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# COX-2 expression is associated with infiltration of inflammatory cells in oral and skin canine melanomas

## Short Running Title : COX-2 and inflammation in canine melanomas

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Keywords: COX-2, canine melanoma, inflammation, macrophages, immunohistochemistry, dog

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# COX-2 expression is associated with infiltration of inflammatory cells in oral and skin canine melanomas

## COX-2 in canine melanomas

### Abstract

Melanoma is a fast-growing tumor in dogs and represents 7% of the total malignant neoplasms from the skin and is the most common tumor found in the oral cavity. In these tumors high expression of COX-2 is associated with a poor prognosis. The aim of this study was to verify if the overexpression of COX-2 is related to the modulation of lymphocytes and if it is associated with the angiogenic and proliferative capacity of the melanoma. Canine melanoma samples ( $n=85$ ) were analyzed by immunohistochemistry to detect the expression of S-100, Melan-A, PNL-2, COX-2, Factor VIII, Ki-67 and immune cells markers (CD3, CD4, FOXP3, and MAC387); and expression levels of MAC387, NOS and CD206 were determined by immunofluorescence. Our study showed a concurrent difference between the expression of COX-2 and inflammatory cell infiltration: Oral melanomas showed positivity for COX-2 in 34% of the cases and this expression was associated with CD3 positivity in the inflammatory infiltrate and angiogenesis; whereas cutaneous melanomas presented positivity for COX-2 in 42% of the cases and this expression was associated with positive staining for CD3, CD4, FOXP3 and MAC387. These markers are associated with inflammatory cells, angiogenesis and proliferation. Interestingly, melanomas were highly infiltrated by FOXP3+ cells, this is related to angiogenesis, whereas CD3, CD4 and MAC387 expression was only associated with cutaneous melanomas. The macrophage profile analysis showed that both oral and cutaneous melanomas with low COX-2 expression have an M1 phenotype, whereas the cases with high COX-2 expression demonstrate a hybrid M1/M2 profile pattern. We concluded that the COX-2 is overexpressed in 42% of cutaneous melanomas and in 34% of oral melanomas, with a direct association with angiogenesis, proliferation, and intratumoral lymphocyte infiltration. We propose that COX-2 is a key regulator of immune cell infiltration and may drive tumor associated macrophage activation.

Keywords: COX-2, canine melanoma, inflammation, macrophages, immunohistochemistry, dog

## Introduction

Melanoma is the most common oral malignancy in dogs. Similar to humans, melanoma is considered an extremely malignant tumor with a high degree of local invasiveness and with a high metastatic propensity to metastasize to the lymph nodes and lungs, as well as to the liver, spleen, brain, heart and bone marrow.<sup>1-3</sup> The treatment for oral and skin melanomas is surgical excision, followed with radiotherapy and chemotherapy, but the efficacy of these methods is poor and the prognosis of 1-year survival is approximately 10%.<sup>1,4</sup> However this outcome is dependent on the size of the initial tumor; when small tumors are treated survival rate is 54% two years after surgery<sup>5</sup>. When larger tumors are taken into account the average survival time decreases to only four months and the death rate is to 100% within two years.<sup>5</sup> Oral melanomas have a worse prognosis than cutaneous melanomas.<sup>5,6</sup>

Given the clinical challenge proposed by melanomas there has been research to identify intrinsic markers of tumor aggressiveness and to evaluate potential prognostic factors, which may be informative in therapeutic planning.<sup>7</sup> The cyclooxygenase-2 (COX-2) is the inducible form of the enzyme that is responsible for prostanoid production: COX-2 converts arachidonic acid into prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), and then the PGH<sub>2</sub> is subsequently converted into a number of prostanoids including prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), prostaglandin F<sub>2</sub> (PGF<sub>2</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostacyclin (PGI<sub>2</sub>) and thromboxane (TXA<sub>2</sub>).<sup>8</sup> Studies investigating the expression cyclooxygenase (COX) enzymes in cancer have shown that overexpression of COX-2 is associated with the carcinogenesis process in several tumor types, including mammary tumors, colon carcinoma, and prostatic carcinoma. COX-2 drive tumor promotion, maintenance and progression to metastatic stages.<sup>9</sup>

In animals, COX-2 expression has been described in different types of neoplasms in dogs, cats and horses. Mammary carcinomas, transitional cell carcinomas, prostatic carcinoma and squamous cell carcinoma in dogs has been the most studied. COX-2 overexpression in these tumors is related to the increased expression of vascular growth factors, and consequently to the angiogenesis process.<sup>10</sup> Another role of COX-2 that has been widely studied is its importance in tumor initiation, which was demonstrated in an in vitro study with human and canine osteosarcoma cell lines. Where cancer stem cells (CSC) have been isolated from tumor cells and treated with COX-2 specific inhibitors, leading to a reduction in the ability to form tumor spheres.<sup>11</sup>

COX-2 expression is observed in neoplastic cells of human melanomas in the primary tumor site and in the corresponding metastases<sup>8</sup> and expression is higher in malignant melanocytic lesions compared to benign lesions. Moreover, under immunohistochemistry analysis, it presents more intense staining in the dermal component of the lesions; this marking pattern varies according to the histological type and between different disease stages.<sup>12</sup> Higher expression of COX-2 in melanomas when compared to melanocytomas is also observed in dogs.<sup>13</sup>

The relationship between COX-2, modulation of immune/inflammatory cell infiltration and angiogenesis has been extensively studied in mammary carcinomas<sup>14,15</sup> and colon tumors.<sup>16</sup> In both canine and human mammary tumors, a poorer prognosis stage is linked to the co-expression of COX-2 and tumor-associated macrophages especially when the later present an M2 profile.<sup>17,18</sup> Additionally, reduced activation of anti-tumor cytotoxic CD8+ T-cell is related to a suppression of antigen-presenting dendritic cells caused by PGE<sub>2</sub>.<sup>6</sup>

Studies that have investigated the relationship between T-helper and regulatory T cells (Tregs) in tumor-infiltrating lymphocytes (TILs) in breast cancer, and showed that T-helper cells infiltration gradually decreases and Tregs cells accumulate with disease progression.<sup>20</sup> Another study of hepatocellular carcinoma associated the higher expression of COX-2 with an increase of Treg infiltration and a decrease of CD4+ T cell frequency.<sup>21</sup> In a recent study in human melanoma, a correlation was shown between the disease-free survival and overall survival in patients expressing low levels of FOXP3 (regulatory T-cell marker) in the primary melanoma.<sup>22</sup>

A study that evaluated the COX-2 expression with angiogenesis, tumor proliferation, presence of macrophages, and T lymphocyte infiltrate in canine melanomas found associations with microvessel density (FACTOR VIII), lymphocyte infiltration and macrophages.<sup>23</sup> However, the association between COX-2 expression and molecular markers of inflammatory infiltrate, angiogenesis and proliferation are not well described in either human or canine. Building on previous reports of canine melanomas and COX-2 expression<sup>13,23</sup> the aim of this study was to define the COX-2 expression profile in the skin and oral canine melanomas, and to characterize mononuclear cell infiltrate and the relationship with angiogenesis. We hypothesized that overexpression of COX-2 in malignant melanocytic neoplasms in dogs may lead to a worse prognosis because it is related to lymphocyte modulation and increases the angiogenic and proliferative capacity of the tumor.

## **Materials and methods**

### *Histologic evaluation and classification*

The tissues obtained for this study consisted of 85 canine melanomas (29 oral and 56 cutaneous) retrieved from the archive of the Comparative Pathology Laboratory, Department of Pathology, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Sections of 4- $\mu$ m sections from formalin-fixed and paraffin-embedded (FFPE) tissues were stained with hematoxylin and eosin (H&E). Each sample was re-examined independently by two pathologists (E.F. and T.S.) to confirm the diagnosis according to the criteria of the International Histological Classification of Tumors of Domestic Animals established by the WHO.<sup>24</sup>

The following clinicopathological features were evaluated the presence of ulceration, degree of pigmentation, the morphology of neoplastic cells (spindle, epithelioid or mixed), mitotic index and tumoral vascular invasion. The mitotic index (MI) was calculated by counting all

the mitoses present in 10 random, non-overlapping high-power fields (HPFs) ( $\times 400$ ) according to the WHO guidelines.<sup>24</sup> A mitotic index was attributed according to the number of mitoses: low mitotic index ( $< 4$  mitoses per 10 HPFs); high mitotic index ( $\geq 4$  mitoses per HPFs).<sup>25</sup> The degree of pigmentation was estimated using a subjective scale from 0 (non-pigmentation), 1 (pigment until 25% of neoplastic cells), 2 (pigmentation in 26-50% of neoplastic cells) and 3 (pigment in  $>50\%$  of neoplastic cells).

