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

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TITLE PAGE

Title: The immune microenvironment of canine melanocytic tumors: an investigation on immunoescape pathways

Short running title: Melanoma immunoescape in dogs

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ABSTRACT

Despite recent promising immunotherapy strategies in human melanoma, studies on the immune environment of canine melanocytic tumors are few. In humans, the activation of immunosuppressive cell subpopulations such as T regulatory cells (Tregs), expressing the factor forkhead box protein P3 (FoxP3), the engagement of surface receptors with immunosuppressive functions, namely cytotoxic T lymphocyte antigen (CTLA-4), and the secretion of molecules inhibiting lymphocytes functions, as indoleamine-pyrrole 2,3-dioxygenase (IDO), are recognized, among others, as immunoescape mechanisms that allow tumor growth and progression.

Aim of our study is to investigate the expression of these immunosuppression markers in canine melanocytic tumors, and to evaluate their possible involvement in tumor biology and progression.

Fifty-five formalin-fixed and paraffin-embedded canine melanocytic tumors (25 oral melanomas; 20 cutaneous melanomas; 10 cutaneous melanocytomas) were selected to investigate the expression of FoxP3, CTLA-4 and IDO by immunohistochemistry and qRT-PCR.

These markers showed a high gene and protein expression in oral melanomas. FoxP3 protein expression was associated with an increased hazard of death (univariate: $P<0.001$; multivariate $P<0.05$). Both gene and protein expression of CTLA-4 was associated with a worse prognosis (univariate: 0.001 and <0.05, respectively). Also, IDO gene and protein expression was associated with an increased hazard of death both at univariate and multivariate analysis ($P<0.001$). FoxP3, CTLA-4 and IDO likely play a role also in canine melanoma immunoescape and progression; moreover, the expression of these molecules could be a helpful

50 prognostic tool in canine melanoma and, by comparative approach, could pave the
51 way to future immunotherapeutic approaches in dogs.
52
53 **KEYWORDS**
54 Melanoma, immunosuppression, FoxP3, CTLA-4, Indoleamine-pyrrole 2,3-
55 Dioxygenase, dogs, prognosis.
56
57 Human melanoma is recognized as one of the most immunogenic tumors; it has
58 been shown that melanoma cells bear a high mutational burden compared to other
59 malignancies, being able to acquire also hundreds of mutations per megabase.[1,32,50]
60 Recently, the tumor heterogeneity has been demonstrated to further contribute to the
61 determination of the host immune response, being also better than tumor mutational
62 burden in predicting immunotherapy outcome.[20,78] Despite melanoma
63 immunogenicity, the host immune response is not effective in controlling tumor
64 progression, being the tumor itself able to model the immune response to its own
65 benefit through the process of immunoediting.[16] It is currently believed that the
66 interplay between the tumor and the immune system can be ideally divided in three
67 phases; the first one described, the elimination phase, allows the development of a
68 tumor specific immunity, providing the host, by homing of specific CD4+ and CD8+
69 cells, with the capacity to eliminate the tumor. The equilibrium phase is characterized
70 by a dynamic balance between tumor cell variants that survived the elimination
71 phase and the host immune system, whereas during the escape phase selected
72 tumor cells can avoid immune detection and elimination.[17,41] The role of different
73 immune cellular populations in the process of immunoediting has been widely
74 investigated in human medicine. This led to the development of new strategies of

