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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-1α (HIF-1α) 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-1α expression was observed in the nucleus, and this localization correlated with the presence or enhanced expression of HIF-1α -regulated genes at the protein level. Multivariate analysis revealed that in dogs given chondroitin sulfate proteoglycan-4 (CSPG4) DNA vaccine, COMMs expressing HIF-1α, 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

	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.

Hypoxia-associated markers in the prognosis of 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-1α (HIF-1α) 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-1α expression was observed in the nucleus, and this localization correlated with the presence or enhanced expression of HIF-1α - regulated genes at the protein level. Multivariate analysis revealed that in dogs given *chondroitin sulfate proteoglycan-4* (*CSPG4*) DNA vaccine, COMMs expressing HIF-1α, 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 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.

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

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

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

human cancers, including melanoma, and primarily contributes to tumor growth, angiogenesis, metastasis, and therapeutic resistance.[8,31]

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

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

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

Material and Methods

Sample collection and clinical data

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

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

Follow-up for the non-vaccinated dogs consisted of clinical evaluation and thoracic radiographs performed every 3 months during the first year and then every 6 months for a minimum of 2 years. Dogs receiving *CSPG4* DNA electro-vaccination 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 guidelines[56] 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 mm^2, 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 colleagues[56] evaluating all available
129 sections and dividing cases into those with ≥50% and those with < 50% pigmented
130 cells. Ki67 was determined as the average number of positive neoplastic cell nuclei
131 per 1 cm^2 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-1α, CAIX, CXCR4, GLUT-1, and VEGF-

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

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

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

Cell line and CoCl2 treatment

A canine malignant melanoma cell line (OLGA), previously established by the authors,[46] was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO2. The OLGA cell line was cultured in chemically induced hypoxia for 12, 24, and 48 hours using a concentration of 200 µM cobalt chloride ($CoCl_2$; Sigma Aldrich, 15862-1ML-F).[42]

Western blot analysis

Proteins from the OLGA cell line, treated with 200 µM of $CoCl_2$ for 12, 24, and 48 hours or untreated (DMSO alone), were extracted in lysis buffer (1% Triton X-100, 10% glycerol, 50mM Tris, 150mM sodium chloride, 2mM EDTA, pH 8.0, and 2mM magnesium chloride) containing Protease Inhibitor Cocktail (P8340 Sigma). Twenty micrograms of total proteins were separated on a 10% SDS-PAGE gel and

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

Immunofluorescence

To investigate the effects of $CoCl_2$ on HIF1-α nuclear translocation and CAIX expression, immunofluorescence against HIF1-α and CAIX was performed on OLGA cells exposed to the highest $CoCl_2$ concentration (200 µM) for 12, 24, and 48 hours. Briefly, $2x10^4$ cells were plated in eight-well chamber slides (Lab-Tek II Chamber Slide System; Nalge Nunc International) until 70% confluence. After treatment, cells were fixed with methanol:acetone (1:1 proportion) for 30 seconds. After washing three times with Tris-HCl (0.1M, pH 7.6), cells were blocked with 10% normal goat serum for 1 hour at room temperature and then incubated overnight at 4°C with antibodies to HIF-1α (1:100) and CAIX (1:200). After washing with Tris-HCl, cells were incubated with a fluorescent secondary Alexa488-conjugated goat anti-rabbit IgG antibody (1:500 dilution, ThermoFisher) and anti-mouse IgG (1:500) for 1 hour in the dark. Subsequently, cell nuclei were stained with DAPI (0.5µg/ml in Tris-HCl, Sigma–Aldrich, USA) for 10 minutes, washed three times with Tris-HCl, and then the sections were covered with mounting medium (PermaFluor, Thermo Scientific) and kept overnight in the dark. Fields were randomly selected by microscope TCS SP8 (Leica Microsystems CMS GmbH Mannehim, Germany) and z-stacks of 10 nm were

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

Statistical analyses

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

RESULTS

Patient and tumor characteristics

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

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

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

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

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

CoCl2 treatment induces HIF-1α accumulation and nuclear translocation

Distinct bands corresponding to HIF-1α (120 kDa) and CAIX (58 kDa) proteins were more pronounced in the Olga cell line treated with $CoCl_2$ at 12 and 24 hours (Fig. 5). In contrast, the expression of these proteins was either negative (HIF-1α) or very low (CAIX) in untreated cells (Fig. 5).

A mild increase of CAIX protein was also present in chemically-induced hypoxic conditions after 24 hours. Under normoxic conditions, HIF-1α was primarily localized to the cytoplasm, with nuclear localization becoming evident only after $CoCl_2$ treatment (Fig. 6). In contrast, chemically induced hypoxia did not yield noticeable effects on CAIX protein.

