Table S2](#)),^{25,29,30,39,44,54}

160 Cell line and CoCl2 treatment

161 A canine malignant melanoma cell line (OLGA), previously established by the
 162 authors,⁴⁶ was cultured in [DMEM-Dulbecco's modified Eagle's](#) medium

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Commented [13]: Please either provide the citation number for the papers listed in Supplemental Table S2 or provide the full citations in the footnotes.

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163 supplemented with 10% fetal bovine serum (~~FBS~~), 1% glutamine, 100 µg/mL
164 penicillin₁ and 100 µg/mL streptomycin at 37°C and 5% CO₂. The OLGA cell line
165 was cultured in chemically induced hypoxia for 12, 24₁ and 48 hours using a
166 concentration of 200 µM cobalt chloride (CoCl₂; Sigma Aldrich, 15862-1ML-F).⁴²

167 **Western blot analysis**

168 Proteins from the OLGA cell line, treated with 200 µM of CoCl₂ for 12, 24₁ and
169 48 ~~h-hours~~ or untreated (DMSO alone), were extracted in lysis buffer (1% Triton X-
170 100, 10% glycerol, 50mM Tris, 150mM sodium chloride, 2mM EDTA, pH 8.0₁ and
171 2mM magnesium chloride) containing Protease Inhibitor Cocktail (P8340 Sigma).
172 Twenty micrograms of total proteins were separated ~~in-on a 10%~~ SDS-PAGE
173 (~~10%~~gel) and transferred onto a 0.2 µm pore-size nitrocellulose membrane (Cytiva;
174 Thermo Fisher Scientific). After washing, membranes were incubated in
175 ~~TBS/BSA~~Tris-buffered saline with 10% (bovine serum albumin) at room temperature
176 for 1 hour and then incubated overnight at 4°C with HIF1-α and CAIX antibodies; α-
177 tubulin was used as a housekeeping protein (~~Supplementary~~ **Supplemental Table**
178 **S1**). Horseradish peroxidase (~~HRP~~) conjugated secondary antibodies were diluted at
179 1:15.000. The chemiluminescence substrate (Clarity ECL Substrate; BIO-RAD
180 Laboratories) was used to produce the light signal, acquired with CL-XPosure films
181 (Thermo Fisher Scientific Inc).

182 **Immunofluorescence**

183 To investigate the effects of CoCl₂ on HIF1-α nuclear translocation and CAIX
184 expression, immunofluorescence against HIF1-α and CAIX was performed on OLGA
185 cells exposed to the highest CoCl₂ concentration (200 µM) for 12, 24₁ and 48 hours.
186 Briefly, 2x10⁴ cells were plated in eight-well chamber slides (Lab-Tek II Chamber

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187 Slide System; Nalge Nunc International) until 70% confluence. After treatment, cells
188 were fixed with methanol:acetone (1:1 proportion) for 30 seconds. After washing
189 three times with Tris-HCl (0.1M, pH 7.6), cells were blocked with 10% normal goat
190 serum for 1 hour at room temperature and then incubated overnight at 4°C with
191 antibodies to HIF-1α (1:100) and CAIX (1:200). After washing with Tris-HCl, cells
192 were incubated with a fluorescent secondary Alexa488-conjugated goat anti-rabbit
193 IgG antibody (1:500 dilution, ThermoFisher) and anti-mouse IgG (1:500) for 1 hour at
194 in the dark. Subsequently, cell nuclei were stained with DAPI (0.5µg/ml in Tris-HCl,
195 Sigma–Aldrich, USA) for 10 minutes, washed three times with Tris-HCl, and then the
196 sections were covered with mounting medium (PermaFluor, Thermo Scientific) and
197 kept overnight in the dark. Fields were randomly selected by Leica TCS SP8 (Leica
198 Microsystems CMS GmbH, Mannheim, Germany) and z-stacks of 10 nm were
199 acquired. Z-stacks were then processed directly with the Leica LAS-X software to
200 produce maximum projection images of each field. To detect co-localization of
201 different fluorochromes, fluorescent signals in different channels were merged to
202 produce multi-color images. Image acquisitions were performed with a resolution of
203 1024_x_1024 pixels with a 200_Hz sampling frequency.

