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**Tumour-infiltrating lymphocytes in canine melanocytic tumours: An investigation on the prognostic role of CD3+ and CD20+ lymphocytic populations**

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**Prognostic significance of tumor-infiltrating lymphocytes (TILs) and their characterization in canine melanocytic tumors.**

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## **Abstract (244)**

The study of the immune response in several types of tumors has been rapidly increasing in recent years with the dual aim of understanding the relationship between neoplastic and immune cells as well as identifying targets for cancer immunotherapy. Despite being considered one of the most immunogenic tumor types, melanoma can progress in the presence of abundant lymphocytic infiltration, therefore suggesting that the immune response is not able to efficiently control tumor growth. The purpose of this study was to investigate whether the density, distribution and grade of tumor-infiltrating lymphocytes (TILs) in 97 canine melanocytic tumors is associated with histologic indicators of malignancy and can be considered a prognostic factor in the dog. As a further step in the characterization of the immune response in melanocytic tumors, an immunohistochemical investigation was performed to evaluate the two main populations of TILs, T-lymphocytes (CD3<sup>+</sup>) and B-lymphocytes (CD20<sup>+</sup>). The results of our study show that TILs are present in a large proportion of canine melanocytic tumors, especially in oral melanomas, and that, differently from humans, the infiltrate is usually mild. The quantity of CD20<sup>+</sup> TILs was significantly associated with some histologic prognostic factors, such as the mitotic count, the

cellular pleomorphism and the percentage of pigmented cells. Remarkably, a high infiltration of CD20<sup>+</sup> TILs was associated with tumor-related death, presence of metastasis/recurrence, shorter overall and disease-free survival, increased hazard of death and of developing recurrence/metastasis, hence representing a potential new negative prognostic factor in canine melanocytic tumors.

**Keywords:** B-Lymphocytes; Dogs; Lymphocytes, Tumor-Infiltrating; Melanoma; Prognosis; T-Lymphocytes

## INTRODUCTION

The incidence of melanoma in human medicine is increasing and no effective therapy is currently available, with tumor-associated mortality being related to metastatic spread in sites distant from the primary tumor.<sup>1</sup> In the last few years, several studies have underlined the similarities between human and canine melanoma in order to investigate the dog as a possible model for the human tumor.<sup>2-6</sup> Canine melanomas, in particular oral melanomas, often have similar clinical presentation, tumor biology and histopathological appearance to their human counterparts; besides, pets and owners share their living environment and may be exposed to the same carcinogens. On the other hand, tumors usually progress more rapidly in animals, shortening data maturation times. In the dog the classical rule that oral/mucosal melanomas are malignant and cutaneous melanomas are relatively benign has been questioned,<sup>7-9</sup> since the prognosis can be variable and sometimes unpredictable. An extensive review on the more commonly adopted and significant prognostic histologic factors has been written by Smedley et al. in 2011,<sup>10</sup> in which well-recognized factors are summarized in a ready-to-use table to classify melanocytic tumors in the diagnostic environment. Recently, the usefulness of tumor thickness as a prognostic factor has been underlined in the canine species: in line to what observed in humans with Breslow thickness, thicker melanocytic tumors seems to have a worse prognosis.<sup>11,12</sup> However, compared to the human medicine, some factors remain to be investigated and there is still the need to find other helpful indicators to better define the prognosis of canine melanocytic tumors.

As an advance in human oncology, the role of the immune response in the development of a tumor and its predisposition to give metastasis has been the object of many investigations.<sup>13–16</sup> Melanomas often develop in an immune cell-rich environment, characterized by the infiltration of several types of inflammatory cells, mostly represented by lymphocytes secreting their cytokines and contributing to an anti-tumor response. Among them, tumor-infiltrating lymphocytes (TILs) have a pivotal role. This term was first introduced by Wallace Clark et al. in 1989<sup>17</sup> and expanded by Clemente et al.<sup>18</sup>: it refers to lymphocytes that infiltrate the tumor disrupting its nests and/or are in direct contact with neoplastic cells.<sup>13,19</sup> An association between the incremental presence of TILs and a more favorable prognosis has been highlighted for the first time by Clark et al. in a study on human skin melanoma.<sup>17</sup> Since then, numerous studies have demonstrated that TILs presence in melanoma is associated with longer overall and disease-free survival and that they can be used as predictors of sentinel lymph node status in patients with cutaneous melanoma.<sup>18–24</sup> On this line, some authors have suggested to include an accurate quantification of TILs in histopathological reporting,<sup>25</sup> although there are conflicting data on the utility of TILs as prognosticators. Indeed, TILs are characterized by an heterogeneous population composed by several types of lymphocytes: most TILs are T lymphocytes, including CD4<sup>+</sup>, CD8<sup>+</sup>, T regulatory cells (Tregs) and  $\gamma/\delta$  T cells, but also B and NK cells are present.<sup>19,26,27</sup> The role of single populations of lymphocytes can be very different and their distribution can deeply vary.<sup>28</sup> The transfer of tumor-infiltrating T cells has been used in adoptive immunotherapy with success in a proportion of melanoma patients.<sup>29–31</sup> However, at the same time, it must be bear in mind that neoplastic cells are able to activate several mechanisms to suppress the immune response both by secreting anti-inflammatory and immunosuppressive cytokines and by recruiting suppressive regulatory T cells (Tregs).<sup>32–35</sup>

The purpose of this study is to analyze the significance of the presence, density, distribution and grade of TILs as well as their principal subpopulations in canine melanocytic tumors. Their associations with histological diagnosis, clinical outcome, presence of metastasis/recurrence, disease-free interval, overall survival and hazard of death or of developing recurrence metastasis of the affected animals were investigated in order to explore their prognostic significance.

## **METHODS**

### **Case selection**

A retrospective study was performed on formalin-fixed, paraffin-embedded tissue samples submitted to the diagnostic laboratories or veterinary teaching hospitals of the Departments of Veterinary Medicine of Perugia and Torino in the period between 2009 and 2016. Inclusion criteria were as follows: (1) diagnosis of mucosal melanoma, cutaneous melanoma or cutaneous melanocytoma, (2) affected dogs had received no therapy (i.e. chemotherapy, radiation) other than surgery before the biopsy, and (3) availability of follow-up information.