DISCUSSION

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

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

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

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

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

We found that CXCR4 expression was associated with an increased Ki-67 index, which is consistent with previous reports in human renal carcinomas[9] and multicentric lymphoma.[63] However, in human cutaneous melanomas, these markers have not been significantly associated.[31,32,60]

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

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

To gain insight into the mechanisms of hypoxia in COMM, the OLGA cell line was treated with $CoCl_2$ to mimic a hypoxic stimulus.[13] We found that *in vitro* hypoxia induction caused an accumulation of HIF-1α protein after treatment. HIF-1α induction and stabilization triggered by hypoxia was further corroborated by immunofluorescence, in which nuclear translocation under $CoCl_2$ treatment to avoid proteasomal degradation was demonstrated.[50] In the nucleus, HIF-1α is known to directly coordinate the transcription of hypoxia-regulating elements.[49] Consistent with the immunohistochemical results, CAIX protein levels were also mildly increased under chemically induced hypoxia. Its accumulation was delayed as compared to HIF-1α, thus supporting the direct role of HIF-1α in the upregulation of this protein.[21]

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

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LEGEND OF THE FIGURES

Figure 1

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

Figure 2

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

Figure 3

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

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

Figure 4

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

Figure 5

Western blot analysis of Olga cells that were untreated (Ctrl) or cells that were treated for 12, 24, or 48 hours with cobalt chloride ($CoCl_2$) to induce hypoxic conditions. HIF-1α, hypoxia-inducible factor-1α; CAIX, carbonic anhydrase IX .

Figure 6

Representative immunofluorescence of primary canine oral malignant melanoma OLGA cell line, either (a-d) untreated or (e-h) treated with 200 μm cobalt chloride ($CoCl_2$) for 24 hours. (a, e) DAPI nuclear staining, blue (b, f) hypoxia-inducible

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

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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)

[a]Excision 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		***n (%)***
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)

[a]Immunohistochemistry not available in the remaining samples

Table 3.
Immunohistochemical scoring of hypoxia-related markers

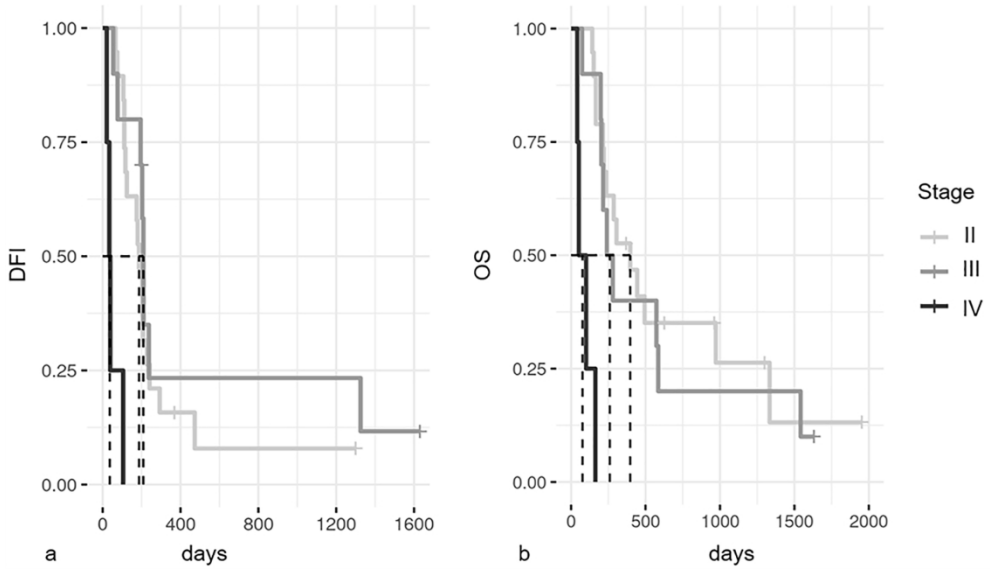
Marker	**IHC Labelling**	***n (%)***	**Total**
HIF-1α	Negative	20 (55.6)	36
	Positive	16 (44.4)	
CAIX	Score 0	9 (29)	31[a]
	Score 1	7 (22.6)	
	Score 2	15 (48.4)	
GLUT-1	Score 0	1 (2.8)	36
	Score 1	12 (33.3)	
	Score 2	23 (63.9)	
CXCR4	Negative	17 (42.2)	36
	Positive	19 (47.2)	
VEGF-A	Score 0	3 (8.3)	36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