#### *Immunohistochemical analysis*

For immunohistochemistry, FFPE sections (4  $\mu\text{m}$  thick) were prepared and the detection of S-100, Melan-A, PNL-2, COX-2, Factor VIII (also named the von Willebrand factor), CD3, CD4, FOXP3, MAC387, and Ki-67 was carried out by peroxidase reaction method with a polymerized secondary antibody (Novolink Polymer Detection System; Leica Biosystems, Newcastle upon Tyne, UK) according to the manufacturer's instructions. Antigen retrieval was performed by pressurized humid heat at 125  $^{\circ}\text{C}$  (Pascal<sup>®</sup> Pressure Cooker; Dako Cytomation, Glostrup, Denmark) for 2 min in citrate buffer pH 6,0 (DakoCytomation Target Retrieval Solution), followed by cooling for 20 min at room temperature. All sections were incubated with the primary specific antibody: S-100 (1:100 dilution, Clone S100; Dako, Glostrup, Denmark), Melan-A (1:100 dilution, Clone A103; Dako, Glostrup, Denmark), Melanoma Antigen (1:100 dilution, Clone PNL2; Santa Cruz Biotechnology, Dallas, Texas, USA), COX-2 (1:80 dilution, Clone SP21; Thermo Fisher Scientific, Waltham, MA, USA), Factor VIII (1:2000 dilution, Polyclonal; Dako, Glostrup, Denmark), CD3 (1:300 dilution, polyclonal antibody, Dako), FOXP3 (1:500 dilution, SP97; Spring Bioscience, Pleasanton, CA, USA), MAC387 (1:400 dilution, Clone MCA874G; AbDSerotec, Kidlington, UK) and Ki-67 (1:50 dilution, Clone MIB-1; Dako, Glostrup, Denmark) for 18h at 4  $^{\circ}\text{C}$ ; for CD4 (1:100, clone DH29A; VMRD, Pullman, WA, USA) the antigen retrieval was performed by means of humid heat (water bath at 98  $^{\circ}\text{C}$ ) for 20 min in citrate buffer pH 6,0 and after repeating the same steps of the others antibodies. The antibody reaction products were observed with the chromogen 3,3' - diaminobenzidine tetrachloride (DAB) at DAB diluent for 3 minutes. After a final washing in distilled water, the sections were counterstained with GIEMSA (for 30 minutes) dehydrated and mounted. Negative controls were sections treated with isotype-matched primary antibodies mouse anti-human IgG and this study also included adequate positive controls: the positive control used was canine mammary tumor for COX-2 and for Factor VIII; epidermis as an internal positive control for Melan-A, PNL-2, and Ki-67, and sections of canine lymph nodes for CD3, CD4, FOXP3, and MAC387.

#### *Quantification of immunoreactivity*

The positivity for S-100, Melan-A, PNL-2<sup>26</sup>, and COX-2<sup>27</sup> was indicated by the presence of distinct brown cytoplasmic labeling and it was used a semiquantitative method previously described.<sup>13,23</sup> The number of COX-2 positive cells was evaluated semi-quantitatively, with the distribution score defined by the estimated percentage of positive cells in 5 fields at 400x magnification: 0 = absence, 1 = fewer than 10% stained cells, 2 = between 10% and 30%, 3 =

between 31% and 60%, 4 = more than 61% stained cells. For staining intensity, values from 0 to 3 were attributed: 0 = absence (-), 1 = weak staining (+), 2 = moderate staining (++) and 3 = strong staining (+++). Distribution score and intensity were multiplied to obtain a total score, which ranged from 0 to 12.<sup>27</sup>

To analyze microvessel density (MVD) structures with or without lumens that were positive for Factor VIII it was considered as individual vessels. Areas of fibrosis, necrosis, and inflammation, and vessels with muscular walls were not counted. Microvessels were counted in the 3 hot spots (identified at low-power magnification - 40x) at a 200x magnification fields (corresponding to approximately 0.76 mm<sup>2</sup>).<sup>28</sup>

To determine the Ki-67 index the area of highest labeling was searched. Ki-67 expression was evaluated by counting 500 tumor cells with the help of a microscopic grid, at high magnification (×400) and the index was expressed as a percentage.<sup>29</sup> For CD3, CD4

FOXP3 and MAC387 evaluation, cells staining positively were counted in three hotspot regions of the tumor and peritumoral, firstly defined at low magnification, in 10 high-power fields, with ×400 magnification. The number expressed for CD3+ T-lymphocytes, CD4+, FOXP3+ and for tumor-associated macrophages (TAMs) represents the mean value ±standard error of mean following a methodology previously used.<sup>30-34</sup>

#### *Immunofluorescence and confocal microscopy*

FFPE sections (3 μm thick) for immunohistochemistry and confocal microscopy were deparaffinised, rehydrated and subjected to heat-induced epitope/antigen retrieval (HIER) for 30s at 125 °C using an antigen retrieval buffer (Trilogy, Cell Marque Corporation) in a pressure chamber (Pascal Pressure Chamber, Dako) and proteinase K, when appropriate. All sections were incubated overnight with the primary specific antibody: S100A8/A9 Complex (1:400 dilution, Clone MAC387-FITC; Abcam, Cambridge, MA, USA), CD206 (1:100 dilution, Clone 3.29B1.10-PE; Beckman Coulter, Marseille, FR) and NOS (1:100 dilution, Polyclonal; Santa Cruz Biotechnology, Dallas, Texas, USA). An unaffected canine lymph node was used as a positive control, while negative controls were sections treated with isotype-matched primary antibodies (rabbit and mouse anti-human IgG, Dako).

Immunofluorescence was performed as described by Rodrigues.<sup>35</sup> Tissue sections were incubated with Alexa Fluor 647 goat anti-rabbit IgG antibody (1:1000, Life Technologies) for detection of inducible nitric oxide synthase (NOS2). Nuclei were counterstained with Hoechst 33342 (Life Technologies). Images were collected using a LSM Zeiss 880 confocal microscope (Carl Zeiss). Samples were excited at 405 nm and observed at 415-480 nm for detection of Hoechst 33342, excited at 488 nm and observed at 500-525 nm for detection of fluorescein isothiocyanate (FITC), excited at 543 nm and observed at 550-630 nm for detection of R-phycoerythrin (R-PE), and excited at 630 nm and observed at 650 nm for detection of Alexa Fluor 647. Zeiss Efficient Navigation (ZEN) software was used for spectral

analysis. Tumors with high COX-2 expression score (n = 5 oral and 5 skin) and low COX-2 expression score (n = 5 oral and 5 skin) with high macrophage counts were evaluated to characterize infiltration. The macrophages expressing MAC387 and NOS2 were considered M1 profile, and the M2 macrophages have the marker profile MAC387 and CD206. The cases with triple-staining (MAC387, NOS, and CD206) were considered mixed profile (M1/M2). All antibodies were previously documented as suitable for detection of epitopes in canine tissues.<sup>34, 36-38</sup>

### *Statistical analysis*

The statistical software InStat (GraphPad Software) version 3.0 was used for statistical analysis. Analysis of variance (ANOVA) was used for analyzing continuous variables. Spearman's correlation test was used to estimate the relationship between the staining patterns of different antibodies used. In all statistical comparisons,  $P \leq 0.05$  was accepted as denoting significant differences.

## **Results**

### *Tumor samples*

A total of 85 melanoma tumors (29 orals and 56 cutaneous) were included in this study and classified accordingly: 23 spindle cells, 53 epithelioid cells and 9 mixed. Of those, 20 has non-pigmentation, 42 with score 1, 7 with score 2 and 16 with score 3 for pigmentation. The mitotic index was evaluated in 74 cases (24 orals and 50 cutaneous), for those 14 orals and 20 cutaneous showed a high mitotic index ( $\geq 4$  mitoses per 10 HPF) (Table1). The MI could not be evaluated on 11 other cases due to high pigmentation.

### *Immunostaining to COX-2, angiogenesis, proliferation, immune cells and melanoma markers*

#### *S-100, Melan-A, PNL-2, Factor VIII, Ki-67, CD3+, CD4+, FOXP3+ in T-lymphocytes and MAC387 macrophages immunostaining*

The immunoreactivity for S100, Melan-A, and PNL-2 was observed as a brown color present in the cytoplasm. In each sample was performed IHQ to S100 and Melan-A, while on the non-pigmentation cases it was also included the PNL-2 antibody on the panel. All samples were positive for S-100 protein, 10 cases (1 skin and 9 oral, all non-pigmentation) were negative for Melan-A and were confirmed the melanoma diagnostic by PNL-2. (Table 2).

For Factor VIII evaluation, vascular cells were considered as positive when cytoplasmic staining was observed and counting was performed using Weidner's method.<sup>39</sup> For Ki-67, the immunoreactivity occurred in the nucleus, appearing in a granular labeling pattern. The CD3, CD4, and FOXP3 immunostaining were observed in the cytoplasm or/and in the cytoplasmic membrane of T-lymphocytes and the diffuse inflammation emerged as the predominant pattern of infiltration. T-lymphocytes contact closely with neoplastic cells and sometimes



were accumulated in peripheral and perivascular clusters. Immunoreaction was considered as positive for anti-macrophage antibodies (MAC387) when cytoplasmic diffuse or granular staining (but no nuclear) was observed and simultaneously demonstrating macrophage morphology. T-lymphocytes and macrophages were located predominately in the intratumoral area, although they were also observed frequently within the periphery of the tumor.

The mean values of inflammatory, angiogenic and proliferation markers are presented in table 3.

#### *COX-2 Immunohistochemistry*

The immunostaining for COX-2 was present in the cytoplasm, nuclear membrane and cytoplasmic membrane, in a diffuse and homogeneous pattern (Figure 1A, 2A). In tumors where positivity of COX-2 was not diffuse, the positive areas also had higher T-lymphocyte and macrophage inflammatory infiltrate. The labeling intensity of COX-2 in the primary tumor and in neoplastic intravascular emboli were similar, however, the extension of COX-2 staining in neoplastic intravascular emboli was always diffuse.

