Tumor-infiltrating lymphocytes in canine melanocytic tumors: an investigation on the prognostic role of CD3+ and CD20+ lymphocytic populations

RUNNING TITLE: TILs in canine melanocytic tumors

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Abstract

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 interactions between neoplastic and immune cells and its importance in cancer pathogenesis and progression, 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 ($CD3^+$) and B-lymphocytes ($CD20^+$). The results of our study show that TILs are present in a large proportion of canine melanocytic tumors, especially in oral melanomas, and that the infiltrate is usually mild. The quantity of $CD20^+$ 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 $CD20^+$ 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, despite advances in understanding its pathogenesis, currently no effective therapy is available and tumor-associated mortality is related to systemic metastatic spread.[1] In the last few years, several studies have underlined the similarities between human and canine melanoma and suggested to investigate the dog as a possible model for the human tumor.[2–8] Canine melanomas, in particular oral melanomas, often have a 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.[9,10] 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, since the prognosis can be variable and sometimes unpredictable.[11–13] An extensive review of the more commonly adopted and significant prognostic histologic factors has been written by Smedley et al. in 2011,[14] in which well-recognized factors are summarized in a ready-to-use table to classify melanocytic tumors histologically. Recently, the usefulness of tumor thickness as a prognostic factor has been highlighted in the canine species: in line with what observed in humans with Breslow thickness, thicker cutaneous melanocytic tumors seem to have a worse prognosis.[15,16] However, compared to 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 metastasize has been widely investigated.[17–20] Human 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.

The definition of TILs was first introduced by Clark et al. in 1989[21] and taken up and developed lately by Clemente et al.[22] TILs are defined as lymphocytes that infiltrate the tumor and that disrupt its nests and/or lymphocytes which are in direct contact with neoplastic cells.[17,23] 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 cutaneous melanoma.[21] 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.[22–28] On this line, some authors have suggested to include an accurate quantification of TILs in histopathological reporting,[29,30] although there are conflicting data on the utility of TILs as prognostic indicators. This is because TILs are indeed composed of very heterogeneous subpopulations of cells ranging from cells with protective functions to cells with immunosuppressive functions. Most TILs are T lymphocytes, including $CD4^+$, $CD8^+$, T regulatory cells (Tregs) and γ/δ T cells, but also B and NK cells are present.[23,31,32] The role of single populations of lymphocytes can be very different and their distribution can vary deeply.[33] The transfer of tumor-infiltrating T cells has been successfully used in adoptive immunotherapy in a proportion of melanoma patients.[34–36] 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).[37–40] All together these data show the complexity of the interaction between tumor cells and tumor-infiltrating inflammatory cells, especially T cells, that are currently the subject of numerous studies.

In veterinary medicine, the investigations on the presence and significance of TILs or their different subpopulations are still few, but the interest and the attention in this field of research is growing as testified by different recent pubblications.[41–45]

The purpose of this study was to analyze the significance of the presence, density, distribution and grade of TILs as well as their two main subpopulations (T and B cells) in canine melanocytic tumors. Their associations with histological diagnosis, clinical outcome, presence of metastasis/recurrence, disease-free survival, 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 in the period between 2009 and 2016. Inclusion criteria were as follows: (1) diagnosis of either 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 formalin-fixed and paraffin-embedded tissue.

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 a telephonic interview with referring veterinarians or through the collection of medical records data from internal cases. The overall survival was the time from first diagnosis/appearance to death for any cause; disease-free survival was the time from first diagnosis/appearance to the first event of recurrent disease or metastasis or death. The recurrence was considered as the reappearance of the tumor at the site of origin after removal. 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 that died because of the tumor was considered “unfavorable”, while that one of patients alive or dead for causes unrelated to melanoma was considered “favorable”.

Histological examination

Some of the samples in this study were included in a previous work on the usefulness of tumor thickness and modified Clark level for the evaluation of canine melanocytic tumors.[15] All samples were histologically evaluated for the parameters having the greater validity for prognostic use in canine melanocytic neoplasia, according to the current literature.[14,15] The parameters evaluated were:

- mitotic count evaluated on 10 contiguous high-power fields (field number of the ocular of 22 mm and a 40x objective), starting from hotspots and avoiding areas with necrosis or severe inflammation;

- tumor thickness;
- modified Clark level;
- nuclear atypia;
- degree of pigmentation, evaluated as percentage of pigmented cells (0%, 50%, and >50%);
- presence of ulceration;
- presence of necrosis;
- cellular pleomorphism (mild/moderate/marked).

TILs evaluation

TILs were firstly evaluated as present/absent in all samples. Additionally, referring to human literature,[23] 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.[17] When lymphocytes were not in direct contact with tumor cells, TILs were considered absent[19] (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.