[a]Immunohistochemical 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	**≥19.5**	
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***		



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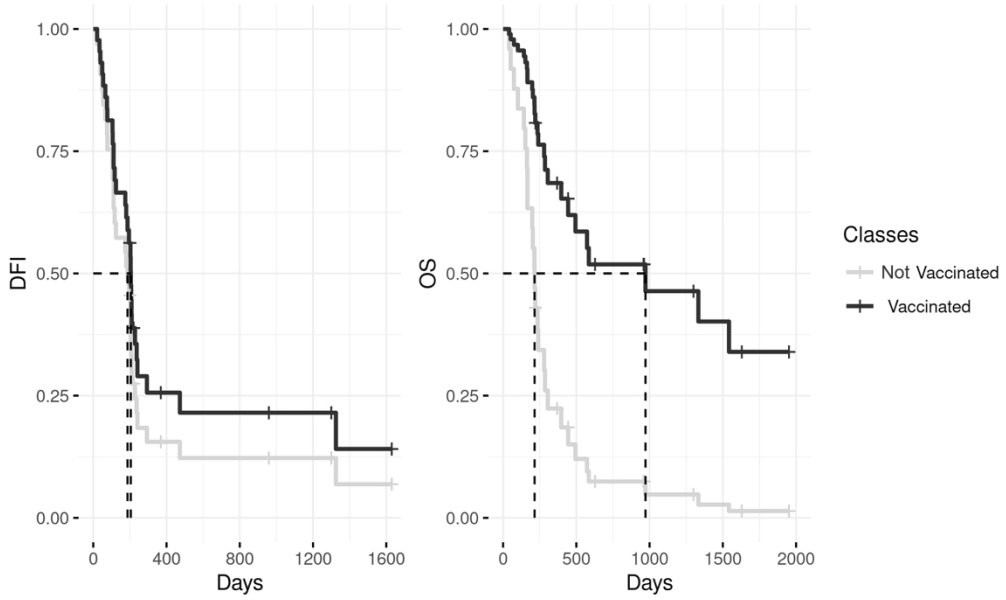

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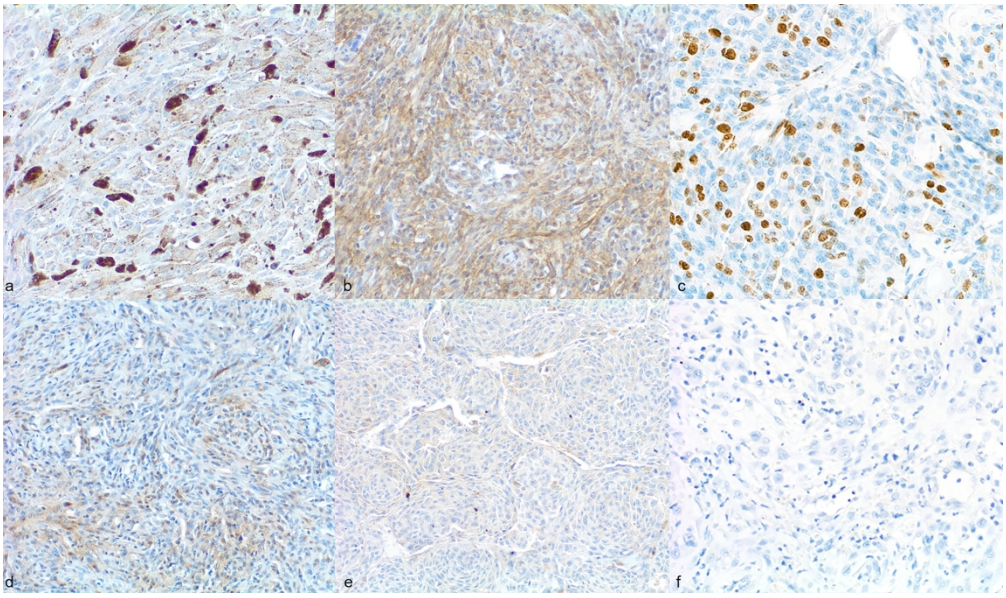