The results showed that COX-2 was expressed in some degree of positivity in 89% of the tumors examined. The immunohistochemistry for COX-2 revealed that 40% of the melanomas had a final score of 6–12. Of the 29 oral melanomas, 34% showed a COX-2 final score of 6–12. Skin melanomas present score of 6-12 in 42% (Table 2).

#### *Correlation between COX-2, Factor VIII, Ki-67, CD3+, CD4+, FOXP3+ T-lymphocytes and MAC387 macrophages*

##### *Oral tumors*

High COX-2 expression demonstrated a statistically significant correlation with Factor VIII ( $p = 0.002$ ) and intratumoral CD3+ ( $p = 0.02$ ) (Table 4). FOXP3+ also demonstrated a significant correlation with CD3+ ( $r = 0.73$ ,  $p = 0.001$ ). The peritumoral infiltrate analysis of high COX-2 expression did not show a statistically significant association with any of the parameters set (Figure 1B-1E, 2B-2E).

##### *Cutaneous tumors*

The high COX-2 expression revealed a statistically significant correlation with Factor VIII ( $p < 0.0001$ ), Ki-67 ( $p = 0.01$ ), intratumoral CD3+ ( $p = 0.0004$ ), CD4+ ( $p = 0.0006$ ), FOXP3+ ( $p = 0.002$ ) and MAC387 macrophages ( $p = 0.02$ ). The analysis of peritumoral infiltrate high COX-2 immunoreaction presented a statistically significant correlation with CD4+ ( $p = 0.004$ ), FOXP3+ ( $p = 0.005$ ) and MAC387 macrophages ( $p = 0.02$ ) (Table 4).

Factor VIII showed correlation with CD3+ ( $r = 0.52$ ,  $p = 0.02$ ) and CD4+ ( $r = 0.54$ ,  $p = 0.01$ ) intratumoral. The FOXP3+ intratumoral demonstrated a statistically correlation with CD3+

( $r= 0.97$ ,  $p < 0.0001$ ), CD4+ ( $r= 0.63$ ,  $p = 0.001$ ) and Factor VIII ( $r= 0.44$ ,  $p = 0.03$ ), as well the peritumoral infiltrate was statistically correlated with CD3+ ( $r= 0.88$ ,  $p < 0.0001$ ), CD4+ ( $r= 0.52$ ,  $p = 0.03$ ) and Factor VIII ( $r= 0.63$ ,  $p = 0.008$ ).

It was also observed that the intratumoral marking for MAC387 macrophages its statistically correlated with Factor VIII ( $r= 0.57$ ,  $p = 0.004$ ), CD3+ ( $r= 0.64$ ,  $p = 0.002$ ) and FOXP3+ ( $r= 0.61$ ,  $p = 0.001$ ). The peritumoral analyzed also demonstrated an association with Factor VIII ( $r= 0.42$ ,  $p = 0.04$ ).

#### *Correlations of clinicopathological features with COX-2, Factor VII, Ki-67, CD3+, CD4+, FOXP3+ T-lymphocytes and MAC387 macrophages immunostaining*

##### *Oral tumors*

Tumors with higher mitotic index showed significant correlation with the histological type (epithelioid and mixed) of the tumor ( $p = 0.03$ ), high COX-2 ( $p = 0.04$ ) and the intratumorals CD3+ ( $r= 0.61$ ,  $p = 0.0005$ ), CD4+ ( $r= 0.60$ ,  $p = 0.003$ ), FOXP3+ ( $r= 0.58$ ,  $p = 0.004$ ) and MAC387 macrophages ( $r= 0.43$ ,  $p = 0.05$ ). The presence of ulceration was statistically associated with pigmentation ( $r= 0.55$ ,  $p = 0.02$ ).

##### *Cutaneous tumors*

The histological type of tumor (epithelioid and mixed) showed significant correlation with high COX-2 ( $p = 0.003$ ) and Factor VIII expression ( $r = -0.35$ ,  $p = 0.007$ ).

The presence of skin ulceration was statistically correlated with high COX-2 ( $p < 0.02$ ), higher FOXP3+ intratumoral ( $r= 0.55$ ,  $p = 0.006$ ) and peritumoral expression ( $r= 0.60$ ,  $p = 0.01$ ), and intratumoral macrophages presence ( $r= 0.46$ ,  $p = 0.02$ ). We also showed that tumors with skin ulceration presents higher expression of CD3+ intratumoral ( $p = 0.01$ ) and peritumoral ( $p = 0.02$ ), CD4+ intratumoral ( $p = 0.05$ ) compared with tumors without skin ulceration.

A higher mitotic index demonstrated significant correlation with high COX-2 ( $p < 0.0001$ ), Factor VIII ( $r= 0.39$ ,  $p = 0.004$ ), pigmentation ( $r= -0.43$ ,  $p = 0.001$ ) and the histological type of tumor ( $r= -0.30$ ,  $p = 0.03$ ). As well as demonstrate that tumors with high MI present higher KI67 expression ( $P = 0.01$ ) in relation to tumors with low MI.

The correlation values of COX-2 with inflammatory, angiogenic, proliferation markers and clinicopathological features are presented in table 4.

##### *M1/M2 macrophage subpopulations in oral and skin tumors*

The NOS2 was expressed in macrophages (MAC387 positive cells with macrophage-like morphology) in 4/5 (80%) skin tumors with low COX-2 expression, while triple staining pattern (MAC387 positive, NOS2 positive, CD206 positive) was expressed in 1/5 (20%) low expression; this profile is consistent with a predominantly M1 profile. For the skin samples presenting a high expression of COX-2 a triple staining pattern (MAC387 positive, NOS2

positive, CD206 positive) was observed in 4/5 (80%), while 1/5 (20%) presented NOS2 in MAC387 positive macrophages showing this immunolabelling profile.<sup>40</sup>

In the 10 oral melanomas (five COX-2 low expression and 5 COX-2 high expression), it was observed in the samples of low expression NOS2 in MAC387 positive macrophages in 3/5 (60%) and in 2/5 (20%) presented the triple staining pattern. For the lesions with high COX-2 expression 1/5 (20%) was observed the CD206 in MAC387 positive macrophages, while in 4/5 cases expressed the triple staining pattern (MAC387 positive, NOS2 positive, CD206 positive) presenting an immunolabelling profile (Figure 3).<sup>40</sup> Statistical analysis was not performed on this data in view of the low number of cases in each group.

## Discussion

Overexpression of COX-2 is associated with enhanced tumorigenesis via inhibition of apoptosis, stimulation of cell cycle progression and the production of angiogenic factors, and may play a role in the regulation of cell proliferation, migration, and invasion. The high expression of COX-2 may also induce tumor cells to secrete growth factors that reshape the microenvironment to support tumor growth and dissemination. The overexpression of COX-2 will increase the production of PGE<sub>2</sub> that may bind receptors on stromal cells promoting a tumor-supportive microenvironment by inducing angiogenesis and aiding immune evasion.<sup>7,11,41</sup>

Increased COX-2 expression was found in various malignant tumors.<sup>11,23,27</sup> However, the question of how elevated COX-2 expression affects canine melanomas remains unanswered. In our study with canine oral and cutaneous melanomas, we found a high COX-2 expression in 34% of oral samples and 42% of cutaneous tumors; this is consistent with the finding of Lavallo *et al*<sup>27</sup>. Lavallo *et al* evaluated the expression of COX-2 in different types of canine mammary carcinomas and found that about 40% presented a high expression of COX-2. Human melanomas behavior is dependent on where it is located, for example acral and skin tumors, and the treatment of these tumors is based on location; mucosal tumors in humans also present a poor prognosis when compared with skin.<sup>42</sup> So, as in humans, the canine melanoma might present a different behavior according to its location and this could be the reason for the variation of COX-2 expression.

Recent results in canine melanomas demonstrated a statistically significant association of high COX-2 levels with angiogenesis (Factor VIII), CD3 T-lymphocytes and macrophage infiltration. These tumors also showed concurrent high COX-2/Factor VIII, high COX-2/CD3, and COX-2/MAC387 that were statistically associated with variables of tumor aggressiveness such as ulceration, vascular invasion, and mitotic index.<sup>33</sup> Accordingly, in our results the increased COX-2 levels are related to increased levels of Factor VIII, CD3 T-lymphocytes and MAC387 macrophages in cutaneous melanomas, unexpectedly the correlation between COX-2/MAC387 was not found in oral melanomas. In human gastric cancer there is an association between down-regulation of COX-2 and inhibition of VEGF, Flt-1, Flk-1/KDR,

angiopoietin-1, tie-2, MMP2 and OPN; all of these proteins are angiogenesis-related molecules and may be related to higher vascular density in tumors with high expression of COX-2.<sup>43</sup> Several studies have demonstrated a correlation between tumor-infiltrating lymphocytes (TILs) and disease recurrence and survival in several neoplasms.<sup>44,45</sup> This lymphocytes polarization is due to the expression of cytokines within the environment.<sup>46</sup> Cases when the neoplastic tissue is ulcerated the inflammatory infiltrate is higher and it is predominantly constituted by degenerated neutrophils and macrophages surrounding the ulcer on the superficial layer. In medium and deep layers the component of the inflammatory infiltrate of the tumor decreases and it becomes composed mostly by lymphocytes and macrophages.<sup>25,30</sup>