Ninety-seven melanocytic tumors (32 oral melanomas, 34 cutaneous melanomas, 31 cutaneous melanocytomas) were selected. For each case, the breed, gender, age, location and major diameter of the tumor (measured during trimming) were obtained from the database. Follow-up information was collected for each case: clinicopathological information was asked through telephonic interview with referring veterinarians or through the collection of medical records data from internal cases. Since the number of recurrence and metastasis were not numerous, we considered them all together (recurrence/metastasis) for statistical analyses. The clinical outcome of dogs died because of the tumor was considered “unfavorable”, while that one of patients alive or dead for causes unrelated to melanoma was considered “favorable”.

### **Histologic examination**

Samples in this study were partially included in a previous study on the usefulness of tumor thickness and modified Clark level for the evaluation of canine melanocytic tumors.<sup>11</sup> All samples were histologically evaluated for the parameters having the greater validity for prognostic use in canine melanocytic neoplasia, according to the current literature.<sup>10,11</sup>

### **TILs evaluation**

TILs were firstly evaluated as present/absent in each of the samples. Additionally, referring to human literature,<sup>19</sup> a four-tier grading system for TILs evaluation, based on the density (mild, moderate, severe) and distribution (focal, multifocal, diffuse) of lymphocytes in the tumor was adopted.<sup>13</sup> When lymphocytes were not in direct contact with tumor cells, TILs were considered absent (Table 1). Final TILs grade was classified as: grade 0: TILs absent; grade 1: mild/moderate focal or mild multifocal TILs; grade 2: marked focal or moderate/severe multifocal or mild diffuse TILs; grade 3: moderate/severe diffuse TILs (Figure 1).

### **Immunohistochemical evaluation of CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs**

Eighty-seven and 77 melanocytic tumors were available for CD3 and CD20 immunohistochemistry, respectively. Immunohistochemistry was performed as previously described, following a modified protocol.<sup>36</sup> Briefly, a commercially available rabbit antibody against CD20 (1:100, RB-9013; Thermo Scientific, Fremont, CA, USA) and a rabbit polyclonal antibody against CD3 (1:200; A 0452; Dako, Glostrup, Denmark) were used. Antigen retrieval was achieved in a preheated Tris-EDTA buffer solution (pH 9.0) for the CD3 antibody, while no antigen retrieval was performed for the CD20 antibody. Endogenous peroxidases were blocked using 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes at room temperature and protein block performed with a commercially available kit (ab93677; Abcam, Cambridge, UK). Slides were incubated with primary antibodies for 1 hour in a humidified chamber at room temperature. Afterwards they were incubated with an ABC ready-to-use kit (ab93677; Abcam, Cambridge, UK) following the manufacturer's instructions. Positive reaction was revealed with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark). Carazzi's hematoxylin was used as a counterstain. Coverslips were mounted with Faramount Mounting Medium (Dako, Glostrup, Denmark). Normal canine lymph node was used as positive control. Negative controls were performed omitting the primary antibody and incubating tissue sections with Tris-phosphate-buffered saline buffer. Only TILs defined according to Clark et al.<sup>17</sup> and Clemente et al.<sup>37</sup> were considered for positivity in this study (CD3<sup>+</sup> or CD20<sup>+</sup> TILs). Considering both markers, tumors were evaluated for:

- Quantity: semiquantitative evaluation of positive cells defined as absent, mild, moderate or severe (Figure 2-3). The quantity of T- and B-lymphocyte was subsequently further grouped into two groups: low (absent and mild) and high (moderate and severe).
- Distribution: distribution of positive cells within the tumor defined as focal, multifocal or diffuse.

### **Statistical analysis**

Descriptive statistics was used for showing data: continuous variables are reported as median with interquartile range (IQR), while categorical variables as absolute and relative frequencies. First, we used graphic tests to verify assumptions of normality; since they were not met, we used non-parametric tests for hypothesis testing. For the analysis of continuous variables, we used Kruskal-Wallis, Mann-Whitney U test and Spearman correlation coefficient ( $\rho$ ); for categorical variables we used chi-square independence test or Fisher's exact test. For multiple comparisons, Bonferroni-adjusted *P* values were reported. The Kaplan-Meier curves and log-rank test were used to compare overall and disease-free survival in accordance with the histological diagnosis, TILs

presence, TILs density, TILs distribution, TILs grade, CD3<sup>+</sup> TILs quantity and CD20<sup>+</sup>. Hazard of death and of developing recurrence/metastasis were further evaluated by the univariate Cox proportional hazard model (Cox regression) for TILs presence, TILs density, TILs distribution, TILs grade, CD3<sup>+</sup> TILs quantity and CD20<sup>+</sup> TILs quantity. Data were analyzed by SPSS 23.0 (SPSS Inc. Chicago, USA) and the software R (R version 3.5.1).<sup>38</sup> A *P* value ≤ 0.05 was considered significant.

## RESULTS

### Clinical data

Ninety-seven canine tumors were included in this study. Sixty-six were melanomas (32 oral and 34 cutaneous) and 31 cutaneous melanocytomas. For 7 dogs, signalment data were unknown. Most of the dogs were mixed breed (n=32/90, 35.6%); the most represented purebreds were German Shepherd (n=9/90, 10.0%), Dachshund (n=6/90, 6.7%), Pinscher (n=4/90, 4.4%), followed by Boxer, Labrador retriever, Yorkshire Terrier, Rottweiler (n=3/90, 3.3% each) and other breeds (n=63/90, 70.0%). Data regarding clinical features according to diagnosis are summarized in Table S1 (Supporting information). Overall, there were 59 males (53 intact and 6 neutered) and 31 females (22 intact and 9 spayed). Age ranged from 1 to 15 years (median=10 years, IQR=8-12 years). Diagnosis was significantly associated with the age of affected animals (*P*<0.0001): dogs with cutaneous melanocytoma were younger compared to dogs with oral melanoma (*P*≤0.05). Among cutaneous tumors, there was a significant association between diagnosis and tumor localization (*P*=0.002): in particular, a higher proportion of melanomas were localized to the digit compared to melanocytomas, whereas a lower proportion of melanomas were localized to the abdomen when compared to melanocytomas (*P*≤0.05, each).

### Histological examination

#### *TILs versus histological diagnosis*

Table 2 shows the results regarding TILs analysis (presence, density, distribution and grade) in the various tumor types. In oral melanomas, they often had a mild density (n=16/25, 64.0%), a multifocal distribution (n=17/25, 68.0%) and a grade 1 (n=18/32, 56.3%). In cutaneous melanomas, TILs were observed in 64.7% of the cases (n=22/34), often had a mild density (n=13/22, 59.1%) with a focal (n=8/22, 36.4%) or multifocal (n=12/22, 54.5%) distribution and a grade 0 (n=14/34,

41.2%) or grade 1 (n=11/34, 32.4%). In melanocytomas, TILs were seen in 61.3% of the cases (n=19/31), where they had a mild density (n=11/18, 57.9%), a focal or multifocal distribution (n=7/19, 36.8% and n=8/19, 42.9%, respectively) and a grade 0 (n=12/31, 38.7%) or grade 1 (n=11/31, 35.5%). No association was found between the presence, density, distribution and grade of TILs and the histological diagnosis (oral melanoma vs cutaneous melanoma vs cutaneous melanocytoma).

#### *TILs versus clinical outcome and recurrence/ metastasis presence*

TILs presence, density, distribution and grade were not associated to clinical outcome or metastatic/recurrent disease (Table 3).

### **Immunohistochemical evaluation of CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs**

#### *CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs versus TILs grade*

The quantity of CD3<sup>+</sup> TILs was associated with TILs grade ( $P=0.003$ ). In particular, most of the cases exhibiting a grade 3 of TILs had severe CD3<sup>+</sup> TILs infiltration; the Spearman correlation coefficient showed a weak correlation between the two variables ( $\rho=0.388$ ;  $P=0.0002$ ). Similarly, the amount of CD20<sup>+</sup> TILs was associated with TILs grade ( $P=0.016$ ). In particular, most of the cases exhibiting a grade 0 of TILs had absent CD20<sup>+</sup> TILs infiltration; the Spearman correlation coefficient showed a weak correlation between the variables ( $\rho=0.291$ ;  $P=0.010$ ) (Table S2, Supporting Information).

#### *CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs versus histological diagnosis*

Data regarding the quantity and distribution of CD3<sup>+</sup> and CD20<sup>+</sup> TILs in the different melanocytic tumors are summarized in Table S3 (Supporting Information). The most interesting result is the association between the histological diagnosis and CD20<sup>+</sup> TILs quantity ( $P<0.001$ ): in cutaneous tumors CD20<sup>+</sup> TILs are more frequently absent (13/26, 50.0% and 18/26, 69.2% for melanoma e melanocytoma respectively) than in oral melanomas (1/25, 4.0%;  $P\leq 0.05$ ). No other association were found.

#### *CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs versus histologic criteria of malignancy*

Data regarding the association between the presence of CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs and the histologic features of malignancy are shown in Table S4 (Supplemental Information). Briefly, a statistically significant association was found between the quantity of CD20<sup>+</sup> TILs and the mitotic

count ( $P<0.001$ ), since it was lower in tumors with CD20<sup>+</sup> TILs absent than severe (median=1.0, IQR=0.0-3.5 and median=15.50, IQR=6.0-41.0, respectively;  $P\leq 0.05$ ). The major diameter ( $P=0.007$ ) was significantly lower in tumors with absent CD20<sup>+</sup> TILs compared to mild, moderate or severe CD20<sup>+</sup> TILs ( $P\leq 0.05$ ). Finally an association between CD20<sup>+</sup> TILs quantity and the percentage of pigmentation ( $P=0.004$ ) or the cellular pleomorphism ( $P=0.001$ ) was also seen.

#### *CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs versus clinical outcome and metastasis/recurrence presence*

CD3<sup>+</sup> TILs quantity was not associated to clinical outcome or metastasis/recurrence presence (Table 4). Conversely, CD20<sup>+</sup> TILs quantity was associated both with clinical outcome ( $P=0.002$ ) and metastasis/recurrence presence ( $P<0.001$ ). Specifically, the frequency of dogs with absent CD20<sup>+</sup> TILs which died because of melanoma was lower (2/19, 10.5%) compared to those alive or dead for causes not related to melanoma (24/43, 55.8%;  $P<0.05$ ). The frequencies of dogs with metastasis/recurrence were higher (7/13, 53.8%) compared to those without metastasis/recurrence (12/46, 2.2%,  $P<0.05$ ) in the group of tumors with moderate CD20<sup>+</sup> TILs infiltration.

#### **Survival analysis**

The follow-up varied from 366 to 3409 days, with a median of 1148 days (IQR=926-1424 days). At the end of the study, 19 dogs were lost at follow-up (19.6%). Twenty-two out of 78 dogs (28.2%) died because of melanoma, while 56 (71.8%) were alive or dead for other causes; 15 dogs (21.1 %) developed recurrence or metastasis and 56 animals did not (78.9%). Overall survival varied depending on histological diagnosis ( $P<0.001$ ), being shorter in oral melanomas compared to cutaneous tumors ( $P<0.05$ ).

#### *TILs and survival*

Kaplan-Meyer curves showed no differences in overall survival nor disease-free survival according to TILs presence, TILs density, TILs distribution or TILs grade ( $P>0.05$ ). Univariate Cox proportional regression analysis confirmed that they were not prognostic factors in our study ( $P>0.05$ ).

#### *CD3<sup>+</sup> TILs and CD20<sup>+</sup> TILs and survival*

Kaplan-Meier curves showed no differences in survival time or disease-free survival according to CD3<sup>+</sup> TILs quantity (Figure 4-5). Univariate Cox proportional regression analysis confirmed that they were not prognostic factors in our study ( $P>0.05$ ).

Overall survival was associated with CD20<sup>+</sup> TILs quantity ( $P<0.001$ ): in particular, dogs with absent CD20<sup>+</sup> TILs quantity had a longer survival time than dogs with moderate CD20<sup>+</sup> TILs quantity ( $P<0.05$ ). Survival probability estimates over time are shown in Table 5. The difference was even more marked when CD20<sup>+</sup> TILs quantity was analyzed as a 2-tier variable (absent and mild grouped together as “low CD20<sup>+</sup> TILs” versus moderate and severe grouped together as “high CD20<sup>+</sup> TILs”; Figure 6): overall survival of dogs with low CD20<sup>+</sup> TILs quantity was significantly longer than dogs with high CD20<sup>+</sup> TILs quantity ( $P<0.001$ ). Moreover, dogs with a high CD20<sup>+</sup> TILs quantity had an hazard of death 5 times greater than dogs with low CD20<sup>+</sup> TILs quantity (HR=5.31, 95%CI=1.98-14.29;  $P=0.001$ ).