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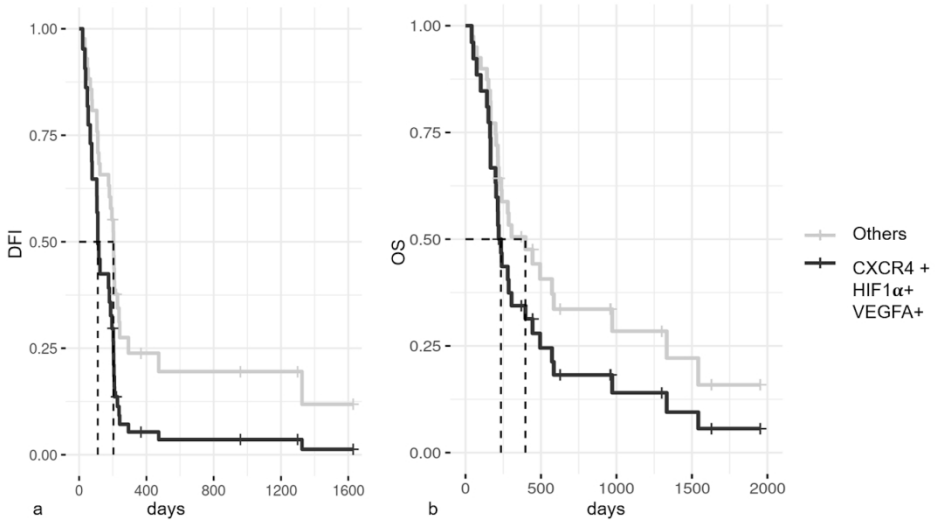

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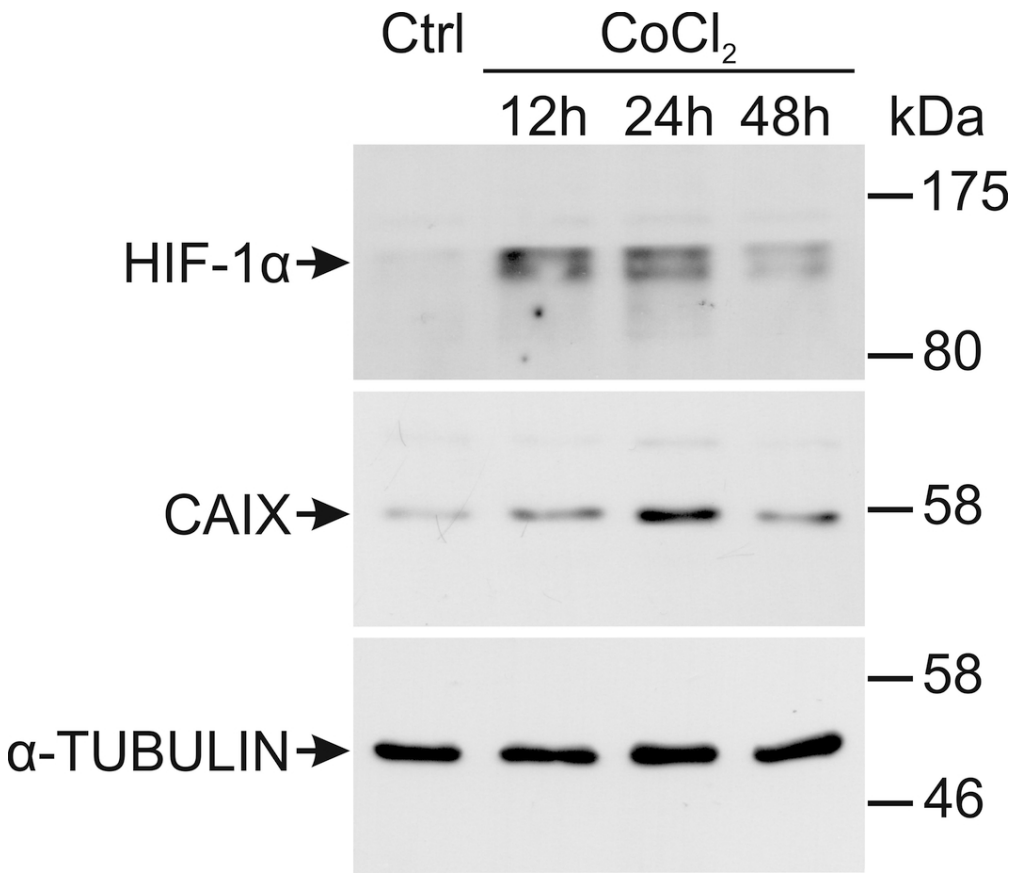

