



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE DELLA RICERCA

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

FoxP3, CTLA-4, and IDO in Canine Melanocytic Tumors

This is the submitted version (pre peer-review, preprint) of the following publication:

Published Version:

FoxP3, CTLA-4, and IDO in Canine Melanocytic Tumors / Porcellato, Ilaria; Brachelente, Chiara; Cappelli, Katia; Menchetti, Laura; Silvestri, Serenella; Sforza, Monica; Mecocci, Samanta; Iussich, Selina; Leonardi, Leonardo; Mechelli, Luca. - In: VETERINARY PATHOLOGY. - ISSN 0300-9858. - ELETTRONICO. - 58:1(2021), pp. 42-52. [10.1177/0300985820960131]

Availability:

This version is available at: <https://hdl.handle.net/11585/801198> since: 2021-02-18

Published:

DOI: <http://doi.org/10.1177/0300985820960131>

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1 Foxp3, CTLA-4, and IDO in canine melanocytic tumors

2

3 AUTHORS: Ilaria Porcellato*, Chiara Brachelente*, Katia Cappelli*, Laura
4 Menchetti*, Serenella Silvestri*, Monica Sforna*, Samanta Mecocci*, Selina Iussich°,
5 Leonardo Leonardi*, Luca Mechelli*

6

7 *Department of Veterinary Medicine, University of Perugia, Italy

8 °Department of Veterinary Sciences, University of Turin, Grugliasco (TO), Italy

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This is the preprint version of:

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Porcellato I, Brachelente C, Cappelli K, et al.

15

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16

Veterinary Pathology. 2021;58(1):42-52.

17

The final published version is available online at: <https://doi.org/10.1177/03009858209601>

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22 **ABSTRACT**

23 Despite promising immunotherapy strategies in human melanoma, there are few
24 studies on the immune environment of canine melanocytic tumors . In humans, the
25 activation of immunosuppressive cell subpopulations, such as regulatory T cells
26 (Tregs) that express forkhead box protein P3 (FoxP3), the engagement of
27 immunosuppressive surface receptors like cytotoxic T lymphocyte antigen (CTLA-4),
28 and the secretion of molecules inhibiting lymphocyte activation, such as
29 indoleamine-pyrrole 2,3-dioxygenase (IDO), are recognized as immunoescape
30 mechanisms that allow tumor growth and progression.

31 The aim of our study was to investigate the expression of these immunosuppression
32 markers in canine melanocytic tumors, and to postulate their possible role in
33 melanoma biology and progression. Fifty-five formalin-fixed, paraffin-embedded
34 canine melanocytic tumors (25 oral melanomas; 20 cutaneous melanomas; 10
35 cutaneous melanocytomas) were selected to investigate the expression of FoxP3,
36 CTLA-4, and IDO by immunohistochemistry and RT-qPCR. All of the tested markers
37 showed high gene and protein expression in oral melanomas and were differently
38 expressed in cutaneous melanomas when compared to their benign counterpart.
39 IDO expression was associated with an increased hazard of death both in
40 univariable and multivariable analyses ($P < 0.001$). FoxP3 protein expression > 6.9
41 cells/HPF was an independent predictor of death ($P < 0.05$). CTLA-4 gene and protein
42 expression were associated with a worse prognosis, but only in the univariable
43 analysis ($P < 0.05$).

44 FoxP3, CTLA-4, and IDO likely play a role in canine melanoma immunoescape.

45 Their expression, if supported by future studies, could represent a prognostic tool in

46 canine melanoma and pave the way to future immunotherapeutic approaches in
47 dogs.

48

49 **KEYWORDS**

50 Melanoma, immunosuppression, FoxP3, CTLA-4, Indoleamine-pyrrole 2,3-
51 Dioxygenase, dogs, prognosis.

52

53 Human melanoma is recognized as one of the most immunogenic tumors.
54 Melanoma cells bear a high mutational burden compared to other malignancies, and
55 can acquire hundreds of mutations per megabase.^{1,33,53} Recently, tumor
56 heterogeneity has been demonstrated to further contribute to the host immune
57 response, and is a better predictor of immunotherapy outcome compared to
58 mutational burden.^{21,83} Despite melanoma's immunogenicity, the host immune
59 response is not effective in controlling tumor progression because the tumor itself is
60 able to mold the immune response to its own benefit through the process of
61 immunoediting.¹⁷ It is currently believed that the interplay between the tumor and the
62 immune system can be divided into three phases. The elimination phase is
63 characterized by the development of a tumor-specific immunity, in which tumor-
64 specific CD4+ and CD8+ cells are able to eliminate the tumor. The equilibrium phase
65 is characterized by a dynamic balance between tumor cell variants that survived the
66 elimination phase and the host immune system. Last, during the escape phase,
67 selected tumor cells avoid immune detection and elimination.^{18,43} The role of different
68 immune cell populations in the process of immunoediting has been widely
69 investigated in humans. This led to the development of new immunotherapy
70 strategies, including immune checkpoint blockade, that target the host's immune