75 immunotherapy, particularly by immune checkpoint blockade, that targets the natural
76 immune system of the host, improving or restoring protective immune functions, as
77 well as inhibiting immunosuppressive pathways activated during the escape phase.
78 This type of tumor treatment has been applied to different types of cancer, especially
79 melanoma, resulting in good and durable response, even in patients with metastatic
80 disease.[9,37]
81 The transcription factor forkhead box protein P3 (FoxP3) is involved in ensuring
82 immune homeostasis,[34] but is also a key-molecule associated with suppression of
83 the activity of cytotoxic T cells in tumor immune response.[30,34] The presence of
84 FoxP3$^+$ Tregs has been associated with a negative prognosis both in human
85 melanoma and other solid cancers.[22,39,55] A recent study also showed that Tregs
86 were less numerous in areas of melanoma regression, confirming their potential role
87 in the establishment of an immunosuppressive environment.[23] Moreover, in a murine
88 melanoma model it was recently shown that a selective FoxP3 depletion achieved
89 through vaccination leaded to the depletion of myeloid-derived stem cells (MDSCs),
90 the reduction of tumor growth, and the improvement in survival rates,[47] supporting
91 the role of Tregs in tumor progression and growth. The presence of FoxP3$^+$ Tregs
92 has been also reported in canine tumors, included melanoma.[10,52,59]
93 Cytotoxic T lymphocyte antigen (CTLA-4), also known as CD152, is a member of the
94 family of immunoglobulin-related receptors expressed on both activated and
95 regulatory T cells (Tregs), responsible for T cells immune regulation and
96 preservation of a normal immune environment. CTLA-4 binds with high affinity B7
97 ligands (CD80 and CD86) on antigen presenting cells (APCs). This leads to the
98 inhibition of T cell response and cycle (T cell exhaustion), and antagonization of the
99 binding of the T cell-stimulating receptor, CD28.[14,15,57,64,75] The importance of CTLA-

100 4 in immune response was stated when a fatal autoimmunity was observed in CTLA-
101 4-deficient mice due to the release of self-reactive T cells, indicating CTLA-4 as a
102 negative regulator of T cell response.[74] Besides, anti-tumor immunity is
103 predominantly mediated by T cells and CTLA-4 has been shown to play a pivotal role
104 in cancer-associated immunoediting, particularly in the escape phase.[64,75] The
105 persistent antigen exposition by melanoma cells and the chronic stimulation of the
106 immune system seems to be critical in the hyperactivation of inhibitory checkpoints
107 on immune cells such as CTLA-4, resulting in a negative feedback on cytotoxic T
108 cells (Figure 1).[24] For its functions, CTLA-4 is also the target of Ipilimumab, an
109 immune checkpoint inhibitor, that proved to be useful in melanoma treatment.[8,33]
110 Another pathway that could contribute to peripheral tolerance and therefore to
111 cancer immunoescape, is mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO),
112 an enzyme with immunosuppressive properties that is postulated to impair the
113 antitumor immune response also in melanoma.[53] IDO can be produced by MDSCs,
114 dendritic cells (DCs), macrophages, and tumor cells themselves, and it is believed to
115 inhibit effector T-cells by depleting tryptophan within the tumor
116 microenvironment.[27,44,46] Tryptophan catabolites (such as L-kynurenine) can
117 suppress the proliferation of activated T cells and, at the same time, both promote
118 the differentiation and activation of Tregs and the CTLA-4 expression.[13] Tregs, in
119 turn, suppress the activation and function of other leukocytes, contributing to the
120 establishment of an immunosuppressive environment and also stimulate IDO
121 production and activation (Figure 1).[43] The inhibition of IDO, in combination with
122 other immunotherapeutic drugs, leads to an improvement in response rate during
123 melanoma therapy.[7] IDO blockade can in fact reduce tumor growth, intratumoral
124 immunosuppression, and stimulate robust systemic antitumor effects.[28,31,42]

125 During the last years, growing evidences point at canine melanomas, particularly the
126 mucosal ones, as a possible predictive preclinical model for human melanoma,[25] but
127 further studies are recommended to better characterize the canine disease, also on
128 the immunological front.
129 Aim of this study is to retrospectively investigate the presence of mechanisms of
130 immunoescape and immunosuppression in canine melanocytic tumors, through the
131 analysis of FoxP3, CTLA-4, and IDO gene and protein expression and to gain more
132 information on the possible similarities with human melanoma immunology.
133

134 **MATERIALS AND METHODS**

135 *Case selection*

136 The retrospective case selection had to meet the following inclusion criteria:
137 - a histological diagnosis of melanoma or melanocytoma,[68] with
138 immunohistochemical positivity for Melan-A and/or PNL2;
139 - availability of follow-up information;
140 - a minimum time to follow-up of 365 days.