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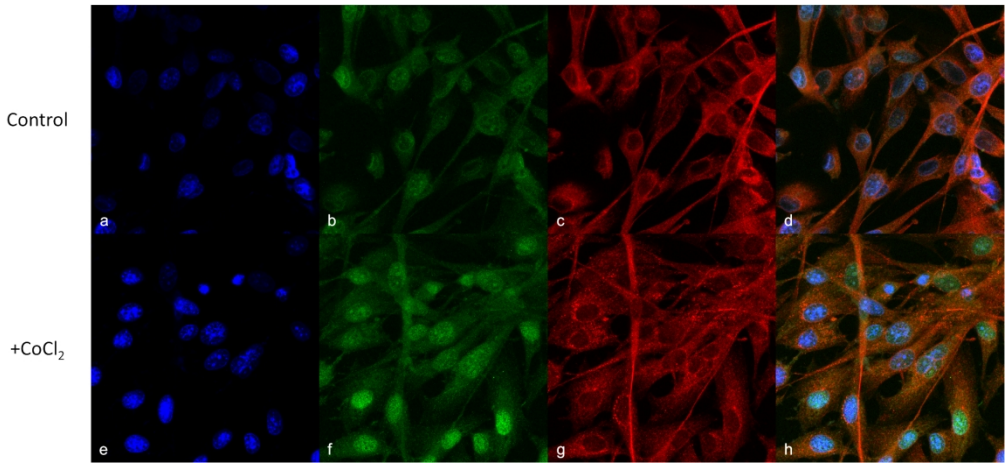

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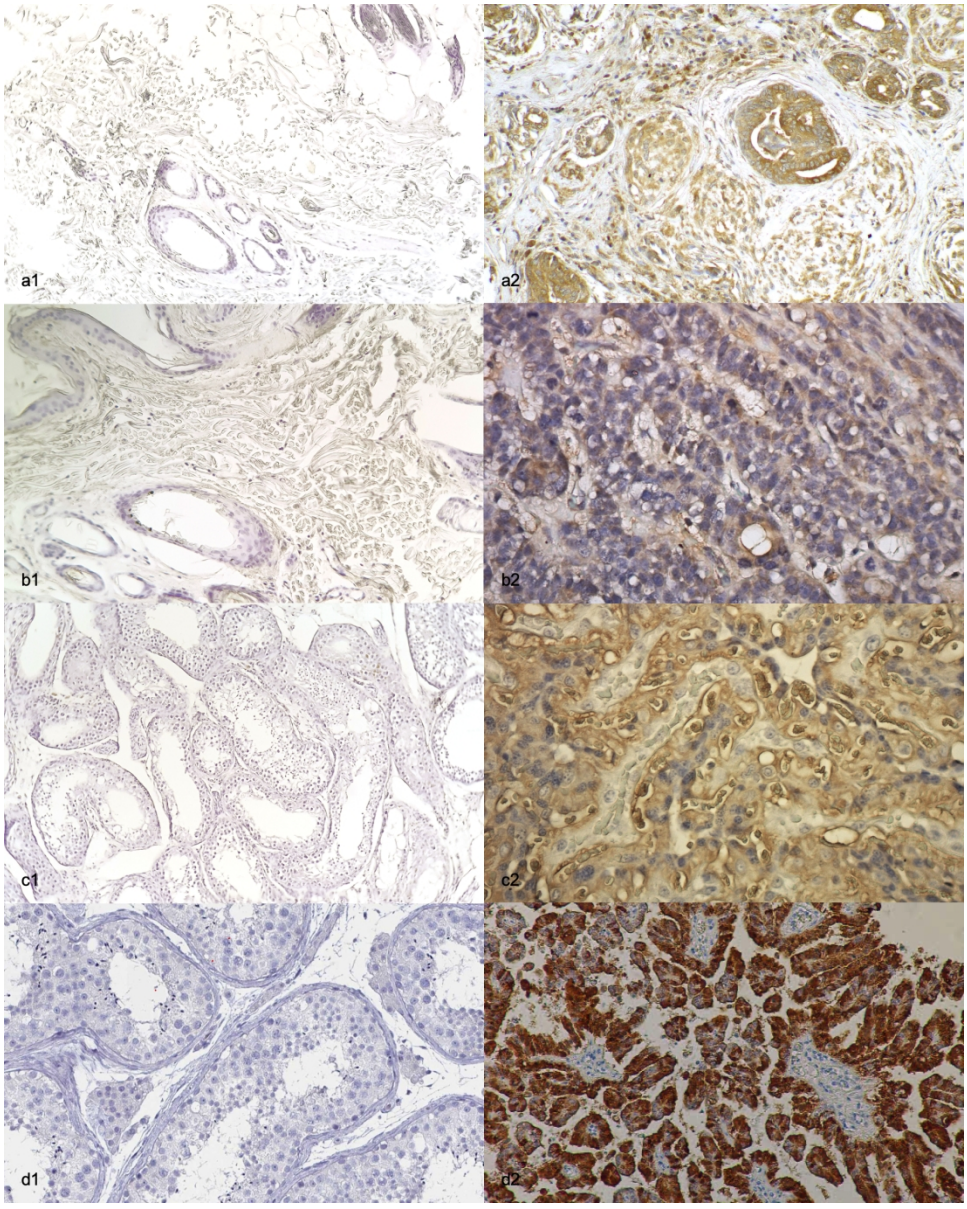
Supplementary Table 1.
Primary antibodies used in this study.