204

205 **Statistical analyses**

206 Fisher's Exact-exact test or the chi-squared test was used to test possible
207 associations among immunohistochemical markers expression and; clinical and
208 histopathological data. Disease-free interval DFI (DFI disease-free interval) was
209 calculated as the time elapsing between surgery and the first detection of
210 metastases and/or local recurrence, while overall survival (OS) (overall survival) was

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211 ~~defined~~ as the period from the day of surgery to the patient's death due to ~~a~~ cancer-
212 related cause. The survival functions of the DFI and OS were estimated with the
213 Kaplan-Meier method, and the comparison of survival function was done by means
214 of the log-rank test. Dogs that died from unrelated causes, were lost to follow-up, or
215 were still alive at the end of the study were right-censored. Univariate and
216 multivariate Cox models were fitted to verify the effects of hypoxia biomarkers on DFI
217 or OS. In multivariate analysis, we considered the following parameters: HIF-1 α -,
218 CXCR4, VEGFA, GLUT1, and CAIX immunohistochemical expression. Statistical
219 significance was set at a 0.05 level. Because the clinical samples belonging to stage
220 I were censored, this stage was omitted. GraphPad Prism 8 (GraphPad Software,
221 San Diego, California) and R statistical software (R Core Team, 2018) were used for
222 statistical analysis.

223

224 RESULTS

225 *Patient and tumor characteristics*

226 A total of 36 dogs with COMM met the inclusion criteria. Clinical and follow-up
227 data, and histopathological and immunohistochemical diagnostic features are
228 provided in Tables 1 and ~~Table 2~~, respectively. Survival analysis (Fig.1) showed that
229 dogs with clinical stage IV had a worse prognosis compared to dogs with clinical
230 stages II and III ($p=0.002$). Similarly, ~~as shown in Figure 2~~, vaccinated dogs had a
231 significantly higher OS time compared to non-vaccinated dogs ($p<0.001$) (Fig. 2).
232 Although not significant, we found that COMMs with a total absence of melanin
233 ~~pigment~~ or less than 25% pigmented neoplastic cells were associated with a lower
234 DFI when compared to tumors with > 50% pigmented neoplastic cells (median DFI:

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235 days 180 vs 210 days, respectively, $p = 0.06$). ~~There~~ The OS was ~~also not~~
 236 significantly ~~association-associated with OS~~ the amount of pigmentation (median OS:
 237 235 days vs 778 days, $p = 0.08$), and none of the other histopathological parameters
 238 showed prognostic significance.

239 ***Overexpression of hypoxic markers negatively affects prognosis in dogs***
 240 ***receiving immunotherapy.***

241 The immunohistochemical scores for HIF-1 α , CAIX, GLUT-1, CXCR4, and
 242 VEGF-A are summarized in Table 3. Representative images are shown in Figure 3.
 243 ~~Interestingly, t~~ The CAIX score was associated with the HIF-1 α score ($p = 0.001$).
 244 Additionally, we found a statistical association between CXCR4 and Ki-67 index ($p =$
 245 0.046) (Table 4). Multivariate analysis revealed that dogs receiving immunotherapy
 246 and overexpressing HIF-1 α , VEGF-A, and CXCR4 had a significantly shorter DFI
 247 (median = 111 days) compared to dogs with the same treatment ~~but that were~~
 248 negative for all three markers or only positive ~~to for~~ 1 or 2 markers (median = 204
 249 days; $p = 0.03$) (Fig. 4), while no significant differences were observed for OS.

250 Multivariate analysis in non-vaccinated patients revealed no statistically
 251 ~~significance-significant differences~~ in OS ~~and or~~ DFI between dogs with concomitant
 252 overexpression of HIF-1 α , VEGF-A, and CXCR4 and dogs negative ~~to for~~ the
 253 markers or expressing only one or two markers.

254 ***CoCl₂ treatment induces HIF-1 α accumulation and nuclear translocation***

255 ~~As illustrated in Figure 5, distinct~~ Distinct bands corresponding to HIF-1 α (120
 256 kDa) and CAIX (58 kDa) proteins were more pronounced in the Olga cell line treated
 257 with CoCl₂ at 12 and 24 hours (Fig. 5). In contrast, the expression of these proteins
 258 was either negative (HIF-1 α) or very low (CAIX) in untreated cells (Fig. 5).

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259 A mild increase of CAIX protein was also present in chemically-induced
260 hypoxic conditions after 24 hours. ~~As depicted in Figure 6, under~~ Under normoxic
261 conditions, HIF-1 α was primarily localized to the cytoplasm, with nuclear localization
262 becoming evident only after CoCl₂ treatment (Fig. 6). In contrast, chemically induced
263 hypoxia did not yield noticeable effects on CAIX protein.