141 Mitotic count was assessed, following a proposed standardized method.[40] A
142 telephonic survey was conducted with the referring veterinarians, to collect data on
143 the clinical tumor staging, the follow-up, the presence of local recurrence, and the
144 cause of death. Disease-free and overall survival were calculated from the day of the
145 sample registration in our Department.
146

147 *Immunohistochemical labeling and evaluation*

148 Samples were cut into 5 µm sections, mounted on poly-L-lysine coated slides,
149 dewaxed and rehydrated. Heavily pigmented tumors were bleached overnight at

150 room temperature with 30% H_2O_2 following a standardized protocol.[52]

151 Immunohistochemistry was performed on serial sections with antibodies against

152 FoxP3 (1:100 dilution; rat monoclonal, Clone FJK-16s; Thermo Fisher, Waltham,

153 Massachusetts, US), CTLA-4 (dilution 1:100; mouse monoclonal, clone F-8; Santa

154 Cruz Biotechnology, Dallas, Texas, US), and IDO (1:50 dilution; rabbit polyclonal;

155 Biorbyt, Cambridge, UK) as previously reported.[52] Tris-EDTA (pH 9.0) was used to

156 perform heat-induced epitope retrieval for CTLA-4. Positive reaction was revealed

157 with 3-amino-9-ethilcarbazole (Dako, Glostrup, Denmark); Mayer's hematoxylin was

158 applied as a counterstain. Reactive canine lymph node was used as a positive

159 control for all the antibodies of this study. Negative controls were run by incubating

160 sections with TBS and omitting the primary antibody and by incubating control-tissue

161 with antibody isotype (only for monoclonal antibodies) to assess the absence of non-

162 specific staining. Positive cells were counted by two operators, in 5 HPF (FN 20),

163 selecting "hot spots" and avoiding areas of necrosis and/or near ulceration; a mean

164 value was then obtained for each case and expressed as the number of positive

165 cells/HPF. The same method was applied for the evaluation of FoxP3, CTLA-4, and

166 IDO positive cells. The expected labeling was nuclear for FoxP3, both membrane

167 and cytoplasmic for CTLA-4, and granular and cytoplasmic for IDO.

168

169 *RNA extraction and Real Time PCR*

170 Three-to-5 (depending on sample size), 8 µm-thick, sections were cut from paraffin

171 blocks. Normal tissue around the tumor was resected and discarded with the help of

172 a sterile scalpel blade or a sterile needle. RNA extraction was performed with a

173 commercial kit (Invitrogen™ PureLink™, FFPE RNA Isolation Kit) following the

174 manufacturer's instructions. Residual genomic DNA was removed from the total RNA

175 by DNase I amplification grade (Thermo Fisher Scientific, Waltham, MA, USA)
176 following manufacturer's specifications. RNA quantity was evaluated by means of
177 both NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA,
178 USA) and Qubit 2.0 Fluorometer (Life Technologies, MA, USA). Total RNA (500 ng)
179 was reverse transcribed using the SuperScript® VILO™ Master Mix (Thermo Fisher
180 Scientific, Waltham, MA, USA), according to the manufacturer's specifications.
181 Successful reverse transcription was confirmed by PCR amplification of the *Canis*
182 *familiaris* GUSβ gene (NM_001003191). Primers on reference genes (GUSβ, HMBS)
183 and on genes of interest were designed on available sequences using the Primer-
184 BLAST suite (Table 1). Whenever possible, primers were located in different exons
185 or at exon–exon junction to minimize inaccuracies due to residual genomic DNA
186 contamination. For each primer pair, a preliminary qRT-PCR assay was performed
187 on a bulk of samples and amplification of non-specific products or primer-dimer
188 artifacts and efficiency (E) were assessed. The qRT-PCR reactions were carried out
189 on CFX96 Touch instrument (BioRad, Hercules, CA) as previously described.[6] Data
190 analysis was carried out with Bio-Rad CFX Manager software (ver. 3.2.2). To
191 analyse gene expression stability of HKGs, geNorm algorithm, included on CFX
192 Manager software (vers. 3.2.2), was applied.[76] geNorm provides a ranking of the
193 tested genes, considering their expression stability, selecting reference genes
194 according to the stability measure M (average pairwise variation of each gene
195 against all others). The expression ratio of the genes of interest was normalized to
196 the relative abundance of the two reference genes using the ΔΔCq method. Non-
197 detects were imputed with GenexPro software (ver.6) to avoid introducing bias as
198 previously reported.[38]
199

200 *Cell line validation statement*

201 Cell line validation work has not been conducted due to no cell lines use in this
202 study.

203

204 *Statistical Analysis*

205 Diagnostic graphics were used to test assumptions and outliers. We analyzed
206 distributions within the categorical variable "breed" using Chi-Square Goodness of
207 FitTests. Differences in number of positive cells and mRNA expression of IDO,
208 FoxP3, and CTLA-4 between diagnoses were analyzed using Kruskal Wallis and
209 Mann-Whitney tests. Values were expressed as medians (Mdn) with interquartile
210 range (IQR). Correlations were evaluated by using Spearman rank correlation
211 coefficient (ρ). Correlation was defined as high when absolute value of $\rho>0.5$,
212 medium when ρ ranged from 0.3 to 0.5, and low when $\rho<0.3$.[19] We used the Life
213 Table method to determine survival probabilities. The differences of survival rate
214 according to diagnosis were evaluated by Kaplan–Meier curve and log-rank test. We
215 used the Cox proportional hazards model to evaluate the influence of parameters on
216 survival. All variables with $P<0.05$ on the univariate analysis were entered into the
217 multivariate model and adjusted for age and mitotic count. We used variance
218 inflation factors (VIF) to identify multicollinearity.[19] The prognostic significance of
219 each variable was expressed as hazard ratio (HR) with corresponding 95%
220 confidence intervals (CIs) and P values. Finally, we used the receiver operating
221 characteristic (ROC) analysis to assess the diagnostic accuracy of the parameters
222 and their cut-off for predicting survival. Optimal cut-off values were determined as
223 points on the curve closest to (0, 1) and by the Youden index. Then, dichotomous
224 variables for each parameter were created based on their cut-off and submitted to

225 Cox regression adjusting for age and mitotic count. All statistical analyses were
226 performed using SPSS 25.0 software (IBM, SPSS Inc., Chicago, IL, USA) and
227 statistical significance was set at $P \leq 0.05$.
228
229 **RESULTS**
230 *Sample population and mitotic count*
231 The final caseload was represented by 25 oral melanomas, 20 cutaneous
232 melanomas, and 10 cutaneous melanocytomas. Thirty-five dogs were male (6/35,
233 17.1% were neutered) and 19 were female (6, 31.6% were spayed). For one animal
234 the age was unknown. Most of the dogs were mixed breed (19/55, 33.9%; $P<0.001$),
235 followed by German Shepherd and Dachshund (5/55, 8.9% each), Labrador
236 Retriever (4/55, 7.1%), and Boxer (3/55, 5.4%). The median age was 11 years
237 (range, 1–16 years). Follow-up at the end of the study ranged from 365 to 1615
238 days, with a median follow-up of 653 days. More than 60% of the study population
239 had a follow-up time longer than 2 years at the end of the study.
240 The 6-month and 1-year estimated survival probabilities are shown in Table S1
241 (Supplemental material). Median survival time for mucosal melanoma was 240 days
242 (IQR= 77-433 days), while it was not reached for cutaneous melanoma. Log-rank
243 test showed lower survival time for dogs with mucosal melanoma compared to dogs
244 with cutaneous melanoma ($P=0.005$). No deaths or recurrences were recorded for
245 the cases of melanocytoma.
246 Mitotic count was higher in oral melanomas (Mdn= 42, IQR=32-61) than in
247 cutaneous melanomas (Mdn= 14, IQR=7-50, $P<0.05$) and melanocytomas (Mdn= 1,
248 IQR=0-2, $P<0.001$).
249

250 *Immunohistochemistry*

251 FoxP3, CTLA-4, and IDO proteins were expressed in all melanomas, both oral and
252 cutaneous, while some cutaneous melanocytoma samples were completely
253 negative. Immunohistochemical labelling revealed that the number of FoxP3-positive
254 nuclei/HPF (Figure 2, A) was higher in oral melanomas, than cutaneous melanomas
255 and cutaneous melanocytomas. Similarly, the number of CTLA-4 positive nuclei/HPF
256 (Figure 2, B) was higher in oral melanomas than in cutaneous melanomas and
257 cutaneous melanocytomas. The number of IDO positive nuclei/HPF was higher in
258 oral melanoma than in cutaneous melanomas and cutaneous melanocytoma.
259 Results are summarized in Figure 5.

260

261 *qRT-PCR*

262 Both *GUSβ* and *HMBS* genes displayed a relatively high stability with M values of
263 0.4, far below the accepted limit of 1.5.[76] The expression level of the three genes
264 was also associated with the histological diagnosis ($P<0.001$). *IDO* and *CTLA4* gene
265 expression was significantly up-regulated in the group of oral melanomas compared
266 to cutaneous melanomas ($P<0.05$) and cutaneous melanocytomas ($P<0.001$). The
267 expression of *FOXP3* was instead upregulated in both oral and cutaneous
268 melanomas, when compared to cutaneous melanocytomas ($P<0.001$). Results of
269 gene expression are summarized in Figure 5.

270

271 *Correlations between parameters*

272 All the examined parameters, both evaluated with immunohistochemistry and qRT-
273 PCR, showed positive correlations between them and with the mitotic count (Table
274 S2, Supplemental material). A strong correlation was observed between $FoxP3^+$

275 cells/HPF and CTLA-4^{+} cells/HPF (ρ=0.709, P<0.01). Also, mitotic count strongly
276 correlated with the protein expression of FoxP3 (ρ =0.706, P<0.01) and CTLA-4 (ρ
277 =0.650, P<0.01), and with gene expression of *IDO* (ρ =0.563, P<0.01).
278
279 *Prognostic significance of IDO, FoxP3, and CTLA-4*
280 In the entire cohort, the univariate Cox analysis (Table 2) showed an increased
281 hazard of death in association with an increased expression of IDO and CTLA-4
282 (P<0.05), both at the protein and mRNA level. The expression of FoxP3 was
283 associated to the hazard of death only when evaluated by immunohistochemistry
284 (P<0.01). Death due to melanocytic tumor was also related to mitotic count (P<0.01)
285 and animal's age (P<0.05). A multivariate model adjusted for age and mitotic count
286 was built, including IDO, FoxP3, and CTLA-4 positive cells/HPF, *IDO* mRNA, and
287 *CTLA4* mRNA. This model showed that only IDO (P<0.01) and FoxP3 (P<0.05)
288 protein expression remained significant, holding constant the other predictors.
289 We investigated the sensibility and specificity associated to the IDO^{+} value of 14.7
290 cells/HPF (optimal cut off reported in our previous study);[52] results shown a 57%
291 sensibility and 79% specificity, in face of an 82% sensitivity and 68% specificity
292 associated with a cut-off value of 8.4 resulted as optimal in the present study group.
293 Table 3 shows the results of Receiver Operator Characteristic analysis. The highest
294 area under the curve (AUC) was found for FoxP3^{+} cells/HPF (AUC=0.849; P<0.001),
295 followed by *CTLA4* mRNA (AUC=0.802; P<0.001) and *IDO* mRNA (AUC=0.798;
296 P<0.001).
297 Then, dichotomous variables were created for each parameter based on their
298 optimal cut-offs (< or ≥ of the cut-off) and analyzed by Kaplan-Meier survival curves
299 (Figure 6) and Cox models adjusting for age and mitotic count (Table 4). All these