71 system to improve or restore protective immune functions, as well as inhibit
72 immunosuppressive pathways activated during the escape phase. This type of
73 treatment has been applied to different types of cancer, especially melanoma,
74 resulting in durable responses, even in patients with metastatic disease.^{10,39}
75 The transcription factor forkhead box protein P3 (FoxP3) is involved in ensuring
76 immune homeostasis,³⁶ but is also a key molecule suppressing cytotoxic T cell
77 activity in anti-tumor immune response.^{31,36} FoxP3 is expressed in Tregs, which
78 have been associated with a negative prognosis both in human melanoma and other
79 solid cancers.^{23,41,61} A recent study also showed that Tregs were less numerous in
80 areas of melanoma regression, confirming their potential role in the establishment of
81 an immunosuppressive environment.²⁴ Moreover, in a murine melanoma model, it
82 was shown that selective FoxP3 depletion achieved through vaccination led to the
83 depletion of myeloid-derived suppressor cells (MDSCs), the reduction of tumor
84 growth, and the improvement in survival rates,⁵⁰ supporting the role of Tregs in
85 tumor progression and growth. The presence of FoxP3⁺Tregs has been also
86 reported in canine tumors, included melanoma.^{11,57,65}
87 Cytotoxic T lymphocyte antigen (CTLA-4), also known as CD152, is a member of the
88 family of immunoglobulin-related receptors expressed on both activated lymphocytes
89 and Tregs and is responsible for T cell regulation and preservation of a normal
90 immune environment. CTLA-4 binds to high-affinity B7 ligands (CD80 and CD86) on
91 antigen-presenting cells (APCs). This leads to the inhibition of T cell responses and
92 proliferation (T cell exhaustion), and antagonizes the binding of the T cell-stimulating
93 receptor, CD28.^{15,16,63,70,80} The importance of CTLA-4 in immune responses was
94 shown when fatal autoimmunity was observed in CTLA-4-deficient mice due to the
95 release of self-reactive T cells, suggesting that CTLA-4 is a negative regulator of T

96 cell response.⁷⁹ Anti-tumor immunity is predominantly mediated by T cells and
97 CTLA-4 has been shown to play a pivotal role in cancer-associated immunoediting,
98 particularly in the escape phase.^{70,80} Persistent antigen exposition by melanoma
99 cells and chronic stimulation of the immune system seems to be critical in the
100 hyperactivation of inhibitory checkpoints on immune cells such as CTLA-4, resulting
101 in the suppression of cytotoxic T cells.²⁵ CTLA-4 is also the target of Ipilimumab, an
102 immune checkpoint inhibitor that is used to treat melanoma.^{9,34}

103 Another pathway that could contribute to peripheral tolerance and therefore to
104 cancer immunoescape, is mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO),
105 an enzyme with immunosuppressive properties postulated to impair the antitumor
106 immune response in melanoma.⁵⁹ IDO can be produced by MDSCs, dendritic cells
107 (DCs), macrophages, and tumor cells, and it is believed to inhibit effector T-cells by
108 depleting tryptophan in the tumor microenvironment.^{28,47,49} Tryptophan catabolites
109 (such as L-kynurenine) can further suppress the proliferation of activated T cells and
110 promote the differentiation and activation of Tregs and CTLA-4 expression.^{14,46}

111 Inhibition of IDO, in combination with other immunotherapeutic drugs, can lead to
112 improved response rates during melanoma therapy.⁸ IDO blockade can reduce
113 tumor growth, intratumoral immunosuppression, and stimulate robust systemic
114 antitumor effects.^{29,32,45} Nevertheless, a recent phase III study did not observe any
115 difference in the group of patients with metastatic melanoma treated with IDO
116 inhibitors when compared to the placebo-treated group.³⁵ Therefore, further studies
117 are required to better understand the role of this enzyme as a potential therapeutic
118 target.

119 During the last few years, growing evidence suggests canine melanomas,
120 particularly mucosal melanomas, might be a predictive a preclinical model for human

121 melanoma.²⁶ Still, further studies are recommended to better characterize the canine
122 disease, including on the immunological front. The aim of this study was to
123 retrospectively investigate the presence of immunoescape mechanisms in canine
124 melanocytic tumors, through the analysis of FoxP3, CTLA-4, and IDO gene and
125 protein expression and to gain more information on the possible similarities between
126 canine and human melanoma immunology.

127

128 **MATERIALS AND METHODS**

129 *Case selection*

130 Cases were retrospectively selected on the following inclusion criteria: histological
131 diagnosis of melanoma or melanocytoma,⁷³ with immunohistochemical positivity for
132 Melan-A and/or PNL2; availability of follow-up information; and minimum follow-up of
133 time 365 days.