Kaplan-Meier curves showed significant differences in disease-free survival ( $P<0.001$ ), being significantly longer when CD20<sup>+</sup> TILs were absent than moderate ( $P<0.05$ ) and when CD20<sup>+</sup> TILs quantity was mild than moderate ( $P<0.05$ ). Probability estimates to not develop recurrence/metastasis over time are shown in Table 6. The difference was even more marked when CD20<sup>+</sup> TILs quantity was analyzed as a 2-tier variable: disease-free survival of dogs with low CD20<sup>+</sup> TILs quantity was significantly longer than dogs with high CD20<sup>+</sup> TILs quantity ( $P<0.001$ ; Figure 7). Moreover, dogs with a high CD20<sup>+</sup> TILs quantity had an hazard of developing recurrence/metastasis 10 times greater than dogs with low CD20<sup>+</sup> TILs quantity (HR=10.27, 95%CI=3.15-33.54;  $P=0.001$ ).

## **DISCUSSION**

This study evaluated TILs in canine melanocytic tumors by morphologically analyzing their density, distribution and grade and immunohistochemically characterizing the two main populations of TILs, CD3<sup>+</sup> and CD20<sup>+</sup> TILs, respectively. The macroscopic and histological features defined by Smedley et al. have been used in this study to define the histological diagnosis of our cases. The samples were divided into three categories: oral melanomas, cutaneous melanomas and cutaneous melanocytomas. Histological diagnosis was significantly associated with overall survival. Cutaneous melanomas, although histologically diagnosed as malignant tumors, had a distinctly

different behavior compared to oral melanomas, as most patients survived for a long time, and only a small proportion died or developed recurrence or metastasis.

Regarding the evaluation and characterization of TILs in canine melanocytic tumors, our results revealed the presence of TILs in a large percentage of melanocytic tumors (68.0%), although the lymphocytic infiltration in all tumors in our series was generally mild and with a multifocal distribution, corresponding to a grade 1 of TILs. This is in contrast with data reported in human literature, where immune cell infiltration is generally very high due to the strong immunogenicity of melanocytic tumors.<sup>39</sup> This reduced infiltration of TILs may be considered peculiar of the canine species, where tumor melanocytes could express a lesser amount of tumor-associated antigens, resulting in a reduced anti-tumor immune response and a lower migration of immune cells in the neoplastic site. Moreover, TILs appear to be especially common in oral melanomas (78.1%). This could be related to the site of origin of the tumor, considering that the oral mucosa has a distinct immune and inflammatory response, which is normally richer in lymphocytes and plasma cells.<sup>40</sup>

No significant association was demonstrated between TILs grade and the histological diagnosis, revealing that tumor-infiltrating lymphocytes are not helpful to define the histological diagnosis in challenging cases or discriminate between cutaneous melanocytomas and melanomas. TILs grade was not associated with the clinical outcome, the presence of metastasis/recurrence, the overall or disease-free survival. This is different from human literature, in which TILs are most commonly associated with a successful outcome and prognosis.<sup>41,42</sup> However, these partially contradicting results could be attributed to the great heterogeneity characterizing the TILs population, which comprises cells that actually protect the host from tumor growth (such as cytotoxic T-lymphocytes, T-memory cells, T-helper 1 lymphocytes, and, partially, B-lymphocytes), together with cells that favor tumor oncogenesis and development (such as Treg lymphocytes, T-helper 17 cells and, partially, B-lymphocytes).<sup>28,43-45</sup> In order to better define the role of the main lymphocyte populations, we used immunohistochemistry to characterize T- and B-lymphocytes.

Our study revealed a statistically significant association between the quantity of CD3<sup>+</sup> TILs or of CD20<sup>+</sup> TILs and the TILs grade, showing that no one of the two populations is clearly prevalent. This results appear in disagreement with human literature, where T-lymphocytes are the most abundant cell population infiltrating melanocytic tumors.<sup>46,47</sup> Another difference with humans is that no statistical association was demonstrated with CD3<sup>+</sup> TILs quantity and any of the features investigated (histological characteristics, clinical outcome, recurrence/metastasis, overall and disease-free survival). In a recent study of meta-analysis on prognostic value of TILs in human

melanoma, it has been shown that a high infiltration of CD3<sup>+</sup> TILs is associated to a lower hazard of death, independently from the tumor type.<sup>42</sup> This finding can lead to the idea that CD3<sup>+</sup> TILs population is mainly composed by antitumor effector T-cells. Our results can be explained because CD3<sup>+</sup> TILs are composed of different subpopulations of lymphocytes including cytotoxic lymphocytes (CD8<sup>+</sup>), T-memory cells (CD8<sup>+</sup>CD45RO<sup>+</sup>), T-helper cells (CD4<sup>+</sup>CCR5<sup>+</sup>, CD4<sup>+</sup>CCR4<sup>+</sup>, CD4<sup>+</sup>CCR4<sup>+</sup>CCR6<sup>+</sup>) and T regulatory cells (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>) with opposite roles.<sup>48,49</sup> It is likely that T-lymphocytes with pro-tumorigenic and anti-tumorigenic role/function/activity are about equally present in the dog, hence CD3<sup>+</sup> TILs are not associated to survival. Indeed, in a previous study, our group demonstrated that Tregs, a subpopulation of T-lymphocytes with immune suppressive function, are frequently present in canine melanocytic tumors and that their infiltration is associated to negative prognosis.<sup>50</sup>

CD20<sup>+</sup> TILs were absent in about half of melanocytic cutaneous tumors (both benign and malignant), while they were present in variable amounts in oral melanomas. The statistical analysis demonstrated an association between the quantity of CD20<sup>+</sup> TILs and some prognostic histologic factors. The positive association between the CD20<sup>+</sup> TILs quantity and the mitotic count or cellular pleomorphism (two negative prognostic factors) as well as the inverse association with percentage of pigmented cells (a distinct feature of well-differentiated cells and a positive prognostic histologic factor) indicate a negative role of B-lymphocytes in canine melanocytic tumors. This role was further confirmed by the statistically significant association of CD20<sup>+</sup> TILs quantity with the unfavorable clinical outcome, the presence of metastasis/recurrence, the shorter overall and disease-free survival. When CD20<sup>+</sup> TILs were analyzed as a 2-tier variable, the difference in overall and disease-free survival was even more evident. Furthermore, the univariate Cox proportional regression analysis showed that the hazard of death and of developing recurrence/metastasis of dogs with a high CD20<sup>+</sup> TILs quantity was greater compared to dogs with low CD20<sup>+</sup> TILs quantity. Although our results needs to be confirmed studying cutaneous and oral melanomas separately in a larger number of animals, we demonstrated that the presence of a high quantity of CD20<sup>+</sup> TILs is a potential new negative prognostic factor in canine melanocytic tumors. In human medicine, the role of lymphocytes B in melanoma is unclear and several studies have shown contradicting results<sup>51-53</sup> 54. It is still a matter of debate how systemic B-cell response or *in situ* B-cell infiltration have an impact on the biological behavior of tumors. In some experimental models, B-lymphocytes infiltration was correlated with tumor growth or progression<sup>52,55</sup>; however, other studies led to opposite results.<sup>52,56</sup> The discrepancy regarding

these results may be related to different functional activities of B cells.<sup>57</sup> On one hand, B lymphocytes can act as antigen-presenting cells, inducing CD4<sup>+</sup> T cell-dependent CD8<sup>+</sup> memory T cells, thus being associated with a better prognosis. However, several studies referred that B cells would have a tumor-promoting role, since they can activate a M2 pro-tumor phenotype in macrophages and stimulate the differentiation of CD4<sup>+</sup> cells in Tregs.<sup>28,58</sup> Another subpopulation of B cells with regulatory functions (Bregs) seems to have a negative prognostic significance, since, in skin cancers, it inhibits tumor-specific immune response.<sup>58–62</sup> Indeed, Bregs can induce Tregs differentiation, CD4<sup>+</sup> apoptosis, CD8<sup>+</sup> anergy as well as suppression of Th1 and Th17 differentiation.<sup>61</sup> However, the role of Bregs is still poorly characterized. Recently, the above mentioned meta-analysis study showed that, in human melanomas, a high infiltration of CD20<sup>+</sup> TILs is indicative of a favorable prognosis, independently from the tumor type. Although the mechanism is still unclear, it can be supposed that CD20<sup>+</sup> TILs would be activated as antitumor effector B-cells, contributing to the direct killing of neoplastic cells.<sup>42,63</sup> Conversely, based on our results, we could speculate that, in canine melanocytic tumors, CD20<sup>+</sup> TILs could function as tumor-promoting cells and that Bregs could play a role in melanoma progression.

Finally yet importantly, it must be remembered that an effective anti-tumoral immune response can be prevented by the selection of a so-called “immune excluded” cancer phenotype. In this phenotype, despite the presence of abundant immune cells in the stroma, factors are selected that hamper the penetration of immune cells in the parenchyma of the tumor (specific cytokines production, vascular factors, stromal proliferation).<sup>16</sup> Our previous results demonstrated novel transcriptional networks promoting collagen metabolism and extracellular matrix remodeling in canine cutaneous melanomas, therefore suggesting that similar mechanisms occur in the canine species.<sup>64</sup>

In conclusion, in this retrospective study we showed that, in melanocytic tumors of the dog, TILs are usually of grade 1, hence less abundant compared to what is described in human melanoma. As additional difference, T-lymphocytes (CD3<sup>+</sup> TILs) and B-lymphocytes (CD20<sup>+</sup> TILs) seems to equally compose TILs population. The presence of CD20<sup>+</sup> TILs is associated with negative histologic prognostic factors such as the mitotic count, the cellular pleomorphism and the pigmentation (inverse association). Furthermore, melanocytic tumors with high infiltrations of CD20<sup>+</sup> TILs have frequently an unfavourable clinical outcome, presence of metastasis/recurrence, shorter overall and disease-free survival. Finally, high CD20<sup>+</sup> TILs quantity is associated to a higher hazard of death and of developing recurrence/metastasis, hence it represents a potential new negative prognostic

factor in canine melanocytic tumors. Further studies are advisable to confirm our findings and investigate B-lymphocyte subpopulations, in order to clarify the role of B-cells in canine melanoma.

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## FIGURE LEGENDS

**Figure 1.** Representative images of TILs grade in canine melanocytic tumors; hematoxylin-eosin, 10x magnification. (A) Cutaneous melanocytoma without TILs grade 0 (absent); (B) Oral melanoma with TILs grade 1: TILs are mild and multifocally distributed, sparse or aggregated in little groups (arrows). (C) Oral melanoma with TILs grade 2: TILs infiltrate is moderate with multifocal distribution (arrows). (D) Oral melanoma with TILs grade 3: TILs are markedly present and have a

diffuse distribution, they often aggregate in large groups (arrows), but they are also scattered infiltrating the neoplastic tissue.

**Figure 2.** Representative images of CD3<sup>+</sup> TILs in canine melanocytic tumors; immunohistochemistry, 20x magnification. (A) Cutaneous melanocytoma with occasional, scattered CD3<sup>+</sup> TILs: they are considered absent. (B) Cutaneous melanoma with scattered CD3<sup>+</sup> positive cells, representative of mild CD3<sup>+</sup> TILs infiltration. (C) Cutaneous melanoma with a moderate quantity of CD3<sup>+</sup> TILs: positive cells can be seen, scattered, among the neoplastic cells. (D) Oral melanoma with a marked infiltration of CD3<sup>+</sup> TILs.

**Figure 3.** Representative images of CD20<sup>+</sup> TILs in canine melanocytic tumors; immunohistochemistry, 20x magnification. (A) Cutaneous melanocytoma without CD20<sup>+</sup> TILs. (B) Cutaneous melanoma with mild CD20<sup>+</sup> TILs. (C) Cutaneous melanocytoma with moderate infiltration of CD20<sup>+</sup> TILs: they tend to be aggregate, but they are also scattered among neoplastic melanocytes. (D) Oral melanoma with marked CD20<sup>+</sup> TILs quantity: positive cells can be seen in a large, central aggregate with abundant, scattered cells at its periphery.

**Figure 4.** Kaplan-Meiers curves of overall survival time in dogs with low and high CD3<sup>+</sup> TILs infiltration (P>0.05).

**Figure 5.** Kaplan-Meiers curves of disease free time in dogs with low and high CD3<sup>+</sup> TILs infiltration (P>0.05).

**Figure 6.** Kaplan-Meiers curves of overall survival time in dogs with low and high CD20<sup>+</sup> TILs infiltration (P>0.05).

**Figure 7.** Kaplan-Meiers curves of disease free time in dogs with low and high CD20<sup>+</sup> TILs infiltration (P>0.05).

## TABLES

**Table 1.** Tumor lymphocytes (TILs) grade depending on TILs density and distribution, according to Azimi et al. (2012).

TILs		Density		
		Mild	Moderate	Severe
Distribution	Focal	1	1	2
	Multifocal	1	2	2
	Diffuse	2	3	3

**Table 2.** Association between tumor-infiltrating lymphocytes (TILs) and histological diagnosis.

TILs	DIAGNOSIS			P value†
	Oral melanoma (n=32)	Cutaneous melanoma (n=34)	Cutaneous melanocytoma (n=31)	
<b>Presence (n,%)</b>				
Absent	7 (21.9)	12 (35.3)	12 (38.7)	0.290
Present	25 (78.1)	22 (64.7)	19 (61.3)	
<b>Density (n,%)</b>				
Mild	16 (64.0)	13 (59.1)	11 (57.9)	0.949
Moderate	7 (28.0)	8 (35.6)	6 (31.6)	
Severe	2 (8.0)	1 (4.5)	2 (10.5)	
<b>Distribution (n,%)</b>				
Focal	5 (20.0)	8 (36.4)	7 (36.8)	0.427
Multifocal	17 (68.0)	12 (54.5)	8 (42.1)	
Diffuse	3 (12.0)	2 (9.1)	4 (21.1)	
<b>Grade (n,%)</b>				
0	7 (21.9)	14 (41.2)	12 (38.7)	0.293
1	18 (56.3)	11 (32.4)	11 (35.5)	
2	4 (12.5)	8 (23.5)	5 (16.1)	
3	3 (9.4)	1 (2.9)	3 (9.7)	

†Chi square or Fisher's exact test.

**Table 3.** Association between tumor-infiltrating lymphocytes (TILs) and outcome or metastasis/recurrence.

TILs	Outcome			Metastasis / Recurrence		
	Dead due to melanoma	Alive or dead for another cause	<i>P</i> value†	Yes	No	<i>P</i> value†
<b>Presence (n, %)</b>						
<b>Absent</b>	4 (18.2)	21 (37.5)	0.099	3 (20.0)	20 (35.7)	0.355
<b>Present</b>	18 (81.8)	35 (62.5)		12 (80.0)	36 (64.3)	
<b>Density (n, %)</b>						
<b>Mild</b>	9 (50.0)	23 (67.5)	0.556	5 (41.7)	23 (63.9)	0.218
<b>Moderate</b>	7 (38.9)	9 (25.7)		5 (41.7)	11 (30.6)	
<b>Severe</b>	2 (11.1)	3 (8.6)		2 (16.7)	2 (5.6)	
<b>Distribution (n, %)</b>						
<b>Focal</b>	4 (22.2)	13 (37.1)	0.536	2 (16.7)	15 (41.7)	0.320
<b>Multifocal</b>	11 (61.1)	16 (45.7)		8 (66.7)	15 (41.7)	
<b>Diffuse</b>	3 (16.7)	6 (17.1)		2 (16.7)	6 (16.7)	
<b>Grade (n, %)</b>						
<b>0</b>	5 (22.7)	21 (37.5)	0.491	4 (27.6)	20 (35.7)	0.714
<b>1</b>	10 (45.5)	23 (41.1)		6 (40.0)	24 (42.9)	
<b>2</b>	5 (22.7)	7 (12.5)		3 (20.0)	7 (12.5)	
<b>3</b>	2 (9.1)	5 (8.9)		2 (13.3)	5 (8.9)	

†Chi square or Fisher's exact test.

**Table 4.** Association between CD3+ TILs and CD20+ TILs quantity or distribution and outcome or metastasis/recurrence.

	Outcome (n, %)		<i>P</i> value†	Metastasis / Recurrence (n, %)		
	Dead for melanoma	Alive or dead for another cause		Yes	No	<i>P</i> value†
<b>CD3<sup>+</sup> TILs quantity</b>						
Absent	2 (11.8)	8(15.1)	0.888	1 (8.3)	8 (15.4)	0.825
Mild	10 (58.8)	25 (47.2)		6 (50.0)	26 (50.0)	
Moderate	4 (23.5)	16 (30.2)		3 (25.0)	14 (26.9)	
Severe	1 (5.9)	4 (7.5)		2 (16.7)	4 (7.7)	
<b>CD3<sup>+</sup> TILs distribution</b>						
Focal	4 (23.5)	3 (6.7)	0.203	2 (16.7)	4 (8.9)	0.786
Multifocal	8 (47.1)	29 (64.4)		7 (58.3)	27 (60.0)	
Diffuse	5 (29.4)	13 (28.9)		3 (25.0)	14 (31.1)	
<b>CD20<sup>+</sup> TILs quantity</b>						
Absent	2 (10.5) <sub>b</sub>	24 (55.8) <sub>a</sub>	0.002	2 (15.4) <sub>a</sub>	24 (52.2) <sub>a</sub>	<0.001
Mild	10 (52.6) <sub>a</sub>	14 (32.6) <sub>a</sub>		3 (23.1) <sub>a</sub>	19 (41.3) <sub>a</sub>	
Moderate	5 (26.3) <sub>a</sub>	4 (9.3) <sub>a</sub>		7 (53.8) <sub>b</sub>	1 (2.2) <sub>a</sub>	
Severe	2 (10.5) <sub>a</sub>	1 (2.3) <sub>a</sub>		1 (7.7) <sub>a</sub>	2 (4.3) <sub>a</sub>	
<b>CD20<sup>+</sup> TILs distribution</b>						
Focal	3 (17.6)	4 (21.1)	0.873	1 (9.1)	2 (9.1)	1
Multifocal	12 (70.6)	14 (73.7)		8 (72.7)	15 (68.2)	
Diffuse	2 (11.8)	1 (5.3)		2 (18.2)	9 (23.1)	

†Chi square or Fisher's exact test.

**Table 5.** Overall survival probability estimates according to CD20<sup>+</sup> TILs quantity in melanocytic tumors.

	Survival probability (median, 95%CI)			
	6 months	1 year	1.5 years	2 years
<b>CD20<sup>+</sup> TILs quantity</b>				
<b>Absent</b>	95.8 (73.9-99.4)	91.7 (70.6-97.8)	91.7 (70.6-97.8)	91.7 (70.6-97.8)
<b>Mild</b>	90.6 (67.3-97.6)	68.6 (42.6-84.7)	62.4 (36.3-80.3)	62.4 (36.3-80.3)
<b>Moderate</b>	60.0 (19.5-85.2)	20.0 (0.9-57.3)	20.0 (0.9-57.3)	n/a†
<b>Severe</b>	100 (100-100)	33.3 (0.8-77.4)	33.3 (0.8-77.4)	33.3 (0.8-77.4)
<b>CD20<sup>+</sup> TILs quantity</b>				
<b>Low</b>	93.5 (81.0-97.8)	81.6 (66.4-90.4)	78.8 (63.0-88.4)	78.8 (63.0-88.4)
<b>High</b>	71.6 (35.0-89.9)	23.9 (3.7-53.7)	23.9 (3.7-53.7)	23.9 (3.7-53.7)

†n/a: not applicable

**Table 6.** Probability estimates of not developing recurrence/metastasis according to CD20<sup>+</sup> TILs quantity in melanocytic tumors.