264

For Peer Review

265 **DISCUSSION**

266 COMM is the most commonly diagnosed malignant tumor occurring in the oral
267 cavity of dogs,^{16,54} and it has a high and rapid metastatic rate, resulting in a poor
268 prognosis.³ Recently, a consensus working group was founded⁵⁵ to identify potential
269 prognostic markers in this neoplasm and to give accurate recommendations for the
270 diagnosis and histopathologic prognostication of canine melanocytic tumors.⁵⁵
271 Although nuclear atypia, MC (mitotic count,) and Ki-67 index are considered the most
272 prognostic factors for COMM, the identification of new markers may improve the ability
273 to prognosticate these neoplasms, as well as aiding in the selection of specific
274 therapies.

275 In this study, we observed that patients with advanced clinical stages (III and
276 IV) had a shorter overall survivalOS compared to stage II patients, which is consistent
277 with existing literature.³ Furthermore, our data confirmed that anti-CSPG4 vaccination
278 significantly increased the overall survival of COMM patients.^{40,46}

279 Interestingly, smaller amounts of melanin in theCOMMs seem to be weakly
280 associated with shorter DFI, suggesting a close association between loss of melanin
281 pigment and tumor dedifferentiation, which may be associated with a more aggressive
282 behavior. This finding doesn't represent a novelty because in 2011, Smedley et al.
283 suggested that a lower level of pigmentation (<50% of pigmented cells) is an
284 unfavorable factor.⁵⁴ In human cutaneous melanoma, decreased pigmentation has
285 also been linked to an aggressive phenotype with implications for prognosis and
286 response to therapy.^{6,53}

287 As previously reported in a number of canine tumor histotypes, a correlation
288 between CAIX and HIF-1 α positivity has been observed, suggesting hypoxia may

289 trigger microenvironment acidosis with metabolic changes in cancer cells growth.³⁶
290 Our findings reinforce the close interaction between these molecules and highlight the
291 strong HIF-1 α -dependent regulation of CAIX as an adaptation of COMM cells to
292 extracellular acidosis.⁶¹ This adaptation may enhance the tumor's ability to survive and
293 grow under adverse conditions.

294 We found that CXCR4 expression was associated with an increased Ki-67
295 index, which is consistent with previous reports in human renal carcinomas⁹ and
296 multicentric lymphoma.⁶³ However, in human cutaneous melanomas, these markers
297 ~~were have~~ not ~~been~~ significantly associated.^{31,32,60}

298 Univariate data analysis for the hypoxic markers did not show any statistical
299 significance for DFI or OS. However, these markers are interconnected in the hypoxic
300 cellular pathway signaling. Hence, we investigated the prognostic value of their co-
301 expression. Multivariate data analysis revealed that concurrent expression of HIF-1 α ,
302 VEGF-A and CXCR4 in COMMs treated with ~~anti-a~~ CSPG4 vaccine is associated with
303 a lower DFI compared to COMMs negative for these markers. This suggests a lower
304 efficacy of ~~anti~~-CSPG4 vaccination in tumors displaying hypoxic features. This finding
305 is in line with the literature, in which hypoxia is known to induce immune-resistance
306 and negatively interferes with immune surveillance of tumors, as well as adoptive
307 immunotherapy.^{2,24,62} Likewise, co-expression of hypoxic markers and hypoxia-related
308 signatures have been documented in several human cancer as predictive of a poor
309 outcome.^{7,26,64} This co-expression triggers the activation of HIF-1 α -dependent
310 pathways, which can lead to tumor growth, angiogenesis, and metastasis.⁸ Regarding
311 the relationship between hypoxic microenvironments and CSPG4 expression, it's very
312 interesting to underscore that in human melanomas, CSPG4 is regulated by hypoxia
313 in vitro and its expression confers resistance to immunotherapy.^{23,41} On the basis of

314 the preliminary data obtained by the authors, we can hypothesize that in dogs, CSPG4
315 is regulated by hypoxia, strengthening COMM as a good model for comparative
316 oncology.³⁴

317 To gain insight into the mechanisms of hypoxia in COMM, the OLGA cell line
318 was treated with CoCl₂ to mimicking a hypoxic stimulus.¹³ We found that *in vitro*
319 hypoxia induction caused an accumulation of HIF-1 α protein after treatment. HIF-1 α
320 induction and stabilization triggered by hypoxia was further corroborated by the results
321 of immunofluorescence, in which nuclear translocation under CoCl₂ treatment to avoid
322 proteasomal degradation was demonstrated.⁵⁰ In the nucleus, HIF-1 α is known to
323 directly coordinate the transcription of HREhypoxia-regulating elements.⁴⁹ Consistent
324 with the immunohistochemical results, CAIX protein levels were also mildly increased
325 under chemically induced hypoxia. Its accumulation was delayed as compared to HIF-
326 1 α , thus supporting the direct role of HIF-1 α in the upregulation of this protein.²¹