134 Mitotic count was assessed following a proposed standardized method.⁴² A
135 telephone survey was conducted with the referring veterinarians, to collect data on
136 the clinical tumor staging, the follow-up, local recurrence, and the cause of death.
137 Disease-free and overall survival times were calculated from the day of sample
138 registration in our departments.

139

140 *Immunohistochemical labeling and evaluation*

141 Samples were cut into 5 µm sections, mounted on poly-L-lysine coated slides,
142 dewaxed and rehydrated. Heavily pigmented tumors were bleached overnight at
143 room temperature with 30% H₂O₂ following a standardized protocol.⁵⁷
144 Immunohistochemistry was performed on serial sections with antibodies against
145 Melan A (pH 9.0 antigen retrieval; 1:150; mouse monoclonal, clones A103-M27C10-

146 M29E3; Abcam, Cambridge, UK), PNL2 (pH 6.0 antigen retrieval; 1:150; mouse
147 monoclonal, clone PNL2; Santa Cruz Biotechnology, Dallas, Texas, US), FoxP3 (pH
148 9.0 antigen retrieval; 1:100 dilution; rat monoclonal, clone FJK-16s; Thermo Fisher,
149 Waltham, Massachusetts, US), CTLA-4 (pH 9.0 antigen retrieval; dilution 1:100;
150 mouse monoclonal, clone F-8; Santa Cruz Biotechnology, Dallas, Texas, US), and
151 IDO (pH 9.0 antigen retrieval; 1:50 dilution; rabbit polyclonal; Biorbyt, Cambridge,
152 UK) with standardized protocols previously reported.⁵⁷ FoxP3 FJK-16s clone is
153 reported to cross-react with canine antigen,⁴⁴ whereas anti-IDO and anti-CTLA4
154 antibodies were stated to cross-react with canine tissue and to be suitable for FFPE
155 material in the datasheet provided by the manufacturer. The labeling pattern
156 observed in the lymph node supported the specificity of both the antibodies
157 (Supplemental Figures S1-3). Tris-EDTA (pH 9.0) was used to perform heat-induced
158 epitope retrieval for CTLA-4. Immunolabeling was revealed with 3-amino-9-
159 ethylcarbazole (Dako, Glostrup, Denmark); Mayer's hematoxylin was applied as a
160 counterstain. Reactive canine lymph node was used as a positive control for all the
161 antibodies of this study (Supplemental Figures S1-3). Negative controls were run by
162 incubating sections with TBS and omitting the primary antibody and by incubating
163 control tissue with isotype-matched antibody (only for monoclonal antibodies) to
164 assess the absence of non-specific labeling. Positive cells were counted by two
165 operators, in 5 HPF (FN 20), selecting "hot spots" and avoiding areas of necrosis
166 and/or near ulceration; a mean value was then obtained for each case and
167 expressed as the number of positive cells/HPF. The same method was applied for
168 the evaluation of FoxP3, CTLA-4, and IDO positive cells. The expected labeling was
169 nuclear for FoxP3, both membrane and cytoplasmic for CTLA-4, and granular and
170 cytoplasmic for IDO.

171 Thirty out of 55 cases were part of the selected cases for our previous study,⁵²
172 whereas 25/55 cases were investigated *ex novo*.

173

174 *RNA extraction and Real Time PCR (RT-qPCR)*

175 Three-to-5 (depending on sample size), 8 µm-thick, sections were cut from paraffin
176 blocks. Normal tissue around the tumor was resected and discarded with the help of
177 a sterile scalpel blade or a sterile needle. RNA extraction was performed with a
178 commercial kit (Invitrogen™ PureLink™, FFPE RNA Isolation Kit) following the
179 manufacturer's instructions. Residual genomic DNA was removed from the total RNA
180 by DNase I, amplification grade, (Thermo Fisher Scientific, Waltham, MA, USA)
181 following the manufacturer's specifications. RNA quantity was evaluated by both
182 NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA)
183 and Qubit 2.0 Fluorometer (Life Technologies, MA, USA). Total RNA (500 ng) was
184 reverse transcribed using the SuperScript® VILO™ Master Mix (Thermo Fisher
185 Scientific, Waltham, MA, USA), according to the manufacturer's specifications.
186 Successful reverse transcription was confirmed by PCR amplification of the *Canis*
187 *familiaris* GUSβ gene (NM_001003191). Primers on reference genes (GUSβ, HMBS)
188 and on genes of interest were designed on available sequences using the Primer-
189 BLAST suite (Table 1). Whenever possible, primers were located in different exons
190 or at exon–exon junctions to minimize inaccuracies due to residual genomic DNA
191 contamination. For each primer pair, a preliminary RT-qPCR assay was performed
192 on a bulk of samples generating a standard curve by using 4-fold serial dilutions.
193 Efficiency (E) with related linear correlation coefficients (R²) and the amplification of
194 non-specific products or primer-dimer artifacts were assessed. The RT-qPCR
195 reactions were carried out on CFX96 Touch instrument (BioRad, Hercules, CA) as