Immunohistochemistry and evaluation of $CD3^+$ TILs and $CD20^+$ TILs

Eighty-seven and 77 melanocytic tumors were available for CD3 and CD20 immunohistochemistry, respectively. Some tumors could not be investigated by immunohistochemistry because tissue blocks were considered not sufficient, due to serial recuts of the slides for previous evaluations. Immunohistochemistry was performed as previously described, following a modified protocol.[46] Briefly, commercially available rabbit polyclonal antibodies against CD20 (1:100, RB-9013; Thermo Scientific, Fremont, CA, USA) and 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_2O_2 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.

Afterward, they were incubated with an ABC ready-to-use kit (ab93677; Abcam, Cambridge, UK) following the manufacturer's instructions. The positive reaction was revealed with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark). Carazzi's hematoxylin was used as a counterstain. Coverslips were mounted with Aquatex® (Merck, Darmstadt, Germany). Normal canine lymph node was used as a 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.[21] and Clemente et al.[22] were considered in this study ($CD3^+$ or $CD20^+$ TILs). Considering both markers, tumors were evaluated and classified by three pathologists (SS, IP, CB) for:

- Quantity: semiquantitative evaluation of positive cells defined as absent (no positive cells or rare positive scattered cells), mild (scattered single positive cells, also in occasional small aggregates), moderate (several single positive cells to medium-sized aggregates) or severe (diffuse infiltration of positive cells or massive aggregates) (Figure 1). The quantity of T- and B-lymphocyte was subsequently further grouped into two groups (2-tier variable): absent and mild grouped together as "low $CD3^+$/$CD20^+$ TILs" versus moderate and severe grouped together as "high $CD3^+$/$CD20^+$ TILs".
- Distribution: distribution of positive cells within the tumor defined as focal, multifocal or diffuse
- Localization: localization of positive cells within the tumor regions defined as at the peripheral margins ($CD3^+$/$CD20^+$ TILs were localized in the peripheral tumor area, but always in close contact with tumor cells), intratumoral ($CD3^+$/$CD20^+$ TILs were localized in the tumor area excluding the peripheral margins), or both localizations (homogeneous presence of $CD3^+$/$CD20^+$ TILs into the tumor).

Statistical analysis

Graphic tests were used to verify assumptions of normality, which were not met. 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. Non-parametric tests were performed for hypothesis testing. For the analysis of continuous variables, we used Kruskal-Wallis, Mann-Whitney U test and Spearman correlation coefficient (ρ); 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, $CD3^+$ and $CD20^+$ TILs quantity. The 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, $CD3^+$ and $CD20^+$ TILs quantity. Data were analyzed by SPSS 23.0 (SPSS Inc. Chicago, USA) and the software R (R version 3.5.1).[47] 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=27/90, 30.0%). 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). The diagnosis was significantly associated with the age of affected animals (*P*<0.001): dogs with cutaneous melanocytoma were younger compared to dogs with oral and cutaneous 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

In oral melanomas, TILs were present in 78.1% of cases; 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). Table S2 shows the results regarding TILs analysis (presence, density, distribution and grade) in the various tumor types.

TILs versus clinical outcome and recurrence/metastasis presence

TILs presence, density, distribution and grade were not associated with clinical outcome or metastatic/recurrent disease (Table S3; Supplemental information).

Immunohistochemical evaluation of CD3+ TILs and CD20+ TILs

$CD3^+$ TILs and $CD20^+$ TILs versus TILs grade

The quantity of $CD3^+$ TILs was associated with TILs grade (*P*=0.003; Table S4, Supporting information). In particular, most of the cases exhibiting a grade 3 of TILs had severe $CD3^+$ TILs infiltration; the Spearman correlation coefficient showed a weak correlation between the two variables (ρ=0.388; *P*=0.0002). Similarly, the amount of $CD20^+$ TILs was associated with TILs grade (*P*=0.016); the Spearman correlation coefficient showed a weak correlation between the variables (ρ=0.291; *P*=0.010).

$CD3^+$ TILs and $CD20^+$ TILs versus histological diagnosis

Data regarding the quantity, distribution and localization of $CD3^+$ and $CD20^+$ TILs in the different melanocytic tumors are summarized in Table S5 (Supporting Information). The most interesting result is the association between the histological diagnosis and $CD20^+$ TILs quantity (*P*<0.001): in cutaneous tumors $CD20^+$ TILs are more frequently absent (n=13/26, 50.0% and n=18/26, 69.2% for melanoma e melanocytoma respectively) than in oral melanomas (n=1/25, 4.0%; *P*≤0.05). No other associations were found.

$CD3^+$ TILs and $CD20^+$ TILs versus histologic criteria of malignancy

Data regarding the association between the presence of $CD3^+$ TILs and $CD20^+$ TILs and the histologic features of malignancy are shown in Table S6 (Supporting Information). Briefly, a statistically significant association was found between the quantity of $CD20^+$ TILs and the mitotic

count (P<0.001), since it was lower in tumors with no $CD20^+$ TILs than in ones with a severe infiltration (median=1.0, IQR=0.0-3.5 and median=15.50, IQR=6.0-41.0, respectively; P≤0.05). The major diameter (P=0.007) was significantly lower in tumors with absent $CD20^+$ TILs compared to mild, moderate or severe $CD20^+$ TILs quantity (P≤0.05). Finally, an association between $CD20^+$ TILs quantity and the percentage of pigmentation (P=0.004) or the cellular pleomorphism (P=0.001) was also seen.