	Disease-free probability (median, 95%CI)			
	6 months	1 year	1.5 years	2 years
<b>CD20<sup>+</sup> TILs quantity</b>				
<b>Absent</b>	91.5 (70.0-97.8)	91.5 (70.0-97.8)	91.5 (70.0-97.8)	91.5 (70.0-97.8)
<b>Mild</b>	95.0 (69.5-99.3)	83.6 (56.8-94.5)	83.6 (56.8-94.5)	83.6 (56.8-94.5)
<b>Moderate</b>	47.6 (12.3-76.9)	23.8 (1.3-62.2)	n/a†	n/a
<b>Severe</b>	66.7 (5.4-94.5)	66.7 (5.4-94.5)	66.7 (5.4-94.5)	66.7 (5.4-94.5)
<b>CD20<sup>+</sup> TILs quantity</b>				
<b>Low</b>	93.1 (80.2-97.7)	88.1 (73.7-94.9)	88.1 (73.7-94.9)	88.1 (73.7-94.9)
<b>High</b>	52.4 (20.1-77.0)	34.9 (6.7-66.4)	17.5 (0.9-51.8)	17.5 (0.9-51.8)

†n/a: not applicable

ILLUSTRATIONS (Supplied as separate files)

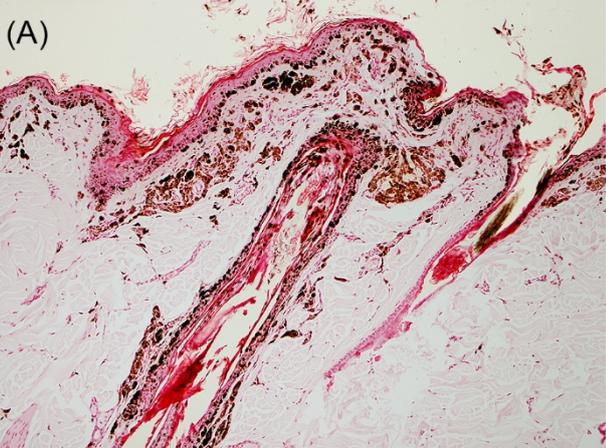


Fig. 1

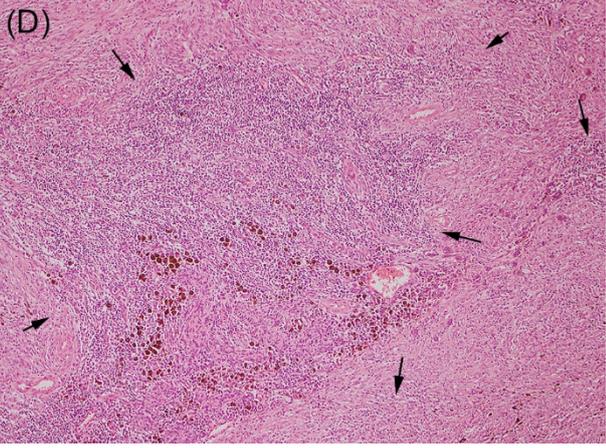
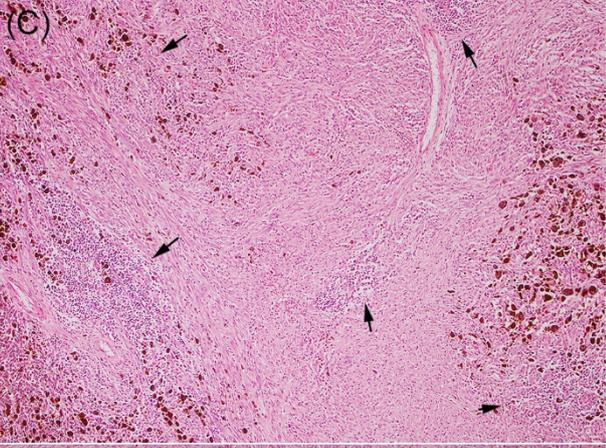
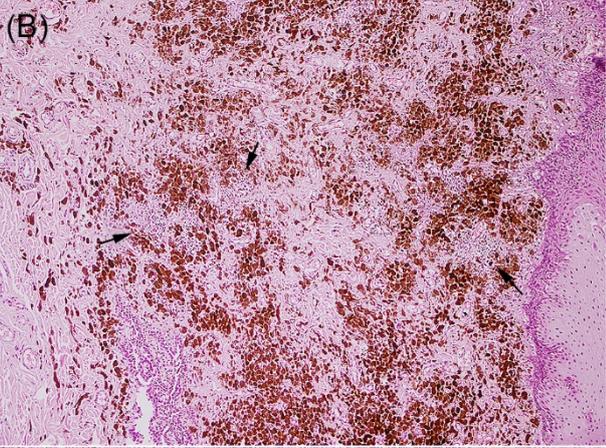
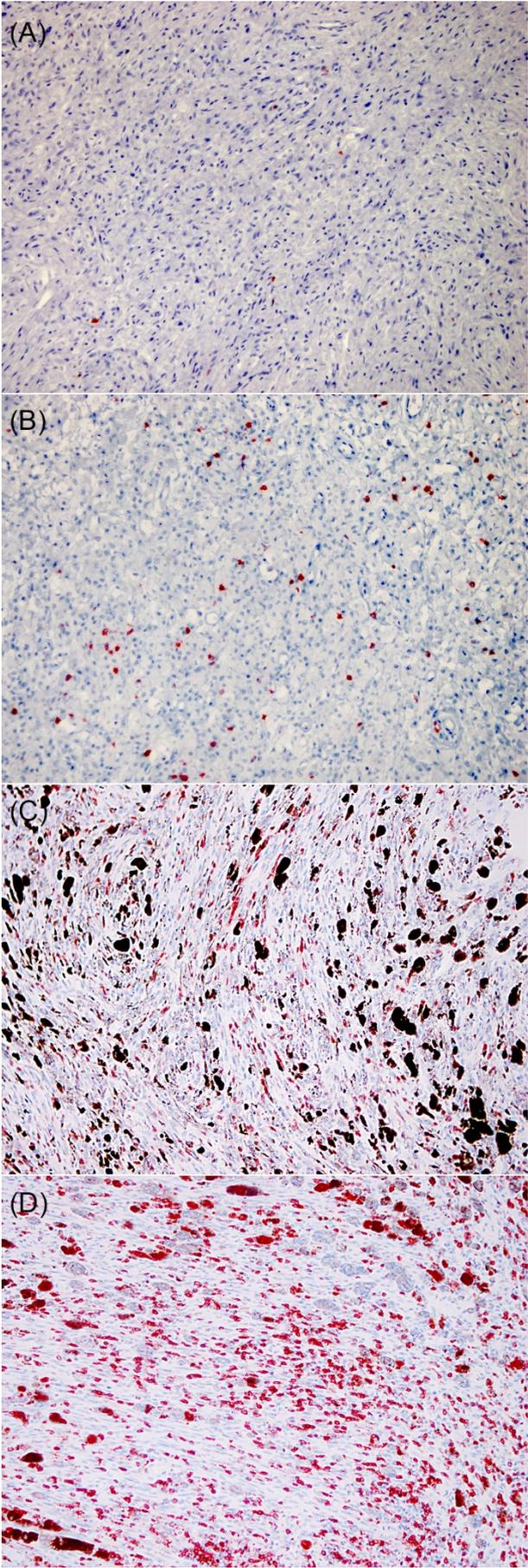
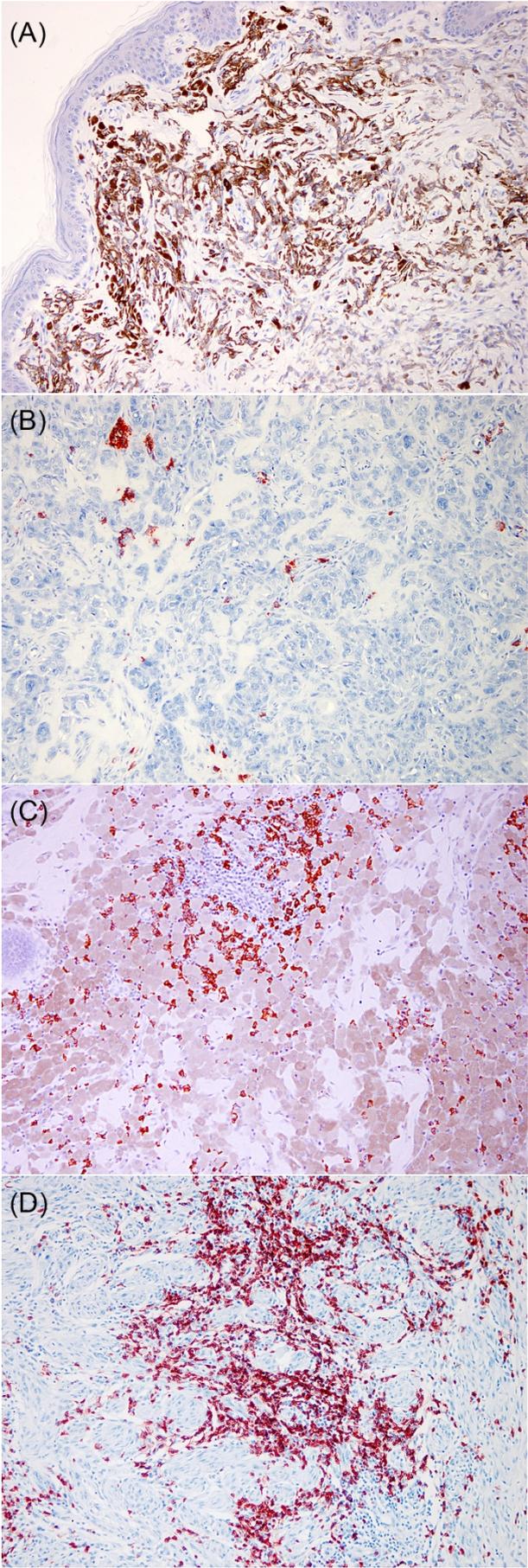