Tumor-Associated Macrophages (TAMs) represent the major inflammatory component of the stroma of many tumors, being able to affect different aspects of the neoplastic tissue. The M1 profile of these TAMs exhibit antitumour activity by mediating host defence against microorganisms and cancer cells; while M2 macrophages have an anti-inflammatory function and is associated with pro-tumoral functions, including the promotion of angiogenesis, matrix remodeling and suppression of adaptive immunity.<sup>40</sup> A correlation between high COX-2 expression and TAMs was associated with poor prognosis in human mammary carcinomas.<sup>17</sup> However, limited information is available for dogs, especially regarding M1/M2 macrophage polarization. Monteiro<sup>18</sup> described a difference in macrophages polarization between benign and malignant mammary tumors: mammary carcinomas present a M2 profile, whereas the benign lesions showed a predominantly M1 profile. Moreover, we demonstrated in our results that macrophages from both oral and cutaneous melanomas with low COX-2 expression showed a predominantly M1 profile, whereas macrophages from tumors with high COX-2 expression present a hybrid M1/M2 profile pattern. The concept of M1 and M2 sub-populations is based on the extremes of a spectrum; macrophages exhibit an M1, M2 or hybrid phenotype in response to a range of signals in different microenvironments.<sup>40</sup>

The COX-2 is also inducible in macrophages and could be expressed in fibroblasts and endothelial cells. A study in humans mammary carcinomas proposed that the over-expression of COX-2 by the tumor was essential for the induction and maintenance of M2-phenotype macrophage polarity, to promote the cell proliferation and survival of the cancer cells by increasing Bcl-2 and P-gp and decreasing Bax. The COX-2 produced by TAMs also induce the expression of COX-2 in breast cancer cells, which in turn promoted M2 macrophage polarization suggesting that COX-2 functions as a key cancer promoting factor by triggering a positive-feedback loop between macrophages and cancer cells. The relation of COX-2 expression between the cancer cells and fibroblasts and endothelial cells is not well elucidated but the COX-2 expression by these inflammatory cells is related to angiogenesis promotion and increase cell migration.<sup>47,48,49</sup>

Furthermore, in our study, the high expression of COX-2 in cutaneous neoplasms was related to KI67, intratumoral CD4, and FOXP3, and also the peritumoral CD4, FOXP3, and MAC387. Stroma-infiltrating CD4+ T-helper cells constitute part of the tumor microenvironment and represents host anti-tumor immunity. Furthermore, studies have demonstrated a decrease of these cells with disease progression,<sup>20</sup> what may explain the absence of these cells in canines oral tumors as this type of tumor is generally diagnosed at advanced stages, as can be observed in the melanoma samples received at LPC where about 55% of animals with oral canine melanoma are TNM stage 3 or 4, while 18% of skin melanomas are in stage 3 or 4. Here we show that CD4+ cells are related to Treg cells in the cutaneous melanomas, suggesting that CD4+ might have an important role in antitumor immunity, although it remains controversial whether or not CD4+ cells promote antitumor response or the tumor growth.

Our results also indicate that there is a high proportional of FOXP3-expressing cells in oral melanomas, and that cutaneous melanomas correlate with Factor VIII, MAC387, and skin ulceration. The Treg-specific transcription factor FOXP3, which is expressed by CD4<sup>+</sup>CD25<sup>high</sup> cells participates in the antitumor immune response by decreasing the T-cell-mediated immune response against the tumor cells.<sup>50</sup> It has been reported that high numbers of circulating Tregs are associated with rapid tumor progression in experimental animal models of melanoma and in patients with melanoma.<sup>51</sup> In canine oral melanomas, infiltration by regulatory T lymphocytes is inversely correlated with infiltration by cytotoxic and helper T lymphocytes.<sup>52</sup> Based on these recent studies our suggestion is that this high FOXP3 expression may be one of the keys factors that can explain the aggressiveness of oral melanomas.

FOXP3 is expressed mainly by CD4<sup>+</sup>CD25<sup>high</sup> cells, the so-called Tregs that are a subpopulation of T cells that have the vital property of downregulating harmful immune-mediated inflammation.<sup>22</sup> However, other T-cell populations have also been described as expressing FOXP3.<sup>32</sup> Therefore, we cannot exclude that some of the FOXP3 cells are activated T cells and not Tregs. As in routine immunohistochemical analysis is not possible to perform double or even triple staining with CD25 and CD4, and as the expected frequency of non-Tregs expressing FOXP3 is very low, we focused our study on single staining for FOXP3.

The higher mitotic index (MI) is one of the most important variables that reflect tumor aggressiveness.<sup>25</sup> The MI for oral melanomas of our study correlated with the histological type of the tumor (epithelioid and mixed), high COX-2, CD3+, CD4+, FOXP3+ protein levels and number of MAC387 positive macrophages. The cutaneous neoplasm also showed correlation of MI with the histological type of the tumor (epithelioid and mixed) and high COX-2, Factor VIII, likewise with KI67 and Factor VIII. Although there is a difference of expression between the oral and skin melanomas, all of these markers are related to the aggressiveness of the tumor and a poor prognosis<sup>20-23,32,33,50,41</sup> and support the importance of the evaluating of the MI. The results showed a lack of correlation among pigmentation

and almost all variables of aggressiveness, both in orals and cutaneous melanomas, suggesting that, as in humans, this feature should be used as additional information and not as malignancy criteria.

### *Conclusions*

The present study support the hypothesis that some of the underlying pathways involved in melanoma progression (including angiogenesis, lymphocytes and macrophage infiltration) might be dependent on COX-2, as we can see in the shift in the phenotype and activation status of macrophages between the tumors that present low or high COX-2 expression.

This is the first study to demonstrate that orals and cutaneous melanomas show a marked difference in COX-2 expression levels and that this may be related to variation in the tumor aggressiveness. Furthermore, melanomas may be differentially infiltrated by lymphocytes depending on their site of origin. The frequency of tumor-infiltrating FOXP3+ cells implies that it may be a marker of prognostic relevance in melanoma.

Our results showed that the behavior between orals and cutaneous melanomas are different in the relation with the COX-2 expression, tumor modulation and inflammatory infiltrate, and our results indicate that despite being classed as the same tumor type that oral and cutaneous melanomas should be considered as independent forms. This is consistent with the human disease.

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### *Conflict of Interest Statement*

None of the authors has any financial or personal relationships that could inappropriately influence the content of this paper. The authors declare no conflict of interest.

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## Tables

**Table 1.** Clinicopathological parameters of the oral and cutaneous melanomas.

	Oral	Cutaneous
Histological Type		
Epithelioid cells	23	30
Spindle cells	3	20
Mixed	3	6
Pigmentation		
Non-pigmentation	12	8
Score 1	12	30
Score 2	1	6
Score 3	4	12
Mitotic Index		
< 4 mitosis	10	30
≥ 4 mitosis	14	20

**Table 2.** Immunohistochemical positivity of oral and cutaneous melanomas by S-100, Melan-A, PNL-2 and COX-2 antibodies. The PNL-2 were used only in non-pigmentation cases (12 oral and 8 cutaneous).

Molecular markers	Oral	Cutaneous
S-100	29/29 (100%)	56/56 (100%)
Melan-A	20/29 (69%)	55/56 (98%)
PNL-2	12/12 (100%)	8/8 (100%)
COX-2		
Score 0	2/29 (7%)	7/56 (12%)
Score 1-5	17/29 (59%)	25/56 (45%)
Score 6-12	10/29 (34%)	24/56 (43%)

**Table 3.** Means values of immunohistochemical markers and identification of the inflammatory cells localization in oral and cutaneous melanoma

Antigen	Oral	Cutaneous
Factor VIII	37,74 ±19,94	38,99 ±22,14
KI67	28,39 ±19,89	25,19 ±14,18
CD3		
Intratumoral	27,68 ±12,25	20,01 ±15,65
Peritumoral	49,3 ±14,10	34,79 ±29,18
CD4		
Intratumoral*	6,86 ±3,07	4,06 ±1,59
Peritumoral*	10,76 ±5,73	5,12 ±3,67
FOXP3		
Intratumoral*	13,25 ±7,50	9,25 ±4,55
Peritumoral	22,34 ±7,23	15,81 ±10,13
MAC		
Intratumoral*	10,50 ±3,76	13,33 ±5,37
Peritumoral	8,05 ±2,39	9,88 ±4,82

\*significate values  $p < 0,05$  by T test.

**Table 4.** Correlation of COX-2 and clinicopathological features, angiogenic, proliferative and inflammatory markers.

	COX-2	
	Oral	Cutaneous
Molecular Markers		
Factor VIII	$r = 0.70^*$	$r = 0.72^*$
KI67	$r = 0.24$	$r = 0.41^*$
CD3		
Intratumoral	$r = 0.58^*$	$r = 0.72^*$
Peritumoral	$r = -0.05$	$r = 0.49$
CD4		
Intratumoral	$r = -0.44$	$r = 0.68^*$
Peritumoral	$r = 0.36$	$r = 0.65^*$
FOXP3		
Intratumoral	$r = -0.28$	$r = 0.62^*$
Peritumoral	$r = 0.13$	$r = 0.65^*$
MAC		
Intratumoral	$r = 0.01$	$r = 0.49^*$
Peritumoral	$r = 0.20$	$r = 0.55^*$
Clinicopathological		
Diagnosis	$r = -0.23$	$r = -0.38^*$
Skin Ulceration	$r = 0.08$	$r = 0.49^*$
Mitotic index	$r = 0.42^*$	$r = 0.59^*$
Pigmentation	$r = -0.38$	$r = -0.09$

\*significate values  $p < 0,05$  by Spearman test.