$CD3^+$ TILs and $CD20^+$ TILs versus clinical outcome and metastasis/recurrence presence

$CD3^+$ TILs quantity was not associated with clinical outcome or presence metastasis/recurrence (Table 2). Conversely, $CD20^+$ 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 $CD20^+$ TILs that died because of melanoma was lower (n=2/19, 10.5%) compared to those alive or dead for causes not related to melanoma (n=24/43, 55.8%; P<0.05). The frequencies of dogs with metastasis/recurrence were higher (n=7/13, 53.8%) compared to those without metastasis/recurrence (n=12/46, 2.2%, P<0.05) in the group of tumors with moderate $CD20^+$ TILs infiltration. No significant association of clinical outcome or presence of metastasis/recurrence with $CD3^+$/$CD20^+$ TILs distribution or localization was seen (P>0.05).

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 (78.9%) did not. Overall survival varied depending on histological diagnosis (P<0.001), being shorter in oral melanomas (median = 258 days, IQR = 180–992 days) compared to cutaneous tumors (medians not reached; P<0.05).

TILs and survival

Kaplan-Meyer curves showed no differences in overall survival or 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; Table S7-S8, Supporting Information).

$CD3^+$ TILs and $CD20^+$ TILs and survival

Kaplan-Meyer curves showed no differences in overall survival or disease-free survival according to $CD3^+$ TILs quantity (Figure S1 and S2, Supporting information). Univariate Cox proportional regression analysis confirmed that they were not prognostic factors in our study (P>0.05; Table S7-S8, Supporting Information).

Overall survival was associated with $CD20^+$ TILs quantity (P<0.001): in particular, dogs with absent $CD20^+$ TILs had a longer survival time than dogs with moderate $CD20^+$ TILs quantity (P<0.05). Survival probability estimates over time are shown in Table 3. The difference was even more marked when $CD20^+$ TILs quantity was analyzed as a 2-tier variable (low $CD20^+$ TILs versus high $CD20^+$ TILs): overall survival of dogs with low $CD20^+$ TILs quantity was significantly longer than dogs with high $CD20^+$ TILs quantity (P<0.001; Figure 2). Moreover, dogs with a high $CD20^+$ TILs quantity had a hazard of death 5 times greater than dogs with low $CD20^+$ TILs quantity (HR=5.31, 95%CI=1.98-14.29; P=0.001; Table S7, Supporting Information).

Kaplan-Meyer curves showed significant differences in disease-free survival (P<0.001), being significantly longer when $CD20^+$ TILs quantity was absent than moderate (P<0.05) and when $CD20^+$ TILs quantity was mild than moderate (P<0.05). Probability estimates to not develop recurrence/metastasis over time are shown in Table 4. The difference was even more marked when $CD20^+$ TILs quantity was analyzed as a 2-tier variable: disease-free survival of dogs with low $CD20^+$ TILs quantity was significantly longer than dogs with high $CD20^+$ TILs quantity (P<0.001; Figure 3). Moreover, dogs with a high $CD20^+$ TILs quantity had a hazard of developing recurrence/metastasis 10 times greater than dogs with low $CD20^+$ TILs quantity (HR=10.27, 95%CI=3.15-33.54; P=0.001; Table S8, Supplemental Information).

DISCUSSION

This study evaluated TILs in canine melanocytic tumors by morphological analysis of their density, distribution and grade and immunohistochemical characterization of the two main populations of TILs, $CD3^+$ and $CD20^+$ 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 represented by 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.

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 similar to data reported in human literature, where immune cell infiltration is common and frequently mild in cutaneous tumors.[23] This low infiltration of TILs may be caused by the low expression of tumor-associated antigens by neoplastic melanocytes, resulting in a reduction of both anti-tumor immune response and migration of immune cells in the neoplastic site.[48] Another possible explanation could be the high tumor mutational burden (TMB) of melanoma also in the canine species, which is reported in human medicine as a possible inhibitor factor towards the infiltration of immune cells.[49] TILs appear to be common especially in oral melanomas (78.1%). This finding could be related to the site of origin of the tumor, since the oral mucosa has a peculiar immune and inflammatory response, usually characterized by a marked infiltration of lymphocytes and plasma cells.[50]

No significant association was demonstrated between TILs grade and the histological diagnosis, indicating that TILs are likely not helpful in defining the histological diagnosis in challenging cases (in particular in discriminating cutaneous melanocytomas and melanomas).