327 In conclusion, our results reinforce the crucial role of a hypoxic
328 microenvironment and acidification in tumor aggressiveness and outcome in COMM,
329 as extensively established in other canine^{14,57} and human cancers.⁵ It becomes critical
330 to develop novel therapeutic strategies considering the hypoxic status of the tumors,
331 and future studies should address the *in vitro* effects of hypoxia in COMM cells and
332 investigate the inhibition of hypoxia-related signaling pathways as potential
333 therapeutic targets. Notably, as shown in this study, it is crucial to consider the
334 interdependent actions of the molecular mechanisms triggered by HIF-1 α and its
335 transcriptional cascade.

336

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489

For Peer Review

490 **LEGEND OF THE FIGURES**

491

492 **Figure 1**

493 Kaplan-Meier survival curves for stage II, III, and IV canine oral malignant
 494 melanomas. (a) Disease-free interval (DFI) and (b) Overall Survival
 495 survival (OS) curves of COMMs patients with respect to clinical stages II, III and IV.
 496 Stage II (median DFI = 187 days, Median OS = 397 days); Stage III
 497 (median DFI = 209 days, median OS = 260 days); Stage IV (median DFI = 36 days,
 498 median OS = 76). p-values: DFI: $p = 0.06$ and OS: $p = 0.002$

499

500 **Figure 2**

501 Kaplan-Meier survival curves of dogs with canine oral malignant melanomas treated
 502 with or without chondroitin sulfate proteoglycan-4 (CSPG4) DNA vaccination. (a)
 503 Disease-free interval (DFI). Vaccinated median DFI = 205 days, not
 504 vaccinated median DFI = 187 days ($p > 0.05$), and (b) Overall Survival (OS).
 505 Vaccinated median OS = 972 days, not vaccinated median OS = 215 days
 506 ($p < 0.001$) curves of COMMs (canine oral malignant melanomas) patients treated with
 507 CSPG4 (chondroitin sulfate proteoglycan-4)/DNA vaccination with respect to
 508 COMMs patients treated with *en-bloc* surgery alone, (median OS: 972 days versus
 509 215 days $p < 0.001$); (median DFI 205 days versus 187 days ($p > 0.05$)).

510

511 **Figure 3**

Commented [29]: Please capitalize 'not' in the key for Figure 2.

Commented [RD30R30]: Ok

512 Melanomas Immunohistochemistry (IHC) for hypoxia-associated factors in canine
 513 oral malignant melanomas, dog. (a) ~~diffuse~~-Diffuse moderate cytoplasmic
 514 immunolabeling for ~~VEGFA~~ (Vascular-vascular endothelial growth factor A (VEGFA)
 515 in amelanotic melanoma. ~~VEGFA~~ IHC. (b) ~~diffuse~~-Diffuse strong cytoplasmic and
 516 membrane immunolabeling for ~~CXCR4~~ (C-X-C chemokine receptor type 4 (CXCR4)
 517 in spindle amelanotic melanoma. ~~CXCR4~~ IHC. (c) ~~multifocal~~-Multifocal nuclear
 518 immunolabeling for ~~HIF-1 α~~ (Hypoxia-hypoxia-inducible factor-1 α (HIF-1 α) in poorly
 519 melanotic melanoma. ~~HIF-1 α~~ IHC. (d) ~~multifocal~~-Multifocal moderate cytoplasmic
 520 immunolabeling for ~~GLUT1~~ (Glucose-glucose transporter 1 (GLUT1) in poorly
 521 melanotic melanoma. ~~GLUT1~~ IHC. (e) ~~diffuse~~-Diffuse weak cytoplasmic
 522 immunolabeling for ~~CAIX~~ (Carbonic-carbonic anhydrase IX (CAIX) in poorly
 523 melanotic melanoma. ~~CAIX~~ IHC. (f) ~~negative~~-Negative reagent control. ~~DAB~~
 524 diaminobenzidine chromogen-, hematoxylin counterstain.