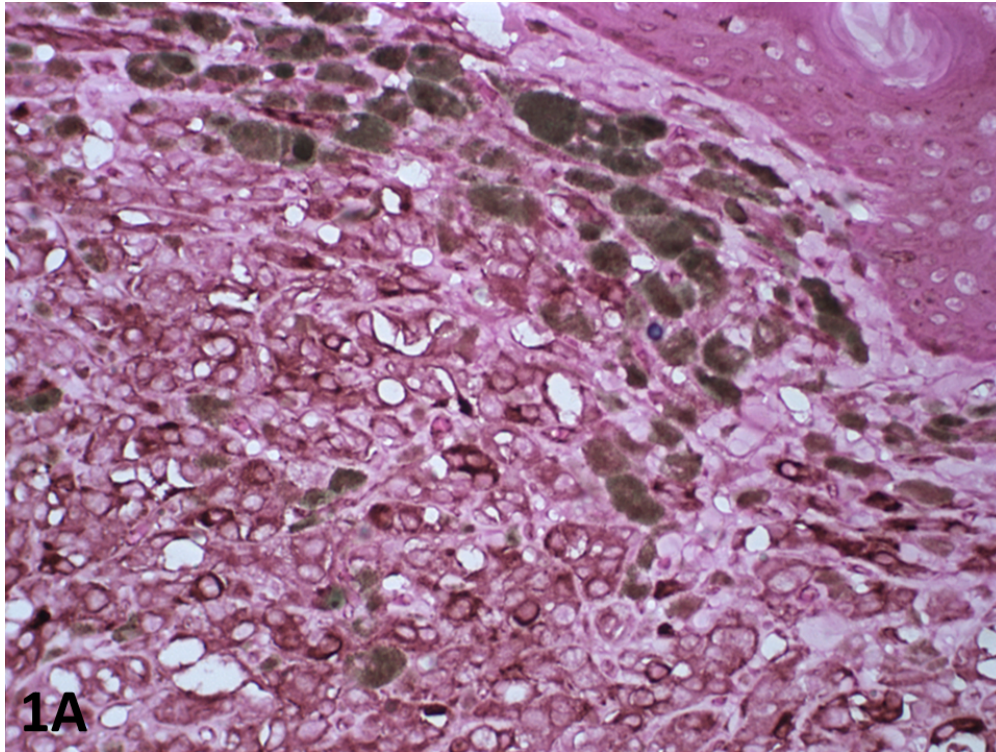
## Figure Legends

**Figures 1-4.** Skin and oral melanomas with COX-2 expression in neoplastic cells - high (1) and low expression (2)- presenting immunolabeling in immune cells. Dog, Immunohistochemistry (IHC), DAB chromogen. **1A.** Skin, IHC staining to COX-2, high expression (score 12) in the cytoplasm of neoplastic cells (presence of melanophages with melanin pigment in green). **1B.** Skin, IHC staining to CD3+ in lymphocytes associated to melanoma with high expression of COX-2. **1C.** Oral, IHC staining to CD4+ in lymphocytes associated to melanoma with high expression of COX-2. **1D.** Oral, IHC staining to FOXP3+ in lymphocytes associated to melanoma with high expression of COX-2. **1E.** Oral, IHC of MAC387 in macrophages associated to melanoma with high expression of COX-2. **2A.** Skin, IHQ staining to COX-2, low expression (score 4) in the cytoplasm of neoplastic cells (presence of melanophages with melanin pigment in green). **2B.** Skin, IHQ staining to CD3+ in lymphocytes (arrow) associated to melanoma with low expression of COX-2. **2C.** Oral, IHQ staining to CD4+ in lymphocytes (arrow head) associated to melanoma with high expression of COX-2. **2D.** Oral, IHQ to FOXP3+ in lymphocytes associated to melanoma with high expression of COX-2. **2E.** Oral, IHC to MAC387 in macrophages associated to melanoma with low expression of COX-2. **3.** Oral, IHQ staining to Factor VIII in endothelial cells in melanoma tumor. **4.** Skin, IHQ staining to KI-67 in nuclei of melanoma's neoplastic cells (melanin pigment in green). All cases were counterstained with GEMSA, obj 40X.

**Figure 5.** Confocal immunofluorescence images of oral melanomas tumors, showing inducible nitric oxide synthase (NOS2; pink), CD206 (red), MAC387 (green) and Hoechst (blue) overlay images distinguishing between different macrophage phenotypes. (A) Low COX-2 expression in melanomas tumors with M1- type population. (B) Low COX-2 expression in melanoma tumor showing a hybrid M1/M2 profile. (C) High expression of COX-2 in oral melanoma tumor a M2 subset pattern. (D) High COX-2 expression in oral melanoma tumor with a mixed macrophage population. Nuclei were revealed by Hoechst staining (blue) and merged images demonstrate the cytoplasmic localisation of the evaluated proteins. Bars = 20 µm. FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin.

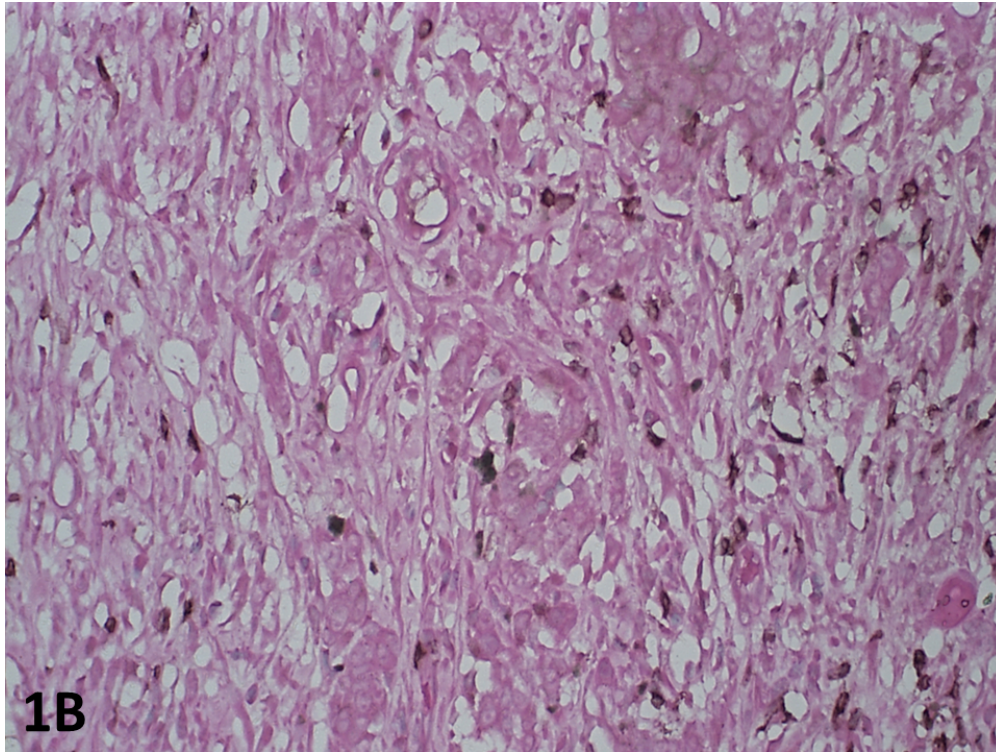
**Supporting Information files.** Supplementary immunohistochemistry table with the IHQ and immunofluorescence results of each case in study.

**Supporting Information figures.** Lymph node with CD4 expression in lymphoid cells presenting immunolabeling in immune cells. Dog, Immunohistochemistry (IHC), DAB chromogen. Obj 40x



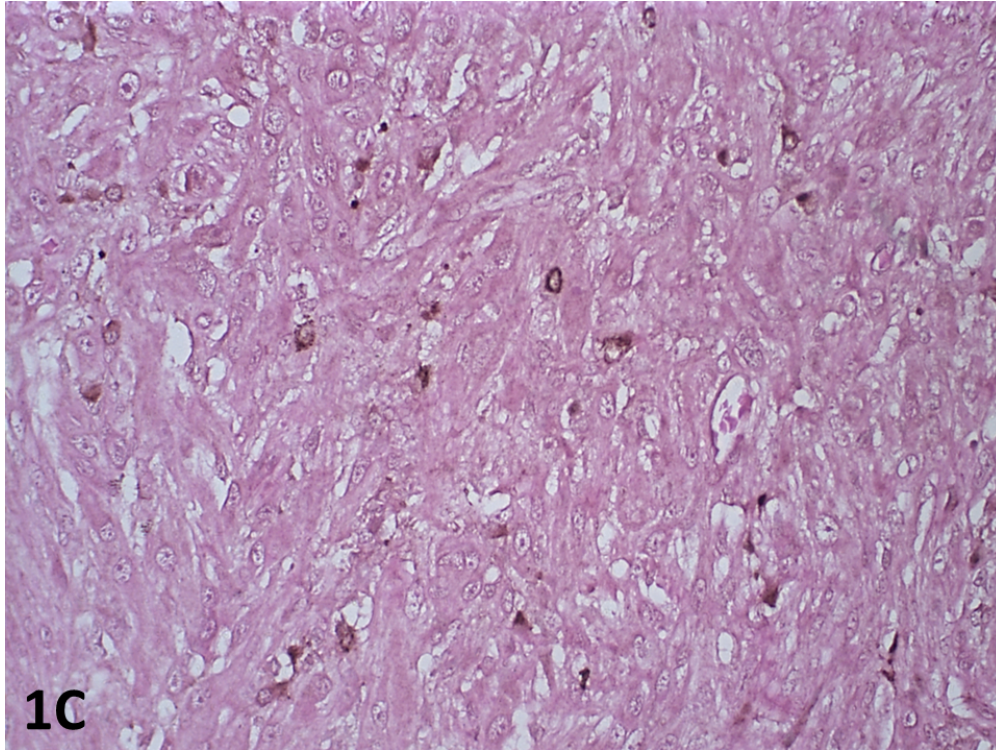
Skin, cutaneous melanoma. IHC staining to COX-2, high expression (score 12) in the cytoplasm of neoplastic cells (presence of melanophages with melanin pigment in green).