Additionally, TILs grade was not associated with the clinical outcome, the presence of metastasis/recurrence, the overall or disease-free survival. These findings are different from human literature: TILs grade and presence are most commonly associated with a better outcome and prognosis and are considered predictors of sentinel lymph node metastasis.[23,51,52] These partially contradicting results could be attributed to the great heterogeneity characterizing the TILs population, which comprises, on one hand, cells that protect the host from tumor growth (such as cytotoxic T-lymphocytes, T-memory cells, T-helper 1 lymphocytes, and, partially, B-lymphocytes), and, on the other hand, cells that instead favor tumor development and progression (such as Treg lymphocytes, T-helper 17 cells and, partially, B-lymphocytes).[33,53–55]

In human literature, T-lymphocytes are the most abundant cell population infiltrating cutaneous melanomas.[56,57] Our study revealed a statistically significant association between TILs grade and the quantity of $CD3^+$ TILs or $CD20^+$ TILs, showing that both of T- and B-lymphocytes compose TILs population, increasing together with them. Differently from humans, no statistical association was

demonstrated between $CD3^+$ TILs quantity and any of the features investigated (histological parameters, clinical outcome, recurrence/metastasis, overall and disease-free survival).

In a recent meta-analysis on the prognostic value of TILs in human melanoma, a high infiltration of $CD3^+$ TILs was associated with a lower hazard of death, independently from the tumor site.[52] This finding suggests $CD3^+$ TILs population as mainly composed of antitumor effector T-cells. Our results could be explained because $CD3^+$ TILs are composed of different subpopulations of lymphocytes including cytotoxic lymphocytes ($CD8^+$), T-memory cells ($CD8^+CD45RO^+$), T-helper cells ($CD4^+CCR5^+$, $CD4^+CCR4^+$, $CD4^+CCR4^+CCR6^+$) and T regulatory cells ($CD4^+CD25^+FOXP3^+$) with opposite roles, that should be properly investigated also in dogs.[58,59] It is likely that T-lymphocytes with a pro-tumorigenic and anti-tumorigenic activity are both present in the dog. This could explain why in our study $CD3^+$ TILs were not associated with survival. Supporting this hypothesis, our group recently observed that Tregs, a subpopulation of T-lymphocytes with immune suppressive function, are frequently present in canine melanocytic tumors and that their infiltration is associated with negative prognosis; increased hazard of death was observed in dogs with a higher number of $FoxP3^+$ cells (T regs) and with a higher percentage of $FoxP3^+$ cells on the total of $CD3^+$ lymphocytes associated with the tumor.[60] Another study described the similarities between human breast cancer and canine mammary tumors demonstrating the association of the CD4+/ CD8+ T-cells ratio with low survival rates, the association of increased Treg cells with poor prognostic factors and the promotion of tumor progression by the selection of a Th2-mediated environment.[61] Recently, it was also observed that the expression of granzyme B^+ TILs was associated with a better prognosis in canine transitional cell carcinomas in face of a non-prognostic number of $CD3^+$ lymphocytes.[42] Hence, since the tumor immune environment is a complex model, further investigations are needed to better characterize $CD3^+$ subpopulations and to better characterize their interaction with canine melanoma cells.

$CD20^+$ TILs were absent in about half of melanocytic cutaneous tumors (both benign and malignant), while they were always observed, in variable amounts, in oral melanomas. The statistical analysis demonstrated an association between the quantity of $CD20^+$ TILs and some prognostic histologic factors. The positive association between the $CD20^+$ TILs quantity and the mitotic count or cellular pleomorphism (two negative prognostic factors) as well as the inverse association with the percentage of pigmented cells (a distinct feature of well-differentiated cells and a positive prognostic histologic factor), would indicate a negative role of B-lymphocytes in canine melanocytic tumors. This role was further confirmed by the statistically significant

association of $CD20^+$ TILs quantity with the unfavorable clinical outcome, the presence of metastasis/recurrence, the shorter overall and disease-free survival. When $CD20^+$ 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 $CD20^+$ TILs quantity was greater compared to dogs with low $CD20^+$ TILs quantity. Although our results need 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 $CD20^+$ 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[62–66] and it is still a matter of debate how systemic B-cell response or *in situ* B-cell infiltration impact on the biological behavior of tumors. In some experimental models, B-lymphocytes infiltration was correlated with tumor growth or progression;[65,66] however, other studies led to opposite results.[63,67] The discrepancy regarding these results may be related to different functional activities of B cells.[68] On one hand, B-lymphocytes can act as antigen-presenting cells, inducing $CD4^+$ T cell-dependent $CD8^+$ 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 an M2 pro-tumor phenotype in macrophages and stimulate the differentiation of $CD4^+$ cells in Tregs.[33,69] Another subpopulation of B cells with regulatory functions (Bregs) seems to have a negative prognostic significance in skin cancers, since it inhibits tumor-specific immune response.[69–73] Indeed, Bregs can induce Tregs differentiation, $CD4^+$ apoptosis, $CD8^+$ anergy as well as suppression of Th1 and Th17 differentiation.[72] 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 $CD20^+$ TILs is indicative of a favorable prognosis, independently from the tumor type. Although the mechanism is still unclear, it can be supposed that $CD20^+$ TILs would be activated as antitumor effector B-cells, contributing to the direct killing of neoplastic cells.[52,74] Conversely, based on our results, we could speculate that, in canine melanocytic tumors, $CD20^+$ 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.[75] In these tumors, despite the presence of abundant immune cells in the stroma, different factors such as specific cytokines production, vascular factors, stromal proliferation are selected and hamper the

penetration of immune cells in the parenchyma of the tumor.[20] In agreement with this theory, novel transcriptional networks promoting collagen deposition and extracellular matrix remodeling in canine cutaneous melanomas were observed, suggesting the presence of similar mechanisms in dogs.[76]