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
HIF-1α	IHC	1:100	A300-286A	Bethyl Laboratories
	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	*% of positive cells* 0 = < 1% 1 = 1-30% 2 = > 30%			Meier et al. (2016)[30]
CSPG4	*% of positive cells* 0 = none 1 = < 1% 2 = 1-10% 3 = 10-33% 4 = 33-66% 5 = > 66%	*Intensity* 0 = negative 1 = weak 2 = intermediate 3 = strong	*Total score [% + Int]* Negative = 0-2 Positive = ≥3	Mayayo et al. (2011) [29]
CXCR4	*% of positive cells* 0 = up to 5% 1 = 10-20% 2 = 21-40% 3 = >40%	*Intensity* 0 = negative 1 = weak 2 = moderate 3= strong	*Total score [% + Int]* Negative = 0-2 Positive = >3	Ren et al. (2016) [44]
GLUT-1	*% of positive cells* 0 = none 1 = up to 50% 2 = > 50%	*Intensity* 0 = negative 1 = weak 2 = strong	*Total score [% x Int]* Negative = 0 Moderate = 1-2 Strong = 4	Petty et al. (2008) [39]
HIF-1α	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	*% of positive cells* 0 = 0-10% 1 = 10-30% 2 = 31-50% 3 = 51-75% 4 = >75%	*Intensity* 0 = negative 1 = yellow 2 = light brown 3= dark brown	*Total score [% x Int]* Negative = 0 Moderate = 1-8 Strong = 9-12	Liu et al. (2017) [25]



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Supplementary Figure 1

Validation of immunohistochemistry (IHC) antibodies on canine tissues. (a, b) Hypoxia-inducible factor-1α (HIF-1α) 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.

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

2

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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-1α (HIF-1α) and its downstream effectors ~~VEGF-A (Vascular~~ vascular endothelial growth factor A (VEGF-A), GLUT1~~)(Glucose transporter 1)~~, C-X-C chemokine receptor type 4 (CXCR4)~~(C-X-C chemokine receptor type 4)~~ , and carbonic anhydrase IX (CAIX) ~~(Carbonic anhydrase IX)~~ are the main regulators of the adaptive response to low oxygen availability. ~~In this study, the~~ The prognostic value of these markers was evaluated in 36 COMMs using immunohistochemistry. Additionally, the ~~in vitro~~ effects of cobalt chloride-mediated hypoxia were evaluated in one primary COMM cell line. HIF-1α expression was observed in the nucleus, and this ~~subcellular~~ localization ~~appeared to~~ correlated with the presence or enhanced expression of HIF-1α -regulated genes at the protein level. Multivariate ~~data~~ analysis revealed that in dogs given *chondroitin sulfate proteoglycan-4* (*CSPG4*) DNA vaccine, COMMs expressing HIF-1α, VEGF-A, and CXCR4 were associated with a shorter disease-free intervals (DFI) ~~(disease free interval)~~ compared to tumors that were negative for these markers (p=0.03), suggesting hypoxia can influence immunotherapy response. Western blotting ~~analysis~~ showed that under chemically induced hypoxia, COMM cells ~~line~~ accumulate~~s~~ HIF-1 α and smaller amounts of CAIX ~~proteins~~. HIF-1α induction and stabilization triggered by hypoxia was ~~further~~ corroborated by immunofluorescence showing its nuclear translocation. These findings reinforce the ~~crucial~~ role of a hypoxic microenvironment in tumor progression and patient outcome in COMM, as previously established in several human and canine cancers.