300 variables, except *CTLA4* mRNA, had a prognostic value related with survival
301 independently of age and mitotic count (P<0.05; Table 4). In particular, FoxP3 ≥ 6.9
302 cells/HPF had a significantly higher hazard of death (HR=12.20, 95%CI=2.37-62.72).
303

304 **DISCUSSION**

305 In human medicine, the growing number of studies on the characterization of
306 melanoma immune environment led to the successful use of immunotherapy,
307 particularly by targeting PD-1 and CTLA-4.[49] In veterinary medicine, the studies on
308 cancer immunity and on the application of immunotherapy are still few.[2,35,52]
309 Canine cutaneous melanomas are usually benign and surgical resection is typically
310 curative, still their behavior can be quite unpredictable, since highly aggressive forms
311 are observed.[18,61,69] On the other hand, mucosal melanomas, particularly oral
312 melanomas, show a malignant behavior with a predisposition to the development of
313 metastasis to lymph nodes and lungs, similarly to human melanomas.[67] At now,
314 different studies suggest the dog as a valuable spontaneous preclinical model in
315 melanoma research, particularly since the common canine oral form has been
316 demonstrated to share numerous similarities with the more rare human
317 disease.[54,66,77]
318 While different aspects of canine melanoma biology have been investigated,
319 [5,6,25,26,29,60,65] the immune environment interacting with this type of tumors is still
320 largely unknown. Therefore, our study aims at investigating the immune environment
321 of canine melanocytic tumors to acquire further information on the mechanisms of
322 immunosuppression and evasion possibly involved in tumor progression; targets of
323 our investigation are in particular FoxP3, CTLA-4, and IDO.

324 The survival time within our group of dogs with oral melanomas showed a mean
325 survival time of 240 days, calculated from the moment of the submission of the
326 sample for histological diagnosis. The mean overall survival within our group is
327 higher then what is described in previous literature, with reports of a mean survival
328 time of 147 days.[70] This result is probably due to a growing attention of the owners
329 towards the health of their dogs, and therefore to an early diagnosis, and also
330 probably because labial melanomas, which are reported to have a longer survival
331 time, were included within our oral melanoma group. This result endorses the
332 necessity for further studies to better characterize oral and mucocutaneous canine
333 melanocyte biology, together with melanoma behavior in association with different
334 site of origin of the primary tumor. Moreover, a detailed description of the anatomical
335 site of origin of the tumor should be provided by the clinician/surgeon at the moment
336 of the submission of the tissue sample for histopathological analysis, in order to gain
337 more precise data to define the prognostic significance of the primary tumor location.
338 FoxP3 is an intracellular molecule involved in Tregs development and function, and
339 considered, at now, their most specific marker.[62] In our study, FoxP3
340 immunohistochemical expression was associated with a higher hazard of death for
341 melanoma, also in the adjusted model. Nevertheless, *FOXP3* gene expression was
342 bordering significance at the univariate Cox analysis (P=0.069), probably due to the
343 use of FFPE material for mRNA extraction, which could have caused a partial
344 degradation of nucleic acids. The survival analysis, based on the cut-off value,
345 indicated that the hazard of death was 12 times higher in dogs with FoxP3≥6.9
346 cells/HPF. These results seem to confirm that, also in dogs, a higher infiltration of
347 FoxP3^{+} cells is associated with a worse prognosis, similarly to what reported in
348 human melanomas.[21,48] The aforementioned cut-off value was similar to the 6.1

349 cells/HPF value previously reported by our group,[52] but showed both higher
350 sensitivity and sensibility. Results from our study seem to confirm that FoxP3 could
351 be a major player in mechanisms of immunoescape that favors tumor growth and
352 progression also in dogs, particularly in oral and cutaneous melanomas.
353 Furthermore, the strong correlation between FoxP3 and CTLA-4, together with the
354 moderate correlation with IDO protein expression, may be in the synergic role of
355 these proteins in the establishment of an immunosuppressed tumor
356 microenvironment. A strong correlation was also evidenced between FoxP3 protein
357 expression and mitotic count, accounting for Treg role in favoring tumor growth. At
358 the moment, the markers to identify Tregs are few and often not completely specific
359 also in human medicine,[56] making the characterization and the definition of the role
360 of this T cell subpopulation, within tumor immune environment, complex and still not
361 completely understood. Also, it must be reminded that the presence of FoxP3$^+$ Tregs
362 could be influenced by tumor site, molecular subtype of the tumor, and tumor
363 stage.[62] Therefore, further investigations should be encouraged to overcome these
364 limits in defining Treg biology and role in cancer, both in veterinary and comparative
365 medicine.
366 During the last few years, immunotherapies with monoclonal antibodies directed
367 against CTLA-4, together with anti-PD1, have revolutionized the treatment of
368 patients with advanced melanoma in human medicine, but still the presence and the
369 role of CTLA-4-expressing cells have been limitedly explored in dogs and in
370 veterinary oncology. In the present study, we found evidence of the expression of
371 this molecule in canine melanocytic tumors. Our results indicate that both CTLA-4
372 immunohistochemical and gene expression were associated with the histological
373 diagnosis and with an increased hazard of death (univariate analysis), similarly to

374 what is reported in human melanoma.[11] However, in the multivariate analysis, CTLA-
375 4 lost its statistical significance, suggesting that CTLA-4 is not an independent
376 predictor. This result, on one hand, is probably due to the association of this marker
377 with other variables, perhaps to be searched within the complexity of the tumor
378 immune environment itself. On the other hand, the association between the protein
379 and gene expression of this marker and the tumor mitotic count, which is considered
380 one of the most affordable prognostic features of canine melanomas,[3,68] seems to
381 corroborate the hypothesis of the immunosuppressive role of this molecule in
382 melanoma growth. A larger study group should be therefore investigated, to gather
383 more information on the role of this molecule in melanocytic tumors of dogs. To our
384 knowledge, this is the first study that focuses on CTLA-4 within canine melanoma
385 microenvironment, in contrast to previous studies aimed at characterizing the
386 expression of this costimulatory molecule in circulating cells during neoplastic
387 disease and in a healthy subject.[71–73] Our results, although preliminary, highlight the
388 presence of this molecule within canine melanoma and open the way to further
389 investigations on the role of CTLA-4-associated pathways in canine oncology.
390 The enzyme IDO can be expressed by different types of cells, in particular MDSCs,
391 DCs, macrophages, but also tumor cells. This enzyme acts both on APCs and T
392 cells, causing immune suppression and therefore facilitating cancer development.[4]
393 Our results show that IDO immunohistochemical expression was an independent
394 predictor of mortality, both at univariate and multivariate Cox proportional regression
395 analysis. The optimal cut-off value for IDO immunohistochemical expression was set
396 at 8.4 cells/HPF, compared to the 14.7 cells/HPF value evidenced in our previous
397 study. In addition to the different characteristics of the sample population, this
398 incongruity can also be explained by the different percentages of sensitivity and

specificity associated with this new cut-off. Indeed, in the present study, the lowest cut-off was associated with a higher sensitivity (82%) in the prediction of death of the dog due to melanoma. Indeed, by setting the IDO cut-off at 14.7 cells/HPF in the case series of the present study, the specificity improves, reaching the value indicated in our previous study (79%), but it is not being balanced by an adequate sensitivity (57%). The greater accuracy indicated by the higher AUC of the present study group, together with the higher number of cases with a complete follow-up (55 vs 52), suggests that the lower cut-off should be preferred. The role of IDO in canine melanoma seems to be similar to what reported in humans, where IDO protein expression is considered to have a prognostic role in both cutaneous melanoma and nodal metastases.[12,51,58] Gene expression, on the other hand, was significant only at the univariate Cox analysis. The loss of significance could be due to the high variability of mRNA expression detected by qRT-PCR; the possible explanation of this result could be, as for *FOXP3*, the use of FFPE material to retrieve mRNA. In fact, even though numerous studies are based on this protocol for studies of gene expression, fresh-frozen tissue should be preferred to avoid partial mRNA degradation.[36] Our results seem to confirm that IDO is involved in canine tumor immunoescape and progression. Also, IDO could be implicated in the activation of Treg cells within canine melanoma microenvironment, as indicated by the moderate correlation between the variables and also by other authors in different models.[45,63] The results of the multivariate analysis confirm that the mitotic count, the number of IDO^+ cells/HPF, and of $FoxP3^+$ cells/HPF can be considered independent prognostic factors, suggesting that, if further confirmed by prospective studies, these markers could be useful in the oncological evaluation of canine melanocytic tumors.

423 Taken together, the results from our study seem to confirm the presence of
424 immunosuppressive tumor microenvironment mechanisms controlled by FoxP3,
425 CTLA-4 and IDO, also in canine melanoma, particularly in the most aggressive oral
426 form. After this retrospective investigation, prospective studies on fresh/frozen tissue
427 aiming at the confirmation of these results, also including and extending our
428 investigation to other immune populations and to metastatic lesions, have been
429 planned. Further investigations on the immune environment of canine melanocytic
430 tumors should be stimulated, aiming both at a better characterization of canine
431 melanoma biology and immune environment, also for comparative purposes, and at
432 a future possible employment of immunotherapeutic strategies also in the canine
433 species.
434

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648

649 Figure 1. Immunosuppressive interplay within melanoma microenvironment. MCs:
650 melanoma cells; MDSCs: myeloid-derived stem cells; DCs: dendritic cells; TAMs:
651 tumor associated macrophages; IDO: indoleamine 2,3 dioxygenase; CTLA-4:
652 cytotoxic T-lymphocyte-associated protein 4. IDO is produced by MDSC, DCs,
653 macrophages, and neoplastic cells. IDO can stimulate CD4+ cells to differentiate into
654 Tregs, causing an upregulation of FoxP3 and can also activate mature Tregs. Tregs,
655 in turn, can induce IDO. The persistent antigen exposition by melanoma cells and
656 the chronic stimulation of the immune system, together with IDO production, seems
657 to be critical in the hyperactivation of CTLA-4, which leads to peripheral cell
658 tolerance.

659

660 Figure 2. Cutaneous melanoma, haired skin, dog, case n.33. Immunohistochemistry
661 for forkhead box P3 (FoxP3) in a sample of oral melanoma, with disseminated cells
662 within the tumor. AEC and hematoxylin.

663

664 Figure 3. Cutaneous melanoma, haired skin, dog, case n.44. Scattered intratumoral
665 CTLA-4^{+} cells (arrows). AEC and hematoxylin.

666

667 Figure 4. Oral melanoma, oral mucosa, dog, case n.16. Peripheral infiltration of IDO^{+}
668 cells with granular cytoplasmic reactivity (likely macrophages). AEC and
669 hematoxylin.

670

671 Figure 5. Box plots of FoxP3, CTLA-4, and IDO, positive nuclei/HPF in the upper
672 panels, and *FoxP3*, *CTLA-4*, and *IDO* mRNA in the lower panels according to

673 diagnosis. ns=not significant, *P<0.05, **P<0.01, ***P<0.001 (multiple comparisons
674 by Mann-Whitney tests).
675
676 Figure 6. Kaplan-Meier survival curves by optimal cut-off values of IDO (8.4
677 cells/HPF), FoxP3 (6.9 cells/HPF), and CTLA-4 (2.2 cells/HPF), and *FOXP3* (35.9
678 mRNA expression level), *CTLA4* (10.1 mRNA expression level), and *IDO* (22.7
679 mRNA expression level).