196 previously described.⁷ Data analysis was carried out with Bio-Rad CFX Manager
197 software (ver. 3.2.2). To analyze gene expression stability of HKGs, geNorm
198 algorithm, included on CFX Manager software (vers. 3.2.2), was applied.⁸¹ geNorm
199 provides a ranking of the tested genes, considering their expression stability,
200 selecting reference genes according to the stability measure M (average pairwise
201 variation of each gene against all others). The expression ratio of the genes of
202 interest was normalized to the relative abundance of the two reference genes using
203 the $\Delta\Delta Cq$ method. Non-detects were imputed with GenexPro software (ver.6) to
204 avoid introducing bias as previously reported.⁴⁰

205

206 *Statistical Analysis*

207 Diagnostic graphics were used to test assumptions and outliers. We analyzed
208 distributions within the categorical variable “breed” using Chi-square goodness of fit
209 tests. Differences in number of positive cells and mRNA expression of IDO, FoxP3,
210 and CTLA-4 between diagnoses were analyzed using Kruskal Wallis and Mann-
211 Whitney tests. Values were expressed as medians with interquartile range (IQR).
212 Correlations were evaluated by using Spearman rank correlation coefficient (ρ).
213 Correlation was defined as high when absolute value of $\rho > 0.5$, medium when ρ
214 ranged from 0.3 to 0.5, and low when $\rho < 0.3$.²⁰ We used the Life Table method to
215 determine survival probabilities. The differences of survival rate according to
216 diagnosis were evaluated by Kaplan–Meier curve and log-rank test. Dogs that died
217 of other causes or were lost to follow-up were considered censored. We used the
218 Cox proportional hazards model to evaluate the influence of parameters on survival.
219 In addition to the univariable analysis, each parameter (IDO (cells/HPF), FoxP3
220 (cells/HPF), CTLA-4 (cells/HPF), IDO mRNA, FOXP3 mRNA, CTLA4 mRNA) was

221 assessed through a multivariable model that also included the diagnosis. We used
222 variance inflation factors (VIF) to identify multicollinearity.²⁰ The prognostic
223 significance of each variable was expressed as hazard ratio (HR) with corresponding
224 95% confidence intervals (CIs) and P values. Finally, we used the receiver operating
225 characteristic (ROC) analysis to assess the diagnostic accuracy of the parameters
226 and their cut-offs for predicting survival. The state variable (positive category) was
227 the tumor-related death. Optimal cut-off values were determined as points on the
228 curve closest to (0, 1) and by the Youden index. Then, dichotomous variables for
229 each parameter were created based on their cut-off and submitted to Cox
230 regression,⁵⁶ also adjusting for diagnosis. All statistical analyses were performed
231 using SPSS 25.0 software (IBM, SPSS Inc., Chicago, IL, USA) and statistical
232 significance was set at $P \leq 0.05$.

233

234

235 **RESULTS**

236 *Sample population and mitotic count*

237 The final caseload was represented by 25 oral melanomas, 20 cutaneous
238 melanomas, and 10 cutaneous melanocytomas. Thirty-five dogs were male (6/35, 17
239 % were neutered) and 19 were female (6, 32% were spayed). For one animal the
240 age was unknown. Most of the dogs were mixed breed (19/55, 34%; $P < 0.001$),
241 followed by German Shepherd and Dachshund (5/55, 9% each), Labrador Retriever
242 (4/55, 7%), and Boxer (3/55, 5%). The median age was 11 years (range, 1–16
243 years). Follow-up at the end of the study ranged from 365 to 1615 days, with a
244 median follow-up of 653 days. More than 60% of the study population had a follow-
245 up time longer than 2 years at the end of the study.

246 The 6-month and 1-year estimated survival probabilities are shown in Supplemental
247 Table S1. Median survival time for mucosal melanoma was 240 days (IQR=77-433
248 days), while it was not reached for cutaneous melanoma or melanocytoma. Log-rank
249 test showed lower survival time for dogs with mucosal melanoma compared to dogs
250 with cutaneous melanoma (P=0.005). No deaths or recurrences were recorded for
251 the cases of melanocytoma.

252 Mitotic counts were higher in oral melanomas (Median=42, IQR=32-61) than in
253 cutaneous melanomas (Median=14, IQR=7-50, P<0.05) and melanocytomas
254 (Median= 1, IQR=0-2, P<0.001). The majority of the cases were treated only with
255 surgical excision (45/55; 81.8%), whereas 6 cases did not receive any treatment
256 (10.1%) and four cases (7.2%) were treated with surgical excision and different
257 chemotherapy protocols (Supplemental Table S2).