254x190mm (96 x 96 DPI)



1B. Skin, cutaneous melanoma. IHC staining to CD3+ in lymphocytes associated to melanoma with high expression of COX-2.

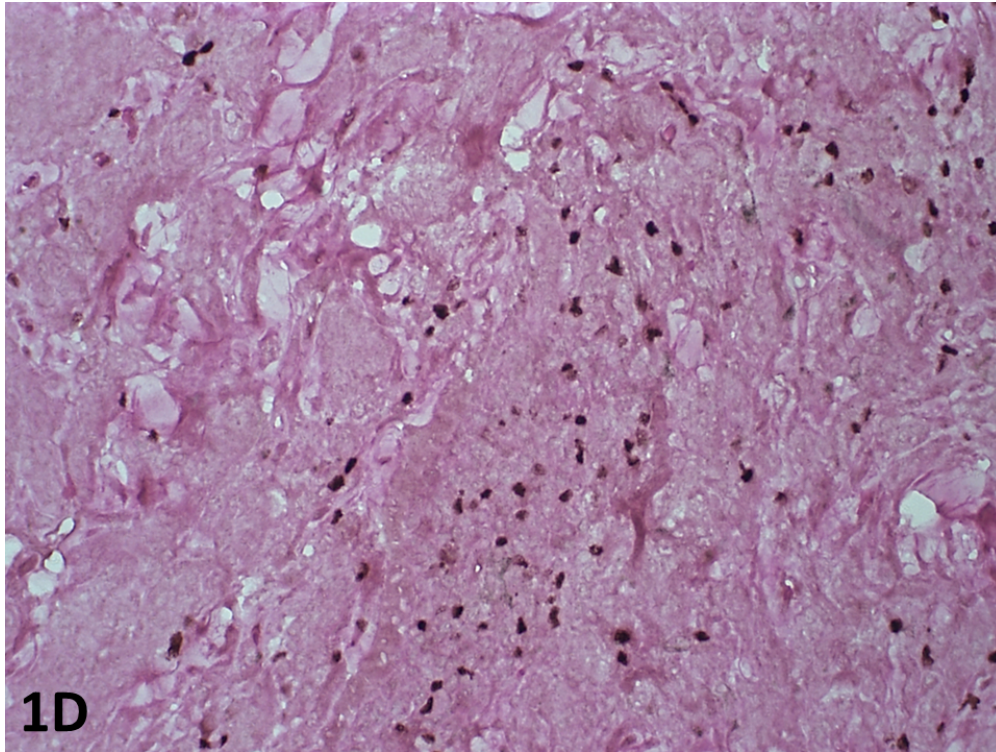
254x190mm (96 x 96 DPI)



1C. Oral melanoma, IHC staining to CD4+ in lymphocytes associated to melanoma with high expression of COX-2.

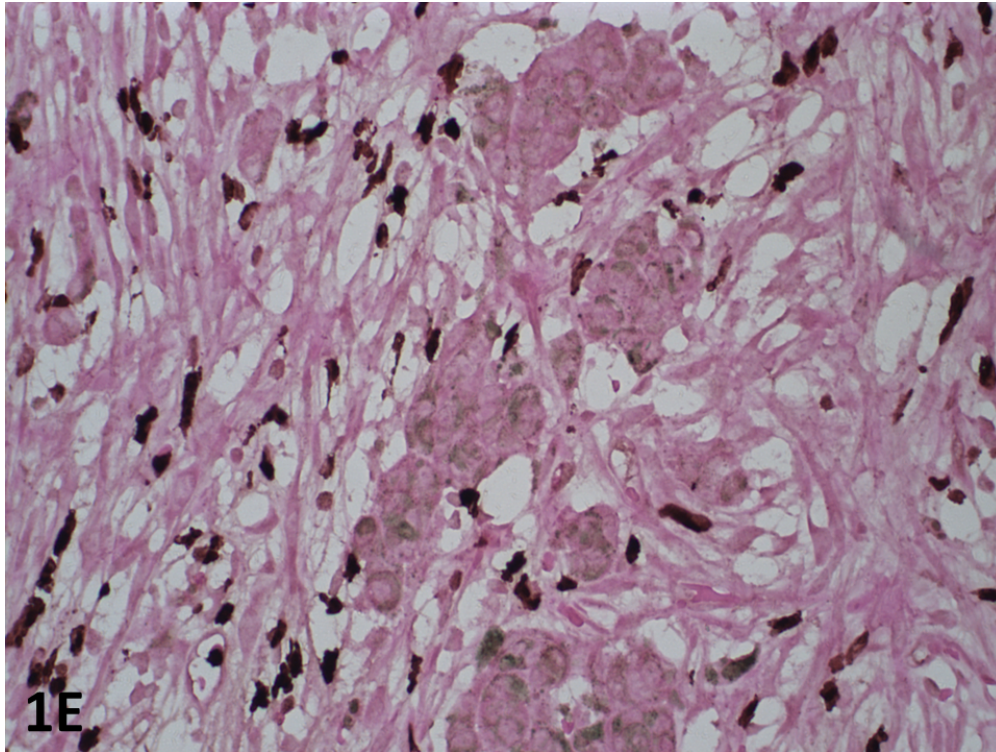
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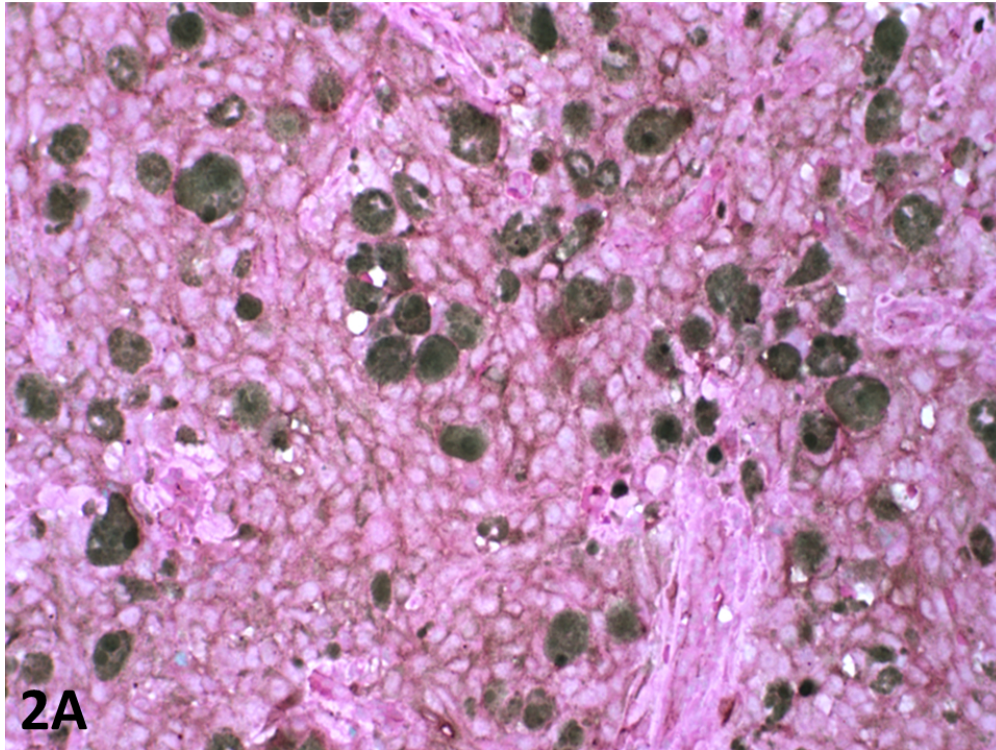
1D. Oral melanoma, IHC staining to FOXP3+ in lymphocytes associated to melanoma with high expression of COX-2.