In conclusion, in this retrospective study we showed that, in canine melanocytic tumors, TILs are usually of grade 1, similarly to what is described in human melanoma. The presence of $CD20^+$ TILs is associated with negative histologic prognostic factors such as the mitotic count, the cellular pleomorphism and is inversely associated with the pigmentation. Furthermore, dogs with melanocytic tumors with high infiltrations of $CD20^+$ TILs are frequently characterized by an unfavorable clinical outcome, presence of metastasis/recurrence, and shorter overall and disease-free survival. Finally, high $CD20^+$ TILs quantity is associated with 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 to investigate B-lymphocyte subpopulations, in order to clarify the role of B-cells in canine melanoma.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

References

1. Balch CM, Gershenwald JE, Soong S-J, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;27(36):6199-6206.
2. Bosenberg M, Arnheiter H, Kelsh R. Melanoma in mankind's best friend. *Pigment Cell Melanoma Res*. 2014;27(1):1.
3. Simpson RM, Bastian BC, Michael HT, et al. Sporadic naturally occurring melanoma in dogs as a preclinical model for human melanoma. *Pigment Cell Melanoma Res*. 2014;27(1):37-47.
4. Gillard M, Cadieu E, De Brito C, et al. Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Cell Melanoma Res*. 2014;27(1):90-102.
5. Atherton MJ, Morris JS, McDermott MR, Lichty BD. Cancer immunology and canine malignant melanoma: A comparative review. *Vet Immunol Immunopathol*. 2016;169:15-26.
6. van der Weyden L, Patton EE, Wood GA, et al. Cross-species models of human melanoma. *J Pathol*. 2016;238(2):152-165.

7. Tarone L, Barutello G, Iussich S, et al. Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy. *Cancer Immunol Immunother CII*. 2019;68(11):1839-1853.

8. Barutello G, Rolih V, Arigoni M, et al. Strengths and Weaknesses of Pre-Clinical Models for Human Melanoma Treatment: Dawn of Dogs' Revolution for Immunotherapy. *Int J Mol Sci*. 2018;19(3):799.

9. Hernandez B, Adissu HA, Wei B-R, Michael HT, Merlino G, Simpson RM. Naturally Occurring Canine Melanoma as a Predictive Comparative Oncology Model for Human Mucosal and Other Triple Wild-Type Melanomas. *Int J Mol Sci*. 2018;19(2).

10. Prouteau A, André C, Prouteau A, André C. Canine Melanomas as Models for Human Melanomas: Clinical, Histological, and Genetic Comparison. *Genes (Basel)*. 2019;10(7):501.

11. Esplin DG. Survival of dogs following surgical excision of histologically well-differentiated melanocytic neoplasms of the mucous membranes of the lips and oral cavity. *Vet Pathol*. 2008;45(6):889-896.

12. Schultheiss PC. Histologic features and clinical outcomes of melanomas of lip, haired skin, and nail bed locations of dogs. *J Vet Diagn Invest*. 2006;18(4):422-425.

13. Spangler WL, Kass PH. The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. *Vet Pathol*. 2006;43(2):136-149.

14. Smedley RC, Spangler WL, Esplin DG, et al. Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. *Vet Pathol*. 2011;48(1):54-72.

15. Silvestri S, Porcellato I, Mechelli L, Menchetti L, Rapastella S, Brachelente C. Tumor Thickness and Modified Clark Level in Canine Cutaneous Melanocytic Tumors. *Vet Pathol*. 2019;56(2):180-188.

16. Scolyer RA, Judge MJ, Evans A, et al. Data Set for Pathology Reporting of Cutaneous Invasive Melanoma. *Am J Surg Pathol*. 2013;37(12):1797-1814.

17. Lee N, Zakka LR, Schatton T. Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. *Pathology*. 2016;48(2):177-187.

18. Gata VA, Lisencu CI, Vlad CI, Piciu D, Irimie A, Achimas-Cadariu P. Tumor infiltrating lymphocytes as a prognostic factor in malignant melanoma. Review of the literature. *J BUON*. 22(3):592-598.

19. Schatton T, Scolyer RA, Thompson JF, Mihm MC. Tumor-Infiltrating Lymphocytes and Their Significance in Melanoma Prognosis. In: *Methods in Molecular Biology (Clifton, N.J.)*. Vol 1102; 2014:287-324.

20. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature*. 2017;541(7637):321-330.

21. Clark WH, Elder DE, Guerry D, et al. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst*. 1989;81(24):1893-1904.

22. Clemente CG, Mihm MC, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer*. 1996;77(7):1303-1310.

23. Azimi F, Scolyer RA, Rumcheva P, et al. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol*. 2012;30(21):2678-2683.

24. Tuthill RJ, Unger JM, Liu PY, Flaherty LE, Sondak VK, Southwest Oncology Group. Risk Assessment in Localized Primary Cutaneous Melanoma. *Am J Clin Pathol*. 2002;118(4):504-511.

25. van Houdt IS, Sluijter BJR, Moesbergen LM, et al. Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression. *Int J cancer*. 2008;123(3):609-615.

26. Mandalà M, Imberti GL, Piazzalunga D, et al. Clinical and histopathological risk factors to predict sentinel lymph node positivity, disease-free and overall survival in clinical stages I–II AJCC skin melanoma: Outcome analysis from a single-institution prospectively collected database. *Eur J Cancer*. 2009;45(14):2537-2545.

27. Donizy P, Kaczorowski M, Halon A, Leskiewicz M, Kozyra C, Matkowski R. Paucity of tumor-infiltrating lymphocytes is an unfavorable prognosticator and predicts lymph node

metastases in cutaneous melanoma patients. *Anticancer Res*. 2015;35(1):351-358.

28. Krynitz B, Rozell BL, Lyth J, Smedby KE, Lindelöf B. Cutaneous malignant melanoma in the Swedish organ transplantation cohort: A study of clinicopathological characteristics and mortality. *J Am Acad Dermatol*. 2015;73(1):106-113.e2.

29. Simonson WTN, Allison KH. Tumour-infiltrating lymphocytes in cancer: implications for the diagnostic pathologist. *Diagnostic Histopathol*. 2011;17(2):80-90.

30. Hendry S, Salgado R, Gevaert T, et al. Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors. *Adv Anat Pathol*. 2017;24(6):235-251.

31. Oble DA, Loewe R, Yu P, Mihm MC. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma. *Cancer Immun*. 2009;9:3.

32. Botti G, Cerrone M, Scognamiglio G, Anniciello A, Ascierto PA, Cantile M. Microenvironment and tumor progression of melanoma: New therapeutic prospectives. *J Immunotoxicol*. 2013;10(3):235-252.

33. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12(4):298-306.

34. Dudley ME, Gross CA, Langhan MM, et al. CD8+ Enriched "Young" Tumor Infiltrating Lymphocytes Can Mediate Regression of Metastatic Melanoma. *Clin Cancer Res*. 2010;16(24):6122-6131.

35. Besser MJ, Shapira-Frommer R, Treves AJ, et al. Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res*. 2010;16(9):2646-2655.

36. Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res*. 2011;17(13):4550-4557.

37. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape. *Curr Opin Immunol*. 2014;27:16-25.

38. Vesely MD, Schreiber RD. Cancer immunoediting: antigens, mechanisms, and implications

to cancer immunotherapy. *Ann N Y Acad Sci*. 2013;1284(1):1-5.

39. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. *Nat Rev Immunol*. 2017;17(11):703-717.

40. Whiteside TL. The role of regulatory T cells in cancer immunology. *ImmunoTargets Ther*. 2015;4:159-171.

41. Sakai K, Maeda S, Yamada Y, et al. Association of tumour-infiltrating regulatory T cells with adverse outcomes in dogs with malignant tumours. *Vet Comp Oncol*. 2018;16(3):330-336.

42. Inoue A, Maeda S, Kinoshita R, Tsuboi M, Yonezawa T, Matsuki N. Density of tumor-infiltrating granzyme B-positive cells predicts favorable prognosis in dogs with transitional cell carcinoma. *Vet Immunol Immunopathol*. 2017;190:53-56.

43. Marcinowska A, Constantino-Casas F, Williams T, Hoather T, Blacklaws B, Dobson J. T Lymphocytes in Histiocytic Sarcomas of Flat-Coated Retriever Dogs. *Vet Pathol*. 2017;54(4):605-610.

44. Franzoni MS, Brandi A, de Oliveira Matos Prado JK, et al. Tumor-infiltrating CD4+ and CD8+ lymphocytes and macrophages are associated with prognostic factors in triple-negative canine mammary complex type carcinoma. *Res Vet Sci*. 2019;126:29-36.

45. McBride R, Sloma EA, Erb HN, Miller AD. Immune Cell Infiltration in Feline Meningioma. *J Comp Pathol*. 2017;156(2-3):162-168.

46. Brachelente C, Affolter VK, Fondati A, et al. CD3 and CD20 Coexpression in a Case of Canine Cutaneous Epitheliotropic T-Cell Lymphoma (Mycosis Fungoides). *Vet Pathol*. 2015:3-6.

47. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

48. Pitcovski J, Shahar E, Aizenshtein E, Gorodetsky R. Melanoma antigens and related immunological markers. *Crit Rev Oncol Hematol*. 2017;115:36-49.