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

Keywords: canine, cell culture, cobalt chloride, hypoxia, ~~in vitro assay~~immunohistochemistry, oral melanoma~~s~~, prognosis

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

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

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

Cancer cells undergo metabolic reprogramming, exhibiting a highly glycolytic phenotype, which is associated with elevated expression of glucose transporter isoform 1 (GLUT1).[12] The metabolic shift increases lactate production. ~~The lactate~~ Lactate is then exported into the extracellular space leading to microenvironment acidification and carbonic anhydrase IX (CAIX) induction. ~~Moreover, hypoxia stimulates cell homing and migration via chemokine-mediated stimuli. C-X-C chemokine receptor type 4 (CXCR4) is overexpressed in various human cancers, including melanoma, and primarily contributes to tumor growth, angiogenesis, metastasis, and therapeutic resistance.[8,31]~~

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

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

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

Material and Methods

Sample collection and clinical data

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

Dogs were clinically staged based on tumor size, regional lymph node~~s~~ involvement, and metastasis (TNM) according to Bergman et al 2007.[3,4] Sixteen out of the 36 COMMs with a chondroitin sulfate proteoglycan-4 (CSPG4)

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immunohistochemical score ≥ 3/8 received *CSPG4* DNA ~~electro~~ electro- vaccination according to the Good Clinical Practice guidelines for animal clinical studies.[29,40,45,59] The remaining 20 cases were treated with surgery alone. Both the Ethics Committee of the University of Turin and the Italian Ministry of Health approved the trial (0004230-20/02/2018-DGSAF-MDS-P).

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

Histological and Immunohistochemical analysis

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

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

Pigmentation was assessed subjectively as described by Smedley and colleagues[56] evaluating all available sections and dividing cases into those with ≥50% and those with < 50% ~~of~~ pigmented cells. Ki67 was determined as the average number of positive neoplastic cell nuclei per ~~area of a~~ 1 cm^2 optical grid reticle at 400x magnification/40x objective (5 grid areas counted) in the highest~~r~~ labelling area with a cut-off value of 19.5.

Immunohistochemistry was performed on 4 µm~~-~~ thick paraffin sections. After blocking peroxidase activity (0.3% ~~H2O2~~ hydrogen peroxide in deionized water for 30 min) and heat-induced antigen retrieval (30 min with citrate buffer at 98°C, pH 6.0), sections were incubated with primary antibodies Ki-67, HIF-1α, CAIX, CXCR4, GLUT-1, and VEGF-A overnight at 4°C (Table 1); the detection was performed using the Vectastain Universal Quick Kit® (Vector Laboratories). Immunolabelled slides were randomized and masked for blinded examination, which was performed by three independent pathologists (SI, ML, LA). In case of disagreements, a consensus was reached using a multi-headed microscope.

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

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

Cell line and CoCl2 treatment

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

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supplemented with 10% fetal bovine serum ~~(FBS)~~, 1% glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO2. The OLGA cell line was cultured in chemically induced hypoxia for 12, 24, and 48 hours using a concentration of 200 µM cobalt chloride ($CoCl_2$; Sigma Aldrich, 15862-1ML-F).[42]

Western blot analysis

Proteins from the OLGA cell line, treated with 200 µM of $CoCl_2$ for 12, 24, and 48 ~~h~~ hours or untreated (DMSO alone), were extracted in lysis buffer (1% Triton X-100, 10% glycerol, 50mM Tris, 150mM sodium chloride, 2mM EDTA, pH 8.0, and 2mM magnesium chloride) containing Protease Inhibitor Cocktail (P8340 Sigma). Twenty micrograms of total proteins were separated ~~in~~ on a 10% SDS-PAGE ~~(10%)~~gel and transferred onto a 0.2 µm pore-size nitrocellulose membrane (Cytiva; Thermo Fisher Scientific). After washing, membranes were incubated in ~~TBS/BSA~~Tris-buffered saline with 10% ~~(~~bovine serum albumin~~)~~ at room temperature for 1 hour and then incubated overnight at 4°C with HIF1-α and CAIX antibodies; α-tubulin was used as a housekeeping protein (~~Supplementary~~ Supplemental Table S1). Horseradish peroxidase ~~(HRP)~~-conjugated secondary antibodies were diluted at 1:15.000. The chemiluminescence substrate (Clarity ECL Substrate; BIO-RAD Laboratories) was used to produce the light signal, acquired with CL-XPosure films (Thermo Fisher Scientific Inc).

Immunofluorescence

To investigate the effects of $CoCl_2$ on HIF1-α nuclear translocation and CAIX expression, immunofluorescence against HIF1-α and CAIX was performed on OLGA cells exposed to the highest $CoCl_2$ concentration (200 µM) for 12, 24, and 48 hours. Briefly, $2x10^4$ cells were plated in eight-well chamber slides (Lab-Tek II Chamber

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

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Statistical analyses

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

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

RESULTS

Patient and tumor characteristics

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

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

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

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

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

CoCl2 treatment induces HIF-1α accumulation and nuclear translocation

~~As illustrated in Figure 5, distinct~~ Distinct bands corresponding to HIF-1α (120 kDa) and CAIX (58 kDa) proteins were more pronounced in the Olga cell line treated with $CoCl_2$ at 12 and 24 hours (Fig. 5). In contrast, the expression of these proteins was either negative (HIF-1α) or very low (CAIX) in untreated cells (Fig. 5).