254x190mm (96 x 96 DPI)



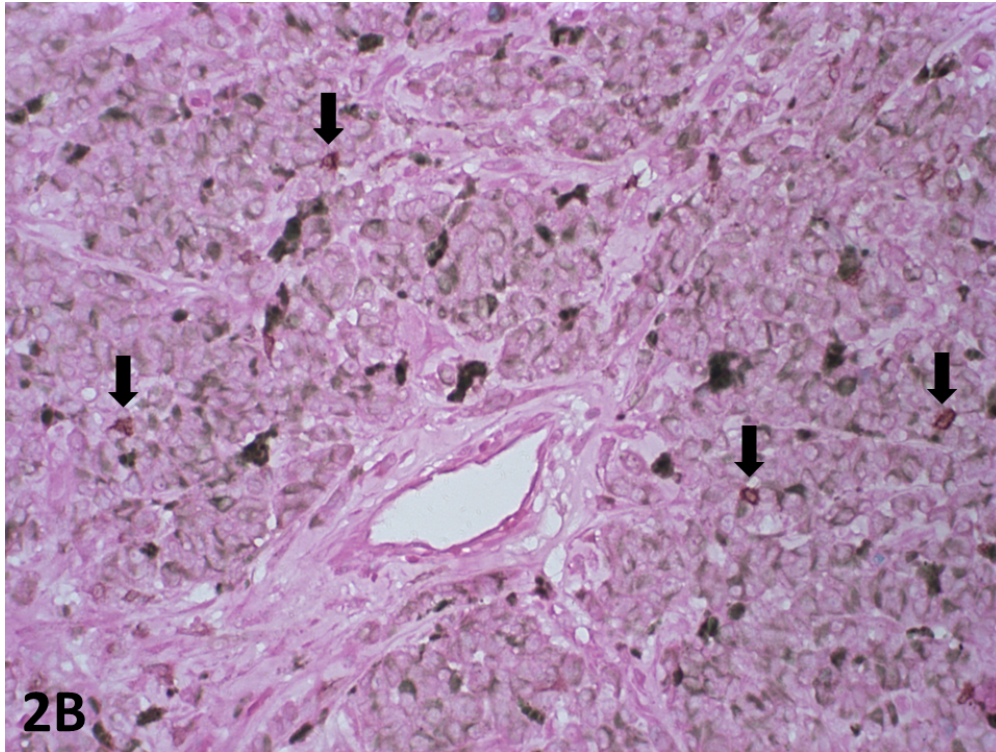
1E. Oral melanoma, IHC of MAC387 in macrophages associated to melanoma with high expression of COX-2.

254x190mm (96 x 96 DPI)



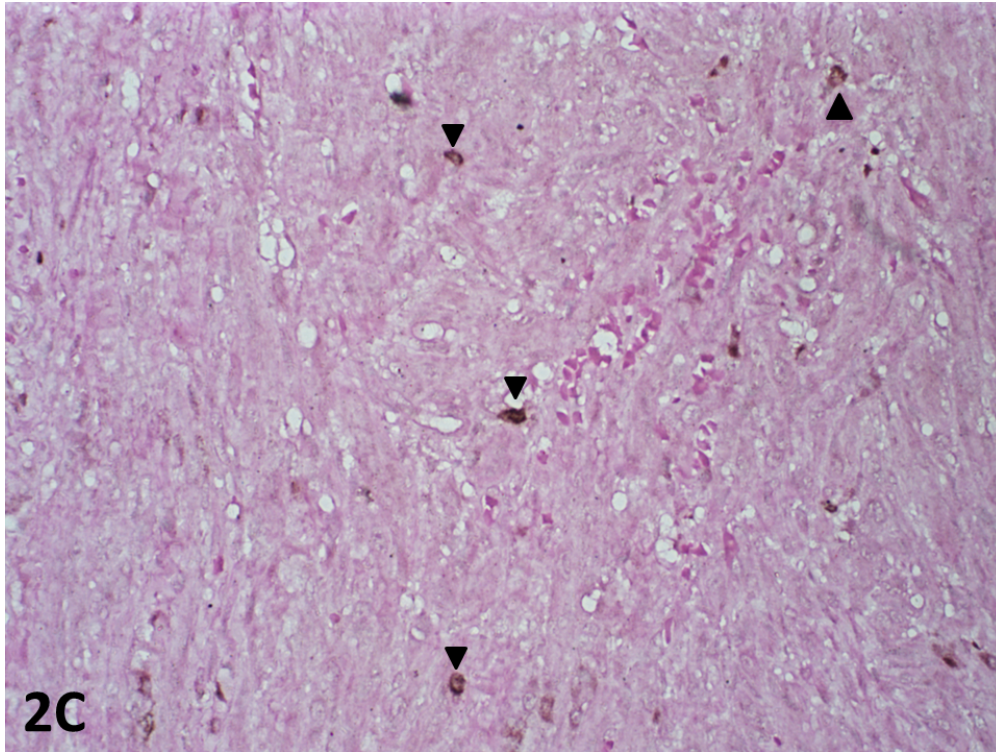
2A. Skin, cutaneous melanoma, IHQ staining to COX-2, low expression (score 4) in the cytoplasm of neoplastic cells (presence of melanophages with melanin pigment in green).

254x190mm (96 x 96 DPI)



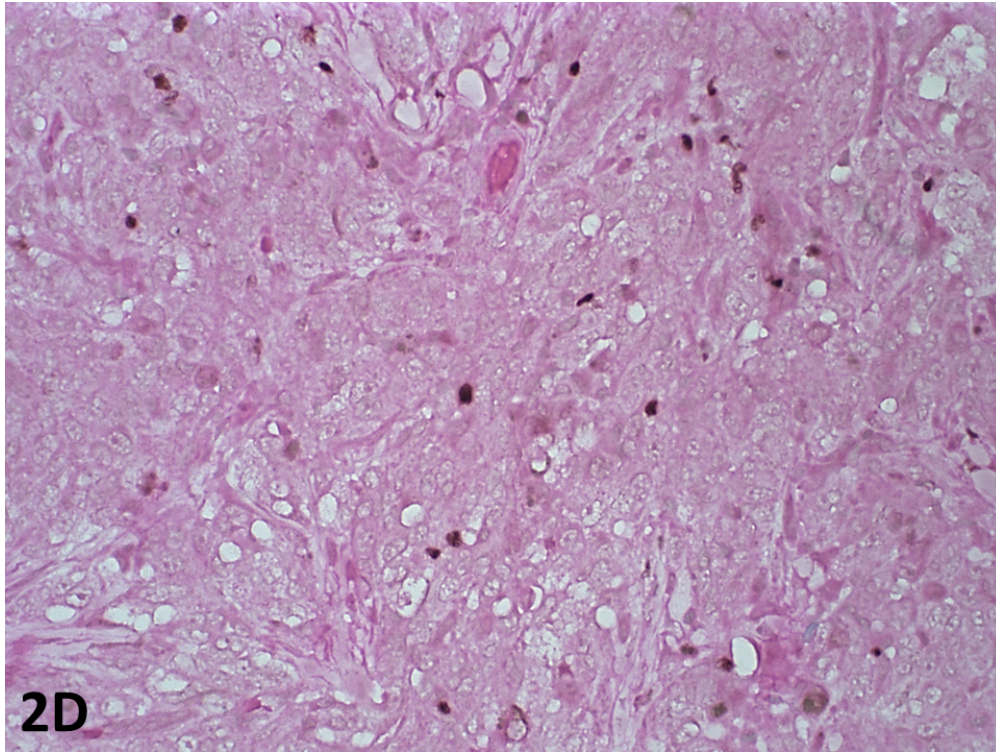
2B. Skin, cutaneous melanoma. IHQ staining to CD3+ in lymphocytes (arrow) associated to melanoma with low expression of COX-2.

254x190mm (96 x 96 DPI)



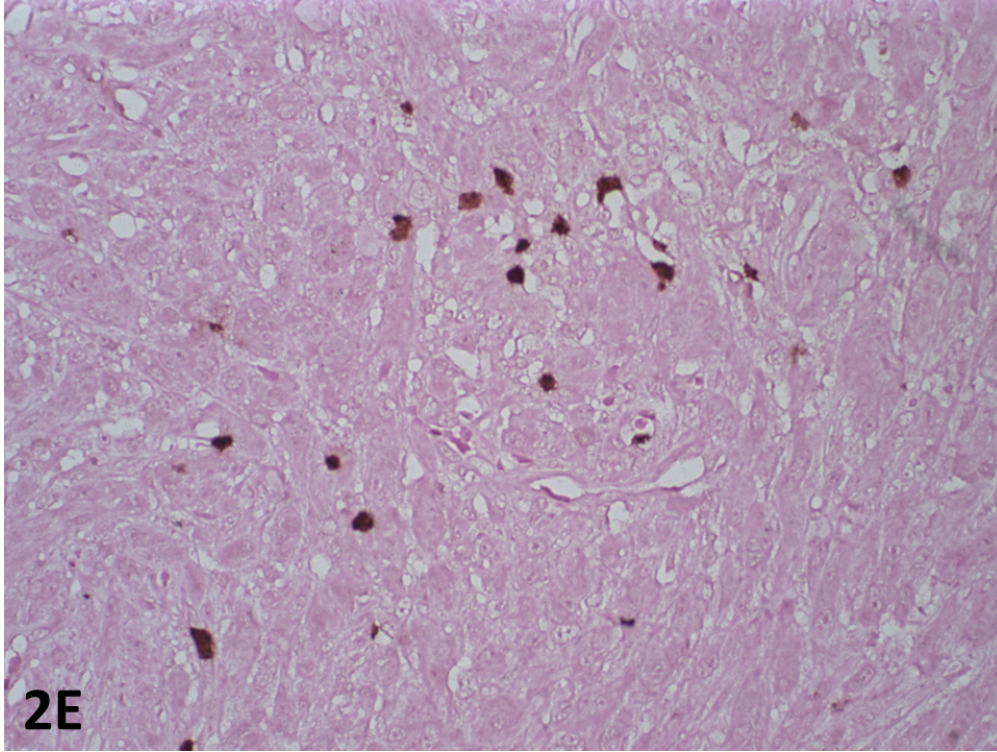
2C. Oral melanoma, IHQ staining to CD4+ in lymphocytes (arrow head) associated to melanoma with high expression of COX-2.