258

259 *Immunohistochemistry*

260 FoxP3, CTLA-4, and IDO positive cells were identified in all oral and cutaneous
261 melanomas, while some cutaneous melanocytoma did not contain any cells
262 expressing these proteins. FoxP3 and CTLA-4 immunolabeling was localized in the
263 nucleus and the cytoplasmic membrane, respectively, in cells with scant cytoplasm,
264 interpreted as lymphocytes. IDO was mostly expressed in the cytoplasm of cells with
265 moderate to abundant cytoplasm (interpreted as histiocytes/macrophages), and
266 rarely in neoplastic cells.

267 Immunohistochemical labeling revealed that the number of FoxP3⁺Tregs (Figs. 1, 2,
268 and 3) was higher in oral melanomas, than cutaneous melanomas and cutaneous
269 melanocytomas. Similarly, the number of CTLA-4 positive cells/HPF (Figs. 4, 5, and
270 6) was higher in oral melanomas than in cutaneous melanomas and cutaneous

271 melanocytomas. The number of IDO positive cells (Figs. 7, 8 and 9) was higher in
272 oral melanoma than in cutaneous melanomas and cutaneous melanocytoma.
273 Results are summarized in Figures 10-12.

274

275 *RT-qPCR*

276 After RNA extraction, samples showed A260/280 ratio ranging from 1.64 to 2.02.
277 Both *GUSβ* and *HMBS* genes displayed relatively high stability with M values of 0.4,
278 far below the accepted limit of 1.5.⁸¹ Linear correlation coefficients (R²) of primer
279 pairs varied from 0.94 to 0.99 and PCR efficiencies (E) ranged between 77.2 and
280 97.7. The expression level of the three genes was also associated with the
281 histological diagnosis (P<0.001). *IDO* and *CTLA4* gene expression was significantly
282 upregulated in the group of oral melanomas compared to cutaneous melanomas
283 (P<0.05) and cutaneous melanocytomas (P<0.001). The expression of *FOXP3* was
284 instead upregulated in both oral and cutaneous melanomas, when compared to
285 cutaneous melanocytomas (P<0.001). Gene expression results are summarized in
286 Figures 13-15.

287

288 *Correlations between FoxP3, CTLA-4, and IDO protein, transcripts and mitotic count*

289 All of the examined parameters evaluated with both immunohistochemistry and RT-
290 qPCR showed positive correlations between them and with mitotic counts
291 (Supplemental Table S3). A strong correlation was observed between FoxP3⁺
292 cells/HPF and CTLA-4⁺ cells/HPF ($\rho=0.709$, P<0.01). Also, mitotic count strongly
293 correlated with the protein expression of FoxP3 ($\rho =0.706$, P<0.01) and CTLA-4 (ρ
294 $=0.650$, P<0.01), and with gene expression of *IDO* ($\rho =0.563$, P<0.01).

295 The correlation between gene expression and immunohistochemical protein
296 expression was moderate for *FOXP3* ($\rho = 0.493$, $P < 0.01$) and *CTLA-4* ($\rho = 0.477$,
297 $P < 0.01$), and moderate-to-low for *IDO* ($\rho = 0.327$, $P < 0.05$).

298

299 *Prognostic significance of IDO, FoxP3, and CTLA-4*

300 The univariable Cox analysis (Table 2) showed an increased hazard of death in
301 association with an increased expression of IDO and CTLA-4, both at the protein and
302 mRNA levels ($P < 0.05$). FoxP3 expression was associated to the hazard of death
303 only when evaluated by immunohistochemistry ($P < 0.01$). Death due to melanocytic
304 tumor was also related to mitotic count ($HR = 1.011$, $95\%CI = 1.003-1.019$; $P < 0.01$)
305 and the animal's age ($HR = 1.222$, $95\%CI = 1.049-1.423$; $P < 0.05$). The models
306 adjusted for diagnosis showed that only IDO protein expression ($P < 0.05$) was an
307 independent factor in the multivariable analysis.

308 We investigated the sensitivity and specificity associated with a previously defined
309 IDO cut-off value of 14.7 cells/HPF.⁵⁷ This cut-off value showed a 57% sensitivity
310 and 79% specificity. In contrast, a cut-off value of 8.4 cells/HPF was identified in this
311 study with an 82% sensitivity and 68% specificity.

312 Table 3 shows the other results of ROC analysis and the optimal cut-offs for
313 predicting melanoma-related mortality. The highest area under the curve (AUC) was
314 found for FoxP3⁺ cells/HPF ($AUC = 0.849$; $P < 0.001$), followed by *CTLA4* mRNA
315 ($AUC = 0.802$; $P < 0.001$) and *IDO* mRNA ($AUC = 0.798$; $P < 0.001$).

316 Dichotomous variables were created for each parameter based on their optimal cut-
317 offs ($<$ or \geq of the cut-off) and analyzed by Kaplan-Meier survival curves (Figures 16-
318 21) and Cox models adjusted for diagnosis (Table 4). Categorizing according to their
319 optimal cut off, FoxP3 ($P < 0.05$) positive cells/HPF had a prognostic value for tumor-

320 related death, independent of the diagnosis of melanocytoma, oral melanoma or
321 cutaneous melanoma.

322

323 **DISCUSSION**

324 In humans, a growing number of studies characterizing the melanoma immune
325 environment has led to the successful use of immunotherapy, particularly by
326 targeting PD-1 and CTLA-4.⁵² In veterinary medicine, there are fewer studies on
327 cancer immunity and on the application of immunotherapy.^{2,37,57,58}

328 Canine cutaneous melanomas are usually benign and surgical resection is typically
329 curative; still, their behavior can be quite unpredictable.^{19,67,74} On the other hand,
330 mucosal melanomas, particularly oral melanomas, show a malignant behavior with a
331 predisposition to the development of lymph node and lung metastasis, similar to
332 human melanomas.⁷³ Several studies suggest the dog is a valuable spontaneous
333 preclinical model in melanoma research since canine oral melanoma shares
334 numerous similarities with the more rare human disease.^{60,72,82}

335 While different aspects of canine melanoma biology have been investigated,
336 ^{6,7,26,27,30,66,71} the immune environment of canine melanoma is still largely unknown.

337 Our study aims to investigate the immune environment of canine melanocytic tumors
338 to acquire further information on the possible mechanisms of immunosuppression
339 and evasion involved in tumor progression; targets of our investigation are in
340 particular FoxP3, CTLA-4, and IDO.

341 The mean survival time of dogs with oral melanomas in this study was 240 days.

342 This is higher than is the previously mean survival time of 147 days.⁷⁵ This
343 discrepancy could be because of earlier diagnosis, and because of the inclusion of
344 labial melanomas in this study, which are reported to have a longer survival time.

345 This result endorses the necessity for further studies to better characterize oral and
346 mucocutaneous canine melanocyte biology, together with melanoma behavior in
347 association with different sites of origin. A detailed description of the tumor's
348 anatomical site of origin of the should be provided by the clinician/surgeon at the
349 time of tissue submission for histopathological analysis; this would enable further
350 evaluationson the prognostic significance of the primary tumor location.

351 FoxP3 is an transcription factor involved in Tregs development and function, and is
352 currently considered their most specific marker and the main regulator of Treg
353 lineage committment.^{3,68} Still, Tregs are a heterogeneous population and better
354 characterization of these cells could include other markers such as CD4, CD25, and
355 CD45RO.⁵⁵ In our study, increased FoxP3 immunohistochemical expression and
356 *FOXP3* gene expression was associated with a higher hazard of death due to
357 melanocytic tumor, but they lost significance when the models were adjusted for
358 diagnosis. However, when FoxP3 expression was categorized according to its
359 optimal cut-off, the survival analysis indicated that the hazard of death was 6 times
360 higher in dogs with FoxP3 \geq 6.9⁺ cells/HPF. These results seem to imply that a higher
361 infiltration of FoxP3⁺ cells could be associated with a worse prognosis in dogs,
362 similar to human melanomas.^{22,51} The aforementioned cut-off value was similar to
363 the 6.1 cells/HPF value previously reported by our group,⁵⁷ but the new cut off
364 showed both higher sensitivity and specificity in this study. It could be postulated that
365 FoxP3 may be a major player in immunoescape mechanisms favoring tumor growth
366 and progression, particularly in oral and cutaneous melanomas. Furthermore, the
367 strong correlation between FoxP3⁺Tregs and CTLA-4, together with the moderate
368 correlation of these two proteins with IDO protein expression, may point at a synergic
369 role of these molecules in the establishment of an immunosuppressed tumor

370 microenvironment. A strong correlation was also evidenced between FoxP3 protein
371 expression and mitotic count, supporting FoxP3⁺Tregs role in favoring tumor growth.
372 The correlation between gene and immunohistochemical protein expression was
373 moderate or moderate-to-low for all tested molecules. This could be due to the
374 higher sensitivity of RT-qPCR when compared to the immunohistochemical
375 quantification, but also to poor mRNA quality in FFPE samples, which directly affects
376 the efficiency of some primer combinations (as low as 77.2%). The authors are
377 aware that these are sub-optimal values however, as reported in a previous study,⁷
378 accuracy in gene expression profile should not be compromised.

379 Markers to identify Tregs are limited and often non-specific,⁶² making the
380 characterization of this T cell subpopulation, within tumor immune environment,
381 difficult and still not completely understood. To overcome this problem, a
382 colocalization of CD4, CD25 and FoxP3 would be useful to correctly identify Tregs
383 subpopulation also in canine melanomas. Moreover, the presence of FoxP3⁺Tregs
384 could be influenced by tumor site, molecular subtype of the tumor, and tumor stage,
385 adding further bias to their evaluation.⁶⁸

386 During the last few years, immunotherapies with monoclonal antibodies directed
387 against CTLA-4 and PD1 revolutionized the treatment of patients with advanced
388 melanoma in human medicine. Still, the role of CTLA-4-expressing cells have been
389 explored in veterinary oncology in a limited manner. In the present study, we
390 described the expression of this molecule in canine melanocytic tumors, suggesting
391 that both CTLA-4 immunohistochemical and gene expression may associated with
392 the histological diagnosis and with an increased hazard of death (univariable
393 analysis), similarly to what reported in human melanomas.¹² However, in the
394 multivariable analysis, CTLA-4 lost its statistical significance, suggesting that CTLA-4

395 may be not an independent predictor. Still, the association between the protein and
396 gene expression of this marker and the tumor mitotic count, which is considered one
397 of the most affordable prognostic factors in canine melanomas,^{4,73} may corroborate
398 the hypothesis of an immunosuppressive role of CTLA-4 in melanoma growth. To the
399 best of our knowledge, this is the first study focusing on CTLA-4 within canine
400 melanoma microenvironment; previous studies aimed at characterizing the
401 expression of this costimulatory molecule in circulating cells during neoplastic
402 disease and in a healthy subject.⁷⁶⁻⁷⁸ Our results, although preliminary, highlight the
403 presence of this molecule within canine melanoma and open the the path for new
404 investigations on the role of CTLA-4-associated pathways in canine oncology.

405 IDO can be expressed by different cell types, in particular MDSCs, DCs,
406 macrophages, and tumor cells. It acts both on APCs and T cells, causing immune
407 suppression and facilitating cancer progression.⁵ Our results show that IDO
408 immunohistochemical expression was an independent predictor of mortality, even
409 when the model was adjusted for diagnosis (melanocytoma, oral melanoma,
410 cutaneous melanoma). The optimal cut-off value for IDO immunohistochemical
411 expression in this study was set at 8.4 cells/HPF, compared to the 14.7 cells/HPF
412 value that was used in our previous study. In addition to the different characteristics
413 of the sample population, this incongruity can also be explained by the different
414 percentages of sensitivity and specificity associated with this new cut-off. In the
415 present study, the lowest cut-off was associated with a higher sensitivity (82%) in the
416 prediction of death due to melanoma. By setting the IDO cut-off at 14.7 cells/HPF in
417 this case series, the specificity improves, reaching the value indicated in our
418 previous study (79%), but it is not balanced by an adequate sensitivity (57%). The
419 greater accuracy is indicated by the higher AUC in the present study, together with

420 the higher number of cases with a complete follow-up (55 vs 52) in this study,
421 suggests that the lower cut-off is preferred.

422 One of the possible limits of our study is related to the fact that we did not stratify our
423 cases by diagnosis. On one hand, the stratification would have led to a drastic
424 reduction in both the sample size and the number of “events” (i.e. deaths) to be
425 included for survival analysis. On the other hand, the diagnosis of
426 melanocytoma/melanoma poses often some doubts in the diagnostic routine of the
427 pathologist, particularly in borderline lesions. By avoiding stratification, the cut-offs
428 set for our markers were defined independent of the histologic diagnosis and
429 pathologist’s the judgment of the. Investigations based on a larger study are required
430 to confirm these results and to better assess these markers’ prognostic value.

431 The role of IDO in canine melanoma may be similar its role in human melanoma,
432 where IDO protein expression has been shown to have a prognostic role in both
433 cutaneous melanoma and nodal metastases.^{13,54,64} Gene expression, on the other
434 hand, was significant only in the univariate Cox analysis. The loss of significance
435 could be because of the high mRNA expression variability detected by RT-qPCR due
436 to the use of FFPE material to retrieve mRNA. In fact, even though numerous
437 studies have used this protocol, fresh-frozen tissue are preferred to avoid partial
438 mRNA degradation.³⁸ Our results suggest that IDO is involved in canine tumor
439 immunoescape and progression, but mechanistic studies are needed to confirm this
440 finding. Also, IDO could be implicated in the activation of Treg cells within the canine
441 melanoma microenvironment, as indicated by the moderate correlation between the
442 variables in this study and in other models.^{48,69}

443 Interestingly, all of the markers tested in our study showed a significant difference
444 between the cutaneous melanomas and cutaneous melanocytomas, suggesting

445 increased activation of immunosuppressive pathways in malignant cutaneous tumors
446 compared to their benign counterpart. This finding, if further confirmed by ongoing
447 investigations, could confirm similar mechanisms of immune evasion in the dog as
448 compared to human species. Furthermore, these markers could be useful in
449 prognosticating canine cutaneous melanocytic tumors. If confirmed by prospective
450 studies, IDO⁺ cells/HPF and the threshold of >6.9 FoxP3⁺ cells/HPF might be useful
451 in the evaluation of canine melanocytic tumors, as evidenced by the multivariable
452 analysis.