525 **Figure 4**

526 Kaplan-Meier survival curves of dogs with canine oral malignant melanomas treated
 527 with chondroitin sulfate proteoglycan-4 DNA vaccination that either co-express
 528 hypoxia-inducible factor-1 α (HIF-1 α), C-X-C chemokine receptor type 4 (CXCR4),
 529 and vascular endothelial growth factor A (VEGFA) or lack the expression of at least
 530 one of protein (others). (a) ~~Disease~~-Disease-free interval (DFI). Patients
 531 overexpressing HIF-1 α , CXCR4, and VEGFA had significant shorter DFIs ($p < 0.05$)
 532 compared to dogs that were negative for all three markers, as well as those positive
 533 to only 1 or 2 markers (others). and (b) Overall ~~Survival~~-survival (OS). There were
 534 no statistically significant differences in OS ($p > 0.05$). -curves of COMMs (canine
 535 oral malignant melanomas) patients vaccinated with CSPG4 (chondroitin sulfate
 536 proteoglycan 4). As shown in the figure, patients overexpressing HIF-1 α (hypoxia-

Commented [31]: Nuclear staining is not evident in this image. While there are darkly labeled areas, it is not clear where those areas are. The recognizable nuclei are not staining.

Commented [32R32]: One approach to address the AE's concern is to provide an image where the signal is less saturated and the chromatin pattern of the nucleus is more apparent.

Commented [RD33R32]: According to suggestions the figure 3 C has been replaced with nuclear staining is more evident

537 inducible factor-1 α), CXCR4 (C-X-C chemokine receptor type 4) and VEGFA
538 (vascular endothelial growth factor A) showed a significant shorter DFI ($p < 0.05$) with
539 respect to negative COMMs for all three markers, as well as those positive to only 1
540 or 2 markers. OS ($p > 0.05$)

541 **Figure 5**

542 Western blot analysis of Olga cells that were untreated (Ctrl) or cells that were
543 treated for 12, 24, or 48 hours with cobalt chloride (CoCl_2) to induce hypoxic
544 conditions. HIF-1 α (hypoxia-inducible factor-1 α), CAIX (Carbonic anhydrase IX)
545 and α -tubulin. 20 μg total proteins were run on a 10% polyacrylamide gel and
546 transferred to a nitrocellulose membrane. Visualization was performed by exposing
547 X-ray films on membranes incubated with secondary HRP-conjugated antibodies.
548 CTRL (not treated sample control)

549 **Figure 6**

550 Representative immunofluorescence staining of primary canine oral malignant
551 melanoma COMMs (canine oral malignant melanomas) OLGA cell line, either (a-d)
552 untreated (panels a-d) or (e-h) treated with 200 μm cobalt chloride (CoCl_2) (panel e-
553 h) for 24 hours. (a, e) Panel a and e show DAPI nuclear staining, in blue. (b, f)
554 panel b and f HIF1- α (hypoxia-inducible factor-1 α (HIF1- α), staining in green. panel
555 e and g (c, g) CAIX (carbonic anhydrase IX (CAIX), staining in red. and panel d and
556 h show the (d, h) merged fluorescent signals of DAPI, HIF1- α , and CAIX.

557

558 **Supplementary Figure 1**

559 Validation of IHC-immunohistochemistry (IHC) antibodies on canine tissues. (a, b)
560 Hypoxia-inducible factor-1 α (HIF-1 α) IHC. (a) haired skinned skin as, negative

561 tissue control. (a1) and (b) Mammary carcinoma-as, positive tissue control. (c-d)
 562 (a2); CXCR4C-X-C chemokine receptor type 4 IHC.; (c) Hhaired skin-as, negative
 563 tissue control. (b1d) and mammary-Mammary carcinoma-as, positive tissue control.
 564 (b2); (e, f) glucose transporter 1 IHC. GLUT-1: (e) testis-Testis,as negative tissue
 565 control. (f) (c1) and placenta-Placenta,as positive tissue control-(c2);-. (g, h) Carbonic
 566 anhydrase IX.CAIX: (g) Ttestis,as negative tissue control. (h) (d1) and renal-Renal
 567 cell carcinoma-as, positive tissue control. (d2)-DAB chromogen, hematoxylin
 568 counterstain.

Commented [34]: Please submit the supplemental figure legend as a separate Word file.

Commented [35R35]: Please label the supplemental figure in the same manner other figures are labeled. I have adjusted the legend accordingly, but please change the letters on the figure.

Commented [RD36R35]: Done in the MAIN DOCUMENT (CLEAN COPY)

Authors' Contributions

573 RDM, CG, designed and performed the experimental design. SI, LM, performed
 574 histological diagnosis; LM, PM, KV performed IHC evaluations; EM, DG, MO
 575 enrolled and surgically treated COMMs
 576 EM* performed statistical analysis.
 577 PA EM** performed in vitro assay, western blot and immunofluorescence.
 578 The manuscript was written by RDM, CG, LM with contribution of LA.

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