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A mild increase of CAIX protein was also present in chemically-induced hypoxic conditions after 24 hours. ~~As depicted in Figure 6, under~~ Under normoxic conditions, HIF-1α was primarily localized to the cytoplasm, with nuclear localization becoming evident only after $CoCl_2$ treatment (Fig. 6). In contrast, chemically induced hypoxia did not yield noticeable effects on CAIX protein.

DISCUSSION

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

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

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

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

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

We found that CXCR4 expression was associated with an increased Ki-67 index, which is consistent with previous reports in human renal carcinomas[9] and multicentric lymphoma.[63] However, in human cutaneous melanomas, these markers ~~were~~ have not been significantly associated.[31,32,60]

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

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

To gain insight into the mechanisms of hypoxia in COMM, the OLGA cell line was treated with $CoCl_2$ to mimic~~king~~ a hypoxic stimulus.[13] We found that *in vitro* hypoxia induction caused an accumulation of HIF-1α protein after treatment. HIF-1α induction and stabilization triggered by hypoxia was further corroborated by ~~the results of~~ immunofluorescence, in which nuclear translocation under $CoCl_2$ treatment to avoid proteasomal degradation was demonstrated.[50] In the nucleus, HIF-1α is known to directly coordinate the transcription of ~~HRE~~hypoxia-regulating elements.[49] Consistent with the immunohistochemical results, CAIX protein levels were also mildly increased under chemically induced hypoxia. Its accumulation was delayed as compared to HIF-1α, thus supporting the direct role of HIF-1α in the upregulation of this protein.[21]

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

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LEGEND OF THE FIGURES

Figure 1

Kaplan-Meier survival curves for stage II, III, and IV canine oral malignant melanomas. (a) ~~Disease~~ Disease-free interval (DFI) ~~and~~. (b) ~~Overall~~ Overall ~~Survival~~ survival (OS) ~~curves of COMMs patients with respect to clinical stages II, III and IV~~. Stage II (median DFI = 187 days, ~~Median~~ median OS: ~~=~~ 397 days); Stage III~~3~~ (median DFI = 209 days, median OS = 260 days); Stage IV4 (median DFI = 36 days, median OS= 76). ~~p values:~~ DFI: p = 0.06 ~~and~~, OS: p = 0.002

Figure 2

Kaplan-Meier survival curves of dogs with canine oral malignant melanomas treated with or without *chondroitin sulfate proteoglycan-4* (*CSPG4*) DNA vaccination. (a) ~~Disease~~ Disease-free interval (DFI). Vaccinated median DFI = 205 days, not vaccinated median DFI = 187 days (p > 0.05). ~~and~~ (b) Overall ~~Survival~~ survival (OS). Vaccinated median OS = 972 days, not vaccinated median OS = 215 days (p<0.001)~~curves of COMMs (canine oral malignant melanomas) patients treated with CSPG4 (chondroitin sulfate proteoglycan-4)/DNA vaccination with respect to COMMs patients treated with *en- bloc* surgery alone, (median OS: 972 days versus 215 days p<0,001); (median DFI 205 days versus 187 days (p>0.05)~~.

Figure 3

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

Figure 4

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

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

Figure 5

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

Figure 6

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

Supplementary Figure 1

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

tissue control. ~~(a1) and~~ (b) M~~m~~ammary carcinoma ~~as,~~ positive tissue control. (c-d) ~~(a2); CXCR4~~C-X-C chemokine receptor type 4 IHC.~~:~~ (c) H~~h~~aired skin ~~as,~~ negative tissue control. (~~b1~~d) ~~and mammary~~ Mammary carcinoma ~~as,~~ positive tissue control. ~~(b2); (~~e, f) glucose transporter 1 IHC. ~~GLUT-1: (~~e) ~~testis~~ Testis,~~as~~ negative tissue control. (f) ~~(c1) and placenta~~ Placenta,~~as~~ positive tissue control ~~(c2);~~ . (g, h) Carbonic anhydrase IX.~~CAIX:~~ (g) T~~t~~estis, ~~as~~ negative tissue control. (h) ~~(d1) and renal~~ Renal cell carcinoma ~~as,~~ positive tissue control. ~~(d2) - DAB chromogen, hematoxylin 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.

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Authors' Contributions

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

EM* performed statistical analysis.

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

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