49. Wang X, Li M. Correlate tumor mutation burden with immune signatures in human cancers. *BMC Immunol*. 2019;20(1):4.

50. van Loon LA, Krieg SR, Davidson CL, Bos JD. Quantification and distribution of lymphocyte subsets and Langerhans cells in normal human oral mucosa and skin. *J Oral Pathol Med*.

1989;18(4):197-201.

51. Burton AL, Roach BA, Mays MP, et al. Prognostic significance of tumor infiltrating lymphocytes in melanoma. *Am Surg*. 2011;77(2):188-192.

52. Fu Q, Chen N, Ge C, et al. Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis. *Oncoimmunology*. 2019;8(7):1593806.

53. Torchinsky MB, Blander JM. T helper 17 cells: discovery, function, and physiological trigger. *Cell Mol Life Sci*. 2010;67(9):1407-1421.

54. Larsen SK, Gao Y, Basse PH. NK cells in the tumor microenvironment. *Crit Rev Oncog*. 2014;19(1-2):91-105.

55. Gooden MJM, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer*. 2011;105(1):93-103.

56. Hussein MR. Tumour-infiltrating lymphocytes and melanoma tumorigenesis: an insight. *Br J Dermatol*. 2005;153(1):18-21.

57. Weiss SA, Han SW, Lui K, et al. Immunologic heterogeneity of tumor-infiltrating lymphocyte composition in primary melanoma. *Hum Pathol*. 2016;57:116-125.

58. Tsuchiya T, Ohshima K, Karube K, et al. Th1, Th2, and activated T-cell marker and clinical prognosis in peripheral T-cell lymphoma, unspecified: comparison with AILD, ALCL, lymphoblastic lymphoma, and ATLL. *Blood*. 2004;103(1):236-241.

59. Brucklacher-Waldert V, Steinbach K, Lioznov M, Kolster M, Hölscher C, Tolosa E. Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. *J Immunol*. 2009;183(9):5494-5501.

60. Porcellato I, Brachelente C, De Paolis L, et al. FoxP3 and IDO in Canine Melanocytic Tumors. *Vet Pathol*. 2019;56(2):189-199.

61. Carvalho MI, Pires I, Prada J, Queiroga FL. A Role for T-Lymphocytes in Human Breast Cancer and in Canine Mammary Tumors. *Biomed Res Int*. 2014;2014:1-11.

62. Erdag G, Schaefer JT, Smolkin ME, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic

melanoma. *Cancer Res*. 2012;72(5):1070-1080.

63. Ladányi A, Kiss J, Mohos A, et al. Prognostic impact of B-cell density in cutaneous melanoma. *Cancer Immunol Immunother*. 2011;60(12):1729-1738.

64. Ladányi A. Prognostic and predictive significance of immune cells infiltrating cutaneous melanoma. *Pigment Cell Melanoma Res*. 2015;28(5):490-500.

65. Hillen F, Baeten CIM, van de Winkel A, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. *Cancer Immunol Immunother*. 2008;57(1):97-106.

66. de Visser KE, Korets L V., Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell*. 2005;7(5):411-423.

67. DiLillo DJ, Yanaba K, Tedder TF. B cells are required for optimal CD4+ and CD8+ T cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice. *J Immunol*. 2010;184(7):4006-4016.

68. Nelson BH. CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol*. 2010;185(9):4977-4982.

69. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci*. 2012;125(23):5591-5596.

70. Somasundaram R, Zhang G, Fukunaga-Kalabis M, et al. Tumor-associated B-cells induce tumor heterogeneity and therapy resistance. *Nat Commun*. 2017;8(1):607.

71. Zhang Y, Gallastegui N, Rosenblatt JD. Regulatory B cells in anti-tumor immunity. *Int Immunol*. 2015;27(10):521-530.

72. Rosser EC, Mauri C. Regulatory B Cells: Origin, Phenotype, and Function. *Immunity*. 2015;42(4):607-612.

73. Mauri C, Menon M. The expanding family of regulatory B cells. *Int Immunol*. 2015;27(10):479-486.

74. Tao H, Lu L, Xia Y, et al. Antitumor effector B cells directly kill tumor cells via the Fas/FasL pathway and are regulated by IL-10. *Eur J Immunol*. 2015;45(4):999-1009.

75. Chen DS, Mellman I. Elements of cancer immunity and the cancer–immune set point.

Nature. 2017;541(7637):321-330.

76. Brachelente C, Cappelli K, Capomaccio S, et al. Transcriptome Analysis of Canine Cutaneous Melanoma and Melanocytoma Reveals a Modulation of Genes Regulating Extracellular Matrix Metabolism and Cell Cycle. *Sci Rep*. 2017;7(1):6386.