453 Taken together, the results from our study seem to confirm the presence of
454 immunosuppressive tumor microenvironment mechanisms controlled by FoxP3,
455 CTLA-4, and IDO in canine melanoma, particularly in the most aggressive oral form.
456 After this retrospective investigation, prospective studies on fresh/frozen tissue
457 aiming at the confirmation of these results, including and extending our investigation
458 to other immune populations and to metastatic lesions, have been planned. Further
459 investigations on the immune environment of canine melanocytic tumors should be
460 initiated, aiming to both better characterize canine melanoma biology and immune
461 environment and to possibly employ immunotherapeutic strategies in the canine
462 species.

463

464 **ACKNOWLEDGEMENTS:**

465 The authors would like to thank Gianluca Alunni, Valeria Migni and Luca Stefanelli
466 for their precious technical assistance.

467

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696

697 **Figure 1-3.** Melanocytic neoplasms, dog. The immunolabeling is nuclear in small
698 cells with scant cytoplasm (lymphocytes). Immunohistochemistry for FoxP3 (AEC
699 chromagen and hematoxylin counterstain). **Figure 1.** Oral melanoma, oral mucosa,
700 dog, case 10. Numerous FoxP3⁺ cells are scattered among neoplastic cells. **Figure**
701 **2.** Cutaneous melanoma, haired skin, dog, case 33. FoxP3⁺ cells are present within
702 the neoplasm. **Figure 3.** Cutaneous melanocytoma, haired skin, dog, case 50. No
703 FoxP3⁺ cells are observed at the center or periphery of the tumor. **Figure 4-6.**

704 Melanocytic neoplasms, dog. Immunolabeling is observed on the cell membrane of
705 small cells with scant cytoplasm (lymphocytes). Immunohistochemistry for CTLA-4.

706 **Figure 4.** Oral melanoma, oral mucosa, dog, case 23. Occasional CTLA-4⁺ cells are
707 present among neoplastic cells and also in lymphocytic aggregates at the periphery
708 of the tumor. **Figure 5.** Cutaneous melanoma, haired skin, dog, case 44. Scattered
709 small aggregates of CTLA-4⁺ cells are present within the neoplasm. **Figure 6.**

710 Cutaneous melanocytoma, haired skin, dog, case 51. No CTLA-4⁺ cells are observed
711 at the center or the periphery of the tumor. **Figure 7-9.** Melanocytic neoplasms, dog.

712 IDO is expressed in the cytoplasm of cells with moderate to abundant cytoplasm
713 (interpreted as histiocytes/macrophages), and rarely in neoplastic cells.

714 Immunohistochemistry for IDO. **Figure 7:** Oral melanoma, oral mucosa, dog, case 8.

715 IDO⁺ cells are numerous and often in multifocal aggregates. **Figure 8.** Cutaneous
716 melanoma, haired skin, dog, case 28. Occasional IDO⁺ cells are scattered among
717 neoplastic cells. **Figure 9.** Cutaneous melanocytoma, haired skin, dog, case 49.

718 Single IDO⁺ cells are occasionally observed in the neoplasm, as highlighted in the
719 inset.

720

721 **Figure 10-12.** Box plots of the number of FoxP3, CTLA-4, and IDO-positive
722 cells/HPF. The horizontal line in the box is the median, the whiskers are 1.5 times
723 the inter-quartile range, and the stars are outliers. ns=not significant, *P<0.05,
724 **P<0.01, ***P<0.001 (multiple comparisons by Mann-Whitney tests).

725 **Figures 13-15.** *FoxP3*, *CTLA-4*, and *IDO* mRNA levels according to diagnosis. The
726 horizontal line in the box is the median, the whiskers are 1.5 times the inter-quartile
727 range, and the stars are outliers. ns=not significant, *P<0.05, **P<0.01, ***P<0.001
728 (multiple comparisons by Mann-Whitney tests).

729

730 **Figure 16-21.** Kaplan-Meier survival curves by optimal cut-off values of FoxP3
731 (Figure 16; 6.9 cells/HPF), CTLA-4 (Figure 17; 2.2 cells/HPF), and IDO (Figure 18;
732 8.4 cells/HPF), and *FOXP3* (Figure 19; 35.9 mRNA expression level), *CTLA4* (Figure
733 20; 10.1 mRNA expression level), and *IDO* (Figure 21; 22.7 mRNA expression level).
734 Vertical hash marks represent censored cases.

735