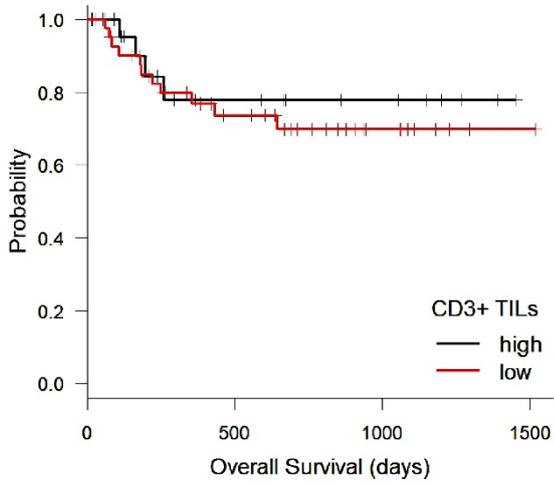
Fig. 2



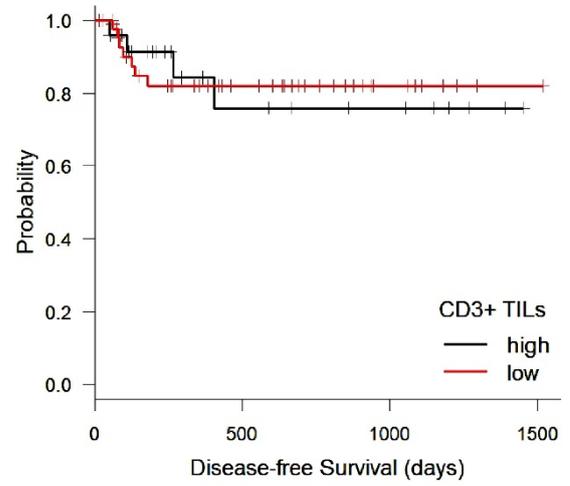
**Fig. 3**



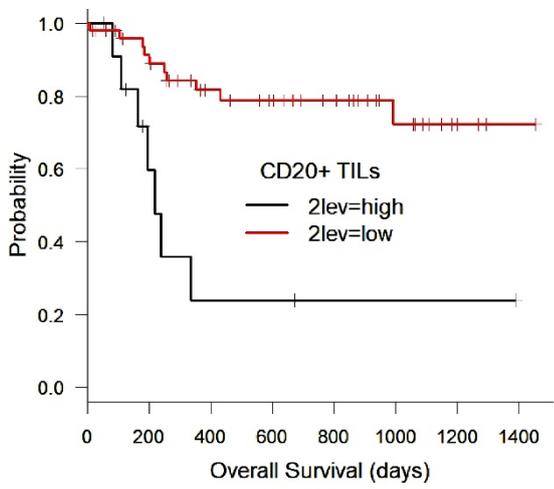
**Fig. 4**



**Fig. 5**



**Fig. 6**



**Fig. 7**