FIGURE LEGENDS

Figure 1. Representative images of TILs grade in canine melanocytic tumors; hematoxylin-eosin, 10x magnification. (A) Cutaneous melanocytoma with TILs grade 0 (absent); (B) Oral melanoma with TILs grade 1: TILs are mild and multifocally distributed, sparse or aggregated in small 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. Representative images of $CD3^+$ TILs in canine melanocytic tumors; immunohistochemistry, 20x magnification. (E) Cutaneous melanocytoma with occasional, scattered $CD3^+$ TILs (arrows); they are considered absent. (F) Cutaneous melanoma with scattered $CD3^+$ positive cells, representative of mild $CD3^+$ TILs infiltration. (G) Cutaneous melanoma with a moderate quantity of $CD3^+$ TILs: positive cells can be seen, scattered, among the neoplastic cells. (H) Oral melanoma with a marked infiltration of $CD3^+$ TILs. Representative images of $CD20^+$ TILs in canine melanocytic tumors; immunohistochemistry, 20x magnification. (I) Cutaneous melanocytoma without $CD20^+$ TILs. (J) Cutaneous melanoma with mild $CD20^+$ TILs. (K) Cutaneous melanocytoma with moderate infiltration of $CD20^+$ TILs: they tend to be aggregate, but they are also scattered among neoplastic melanocytes. (L) Oral melanoma with marked $CD20^+$ TILs quantity: positive cells can be seen in a large, central aggregate with abundant, scattered cells at its periphery.
infiltration (P>0.05).

Figure 2. Kaplan-Meier curves of overall survival time in dogs with low and high $CD20^+$ TILs infiltration (P<0.001).

Figure 3. Kaplan-Meier curves of disease-free survival time in dogs with low and high $CD20^+$ TILs infiltration (P<0.001).

Figure S1. Kaplan-Meier curves of overall survival time in dogs with low and high $CD3^+$ TILs infiltration (P>0.05).

Figure S2. Kaplan-Meier curves of disease-free survival time in dogs with low and high $CD3^+$ TILs

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 CD3+ TILs and CD20+ TILs quantity or distribution and outcome or metastasis/recurrence.

	Outcome (n, %)			**Metastasis / Recurrence (n, %)**		
	Dead for melanoma	**Alive or dead for another cause**	***P* value†**	**Yes**	**No**	***P* value†**
$CD3^+$ TILs quantity						
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)	
$CD3^+$ TILs distribution						
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)	
$CD20^+$ TILs quantity						
Absent	2 (10.5) $_b$	24 (55.8) $_a$	**0.002**	2 (15.4) $_a$	24 (52.2) $_a$	**<0.001**
Mild	10 (52.6) $_a$	14 (32.6) $_a$		3 (23.1) $_a$	19 (41.3) $_a$	
Moderate	5 (26.3) $_a$	4 (9.3) $_a$		7 (53.8) $_b$	1 (2.2) $_a$	
Severe	2 (10.5) $_a$	1 (2.3) $_a$		1 (7.7) $_a$	2 (4.3) $_a$	
$CD20^+$ TILs distribution						
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 3. Overall survival probability estimates according to $CD20^+$ TILs quantity in melanocytic tumors.

	Survival probability (median, 95%CI)			
	6 months	**1 year**	**1.5 years**	**2 years**
$CD20^+$ TILs quantity				
Absent	95.8 (73.9-99.4)	91.7 (70.6-97.8)	91.7 (70.6-97.8)	91.7 (70.6-97.8)
Mild	90.6 (67.3-97.6)	68.6 (42.6-84.7)	62.4 (36.3-80.3)	62.4 (36.3-80.3)
Moderate	60.0 (19.5-85.2)	20.0 (0.9-57.3)	20.0 (0.9-57.3)	n/a†
Severe	100 (100-100)	33.3 (0.8-77.4)	33.3 (0.8-77.4)	33.3 (0.8-77.4)
$CD20^+$ TILs quantity				
Low	93.5 (81.0-97.8)	81.6 (66.4-90.4)	78.8 (63.0-88.4)	78.8 (63.0-88.4)
High	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 4. Probability estimates of not developing recurrence/metastasis according to $CD20^+$ TILs quantity in melanocytic tumors.

	Disease-free probability (median, 95%CI)			
	6 months	**1 year**	**1.5 years**	**2 years**
$CD20^+$ TILs quantity				
Absent	91.5 (70.0-97.8)	91.5 (70.0-97.8)	91.5 (70.0-97.8)	91.5 (70.0-97.8)
Mild	95.0 (69.5-99.3)	83.6 (56.8-94.5)	83.6 (56.8-94.5)	83.6 (56.8-94.5)
Moderate	47.6 (12.3-76.9)	23.8 (1.3-62.2)	n/a†	n/a
Severe	66.7 (5.4-94.5)	66.7 (5.4-94.5)	66.7 (5.4-94.5)	66.7 (5.4-94.5)
$CD20^+$ TILs quantity				
Low	93.1 (80.2-97.7)	88.1 (73.7-94.9)	88.1 (73.7-94.9)	88.1 (73.7-94.9)
High	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