254x190mm (96 x 96 DPI)



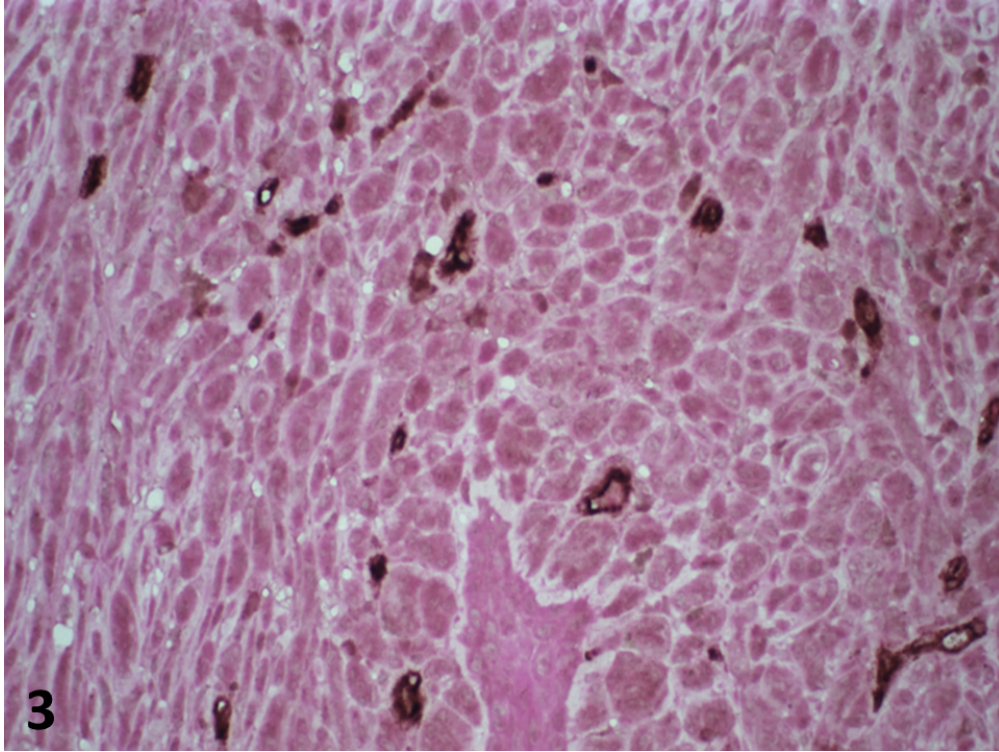
2D. Oral melanoma, IHQ to FOXP3+ in lymphocytes associated to melanoma with high expression of COX-2.

254x190mm (96 x 96 DPI)



2E. Oral melanoma, IHC to MAC387 in macrophages associated to melanoma with low expression of COX-2.

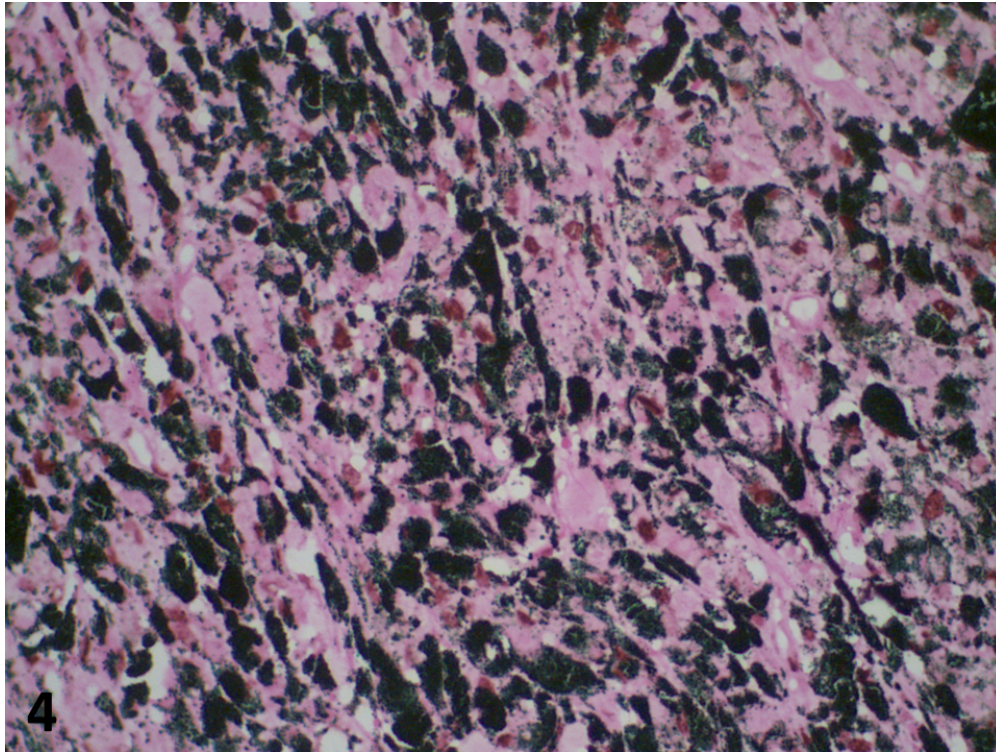
254x190mm (96 x 96 DPI)



3. Oral melanoma, IHC staining to Factor VIII in endothelial cells in melanoma tumor.

254x190mm (96 x 96 DPI)





4. Skin, cutaneous melanoma. IHQ staining to KI-67 in nuclei of melanoma's neoplastic cells (melanin pigment in green).

254x190mm (96 x 96 DPI)

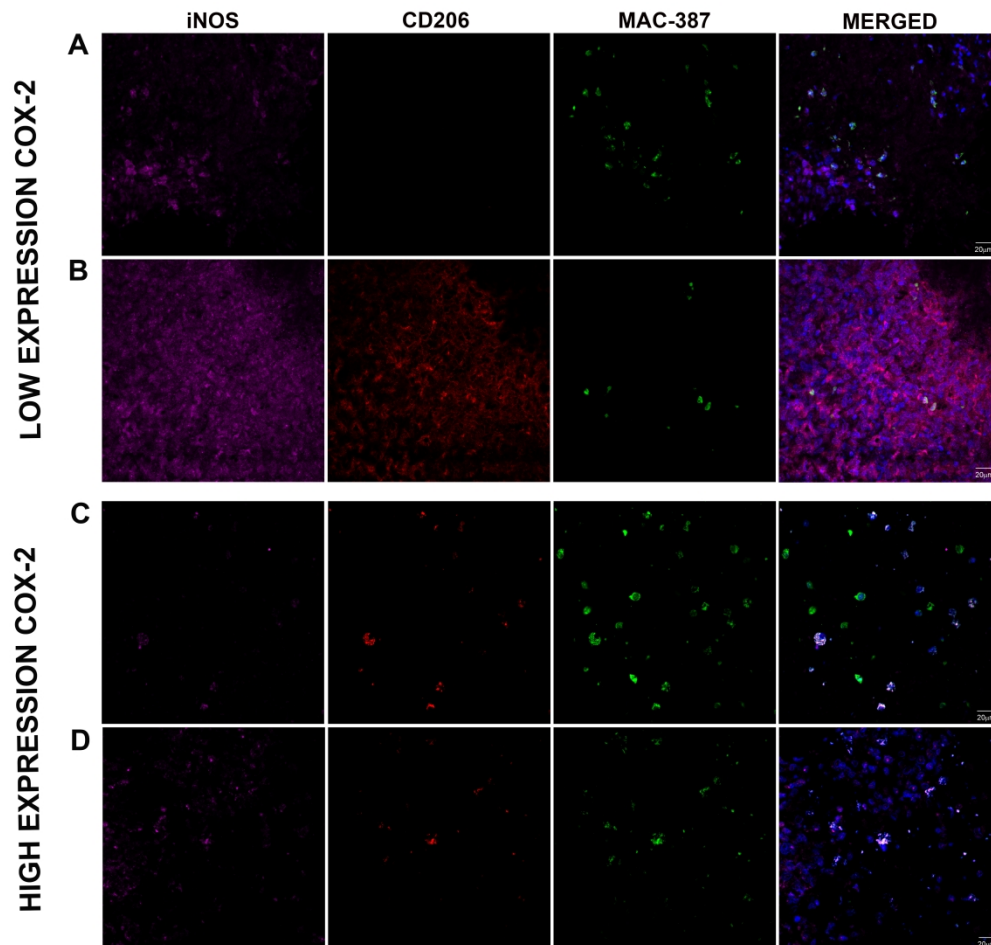


Figure 5. Confocal immunofluorescence images of oral melanomas tumors, showing inducible nitric oxide synthase (NOS2; pink), CD206 (red), MAC387 (green) and Hoechst (blue) overlay images distinguishing between different macrophage phenotypes. (A) Low COX-2 expression in melanomas tumors with M1- type population. (B) Low COX-2 expression in melanoma tumor showing a hybrid M1/M2 profile. (C) High expression of COX-2 in oral melanoma tumor a M2 subset pattern. (D) High COX-2 expression in oral melanoma tumor with a mixed macrophage population. Nuclei were revealed by Hoechst staining (blue) and merged images demonstrate the cytoplasmic localisation of the evaluated proteins. Bars = 20  $\mu$ m. FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin.